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VOLUME 91

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Academic Press is an imprint of Elsevier

Elsevier Academic Press
525 B Street, Suite 1900, San Diego, California 92101-4495, USA
84 Theobald's Road, London WC1X 8RR, UK

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ISBN-13: 978-0-12-022491-3
ISBN-10: 0-12-022491-7

PRINTED IN THE UNITED STATES OF AMERICA
06 07 08 09 9 8 7 6 5 4 3 2 1

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A Reappraisal of Humoral Immunity Based on Mechanisms of Antibody-Mediated Protection Against Intracellular Pathogens

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Abstract

Sometime in the mid to late twentieth century the study of antibody-mediated immunity (AMI) entered the doldrums, as many immunologists believed that the function of AMI was well understood, and was no longer deserving of intensive investigation. However, beginning in the 1990s studies using monoclonal antibodies (mAbs) revealed new functions for antibodies, including direct antimicrobial effects and their ability to modify host inflammatory and cellular responses. Furthermore, the demonstration that mAbs to several intracellular bacterial and fungal pathogens were protective issued a serious challenge to the paradigm that host defense against such microbes was strictly governed by cell-mediated immunity (CMI). Hence, a new view of AMI is emerging. This view is based on the concept that a major function of antibody (Ab) is to amplify or subdue the inflammatory response to a microbe. In this regard, the "damage-response framework" of microbial pathogenesis provides a new conceptual viewpoint for understanding mechanisms of AMI. According to this view, the ability of an Ab to affect the outcome of a host–microbe interaction is a function of its capacity to modify the damage ensuing from such an interaction.

ADVANCES IN IMMUNOLOGY, VOL. 91 0065-2776/06 $35.00

DOI: 10.1016/S0065-2776(06)91001-3

In fact, it is increasingly apparent that the efficacy of an Ab cannot be defined either by immunoglobulin or epitope characteristics alone, but rather by a complex function of Ab variables, such as specificity, isotype, and amount, host variables, such as genetic background and immune status, and microbial variables, such as inoculum, mechanisms of avoiding host immune surveillance and pathogenic strategy. Consequently, far from being understood, recent findings in AMI imply a system with unfathomable complexity and the field is poised for a long overdue renaissance.

1. Introduction

The classical view of antibody-mediated immunity (AMI) is that specific antibody (Ab) produced during the immune response to a microbial infection helps to clear the microbe by enhancing the efficacy of innate immune mechanisms and then confers immunity to subsequent encounters with the microbe. Consistent with this view, historically established mechanisms of AMI include viral and toxin neutralization, complement activation, phagocytosis, and antibody-dependent cellular cytotoxicity (ADCC) (Janeway *et al.*, 2001). The correlation between a defined amount of serum Ab and immunity against certain viral, bacterial, and toxin-mediated diseases provided proof that AMI is protective (Robbins *et al.*, 1995). Unfortunately, this tidy view of AMI does not apply to many infectious diseases, particularly those caused by intracellular pathogens such as *Mycobacterium tuberculosis* and *Listeria monocytogenes*. In fact, it is difficult to establish a role for AMI in host defense against many pathogenic microbes based on correlations between serum Ab levels and disease prevention and/or efficacy of passive Ab administration (Casadevall, 2004). The difficulty in establishing a role for AMI against intracellular microbes, evidence that the effective tissue response against many intracellular bacteria and fungi is granuloma formation, and that individuals with defects in cell-mediated immunity (CMI) are at increased risk for disease with such microbes, led to the paradigm that AMI and CMI have dichotomous roles, whereby AMI protected against extracellular and CMI protected against intracellular pathogens, respectively (Casadevall, 2003). However, studies with monoclonal antibodies (mAbs) and mice deficient in B cells and Fc receptors suggest that AMI is remarkably complex and poorly understood and that the time is ripe not only just for a paradigm shift but also for a major rethinking of the role of AMI in health and disease. Consequently, the reevaluation of AMI for intracellular pathogens is serving as a major catalyst for revising certain long-held concepts in immunological thought.

2. Intracellular and Extracellular Pathogenic Microbes: How Distinct Are They?

When immunologists consider the relative efficacy of AMI and CMI against a microbe, they often focus on whether it is an intracellular or extracellular pathogen. A major impetus for the classification of microbes as intracellular or extracellular was to ground the understanding of host defense against microbes with different pathogenic strategies in known and emerging immunological mechanisms. Based on what were believed to be fundamental mechanisms of AMI and CMI, AMI was viewed as the essential mediator of protection against extracellular microbes. As such, AMI was considered to be incapable of protecting against intracellular microbes because immunoglobulins are largely confined to the extracellular space. On the other hand, the discoveries that T cells only recognize antigen (Ag) in the context of Ag-presenting molecules and that infected cells express microbial Ags on their surface provided a mechanistic rationale for separating the roles of AMI and CMI based on the availability of microbial ligands and microbial localization during infection. The paradigm of a duality in function for AMI and CMI that has dominated thinking in immunology since the 1960s made sense in light of the inability to demonstrate efficacy of AMI against many intracellular pathogens and the prevailing understanding of mechanisms of Ab and T cell function. By the late twentieth century, this view of a division of labor for AMI and CMI was rather universally accepted, leading to it being used as the intellectual and basic scientific framework for research on host defense and vaccine design against many pathogenic microbes. However, closer scrutiny of the concept that AMI protects against extracellular pathogens and CMI protects against intracellular pathogens reveals numerous flaws in this paradigm.

A central problem in separating microbes into extracellular and intracellular groups has been the ambiguity, uncertainty, and inconsistency of these designations. First, the classification of microbes as intracellular or extracellular is almost exclusively applied to pathogenic bacteria and fungi. Paradoxically, viruses are usually not considered within the rubric of intracellular pathogens, although they have an absolute requirement of intracellular replication. Similarly, certain protozoa with intracellular phases in their growth cycles, such as *Plasmodium* spp., are not usually viewed through the intracellular versus extracellular immunological lens. In fact, microbes that are not full-time intracellular or extracellular inhabitants pose a problem for the AMI/CMI duality, since AMI is known to be effective against many viral diseases and is acknowledged as an important component of protection against *Plasmodium*-related diseases (Pleass and Holder, 2005). Second, all microbes, with the possible exception of endogenous retroviruses, have an extracellular phase

during which they exist outside the cell membrane. Even microbes capable of cell-to-cell spread, such as *L. monocytogenes* and *Shigella flexneri*, inhabit extracellular spaces when they first infect a host. Of relevance, AMI can be active and effective during this period, even if brief, as evidenced by the finding that Ab-mediated protection for the obligate intracellular pathogen *Ehrlichia chaffeensis* occurs during the brief period of extracellular life phase (Li and Winslow, 2003). Third, some authorities base their definitions of intracellular and extracellular on whether replication occurs predominantly in the intracellular or extracellular space. Such distinctions are often based on either *in vitro* observations of infected monolayers or pathological examination of infected tissues. Hence, the fungus *Histoplasma capsulatum* is considered an intracellular pathogen because it is found almost exclusively inside macrophages in infected tissues. On the other hand, *Streptococcus pyogenes* and *Staphylococcus aureus* are never considered intracellular pathogens, despite the fact that both have been found to persist in phagocytes (Gresham *et al.*, 2000; Medina *et al.*, 2003). Fourth, encapsulated organisms, such as *Streptococcus pneumoniae*, *Neisseria meningiditis*, and *Haemophilus influenzae*, are often considered extracellular pathogens because their capsules are antiphagocytic *in vitro*, which allows them to survive in the extracellular space (Collins, 1979). However, each of these organisms is often found inside phagocytic cells in tissue. In fact, the presence of a capsule cannot be used as definitive criterion for intracellular versus extracellular classification. The pathogenic fungus *Cryptococcus neoformans* has a large polysaccharide capsule yet replicates inside macrophages *in vivo* and *in vitro* (Feldmesser *et al.*, 2000) and, as noted above, *S. pyogenes* and *S. aureus* persist without being killed in neutrophils (Gresham *et al.*, 2000; Medina *et al.*, 2003). On the other hand, *M. tuberculosis* has an outer polysaccharide capsule, yet is considered a prototypic intracellular pathogen (Daffe and Etienne, 1999).

The classification of microbes as intracellular or extracellular pathogens lacks rigorous definitional boundaries and on close examination reflects a certain degree of circular reasoning. For example, one argument for the paradigm that AMI protects against extracellular pathogens whereas CMI protects against intracellular pathogens was that AMI could not be demonstrated to protect many intracellular pathogens, despite serious limitations in the methodologies available for evaluating AMI (Casadevall, 2004). This led to the tendency to use a lack of AMI against a microbe as a criterion for classifying it as an intracellular pathogen from an immunological perspective. In fact, it was argued that one criterion for assessing the efficacy of CMI in host defense was demonstrating lack of protection by AMI (Mackaness, 1977). However, in retrospect it is clear that serious limitations in the available methodologies for evaluating AMI, including their dependence on heterogeneous, impure reagents, made

it impossible to conclude that negative results meant that AMI was ineffective (Casadevall, 2004). Furthermore, it is noteworthy that the AMI versus CMI paradigm for extracellular and intracellular pathogens, respectively, was derived from the examination of a relatively small number of microbes. For example, the enormously authoritative and influential reviews of Mackaness and Collins that posited the importance of CMI for intracellular pathogens were focused on a small subset of pathogenic microbes such as *Mycobacterium* spp., *L. monocytogenes*, and *Salmonella* spp. (Collins, 1979; Mackaness, 1971, 1977). Nonetheless, it is noteworthy that the proposal that CMI was protective against these organisms emerged from a struggle to establish a role for CMI in the mid-twentieth century immunological world that often equated Ab with immunity. Hence, the focus on a few organisms for which AMI could not be demonstrated made sense in the context of establishing the field of cellular immunity. As such, the investigators who pioneered those studies left us a legacy of outstanding science and a greater understanding of host defense. However, the problem arose when the principles derived from a few microbes were generalized to the larger set of pathogenic microbes. There have always been microbes with intracellular pathogenic strategies for which AMI appeared to be important, including *Bacillus anthracis* and *Legionella pneumophilia*. AMI is protective against anthrax (Beedham *et al.*, 2001; Little *et al.*, 1997), although *B. anthracis* is not considered an intracellular pathogen in an immunological context. Although *B. anthracis* is a free-living spore that does not require a host for survival, it replicates within macrophages after escaping from the phagosome to the cytosol (Dixon *et al.*, 2000), making it a classical intracellular pathogen by most definitions. AMI also contributes to host defense against *L. pneumophilia* (Brieland *et al.*, 1996; Eisenstein *et al.*, 1984a; Rolstad and Berdal, 1981), a free-living bacterium in water sources capable of replicating inside macrophages and amoebae. These examples and the aforementioned ability of gram-positive organisms to persist in phagocytes illustrate that neither the ability for intracellular replication nor phylogenetic derivation nor the relative efficacy of AMI and CMI can be used as a singular or definitive criterion for designating a microbe as extracellular or intracellular. Consequently, the distinction between intracellular and extracellular, as the terms are most commonly used, appears to be more microbe specific, than based on shared or common microbiologic or pathogenic characteristics. Hence, our view is that the paradigm of a dichotomous role for AMI and CMI for extracellular and intracellular microbes, respectively, is logically inconsistent and inadequate to serve as a pillar of immunological theory and thought. Nonetheless, the separation of microbes into intracellular and extracellular pathogens has formed the basis of much immunological thought and was central to the development of current views of the relative efficacy of AMI and CMI (Collins, 1974;

Mackaness, 1971). Therefore, this chapter will discuss AMI from the vantage point of this distinction. Cognizant of the limitations of the term intracellular, we will use it to refer to microbes that have significant intracellular growth phases and for which CMI is generally considered to be the primary host defense mechanism.

3. Components of AMI

The term AMI is used here to encompass all the protective effects associated with Ab, including those mediated by "naturally occurring Ab," passively transferred Ab, and acquired Ab (Ab generated by an immune response). When considering the function of AMI, it is worthwhile to remember that serum contains a high concentration of immunoglobulin proteins that include many different microbial and self-specificities and isotype compositions. This immunoglobulin pool reflects the host response to endogenous microbiota as well as the immunological memory of the host for a variety of acquired microbial agents. Understanding the role of AMI in protection against infectious diseases involves developing an appreciation for the differences in function between Abs referred to as "naturally occurring," those that are passively administered, and those that are induced by a specific agent.

The term naturally occurring is inexact and vague. This designation was probably meant to differentiate preexisting Ab from that generated during an immune response, which is often referred to as "specific" or "acquired" Ab. In fact, a rise in titer in serological assays is sometimes used to try to distinguish naturally occurring Ab from specific Ab produced in response to a specific agent. Important caveats to this approach are that the heterogeneous nature of serum precludes knowing whether the Abs detected before contact with an agent recognize the same determinants as those that are detected afterward and that many methods of detecting Ab can measure some degree of reactivity with the agent of interest. Issues of detection notwithstanding, a problem with the term naturally occurring Ab is that the actual agent/s that elicited such Abs is/are essentially unknown. Further, the naturally acquired Ab repertoire also consists of Abs that can be shown to be cross-reactive with a multitude of microbial and even self-determinants, making their origin even more elusive. In this chapter, we will use the term naturally occurring to refer to preexisting Abs that are found in the serum of a host prior to contact with a new microbial agent and/or immunogen.

Naturally occurring Ab has the capacity to bind pathogenic microbes even when the host has not encountered the microbe in question. Although the interaction of naturally occurring Ab with a microbe is often a low-affinity interaction, the principle of mass action is likely to enable some immunoglobulin

binding to microbial surfaces since the concentration of immunoglobulins in serum is high. Since Ab binding is sufficient to induce B cell activation, low-affinity interactions between an Ab and Ag have the potential to induce biologically relevant Ab activity. Contrary to prevailing thought, it has been proposed that low-affinity Abs have a better potential to discriminate between, and as such be more specific for given antigenic determinants, since they are more likely to dissociate than high affinity Abs (Van Regenmortel, 1998).

Consistent with a biological role for such Abs, there is increasing evidence that naturally occurring Abs provide a key layer of early protection against many pathogenic microbes by virtue of their capacity for low-affinity interactions. The importance of preexisting Abs in resistance to numerous pathogens, including bacteria, viruses, and parasites, in animal models of infectious diseases has been increasingly recognized (Boes *et al.*, 1998a; Brown *et al.*, 2002; Couper *et al.*, 2005; Rajan *et al.*, 2005). Some of these models rely on the use of secretory IgM (sIgM)-deficient mice, which have a defect in IgM secretion that results in their having normal serum levels of other isotypes but no serum IgM (Boes *et al.*, 1998b). Preexisting IgM is crucial for resistance to pneumococcus in mice (Brown *et al.*, 2002), despite the fact that serum levels of IgG are considered a surrogate for vaccine efficacy against this microbe. IgM derived from a defined repertoire of memory B cells has been strongly implicated in protection against pneumococcal disease in humans (Carsetti *et al.*, 2005; Kruetzmann *et al.*, 2003; Shi *et al.*, 2005). This population of B cells is reduced in patients at high risk for pneumococcal disease, including HIV-infected individuals and the elderly (Chong *et al.*, 2004; Shi *et al.*, 2005). The activity of germline and/or early acquired IgM against viral pathogens, such as influenza and *West Nile virus*, and other agents, such as pneumococcus (Brown *et al.*, 2002; Diamond *et al.*, 2003; Harada *et al.*, 2003; Mehlhop *et al.*, 2005), implicates naturally occurring IgM as an important component of innate immune responses to and complement-mediated protection against these agents. One mechanism by which naturally occurring AMI could contribute to host defense is by amplifying complement activation and providing opsonins. This function suggests that AMI could play a proinflammatory role, shortening the response time when the host encounters a pathogenic microbe. On the other hand, IgM has been shown to inhibit complement activation without compromising opsonic activity, in some instances by blocking classical but not alternative complement pathway activation (Walpen *et al.*, 2004; Werwitzke *et al.*, 2005). Given that our appreciation of the importance of naturally occurring Abs in host defense against infectious diseases is in its infancy, future studies are likely to reveal a fuller understanding of the mechanisms that govern the efficacy of naturally occurring AMI in host defense.

Passively administered Ab confers a form of AMI that is different than either naturally occurring or AMI that is induced by a specific Ag/agent. Most passively administered Abs have either known specificity or protective properties that are already known to confer a state of immediate immunity. However, pooled, nonspecific immunoglobulin preparations are in use as anti-inflammatory therapy for diseases as diverse as inflammatory myopathies and streptococcal toxic shock (Dalakas, 2003; Norrby-Teglund *et al.*, 2003). The efficacy of nonspecific immunoglobulin in toxic shock syndrome has been attributed to toxin neutralization (Darenberg *et al.*, 2003). IgM-enriched immunoglobulin preparations (pentaglobulin) were found to be cost effective in treatment of severe septic shock (Neilson *et al.*, 2005), an effect that could reflect the ability of certain IgMs to bind endotoxin (Bennett-Guerrero *et al.*, 1997; Maury *et al.*, 2003) or to inhibit complement activation (Rieben *et al.*, 1999; Walpen *et al.*, 2004). When used as antimicrobial therapy against experimental infection, passive Ab is most effective when administered before microbial challenge. In fact, passive Ab is often ineffective against established infection. Hence, passive Ab preparations must be able to be effective in the context of early innate and cellular immune responses. Passive Ab is often ineffective against established infection. This raises a fundamental problem that has never been adequately explained, since Ab presumably contributes to host defense in natural infection even though it is made in response to infection. For example, clinical improvement from pneumococcal pneumonia in the preantibiotic era was associated with the appearance of specific serum Ab. One explanation for this phenomenon, which is consistent with the observation that recovery from many infections occurs sooner than the time it takes for a specific Ab response to develop, is that the Abs that protect against acute infection are part of the natural, preexisting repertoire and that these Abs are different from acquired Abs, protect against reinfection, or downregulate the inflammatory response by engaging inhibitory Fc receptors. In these scenarios, naturally occurring AMI would cooperate with innate immune mechanisms and the nascent CMI response to contain infection, while the secondary IgG response is made later for long-lasting immunity (Fig. 1). Hence, the efficacy of passively administered specific Ab in a naïve host may recapitulate conditions that mimic the immune response or reencounter with a microbe more than the naïve response.

Specifically induced or acquired AMI involves the production of IgM, IgA, or IgG in response to a microbial agent or immunization. A paradoxical observation involving AMI is that specific IgG is often made after the host has recovered. In fact, a rise in serum IgG titer is a time-honored method for diagnosing many infectious diseases. This observation begs the question of why IgG is made after recovery from most infectious diseases. Invoking a need to

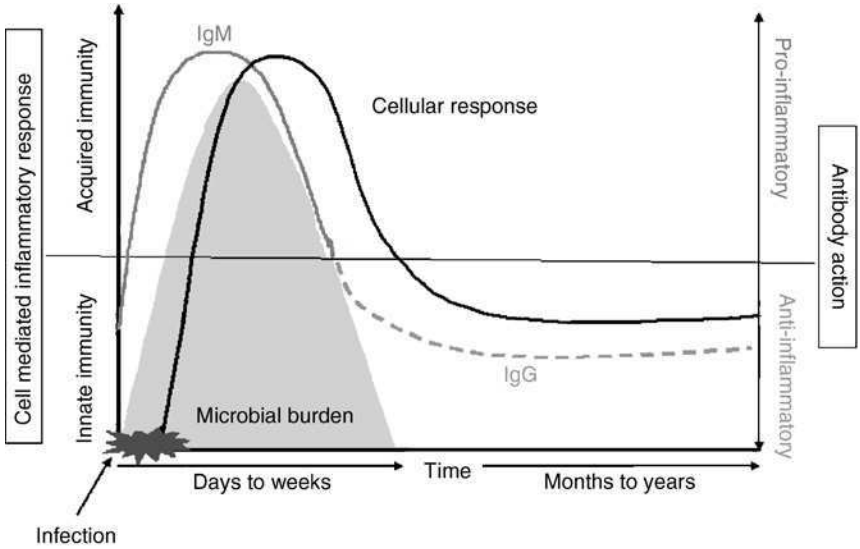

Figure 1 The proposed role of Ab as an regulator of the inflammatory and cellular response (Casadevall and Pirofski, 2003). The scheme is idealized for a host–microbe interaction whereby the immune system can contain and eradicate the microbe. The left hand *y*-axis depicts the cellular inflammatory response to a microbe. The right hand *y*-axis depicts Ab action shown as pro-inflammatory and anti-inflammatory effects. In this schema, IgM functions predominantly in a proinflammatory role, which augments the innate cellular immune response to and pathogen clearance, and IgG functions predominantly in an anti-inflammatory role, which decreases the cell-mediated inflammatory response that follows pathogen clearance. This is depicted on the right hand *y*-axis. According to this view, Ab helps to confer the state of immunity by downregulating the CMI of a primed host on a subsequent encounter with the same microbe (Casadevall and Pirofski, 2003).

prevent recurrences is a somewhat unsatisfactory answer if the initial innate and cellular response was adequate to clear the first bout of disease. Nevertheless, an adaptive response that avoids recurrent bouts of a particular disease would have a significant survival advantage. The presence of serum IgG is a marker of immunity for many infectious diseases, even if IgG may not have been responsible for control of the initial infection.

4. AMI in the Context of the "Damage-Response Framework"

Given that AMI is a host defense mechanism against pathogenic microbes, an attempt to understand its function should account for Ab action in the context of principles of microbial pathogenesis. Unfortunately, until recently we lacked a unified theory that incorporated the contribution of the host response as well

as the microbe into microbial pathogenesis. We have proposed the damage-response framework (Casadevall and Pirofski, 1999, 2003) as a unified theory of microbial pathogenesis. This theory is grounded by the proposal that the common denominator in all cases of microbial pathogenesis, irrespective of the causative microbial agent, is damage to the host. This view provides a universal, yet flexible, construct to account for microbial pathogenesis without the need for separate categories for different types of microbes. According to the damage-response framework, damage is defined as a perturbation of host homeostasis that disrupts or alters tissue integrity, function, physiology, biochemistry, or hemodynamics or cellular function, secretion or inflammation.

The damage-response framework is built on the following three tenets: (1) microbial pathogenesis is the outcome of an interaction between a host and a microbe, (2) the relevant outcome of host–microbe interaction is damage in the host, (3) damage can be the result of host factors, microbial factors, or both (Casadevall and Pirofski, 1999, 2003). Consequently, the outcome of host–microbe interaction can be plotted on a u-shaped curve, whereby the *y*-axis represents the amount of host damage and the *x*-axis represents the host response from weak to strong going from left to right (Fig. 2). The terms weak and strong are relative and include both quantitative and qualitative parameters (Casadevall and Pirofski, 1999). Although we recognize that plotting an immune response along a single axis is an oversimplification of an enormously

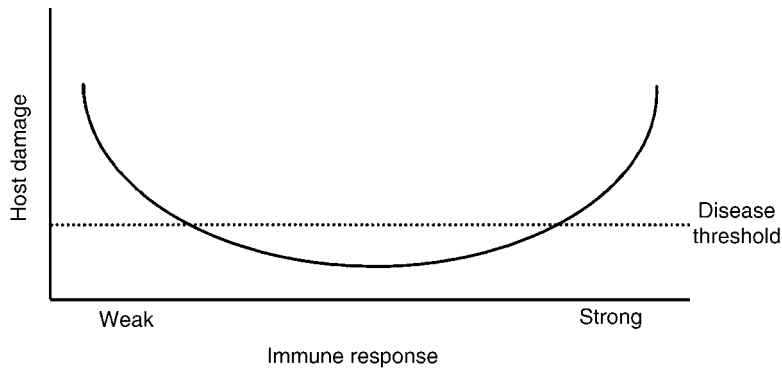

Figure 2 The basic curve of microbial pathogenesis as proposed by the damage-response framework is a parabola whereby host damage from the host–microbe interaction occurs primarily at the extremes of immune response (Casadevall and Pirofski, 1999, 2000, 2003). One can modify this curve to generate classify known pathogens into six groups (Casadevall and Pirofski, 1999). The curve can also be modified to account for commensal host–microbe interactions. Most of the classical intracellular pathogens considered in this chapter are class 3 pathogens (Casadevall and Pirofski, 1999) and are represented by the curve shown here.

complex process, the continuum from weak to strong provides a first approximation for developing systematic approach to understanding microbial pathogenesis. Certain infectious diseases are the result of excessive (strong, inappropriate) immune responses that require downregulation of inflammation, whereas others are the result of insufficient (weak, inappropriate) immune responses that require bolstering. Hence, it is logical to assess Ab function based on its impact on host damage and the nature of the immune response to a microbial agent.

The damage-response framework is a new schema for understanding the role of AMI in health and disease. In the context of the damage-response framework, a protective Ab is one that shifts the curve depicting host damage as a result of the immune response in favor of the host by reducing damage. This can occur as a result of Ab-mediated reduction of an exuberant inflammatory response (shift to the left) or enhancement of a weak inflammatory response (shift to the right) (Fig. 3). Often, host damage in the setting of a weak immune response is a result of microbial factors, whereas damage in the setting of a strong immune response reflects an excessive inflammatory response. Hence, in the setting of a weak host response to a microbe, a protective Ab might enhance the immune response. This could be mediated by an Ab with the ability to augment the inflammatory response. This type of phenomenon was described for an IgM to the polysaccharide capsule of *S. pneumoniae*, which promoted earlier recruitment of neutrophils to the lungs of mice with pulmonary infection (Burns *et al.*, 2005). However, later in the course of infection, when control mice had high levels of proinflammatory mediator expression, the same Ab led to downregulation of chemokine expression in lung. Hence, Abs can mediate protection by downregulating the immune response in the setting of strong immune responses, during which high levels of inflammatory mediators are major contributors to host damage. Along these lines, Ab-mediated protection to *C. neoformans* in mice has been associated with downregulation of IFN-γ and, in some mouse strains, increased levels of IL-4 and IL-10 (Feldmesser *et al.*, 2002; Rivera and Casadevall, 2005; Rivera *et al.*, 2002, 2005). However, in certain conditions, Ab administration to mice with chronic *C. neoformans* infection can result in catastrophic cardiovascular collapse associated with release of platelet-activating factor and other proinflammatory mediators (Lendvai *et al.*, 2000; Savoy *et al.*, 1997). Furthermore, experiments with human cells *in vitro* have clearly demonstrated the same type of antibodies can promote the release of proinflammatory mediators under certain conditions (Vecchiarelli and Casadevall, 1998; Vecchiarelli *et al.*, 1998a,b) (52–54). Consequently, a given Ab can mediate proinflammatory or anti-inflammatory changes depending on the specific host–microbe interaction.

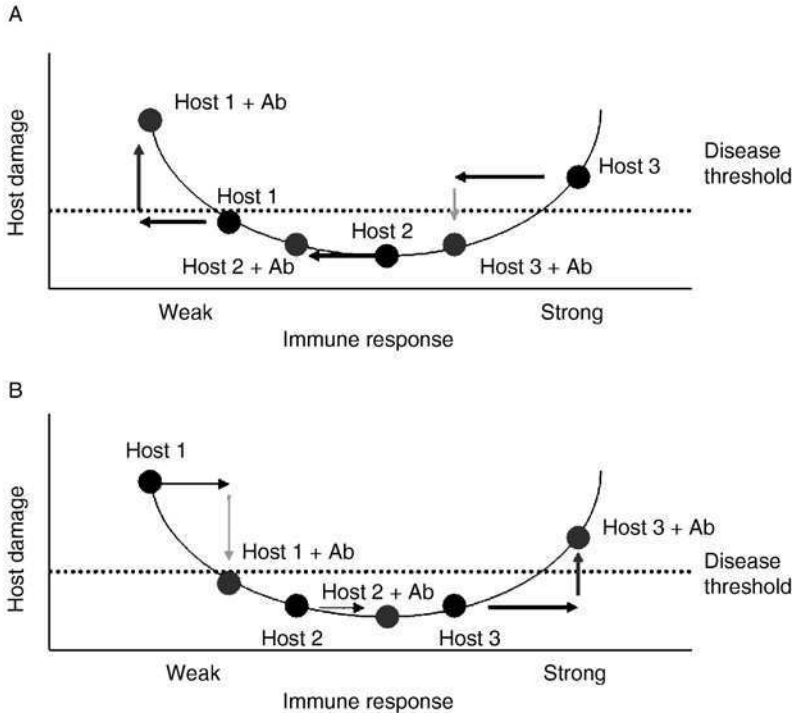

Figure 3 Conceptual representation of Ab that enhances or diminishes the immune response in the context of the damage-response framework of microbial pathogenesis. (A) An Ab that diminishes the immune response would be protective for host 3, disease enhancing for host 1, and have no clinical effect on host 2. (B) An Ab that enhances the immune response would be protective for host 1, disease enhancing for host 3, and have no clinical effect on host 2.

In contrast to protective Abs, a disease-enhancing Ab would shift the curve such that host–microbe interaction resulted in greater host damage. In this regard, immune complexes to *Leishmania* have been proposed to contribute to virulence by promoting the secretion of IL-10 that downregulates the immune response (Miles *et al.*, 2005), thereby shifting the curve to the left. However, Ag–Ab complexes can also shift the curve to the right as evidenced by the observation that Ab administration to certain mice with chronic *C. neoformans* infection caused cardiovascular collapsed from secretion of the inflammatory mediator platelet-activating factor (Lendvai *et al.*, 2000; Savoy *et al.*, 1997). Hence, it is increasingly apparent that, rather than being inherently good or bad, the effects of Abs are either beneficial or deleterious in a host, depending on the type of microbe–host interaction, including the setting in which damage

occurs as a function of the host immune milieu and response. A logical extension of this concept is that an Ab that is protective in one host may not be protective in another if the nature of their immune responses to the relevant agent places them on different parts of the damage-response curve (Fig. 3). These concepts have important ramifications for vaccine design since vaccine efficacy could depend on enhancement of the immune response for those with weak immune responses, but enhanced responses could be detrimental in those who naturally generate strong immune responses. An excessive inflammatory response mediated by immune complexes could have been in part responsible for the failure of the killed measles vaccine, which was associated with the development of atypical measles (Polack *et al.*, 1999). Hence, more than one type of vaccine may be needed to prevent infectious diseases that can develop in the setting of either weak or strong immune responses.

Toxin-mediated diseases are viewed by the damage-response framework as a special case whereby damage occurs across a range of host responses. Bacterial toxins, such as those produced by *Clostridium tetani* and *Corynebacterium diphtheriae*, cause host damage without eliciting a significant immune response as evidenced by the fact that neither tetanus nor diphtheria confers immunity to recurrent disease (Spenney *et al.*, 1971). AMI protects against these diseases through toxin neutralization, a phenomenon that would reduce the incidence of disease without necessarily altering the prevalence of infection.

Another important principle of the damage-response framework is that the state of the host–microbe interaction is a function of time (Casadevall and Pirofski, 2003). The damage-response framework sees no fundamental difference between the states of colonization, commensalism, latency, and disease except for the amount of host damage that results from the host–microbe interaction over time (Casadevall and Pirofski, 2003). Hence, Abs that are protective in some host–microbe interactions could prevent host damage that would otherwise lead to progression from colonization or latency to disease. This provides a functional explanation for how vaccines for microbes that often exist in a state of colonization prevent disease; protective Ab responses would control host damage, keeping it below the threshold that would result in disease. Abs that promote or maintain a state of latency could contribute to protective responses because they decrease the likelihood that disease will ensue. Although Abs that maintain latency have not been identified, they have not been looked for, raising the question of whether certain naturally occurring and/or specifically induced Abs that bind latent microbes, such as *M. tuberculosis*, herpesviruses, or *C. neoformans*, have a role in maintaining latency. This concept is supported in principle by serological studies that showed a decline in IgM to cryptococcal capsular polysaccharide in individuals

at high risk for the development of disease (Fleuridor *et al.*, 1999; Subramanian *et al.*, 2002). Further, the demonstration that vaccination of elderly individuals against varicella-zoster virus prevented herpes zoster suggests that the elicited Abs helped to maintain the latent and prevent the development of disease (Oxman *et al.*, 2005). Perhaps Abs similar to those that were elicited by the vaccine are already present in individuals who do not develop herpes zoster.

In contrast to Abs that mediate protection, Abs that enhance disease or are deleterious are those that contribute to progression of damage and/or disease. Examples of such Abs are those that are part of damaging immune complexes or those that enhance uptake of microbes, which improves their ability to replicate and damage host cells, such as certain Abs to *Dengue virus* (Sullivan, 2001). In summary, the damage-response framework provides a flexible schema by which to characterize Ab efficacy, which, despite the complexity of the interactions that underlie host–microbe relationships and Ab function, can be reduced to assessment of how Ab affects two relationships: host damage as a function of the host immune response and host damage as a function of time.

5. Abs as Enhancers of Innate Immunity

The importance of naturally occurring Abs, predominantly of the IgM isotype, in enhancing innate immune responses to a multitude of pathogens is being increasingly recognized. In addition to the aforementioned animal models in which microbial virulence is reduced in the absence of serum IgM (see earlier), naturally occurring IgMs enhance complement-mediated and complement-independent antimicrobial mechanisms. For example, naturally occurring swine IgM promoted complement-mediated lysis of pseudorabies virus–infected cells (Hayashi *et al.*, 2005) and naturally occurring human serum IgM that lacked the ability to promote complement-mediated lysis *in vitro* protected infant rats against *N. meningiditis* serogroup B, a serogroup against which an effective vaccine has not yet been developed (Toropainen *et al.*, 2005). The mechanism by which such protection is mediated is not known. However, in light of the discovery that the efficacy of a nonopsonic IgM to pneumococcal polysaccharide was associated with downregulation of the proinflammatory response to pulmonary pneumococcus infection (Burns *et al.*, 2005), a similar mechanism could be operative against other microbes. Such a mechanism appears to allow innate host defense mechanisms to combat the microbe, while reducing the damage that might result from the inflammation induced by this process. Consistent with the idea that naturally occurring Abs can regulate the inflammatory response, natural IgM was found to promote granuloma formation in an experimental model of filarial infection (Rajan *et al.*, 2005). One clue to the mechanism by which some such Abs might work

is the discovery of a natural IgM Ab that enhanced the Ag-presenting ability of dendritic cells by binding to B7 (Radhakrishnan *et al.*, 2003), suggesting that an Ab ligand can link receptors of innate and acquired immunity. Another mechanism by which naturally occurring Abs could enhance the potential of innate immunity to microbial pathogens is through the catalytic activity that has been demonstrated for some such molecules (Nathan, 2002; Paul *et al.*, 2005). The ability of certain Abs to mediate catalysis has been attributed to variable region nucleophilic sites with the capacity for covalent binding (Paul *et al.*, 2005). Although whether the rate of catalysis mediated by catalytic Abs is sufficient to confer biological activity *in vivo* has been debated, higher levels of catalytic IgG correlated with better survival in patients with septic shock (Lacroix-Desmazes *et al.*, 2005).

6. Abs as Direct and Indirect Effector Molecules

Abs can promote host defense by direct or indirect mechanisms (Table 1). Direct Ab functions are those that are manifest when an Ab binds a microbe and/or a microbial component and mediates an antimicrobial or antitoxin effect. Direct Ab functions include those classically associated with AMI such as complement activation, agglutination; toxin and viral neutralization. With the exception of toxin and viral neutralization, these direct effects are facilitated by Abs but mediated in concert with other components of the immune system. However, a considerable body of evidence has accumulated indicating that certain Abs can mediate direct effects against bacteria and fungi by themselves. Specific IgM can be bactericidal to *Borrelia* in the absence of complement by killing the bacteria through surface effects (Connolly and Benach, 2001; Connolly *et al.*, 2004). Abs that mimic the action of a yeast killer toxin have been shown to be directly microbicidal to a variety of different classes of microbes, including *Leishmania* spp. (Savoia *et al.*, 2002), *Candida albicans* (Polonelli *et al.*, 1996), *Aspergillus* (Torosantucci *et al.*, 2005) and *M. tuberculosis* (Conti *et al.*, 1998). Abs to *C. albicans* surface Ags inhibited hyphal formation and growth (Moragues *et al.*, 2003). In fact, a single mAb to *C. albicans* has been shown to mediate multiple antifungal effects including inhibition of germination and attachment to host cells in addition to having direct candidicidal activity *in vitro* (Moragues *et al.*, 2003). A list of direct Ab effects is provided in Table 2.

In contrast, indirect Ab functions are antimicrobial effects mediated through actions with effector cells and/or by changes in the inflammatory and immune response. Indirect Ab functions classically associated with AMI are phagocytosis and ADCC. AMI can have profound effects on the inflammatory response through a variety of mechanisms that include activation of

Table 1 Direct and Indirect Antimicrobial Activities of Abs

Ab mechanism and/or action	Type	Reference
Opsonization	Direct	Janeway *et al.*, 2001
Complement activation	Direct	Janeway *et al.*, 2001
Viral neutralization	Direct	Janeway *et al.*, 2001
Toxin neutralization	Direct	Janeway *et al.*, 2001
ADCC	Direct	Janeway *et al.*, 2001
Bactericidal	Direct	Connolly and Benach, 2001; Connolly *et al.*, 2004; Goel and Kapil, 2001
Fungistatic	Direct	Moragues *et al.*, 2003; Rosas *et al.*, 2001; Torosantucci *et al.*, 2005
Interference with antigen release	Direct	Martinez *et al.*, 2004
Interference with biofilm formation	Direct	Martinez and Casadevall, 2005
Interference with iron acquisition	Direct	Fitzgerald and Rogers, 1980
Generation of oxidants	Direct	Wentworth *et al.*, 2002
Oxidative burst	Indirect	Johnston *et al.*, 1976; Mozaffarian *et al.*, 1995
Changes in cytokine expression	Indirect	Anderson and Mosser, 2002; Gerber and Mosser, 2001; Marsh *et al.*, 1994, 1995, 1997, 1998; Vecchiarelli *et al.*, 1998b
Release of prostaglandins	Indirect	Neuwirth *et al.*, 1988
Changes in costimulatory molecule expression	Indirect	Vecchiarelli *et al.*, 1998c
Changes in FcγR expression	Indirect	Rivera and Casadevall, 2005
Enhancement of lysosome-phagosome fusion	Indirect	Armstrong and Hart, 1975

inhibitory FcR receptors, modulating the release of proinflammatory and anti-inflammatory cytokines, promoting release of prostaglandins, and clearance of microbial molecules with immunomodulatory effects (Casadevall and Pirofski, 2003). In addition, intravenous immunoglobulin (IVIG) has been shown to induce neutrophil apoptosis via an Fc receptor-dependent H_2O_2-dependent pathway (Takeshita *et al.*, 2005), a function that could contribute to its anti-inflammatory activity. Indirect Ab functions may be beneficial or detrimental to the host, depending on the type of host–microbe interaction. Ab effects that reduce host damage due to the inflammatory response can be expected to translate into Ab-mediated protection, whereas proinflammatory changes that increase damage can be expected to result in no protection or disease-enhancing

Table 2 Facultative and Obligate Intracellular Pathogens for Which Ab Can Affect the Outcome of Experimental Infection

Organism[a]	Reference[b]
Anaplasma marginale	Tebele *et al.*, 1991
Brucella abortus	Bowden *et al.*, 1995; Elzer *et al.*, 1994
Chlamydia spp.	Cotter *et al.*, 1995; Zhang *et al.*, 1987
Cryptococcus neoformans	Dromer *et al.*, 1987; Fleuridor *et al.*, 1998; Mukherjee *et al.*, 1992; Sanford *et al.*, 1990
Ehrlichia chaffeensis	Kaylor *et al.*, 1991; Li *et al.*, 2001, 2002
Histoplasma capsulatum	Nosanchuk *et al.*, 2003
Legionella pneumophilia	Brieland *et al.*, 1996; Eisenstein *et al.*, 1984a
Leishmania spp.	Anderson *et al.*, 1983; Savoia *et al.*, 2002
Listeria monocytogenes	Edelson and Unanue, 2001; Edelson *et al.*, 1999
Mycobacteria tuberculosis	Chambers *et al.*, 2004; Hamasur *et al.*, 2003, 2004; Pethe *et al.*, 2001; Teitelbaum *et al.*, 1998; Williams *et al.*, 2004
Rickettsia typhi	Gambrill and Wisseman, 1973
Salmonella spp.	Eisenstein *et al.*, 1984b; Ornellas *et al.*, 1970; Robbins and Robbins, 1984; Watson *et al.*, 1992
Shigella flexneri	Phalipon *et al.*, 1995
Toxoplasma gondii	Cha *et al.*, 2001; Johnson and Sayles, 2002; Johnson *et al.*, 1983; Mineo *et al.*, 1994; Pavia *et al.*, 1992

[a]The strength of the evidence for the protective role of AMI varies for the different pathogens listed below.

[b]Not a complete listing.

effects. Although the distinctions between direct and indirect effects are somewhat artificial and simplistic and there is some overlap of these effects, they provide a useful construct to categorize mechanisms of AMI. Nonetheless, it must be recognized that the interrelatedness of components of the immune system is such that any action mediated by an Ab is likely to affect other aspects of the immune response.

Considering Ab-mediated effects to be part of direct or indirect effector categories can provide clues as to why it has been so difficult to demonstrate the efficacy of AMI against intracellular pathogens. Historically, immunological concepts of Ab-mediated protection have largely focused on direct Ab effects that apply primarily to toxins and extracellular microbial pathogens, with less emphasis on considering indirect effects of Ab action, such as modulation of the inflammatory response. This was undoubtedly a consequence of the fact that the tools for studying mediators of inflammation have become available relatively recently. Furthermore, the view that Ab molecules were confined to the extracellular space by cell membranes encouraged the view that AMI was not a major contributor to host defense against intracellular pathogens.

However, when indirect Ab functions are taken into account, it is clear that there are numerous mechanisms by which AMI can affect the outcome of host–microbe interactions with intracellular pathogens.

An important burgeoning role for AMI is found in the emerging understanding that Ab can be required for resistance to reinfection, even though it may not be required for primary resistance. For example, specific Ab mediated resistance to reinfection with *Candida*, although B cell-deficient mice were resistant to primary infection (Montagnoli *et al.*, 2003). This finding was attributed to the ability of Ab to prime dendritic cell–mediated antifungal immunity. This is consistent with the concept that AMI is important for the establishment and maintenance of certain memory responses, particularly those that depend on $CD8^+$ T cells. The memory response to *Helicobacter pylori* and cytotoxic lymphocytes (CTLs) to *Lymphocytic choriomeningitis virus* (LCMV) is enhanced by B cell presentation and or activation (Azem *et al.*, 2005; Homann *et al.*, 1998; Klenerman, 2004; Matter *et al.*, 2005). Similarly, B cells, which play an insignificant role in the primary response, were required for the development of a memory response to *L. monocytogenes* Shen *et al.*, 2003) and reinfection with *Francisella tularemia* (Bosio and Elkins, 2001). The demonstration of B cells in granulomatous skin lesions of patients with *Coccidiodes immitis* underscores the importance of B cells in the immune response to fungi, microbes that were formerly believed to depend solely on CMI, and supports their emerging role as immunoregulators (Li *et al.*, 2005). The exact role of immunoglobulin in these processes remains to be determined.

7. AMI as a Regulator of the Inflammatory Response

As discussed previously, Ab can be a positive or negative regulator of the inflammatory response. The ability of an Ab to function in a positive or negative regulatory capacity is a function of the Ab isotype, amount, and specificity. Proinflammatory activities of Abs are complement activation, FcR engagement with the release of proinflammatory mediators such as cytokines, chemokines, platelet-activating factor, and chemokines, neutralization of microbial components that interfere with an inflammatory response, and the capacity to promote phagocytosis and enhance Ag presentation. For IgG, many proinflammatory and anti-inflammatory functions are mediated by interaction with activating (FcγRI and FcRγRIII) and inhibitory (FcγRII) FcγRs (Ravetch and Bolland, 2001; Ravetch and Lanier, 2000). These receptors have different affinities for the various IgG subclasses (Nimmerjahn and Ravetch, 2005). Consequently the proinflammatory or anti-inflammatory activity of a given isotype is in part inherent, depending on the type of receptor with which it interacts. In mice, IgG1 interacts exclusively with FcRγRIII, whereas a new

fourth IgG FcR has been described (FcγRIV) that has specificity for IgG2a and IgG2b, but does not bind to IgG1 or IgG3 (Nimmerjahn *et al.*, 2005). The inhibitory (FcγRII) FcR has been implicated in the anti-inflammatory effect of IVIG and innate resistance to pneumococcus (Clatworthy and Smith, 2004). There is an emerging literature showing that the types of Fc receptors activated can have a profound effect on the development of immune responses. In this regard, differences in the degree of stimulation of activating and inhibitory receptors on dendritic cells may tilt the response toward tolerance or immunity (Boruchov *et al.*, 2005). Specific IgM probably has a greater proinflammatory capacity than specific IgG by virtue of its greater complement-activating activity and the absence of inhibitory IgM Fc receptors. However, IgM also has anti-inflammatory effects, which could in part be due to the ability of certain naturally occurring IgMs to neutralize endotoxin, clear apoptotic cells, and/or to inhibit classical complement pathway activation (Peng *et al.*, 2005; Reid *et al.*, 1997; Reith *et al.*, 2004; Rieben *et al.*, 1999; Walpen *et al.*, 2004).

Anti-inflammatory activities of Abs include their ability to reduce a microbial inoculum by promoting microbial clearance by phagocytosis, FcγR engagement to produce anti-inflammatory cytokines such as IL-10 (Tripp *et al.*, 1995), and binding to proinflammatory microbial components such as lipopolysaccharide (LPS). Acute LCMV infection was attenuated by an Ab-mediated reduction in T cell-mediated host damage that was associated with a reduction in viral replication (Wright and Buchmeier, 1991). IgG is probably a more anti-inflammatory Ig class than IgM by virtue of its ability to engage the inhibitory FcγR and its requirement for multiple molecules in activating complement. Consistent with this property, IgG administration is commonly used clinically to treat inflammatory conditions. However, IgM (pentaglobulin) was beneficial in patients after abdominal surgery and in those with septic shock (Buda *et al.*, 2005; Pul *et al.*, 2002; Reith *et al.*, 2004). In summary, IgM and IgG can each be proinflammatory or anti-inflammatory depending on their amount, specificity, and access to FcRs. Proinflammatory and anti-inflammatory functions of Ig are listed in Table 3. The ability of AMI to function in both proinflammatory and anti-inflammatory roles, depending on the variables mentioned above, implies that it is an integral part of the host response and that its net effect will be a function of the conditions that prevail for the relevant host–microbe interaction.

Another mechanism by which Ab can function as an immunodulator is by the ability of certain Abs to alter the immune response to an Ag when they are complexed with that Ag (Brady, 2005). This phenomenon has been extensively studied with mAbs to the *Streptococcus mutans* Ag P1. Complexes of mAb and P1 altered the isotype and specificity of the serum Ab response to P1 when administered mucosally or systemically (Brady *et al.*, 2000; Oli *et al.*, 2004).

Table 3 Effect by Which Ab Can Affect Inflammatory Responses

Effect	Mechanism(s)	Outcome proinflammatory	Anti-inflammatory
Complement activation	Phagocytosis Microbial damage Production of proinflammatory complement split products	Increased recruitment of inflammatory cells Microbial damage releases proinflammatory products	Reduction of inoculum IgM-mediated reduction of complement activation
Direct antimicrobial effects	Bactericidal activity Fungistatic activity Inhibition of biofilm formation	Microbial damage releases proinflammatory products	Reduction of inoculum
Formation of Ag–Ab complexes	FcγR cross-linking Complement activation Immunization	Release of proinflammatory mediators such as cytokines and platelet activation factor	Removal of antigens with immunomodulatory effects Release of anti-inflammatory cytokines such as IL-10 Inhibition of proinflammatory cytokines such as IL-12
FcγR activation	Cellular signal transduction following interaction with activating and inhibitory FcγR	Release of proinflammatory mediators such as cytokines, prostaglandins, and platelet activation factor Phagocytosis Enhanced antigen presentation Oxidative burst Expression of costimulatory molecules Reduced inoculum	Release of anti-inflammatory cytokines such as IL-10 Inhibition of proinflammatory cytokines such as IL-12

The mechanism(s) responsible for this phenomenon are not fully understood. mAb-directed alterations in the Ab response, which depended on the amount, isotype, and specificity of the P1-reactive mAb (Oli *et al.*, 2004), could reflect alterations in P1 processing and presentation since mAb binding to P1 induced changes in proteolytic cleavage of P1 (Rhodin *et al.*, 2004). Such a mechanism predicts that mAb–Ag complexes could broaden the response to the Ag to include determinants that induce a more heterogenous array of Abs (Nie *et al.*, 1997), perhaps reactive with a larger number of determinants and or with more favorable biological activity. In this regard, complexes of induced Ab with residual or newly introduced Ag could drive the Ab response toward cryptic or determinants that are underrepresented or poorly antigenic on the native Ag. The possibility that the response to some vaccines may be enhanced by this mechanism is suggested by evidence that passive Ab therapy can drive somatic mutation and affinity maturation of Abs to its relevant Ag (Song *et al.*, 1999). In summary, Ab-mediated immunomodulation is a multifacted function that can depend on Fc dependent or independent, T cell dependent or independent mechanism (Brady, 2005), or as yet unknown processes that may converge in their ability to alter the Ag determinant and/or Ag signaling on which the immune response depends.

There is overwhelming evidence from many systems that Ab and CMI cooperate and are interdependent. For both *Francisella tularensis* (Rhinehart-Jones *et al.*, 1994) and *C. neoformans* (Yuan *et al.*, 1997), the efficacy of passive Ab is dependent on both IFN-γ and T cells. For *Salmonella typhimurium*, the efficacy of passive Ab correlates with the inherent resistance of the mouse strain, suggesting a dependence on cellular and/or innate immune mechanisms for Ab action (Eisenstein *et al.*, 1984b). Similarly, the efficacy of passive Ab against Friend leukemia virus is dependent on the major histocompatibility complex (MHC) type of the host because of a requirement for both CD4 and CD8 T cells (Hasenkrug and Chesebro, 1997; Hasenkrug *et al.*, 1995), but sterilizing immunity requires the presence of neutralizing Ab (Messer *et al.*, 2004). For LCMV, sterilizing immunity required the action of both $CD8^+$ T cells and neutralizing Ab (Baldridge *et al.*, 1997). Synergy between AMI and CMI has also been described in the resolution of lymphoma (Vasovic *et al.*, 1997). Interdependency, cooperation, and the ability of Ab to affect the development of cellular responses suggest that for some systems the relative contribution of AMI and CMI is not easily separable. In fact, one could argue that attempts to separate these components through reductionistic experimental approaches may fail to yield an accurate and comprehensive view of the depth of host defense mechanisms. If this is the case new integrative approaches, perhaps including mathematical modeling, may be needed to achieve a better and more predictive understanding of AMI.

8. Dose-Response Conundrum

Early investigators noted that the efficacy of passive Ab therapy did not obey the law of multiple proportions (Goodner and Horsfall, 1935). Classic studies of passive Ab protection against *S. pneumoniae* revealed that the outcome of a passive Ab protection experiment was critically dependent on the amount of Ab administered (Felton, 1928; Goodner and Horsfall, 1935). The amount of Ab below which no protection occurred for a given inoculum was known as the "limiting titer zone," a phenomenon that could be understood in the context of a requirement for a certain amount of Ab in mediating protection. Furthermore, it was known that no amount of Ab would protect against massive inocula, and this inoculum was known as the German word "*Schwellenwert*" that translates to "threshold." Presumably the *Schwellenwert*-infective dose was so overwhelming that AMI was ineffective. Perhaps the most perplexing aspect of the mouse protection test for *S. pneumoniae* was a "prozone" phenomenon whereby the admistration of large amounts of Ab was accompanied by diminished or abolished protection. For *S. pneumoniae*, the prozone was shown to be caused by a reduction in phagocytosis at very high-Ab concentrations resulting in unchecked bacterial replication (Goodner and Horsfall, 1936). "Prozone-like" phenomena have been demonstrated in other systems, including Ab effects against viruses, bacteria, parasites, fungi, and even cancer cells *in vitro* and *in vivo* (Asano *et al.*, 1982; Flavell *et al.*, 1995; Kozel *et al.*, 2004; Lieberman *et al.*, 1988; Lowell *et al.*, 1980; Parker *et al.*, 1995; Peeling *et al.*, 1984). Prozone-like effects were reproduced using mAbs in two models of murine *C. neoformans* infection (Taborda and Casadevall, 2001; Taborda *et al.*, 2003). For *C. neoformans*, at least three mechanisms have been demonstrated by which high Ab concentrations produce prozone-like effects. First, a high concentration of Ab on the fungal capsule can interfere with nitrogen-derived oxidants that are used by phagocytic cells for microbicidal activity. Second, the cytokine response at high- and low-Ab doses is markedly different, and this effect was shown to be isotype related using a family of variable-gene identical mAbs that differed in constant region. Third, the interaction of complement with *C. neoformans* cells differs at high- and low-Ab concentrations such that the Ab amount can affect the likelihood of phagocytosis by the complement or Fc receptor.

The dependence of Ab efficacy on concentration, and the fact that high concentrations of Ab can render an Ab that was protective nonprotective, suggests the need for caution in drawing negative conclusions about the relative efficacy of AMI, unless a careful dose-response study over a range of Ab amounts is undertaken. The fact that Ab efficacy depends on both the Ig concentration and microbial innoculum (and possibly burden) suggests that

AMI is most effective along a relatively narrow range of Ab concentrations, especially in passive Ab experiments (Casadevall, 2004). Furthermore, since the amount of Ig produced during an immune response changes with time as a function of the rate of Ab production and consumption, and the microbial burden changes with time as a function of microbial and host characteristics that govern replication, infection, and the immune response, it is conceivable that Ab efficacy changes with time as a function of the Ig to microbe ratio. Hence, rather than being a static or stable characteristic, the ability of an Ab to mediate protection is likely to be dynamic, changing as a function of time, the host response, available host receptors and inflammatory mediators, and the state of the microbe in the host.

9. Ab-Mediated Protection Against Intracellular Pathogens

By the late twentieth century, the struggle between the cellularists and humoralists that began with the Ehrlich and Metchnikov debate on the relative importance of CMI and AMI nearly a century earlier (Silverstein, 1979) had settled into a sort of détente whereby each arm of the immune response was assigned a specific role in host defense against certain types of microbes. In this dichotomous view of immune function, AMI was considered to have a key role in protection against extracellular organisms, toxins, and certain types of viruses, while CMI protected against intracellular pathogens. A central problem in this division of labor was the common conclusion that negative data in Ab protection studies implied that Ab had no role in host protection against the relevant microbe (Casadevall, 1998, 2003, 2004). In the past decade, the results of studies with mAbs to various intracellular pathogens have challenged this assumption and established new functions for AMI (Casadevall, 1998, 2003, 2004; Casadevall and Pirofski, 2004). We will consider developments in AMI for several intracellular pathogens. Our goal is to highlight mechanisms by which AMI can protect without being exhaustive. We recognize that in selecting certain microbes for detailed discussion we regretfully will not cover seminal work in certain fields. For those microbes that are not covered in depth, such as *Salmonella* spp., *L. pneumophila*, *S. flexneri*, and others, we provide references in Table 2.

9.1. *Cryptococcus neoformans*

Like many other intracellular pathogens, such as *M. tuberculosis* and *L. monocytogenes*, it was not possible to assign an important role for AMI against *C. neoformans* by either passive administration of immune sera or demonstrating reduced susceptibility in the presence of *C. neoformans*-reactive serum Ab

(reviewed in Casadevall, 1995). Hence, by the late 1980s the prevailing view was that AMI had no role in protection and that host defense was the exclusive domain of CMI. This view was supported by the lack of association of cryptococcosis with B cell defects and the high prevalence of AIDS-related cryptococcosis in patients with CD4 counts <200 cells/cm^3. However, when mAbs were used in passive immunization studies some Abs were found to be protective (Dromer *et al.*, 1987; Fleuridor *et al.*, 1998; Mukherjee *et al.*, 1992; Sanford *et al.*, 1990). Furthermore, studies with individual mAbs revealed that there were protective, nonprotective, and even some disease-enhancing Abs (Maitta *et al.*, 2004; Mukherjee *et al.*, 1995). Ab-mediated protection against *C. neoformans* was shown to be dependent on such Ig-related variables as Ab amount (Dromer *et al.*, 1987), isotype (Yuan *et al.*, 1995, 1998), specificity of Ig (Mukherjee *et al.*, 1995). On the other hand, Ab-mediated protection was also dependent on host factors such as T cells (Yuan *et al.*, 1997), B cells (Rivera *et al.*, 2005), the presence of inducible nitric oxide (Rivera *et al.*, 2002), and both Th1- and Th2-associated cytokines (Beenhouwer *et al.*, 2001). IgM, but not IgG, required complement (Fleuridor *et al.*, 1998; Shapiro *et al.*, 2002). Hence, the outcome of Ab protection studies was determined by the interaction between Ab characteristics and immune parameters of the host such that certain Abs were protective in certain host immune milieus but not others and vice versa.

At present, our understanding of the factors that govern cryptococcal pathogenesis remains insufficient to consistently predict which Ab characteristics are required for protection in a given host immune or inflammatory milieu. However, it is reasonable to predict that Abs that require CD4$^+$ T cells to function might not be effective in HIV-infected individuals and that Fc receptor polymorphisms could affect the efficacy of Abs that bind the relevant receptor. In this regard, individuals who are homozygous for a low-affinity receptor for (human) IgG2 are more susceptible to menigococcal sepsis (Domingo *et al.*, 2004; van Sorge *et al.*, 2003). Further, available data suggest that Abs that mediate protection in wild-type mice fail to do so in mice with dysregulated cytokine responses such as NO-, cytokine-, and B cell-deficient mice (Beenhouwer *et al.*, 2001; Feldmesser *et al.*, 2002; Rivera *et al.*, 2002, 2005). Hence, the interplay between the host immune response and the way in which a given Ab affects the inflammatory response can govern whether an Ab will reduce host damage sufficiently to be protective.

9.2. *Mycobacterium tuberculosis*

Numerous studies over the past century found evidence for and against a role for AMI against *M. tuberculosis* (Glatman-Freedman, 2003; Glatman-Freedman

and Casadevall, 1998), yet by the 1990s the prevailing view was that AMI had little or no role in host defense. Consistent with this notion, B cell-deficient mice did not manifest great susceptibility to *M. tuberculosis* or *M. avium* infection (Bosio *et al.*, 2000; Johnson *et al.*, 1997; Sangari *et al.*, 2001; Vordermeier *et al.*, 1996), with the caveat that negative studies in this type of system cannot be used to exclude a role for AMI (Casadevall, 2004). However, in 1998 an mAb to the arabinomannan component of the mycobacterial surface was shown to mediate protection when coadministered with mycobacteria by the intratracheal route (Teitelbaum *et al.*, 1998). Subsequently, four independent groups have confirmed that different mAbs can mediate protection against mycobacteria in mouse models of infection (Chambers *et al.*, 2004; Hamasur *et al.*, 2004; Pethe *et al.*, 2001; Williams *et al.*, 2004). Protective mAbs to mycobacteria include those recognizing polysaccharide and protein Ags, indicating that different types of Ags have the potential to elicit useful AMI. Furthermore, one report showed that an F(ab) derived from an mAb to arabinomannan could mediate protection, implying that for certain Abs protection could be Fc independent (Hamasur *et al.*, 2004). The mechanism by which an F(ab) can mediate protection is uncertain. However, there are precedents for F(ab)-mediated protection against other bacterial and fungal pathogens (Matthews *et al.*, 2003; Ramisse *et al.*, 1996) through mechanisms that may include direct antimicrobial effects or immunization-type phenomena (Brady, 2005). The ability of AMI to protect against *M. tuberculosis* is further supported by the demonstration that polysaccharide-protein conjugate vaccines constructed with oligosaccharides from lipoarabinomannan elicited immune responses were protective in mice and comparable to BCG (Hamasur *et al.*, 2003). In another study, mice immunized with arabinomannan conjugated to recombinant *Pseudomonas aeruginosa* exoprotein A had a lower lung bacterial burden at day 7 of infection (Glatman-Freedman *et al.*, 2004).

Despite strong evidence that certain Ab responses can protect against mycobacteria, the mechanism of Ab action has not been fully elucidated. Mycobacterial polysaccharides are immunomodulators. Hence, the ability of specific Ab to promote clearance could confer an immunological benefit (Glatman-Freedman *et al.*, 2000; Schwebach *et al.*, 2001). Another potential mechanism includes modification of the outcome of intracellular infection by specific Ab, since phagocytosis in the presence of specific Ab was reported to promote the fusion of lysosomes with mycobacterial-containing phagosomes (Armstrong and Hart, 1975). Ab-mediated internalization of *M. tuberculosis* was shown to be associated with high Ca^{2+} concentrations that promoted phagosomal maturation and intracellular killing of mycobacteria (Malik *et al.*, 2000). This effect was different than complement-mediated phagocytosis and suggested that engagement of certain FcR could reverse mycobacterial

inhibition of Ca^{2+} fluxes that are associated with intracellular survival (Malik *et al.*, 2000).

Ab-mediated effects on intracellular survival and/or clearance of mycobacterial products can enhance the immune response, suggesting that Ab-mediated protection translates into reduced host damage. The possibility that Ab-mediated protection is associated with a reduction in the inflammatory response comes from the observations that mice given passive IgG3 had differences in the histology of lung inflammation and that B cell- and IgA-deficient mice (Rodriguez *et al.*, 2005) infected with *M. tuberculosis* manifested different immune responses.

The ability of certain mAbs to mediate protection against *M. tuberculosis* is in contrast to the historical difficulty in consistently demonstrating protection in passive Ab studies or in associating Ab responses with immunity to tuberculosis (Glatman-Freedman, 2003; Glatman-Freedman and Casadevall, 1998). However, the finding that some mAbs are protective while others are non-protective (Teitelbaum *et al.*, 1998) suggests that like the situation for *C. neoformans* the problem in demonstrating the efficacy of AMI against *M. tuberculosis* could reflect heterogeneity in and the complex nature of the Ab response. In fact, a serological study of Abs to arabinomannan in human sera revealed quantitative and qualitative differences in individual responses (Glatman-Freedman *et al.*, 2004). It is likely that differences in mechanisms of Ab action will be discovered for Abs to *M. tuberculosis*, since the heterogenous serum response is associated with resistance to disease in most individuals who experience an infection. However, understanding of the role of AMI in human *M. tuberculosis* infection must await the use of more sophisticated serological tools that can measure quantitative and qualitative aspects of the Ab response and establish correlations between serological responses and clinical endpoints ranging from latency to disease.

9.3. *Ehrlichia chaffeensis*

Several studies have conclusively established a role for AMI in host protection against *E. chaffeensis*, an obligate intracellular bacterium that infects monocytes and macrophages (Li and Winslow, 2003; Li *et al.*, 2002; Winslow *et al.*, 2000). *E. chaffeensis* infection is cleared in C57Bl/6 mice rapidly but produces lethal infection in severe combined immunodeficiency (SCID) mice. Passive administration of immune serum led to transient clearance of infection in SCID mice, implying the ability of specific Ab to control and eradicate this organism without T cell help (Winslow *et al.*, 2000). Most striking was the ability of immune serum to control established infection, although this effect was transient and required repeated administration for maintenance (Winslow

et al., 2000). Subsequent studies established that Ab-mediated protection could be conferred by passive administration of mAbs to the *E. chaffeensis* outer membrane protein 1-g (OMP-1g) and that Abs of this specificity were present in immune sera from both humans and mice (Li *et al.*, 2001). The comparison of mAb-mediated protection revealed isotype-related differences in efficacy with IgG2a > IgG3 = IgG2b ≫ IgM in a set of variable region matched Abs that recognized a linear epitope in the first hypervariable domain of OMP-1g (Li *et al.*, 2001, 2002). Ab efficacy was also found to be directly associated with half-life and picomolar affinity (Li *et al.*, 2002). The consistency of these observations became apparent when free *E. chaffeensis* was demonstrated in the serum of infected mice, implying the existence of an extracellular phase during which this obligate intracellular bacterium spread from cell to cell and was susceptible to AMI (Li and Winslow, 2003). Hence, the emerging story for AMI to *E. chaffeensis* indicates that a different mechanism than that described for other intracellular pathogens, which relies on the bactericidal capacity of Ab in serum, is responsible for Ab efficacy. However, there is also evidence that specific Ab to *E. chaffeensis* can modify the cytokine expression of host cells, suggesting that, like that to *C. neoformans*, Ab-mediated protection may be due to changes in the inflammatory response (Lee and Rikihisa, 1997).

Passive Ab administration is also protective against another *Erlichia* species *E. risticci*, an obligate intracellular bacterial pathogen of horses (Kaylor *et al.*, 1991). For this microbe, the F(ab) of horse immune serum blocked bacterial entry, while intact IgG allowed internalization of the host cell via the Fc receptor, which interfered with intracellular growth of the bacterium (Messick and Rikihisa, 1994).

9.4. *Listeria monocytogenes*

Immunological studies of host defense against the facultative intracellular gram-positive bacterium *L. monocytogenes* helped to formulate the paradigm whereby protection against intracellular bacterial microbes was the exclusive domain of CMI (Mackaness, 1971, 1977). For *L. monocytogenes*, passive Ab transfer experiments using immune serum did not provide protection (Miki and Mackaness, 1964). Comparison of the outcome of infection in B cell-deficient and normal mice suggested a role for B cells in the establishment of CMI that was independent of Ab production (Matsuzaki *et al.*, 1999). However, passive administration of an mAb to listeriolysin O (LLO) to mice before challenge with *L. monocytogenes* mediated protection by prolonging survival and reducing the tissue bacterial burden (Edelson *et al.*, 1999). One peculiar aspect of this phenomenon was the requirement for relatively high

Ab doses to achieve protection. The mechanism of Ab-mediated protection involved neutralization of LLO inside macrophages preventing passage of the bacteria from the phagosome to the cytoplasm (Edelson and Unanue, 2001). Hence, the requirement for large Ab doses was explained by the need to achieve high enough serum concentrations to deliver sufficient immunoglobulin to mediate toxin neutralization. Consistent with this mechanism, Ab-mediated protection was not dependent on FcγR (Edelson and Unanue, 2001). Abs with LLO-neutralizing activity were not found in the serum of infected animals, implying that this determinant was not antigenic in the course of experimental infection. Whereas this example of Ab-mediated protection could be explained by the classical mechanism of toxin neutralization, it extends this mechanism to phagosomal spaces, underscoring that AMI is not limited to the extracellular space.

9.5. *Histoplasma capsulatum*

This fungus is a facultative intracellular pathogen that is almost always found inside macrophages in tissue. Numerous studies have failed to demonstrate a role for AMI against *H. capsulatum* in mice passively immunized with immune sera (Tewari *et al.*, 1977) or B cell deficiency (Allendoerfer *et al.*, 1999). In contrast, there is overwhelming evidence that CMI is critical for host defense (Deepe and Seder, 1998). However, when the potential role of AMI was investigated by generating mAbs to *H. capsulatum* surface Ags, an mAb was identified that mediated protection when administered prior to experimental infection in mice (Nosanchuk *et al.*, 2003). The Ag recognized by this mAb was a histone-like protein that is expressed on the surface of fungal cells. Although the mechanism of Ab action was not fully clarified, there was evidence that it was opsonic *in vitro* and that Ab-treated mice had altered inflammatory responses, as shown by changes in tissue histology and cytokine expression (Nosanchuk *et al.*, 2003). Passive Ab was most effective when given with small amounts of amphotericin B, an antifungal agent that is a potent immunomodulator by virtue of its ability to stimulate Toll-like receptors (Nosanchuk *et al.*, 2003). This observation is consistent with the view that Ab-mediated protection in this system was a result of alterations in the inflammatory response (Nosanchuk *et al.*, 2003).

9.6. *Toxoplasma gondii*

Toxoplasma gondii is an intracellular pathogen that is able to infect all mammalian cells. After the parasite gains entrance to the cell, it forms a parasitophorous vacuole that effectively shields it from host cellular antimicrobial mechanisms.

Numerous studies have established the potential efficacy of AMI in protection against *T. gondii*. For this microbe, the evidence that AMI contributes to host defense includes studies showing greater susceptibility in hosts with impaired AMI, demonstration of protection in passive transfer studies, and association of vaccine-mediated protection with AMI (Pavia *et al.*, 1992). B cell-deficient mice (μMT) were significantly more susceptible to toxoplasmosis than wild-type mice and could be protected by the administration of polyclonal rabbit immune sera (Kang *et al.*, 2000). CD4-deficient mice manifest greater susceptibility to *T. gondii* that was ameliorated by the transfer of immune sera (Johnson and Sayles, 2002).

Several studies suggest possible mechanisms for Ab-mediated protection and a high likelihood that there are multiple Ags that can elicit protective and nonprotective Abs to *T. gondii*. Secretory IgA reactive with a 46-kD Ag was shown to inhibit the enterozyte infection *in vitro* (Mack and McLeod, 1992). However, not all specific IgG is protective, since other studies have shown no reduction in the ability of *T. gondii* to replicate in macrophages when opsonized by IgG (Fadul *et al.*, 1995). An mAb to a 97-kD Ag inhibited intracellular replication of *T. gondii* in macrophages by a complement-independent mechanism that did not involve interference with internalization or attachment (Mineo *et al.*, 1994). However, complement may be important for the action of certain *T. gondii*-specific Abs. mAbs to the dense granular proteins of *T. gondii* mediate protection when tachyozites were incubated with Ab and complement prior to murine infection while Ab alone had no effect (Cha *et al.*, 2001). Other protective mAbs recognize different Ag of 35 and 14 kD (Johnson *et al.*, 1983).

9.7. *Chlamydia* spp.

Chlamydia trachomatis in an intracellular pathogen is the leading cause of sexually transmitted disease. For this organism, there is overwhelming evidence that both CMI and AMI contribute to host defense. The appearance of serum Abs correlates with clearance of experimental *C. trachomatis* infection in rabbits (Rank *et al.*, 1979) and serum Ab is a marker of immunity (Murray *et al.*, 1973; Rank and Barron, 1983). The presence of IgA in human vaginal secretions demonstrates a striking inverse correlation with the likelihood of cervical recovery of the organism in women (Brunham *et al.*, 1983). Passive transfer of immune serum protected guinea pigs against experimental genital infection (Rank and Batteiger, 1989). Passive administration of mAbs to the *C. trachomatis* outer membrane protein mediated protection against lethal infection in mice and neutralized chlamydial infectivity in a monkey model of

opthalmitis (Zhang *et al.*, 1987). In contrast, individual mAbs had variable efficacy in passive transfer studies (Cotter *et al.*, 1995), perhaps suggesting the need for multiple Ab specificities and isotypes to fully protect against *C. trachomatis* in various tissue compartments. However, studies with B cell-deficient mice have shown that Ab is not required for resolution of infection or resistance to reinfection (Ramsey *et al.*, 1988; Williams *et al.*, 1987). Hence, AMI contributes to host defense against *C. trachomatis* in the context of other antichlamydial immune mechanisms that cooperate and work in parallel.

10. Protective Efficacy of an Ab Molecule

Given that the efficacy of an Ab depends on its specificity, isotype, affinity, and the immune status and genetic background of the host, one cannot classify an Ab as protective, nonprotective, or disease enhancing solely on the basis of Ig structure. In fact, for each microbe Ab-mediated protection might be thought of as a complex function of: (1) Ab variables such as isotype, specificity, and amount; (2) host variables such as genetic background, immunization status, and immune competence; and (3) microbial variables such as virulence factors, inoculum, and pathogenic strategy. Furthermore, it is likely that this function will be different for each pathogenic microbe. For example, IgG3 to capsular polysaccharide is protective against *M. tuberculosis* in BALB/c and C57Bl/6 mice (Teitelbaum *et al.*, 1998) but not against *C. neoformans* (Yuan *et al.*, 1997). However, the same IgG3 that was not protective in C57Bl/6 or 129/Sv mice against *C. neoformans* was protective against experimental cryptococcosis in C57Bl/6 × 129/Sv mice (Rivera and Casadevall, 2005). In a polyclonal response, the efficacy of AMI can be expected to be a function of the combined effects of individual Ab molecules, each with its own protective function based on the characteristics listed earlier. Whether the net effect of each component on protective function is additive or multiplicative is unknown. Considering that the immune response to pathogenic microbes includes Abs to many Ags differing in the predominant isotype and amount, one can easily envision unfathomable complexity that becomes even more daunting if one considers host genetic variation in an outbred species. Clearly, defining protective efficacy of an Ab molecule in a predictive fashion is currently beyond the state of immunological science and may not be possible with current reductionistic approaches to scientific problems. Nevertheless, we remain optimistic that as the variables that impact Ab-protective efficacy are identified it may be possible to define algorithms that provide predictive information.

11. Some Emerging Concepts

1. Abs are both proinflammatory and anti-inflammatory and mediate some of their effects by modulating both innate and adaptive cellular responses.

2. Protective Abs can probably be made against many if not all pathogens for which current methods cannot demonstrate a clear role for AMI in host defense. The most efficient way to achieve this is to generate mAbs to the microbe in question with the caveat that immunological knowledge is insufficient to predict the Ab characteristics that will be protective. Therefore, in most instances, determining the efficacy of Ab remains an empiric rather than predictive discipline.

3. The inability to demonstrate a role for AMI against a particular pathogen using the classical methods of passive Ab administration and correlation of Ab titer with immunity does not rule out a role for AMI in protection or pathogenesis.

4. The efficacy of an Ab cannot be defined solely from the molecular characteristics of the Ig molecule or independently of the host in which it is tested.

5. Ab-mediated protection can be associated with enhanced or reduced inflammatory responses depending on the microbe in question.

6. Given the strong dependence of Ab function on the quantity and the nature of the host immune response, it is likely that for some microbes the function of AMI differs early and late in infection or in the context of reinfection.

7. Protective Abs can be used as probes in reverse vaccinology approaches to identify epitopes and design vaccines that induce Abs that mediate protection. Examples of this approach are provided by *C. neoformans* (Devi, 1996), *C. albicans* (Han *et al.*, 1999), and *M. tuberculosis* (Hamasur *et al.*, 2003) in which the identification of protective mAbs led to the identification of an Ag that elicited a protective Ab response that was then used to make an effective conjugate vaccine.

8. The relative contributions of AMI and CMI to host defense and microbial clearance may be inseparable for certain, particularly, intracellular pathogens, suggesting the need for new models and systems to identify and characterize mechanisms of Ab action.

Acknowledgments

This work was supported by grants from the National Institutes of Health AI 033142, AI 033774, HL 059842 (AC) and AI 035370, AI 045459, AI 044374 (CP).

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Accessibility Control of V(D)J Recombination

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Abstract

Mammals contend with a universe of evolving pathogens by generating an enormous diversity of antigen receptors during lymphocyte development. Precursor B and T cells assemble functional immunoglobulin (Ig) and T cell receptor (TCR) genes via recombination of numerous variable (V), diversity (D), and joining (J) gene segments. Although this combinatorial process generates significant diversity, genetic reorganization is inherently dangerous. Thus, V(D)J recombination must be tightly regulated to ensure proper lymphocyte development and avoid chromosomal translocations that cause lymphoid tumors. Each genomic rearrangement is mediated by a common V(D)J recombinase that recognizes sequences flanking all antigen receptor gene segments. The specificity of V(D)J recombination is due, in large part, to changes in the accessibility of chromatin at target gene segments, which either permits or restricts access to recombinase. The chromatin configuration of antigen receptor loci is governed by the concerted action of enhancers and promoters, which function as accessibility control elements (ACEs). In general, ACEs act as conduits for transcription factors, which in turn recruit enzymes that covalently modify or remodel nucleosomes. These ACE-mediated alterations are critical

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ADVANCES IN IMMUNOLOGY, VOL. 91 0065-2776/06 $35.00

DOI: 10.1016/S0065-2776(06)91002-5

for activation of gene segment transcription and for opening chromatin associated with recombinase target sequences. In this chapter, we describe advances in understanding the mechanisms that control V(D)J recombination at the level of chromatin accessibility. The discussion will focus on cis-acting regulation by ACEs, the nuclear factors that control ACE function, and the epigenetic modifications that establish recombinase accessibility.

1. Introduction

As mammals, our major defense against an ever-changing constellation of pathogens is provided by B and T lymphocytes, which bear clonally distributed antigen receptors. An enormous diversity of B and T cell receptors (BCR and TCR) is generated during lymphocyte development in an antigen-independent manner. The large repertoire of lymphocytes, each bearing a signature antigen-binding specificity, is poised to recognize pathogens and signal for their elimination by host effector functions.

The ability of lymphocytes to generate such an enormous diversity of antigen receptors ($>10^8$ in healthy individuals), coupled with known restrictions on our genomic complexity, confounded explanation for decades. In the mid-1970s, Susumu Tonegawa and colleagues discovered that unlike other known genes, those encoding for immunoglobulin (Ig) proteins were inherited in a nonfunctional form. The variable region exons of Ig and TCR genes must be assembled from arrays of variable (V), diversity (D), and joining (J) gene segments via somatic recombination (Brack *et al.*, 1978; Weigert *et al.*, 1978). This genetic reorganization occurs only in precursor, receptor-negative lymphocytes and is an integral component of their program for ordered development. The assembly of all antigen receptor genes is mediated by a single V(D)J recombinase consisting of the RAG-1 and RAG-2 proteins, which serve as its key enzymatic components (Oettinger *et al.*, 1990; Schatz *et al.*, 1989). The RAG complex targets conserved recombination signal sequences (RSSs) flanking all Ig and TCR gene segments (Sakano *et al.*, 1979).

Although the generation of receptor diversity by V(D)J recombination is beneficial, it is also an inherently dangerous process. Defects in V(D)J recombination can cause immunodeficiencies or chromosomal translocations that lead to lethal lymphoid malignancies (Bassing *et al.*, 2002; Kuppers and Dalla-Favera, 2001). With regards to the latter aberration, cryptic RSSs and unusual DNA structures can serve as RAG targets leading, in some cases, to the translocation of protooncogenes into highly expressed antigen receptor loci (Raghavan *et al.*, 2005). Thus, normal immune development requires the stringent regulation of recombinase targeting, which is controlled at several levels, including: (1) tissue specificity (e.g., precursor B cells rearrange only Ig,

not TCR loci), (2) locus specificity (e.g., TCRβ rearrangements occur prior to TCRα rearrangements), and (3) allelic exclusion (only one functional allele is produced for each Ig and TCR gene).

Early insights into the molecular mechanisms controlling antigen receptor gene assembly came from the discovery that unrearranged (germline) gene segments are transcribed coincident with their recombination (Van Ness *et al*., 1981; Yancopoulos and Alt, 1985). These observations led to the hypothesis that V(D)J recombination is regulated by changes in chromatin that permit or deny access of nuclear factors to gene segments. In nonlymphoid cells, Ig and TCR loci reside in closed chromatin, which is inaccessible to the transcription and recombinase machinery. However, at the appropriate stage of lymphocyte development, chromatin associated with specific clusters of gene segments open and become targets for transcription/recombination. The links between gene expression and recombination suggested that transcriptional control elements within antigen receptor loci might also serve to regulate chromatin accessibility at neighboring gene segments. Consistent with this model, targeted deletion of promoters or enhancers from antigen receptor loci severely impairs their recombination in cis (Dudley *et al*., 2005; Krangel, 2003; Oltz, 2001; Schlissel, 2003). Thus, the biologic action of V(D)J recombinase is tightly regulated by promoters/enhancers, which serve as accessibility control elements (ACEs) to guide antigen receptor gene assembly and lymphocyte development.

In this chapter, we focus on the genetic and epigenetic mechanisms that control V(D)J recombination at the level of chromatin accessibility to RAG proteins. In recent years, the field has witnessed tremendous strides in efforts to: (1) identify the relevant ACEs that direct V(D)J recombination, (2) understand how ACEs function independently or in concert to regulate recombinase accessibility, and (3) dissect the role of transcription, transcription factors (TFs), chromatin modifications, and nuclear localization in orchestrating the stepwise rearrangement of gene segment clusters. Together, these advances contribute to a deeper understanding of how precursor lymphocytes properly target dangerous recombination events while avoiding aberrations that produce autoimmunity, immunodeficiencies, and lymphoid tumors.

2. V(D)J Recombination: A Mechanistic Perspective

V(D)J recombination is mediated by RSSs that directly flank all Ig and TCR gene segments. Each RSS contains a conserved palindromic heptamer and an AT-rich nonamer, which are separated by a nonconserved spacer of 12 or 23 bp in length. Under physiologic conditions, recombination requires two gene segments flanked by a 12- and 23-bp RSS (Sakano et al., 1979). Experiments conducted with artificial substrates have demonstrated that: (1) V(D)J recombinase is restricted

to precursor lymphocytes (Lieber *et al.*, 1987), (2) all Ig and TCR genes are assembled by a single recombinase activity (Yancopoulos *et al.*, 1986), and (3) the tissue-specific components of V(D)J recombinase are encoded by a pair of linked genes, termed recombination-activating genes 1 and 2 (RAG-1 and -2) (Oettinger *et al.*, 1990; Schatz *et al.*, 1989). Early functional experiments with RAG expression vectors showed that RAG-1/2 is sufficient to confer recombinase activity to any cell type tested (Oettinger *et al.*, 1990; Oltz *et al.*, 1993). Accordingly, loss of RAG function by targeted deletions in mice or natural mutations in humans produce a severe combined immunodeficiency (SCID) due to an inability to initiate V(D)J recombination (Mombaerts *et al.*, 1992; Schwarz *et al.*, 1996; Shinkai *et al.*, 1992).

The advent of *in vitro* V(D)J recombination systems produced a bounty of data that support the following model for recombination by RAG proteins (Fig. 1) (Eastman *et al.*, 1996; McBlane *et al.*, 1995). First, the RAG-1/2 complex binds to an RSS, with initial contact between RAG-1 and the nonamer sequence (Swanson and Desiderio, 1998). Association of RAG-1 with RAG-2 enhances contact between recombinase and the heptamer (Swanson and Desiderio, 1999). The stoichiometry of active RAG complexes *in vivo* remains unclear, however, current evidence suggests that RAG first binds to a 12-bp RSS and introduces a single-strand nick precisely at heptamer/coding border (Curry *et al.*, 2005; Eastman *et al.*, 1996; van Gent *et al.*, 1996). The RAG-nick complex then searches for a 23-bp RSS, forming a synapse, and introduces a similar nick at the second RSS (Curry *et al.*, 2005; Jones and Gellert, 2002; Mundy *et al.*, 2002). The liberated hydroxyl groups then attack the opposing phosphate backbones at each RSS to generate a pair of blunt signal ends (SE) and sealed hairpins at the coding ends (CE) (Roth *et al.*, 1992). *In vitro* studies indicate the existence of a postcleavage complex, which contains the RAG proteins as well as the CEs and SEs (Agrawal and Schatz, 1997; Hiom and Gellert, 1998). This complex is transient in nature and dissolves rapidly to generate an SE complex (SEC) that retains bound RAG proteins and CEs as free DNA hairpins.

Formation of the SEC and CEs represents the endpoint of RAG-dependent events *in vitro*. Completion of V(D)J recombination requires resolution of DNA ends to rescue the chromosome and generate coding joins (CJs). Studies of V(D)J recombination in CHO mutant cells engineered to express RAG proteins revealed an important role for the ubiquitous double-strand break repair machinery in the resolution of both SEs and CEs (Pergola *et al.*, 1993; Taccioli *et al.*, 1993). Together with subsequent studies, the following model has emerged for the resolution of V(D)J breaks by the non-homologous end-joining (NHEJ) repair pathway (Dudley *et al.*, 2005). Free ends are first recognized by a heteromeric complex of KU proteins, which in turn recruit the catalytic component of DNA-dependent protein kinase (DNA-PKcs).

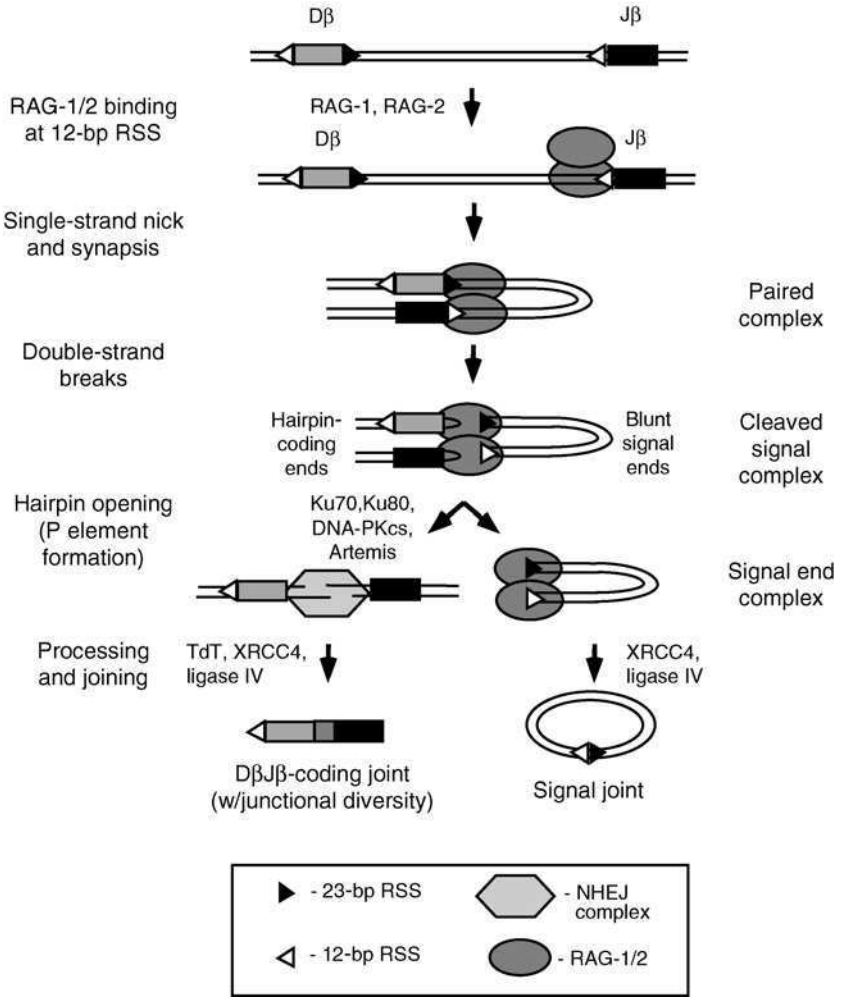

Figure 1 General V(D)J recombination mechanism. The mechanism is exemplified for a portion of the TCRβ locus and shows rearrangement of a single Dβ/Jβ pair. RSSs are represented by black and white triangles and coding segments are depicted as black or gray rectangles. In brief, the RAG-1/2 recombinase complex (gray ovals) forms a synapse with two compatible RSSs, introduces double-strand breaks at the RSS/coding border, and the breaks are resolved by the NHEJ machinery as imprecise CJs and precisely fused signal joins. Refer to text for a detailed description of the process.

Activated DNA-PKcs phosphorylates numerous targets that control cell cycle progression (e.g., p53) and subsequent DNA repair. These include the Artemis protein and the variant histone, H2AX, which is phosphorylated over a broad region surrounding the break (Chen *et al.*, 2000). Phosphorylation of Artemis activates its endonuclease activity, which is critical for opening hairpins at CEs (Ma *et al.*, 2002) and creating palindromic sequences (P elements) at many V(D)J junctions (Lafaille *et al.*, 1989). Moreover, endonuclease activity associated with Artemis generates further diversity at CJs via the random deletion of nucleotides from exposed ends (Ma *et al.*, 2002). The precursor lymphocyte-specific protein, terminal deoxynucleotidyl transferase (TdT), enhances junctional diversity through the random addition of nucleotides at CEs (Komori *et al.*, 1993).

Final resolution of both CEs and SEs is achieved following the recruitment of XRCC4, which binds to and activates DNA ligase IV (Grawunder *et al.*, 1997; Li *et al.*, 1995). Studies suggest that an additional repair factor may facilitate V(D)J recombination *in vivo* (Dai *et al.*, 2003). Notwithstanding, the end result of the repair process is a highly modified CJ, which enhances sequence diversity at the CDR3 region of Ig and TCR proteins. The exposed CJs are resolved rapidly by the NHEJ machinery, whereas SEs are resolved slowly and the resultant SJs are usually deleted from the genome as episomal circles (Hesslein and Schatz, 2001).

Mouse knockouts confirmed the *in vivo* relevance of these cell model studies on NHEJ repair. In addition to radiosensitivity, mice harboring null mutations of KU, DNA-PKcs, Artemis, XRCC4, or DNA ligase IV all exhibited a SCID phenotype due to defects in the formation of CJs or opening of hairpins (reviewed in Dudley *et al.*, 2005). Dual deletion of most NHEJ components and p53 produced mice with aggressive lymphocytic tumors exhibiting chromosomal translocations that are hallmarks of defective V(D)J recombination (Dudley *et al.*, 2005; Gao *et al.*, 2000). Recombinase activity and NHEJ are also coupled via changes in RAG protein stability during the cell cycle. Specifically, RAG-2 is phosphorylated, ubiquitinated, and rapidly degraded in dividing cells (Jiang *et al.*, 2005; Lin and Desiderio, 1993). This cell cycle-dependent control restricts recombinase activity to resting G_0/G_1 cells, in which the NHEJ mechanism of DNA repair predominates (Lee and Desiderio, 1999). The importance of this regulatory mechanism was confirmed in mice that express a phosphodefective mutant of RAG-2 in thymocytes. These mutant animals possessed high levels of TCR signal ends in cycling pre-T cells and exhibited defective TCR joins that were reminiscent of those from NHEJ-deficient mice (Jiang *et al.*, 2004). Together, these *in vivo* studies underscore the importance of proper targeting, regulation, and constraint of V(D)J recombination during the stepwise process of lymphocyte development.

3. Genomic Architecture of Ig and TCR Loci

The Ig and TCR components of antigen receptors are encoded by seven distinct genetic loci. The B cell antigen receptor is a tetrameric structure composed of two identical Ig heavy chains (IgH) covalently linked to their partner light chains (IgL, either Igκ or Igλ). Two distinct classes of T cells exist, which express either a TCRβ/TCRα or TCRγ/TCRδ heterodimers. In contrast to the split nature of gene segments that comprise variable exons, the constant regions of antigen receptor genes exhibit a normal exon/intron structure. Each of the exons encode for a single Ig-fold domain, a β-barrel structure that is commonly found in many surface receptors.

The mouse IgH locus spans a region on chromosome 12 of ~3 megabases (Mb) (Chevillard *et al*., 2002). The constant region coding exons, ordered Cμ, Cδ, Cγ3, Cγ1, Cγ2b, Cγ2a, Cε, and Cα are spread over a region of ~200 kb at the 3′ end of the locus (Fig. 2). Four JH gene segments are positioned in a small cluster located 7.5 kb upstream of Cμ-coding exons and the 13 DH segments are located in a linear array further upstream. Approximately 150 VH segments are dispersed over a 1 Mb region upstream from the DH cluster. In mouse, these VH segments are arranged in families that share a high level of sequence similarity. The D-proximal family, termed 7183, is preferentially used in IgH rearrangements by pro-B cells (Malynn *et al*., 1990). The most distal VH family (J558) is the largest and predominates the peripheral B cell repertoire (Chevillard *et al*., 2002). The DH gene segments are flanked by 12-bp RSSs on both sides, while the VH and JH segments each have 23-bp RSSs. In keeping with the 12/23 rule, this RSS composition precludes VH→JH joining and ensures the inclusion of a DH element in all IgH joins. However, studies have demonstrated that, on targeted deletion of all DH gene segments, direct VH→JH recombination can occur, albeit at low levels (Koralov *et al*., 2005).

The Igκ locus is composed of ~140 Vκ and 4 functional Jκ gene segments, which are spread over 3 Mb on mouse chromosome 6 (Kirschbaum *et al*., 1998; Thiebe *et al*., 1999). A single Cκ exon lies 2.5 kb downstream of the Jκ cluster. A subset of Vκ gene segments are in a reverse transcriptional orientation relative to the Jκ segments. As such, rearrangements involving these segments occur via large-scale inversion of DNA between the selected Vκ/Jκ segments rather than the usual deletion mechanism of joining (Gorman and Alt, 1998). In addition to the RSSs associated with Vκ and Jκ gene segments, consensus RSSs are positioned downstream of the Jκ cluster (Muller *et al*., 1990). These RSSs can recombine with Vκ gene segments to inactivate the targeted Igκ allele during the process of receptor editing (Section 4).

The mouse Igλ locus spans about 200 kb on chromosome 16 and harbors three distinct cassettes of Vλ/Jλ gene segments and Cλ exons (Gorman and

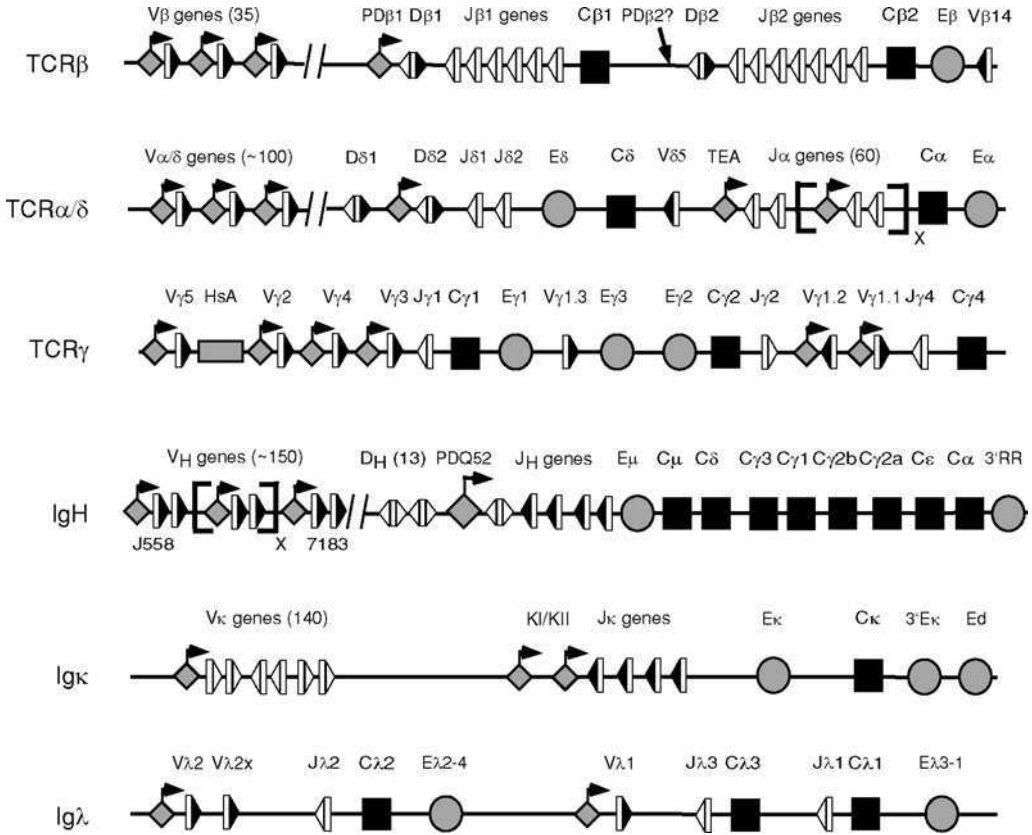

Figure 2 Schematic depiction of mouse Ig and TCR loci (not to scale). Gene segments are represented by rectangles and RSSs are depicted as triangles (23 bp, black and 12 bp, white). Transcriptional promoters and enhancers are shown as gray diamonds and circles, respectively, and constant regions as black squares. Estimated numbers of gene segments are displayed above the represented V, D, and J regions. For the IgH locus, the most proximal (7183) and distal V_H families (J558) are shown.

Alt, 1998). Only two of the three Vλ gene segments (Vλ1 and Vλ2) are used predominantly in developing B cells. In cells that fail to express functional Igκ genes, these Vλ segments rearrange preferentially to their most proximal Jλ-Cλ clusters (Vλ2 with Jλ2 and Vλ1 with Jλ1/Jλ3) (Reilly *et al*., 1984). As a result, the repertoire of mouse Igλ rearrangements is far more restricted than that observed for the Igκ locus.

The TCRβ locus spans ~1 Mb on mouse chromosome 6 (Glusman *et al*., 2001). The 5′ region of the locus is composed of 35 Vβ segments, 14 of which are nonfunctional pseudogenes. The 3′ region of the locus harbors two DβJβ clusters, each containing one Dβ and six functional Jβ segments. Coding exons for the TCRβ constant region reside downstream of each DβJβ cluster (Cβ1 or Cβ2). Finally, a single Vβ element, called Vβ14, lies downstream of Cβ2 and rearranges by an inversional mechanism. All Vβ gene segments are flanked on their 3′ sides by a 23-bp RSS, while the Jβ elements are bordered by 12-bp RSSs. The two Dβ elements are flanked by a 12- and 23-bp RSS on their 5′ and 3′ sides, respectively. In theory, this RSS arrangement should permit direct Vβ→Jβ recombination. However, these joins are rarely observed *in vivo* due to undefined constraints of the recombination process, termed "beyond 12/23 restriction" (Section 5) (Bassing *et al*., 2000; Jung *et al*., 2003; Tillman *et al*., 2003).

The gene segments encoding mouse TCRα and TCRδ are intermingled in a single locus spanning 1.5 Mb on chromosome 14 (Bosc and Lefranc, 2003; Glusman *et al*., 2001). In total, the locus contains over 100 V segments, some of which rearrange only with Jα gene segments, some with only DδJδ joins, and some contribute to both the TCRα and TCRδ repertoires (Krangel *et al*., 2004). A pair of Dδ and Jδ segments lies between the V cluster and the Cδ-coding region. Further downstream of Cδ lie 60 Jα gene segments followed by the Cα-coding region.

The TCRγ locus is distributed across a short region of DNA (~200 kb) on mouse chromosome 13 (Glusman *et al*., 2001). This locus consists of seven Vγ gene segments and one Vγ pseudogene interspersed among three functional Jγ-Cγ units and one nonfunctional Jγ-Cγ unit. All of the TCRγ gene segments are positioned in the same transcriptional orientation, with Vγ segments flanked by 23-bp RSSs and Jγ gene segments flanked by 12-bp RSSs.

4. Regulation of Antigen Receptor Gene Assembly During Lymphocyte Development

The generation of functional B and T lymphocytes requires the precise orchestration of antigen receptor gene assembly and a highly ordered program of cellular differentiation (Busslinger, 2004; Rothenberg and Taghon, 2005).

Both lineages derive from pluripotent stem cells in adult bone marrow, which differentiate into common lymphoid progenitor (CLP) cells. These progenitors lack lymphocyte surface markers, but can differentiate into both B and T cells in radiation chimeras. B lymphopoiesis occurs in the liver during fetal development but continues in the bone marrow of adults. In contrast, T cell progenitors migrate from the bone marrow and complete their development in the thymus. B and T cell precursors initially lack surface antigen receptors but, on their commitment, they rapidly initiate the program of V(D)J recombination at either Ig or TCR loci. This ordered process is an integral component of developmental pathways, with the protein products from each step guiding cellular differentiation and subsequent steps of gene assembly. The end result of this genetic program is the acquisition of Ig or TCR expression and a signature antigen-binding specificity on each lymphocyte clone. We now examine the processes of lymphocyte development more closely in the context of ordered antigen receptor gene assembly.

To initiate V(D)J recombination, precursor lymphocytes must first express the tissue-specific components of recombinase—the RAG genes. The RAG-1/2 genes are located ~15 kb apart on chromosome 2 in mouse and are under the transcriptional control of multiple *cis*-acting elements. These elements work in concert to repress RAG expression in nonlymphoid cells and activate expression in precursor B and T lymphocytes (Hsu *et al.*, 2003; Yu *et al.*, 1999). Studies have shown that RAG-1/2 expression initiates in CLPs and a significant portion of these cells target the IgH locus for DH→JH recombination (Borghesi *et al.*, 2004). This expression pattern likely explains the presence of DHJH joins in thymocytes (Born *et al.*, 1988). However, neither the ordered assembly nor the cell-type specificity of V(D)J recombination can be explained simply by RAG expression patterns because both genes are expressed at varying levels throughout all stages of precursor lymphocyte development.

Should a CLP fully commit to the B cell lineage, its subsequent development can be tracked using a combination of surface marker expression and the rearrangement status of Ig loci (Fig. 3A). The first developmental stage, termed a pro-B cell, is identified by expression of the lineage marker B220 and the CD43 surface protein. Pro-B cells can be categorized further into fractions A–C on the basis of BP1 and HSA expression (Li *et al.*, 1996). Fraction A/B cells first target the IgH locus for DH→JH recombination, which almost always occurs on both alleles. Fraction B cells then initiate VH→DHJH recombination, which appears to be a less efficient process and targets each allele sequentially (Hardy *et al.*, 1991). Because all recombination events are accompanied by the random addition and deletion of nucleotides, only one in three VHDHJH junctions will be inframe. Thus, two-third of pro-B cells fail to generate a functional join on the first IgH allele and proceed to

rearrange the second allele. Pro-B cell clones that fail to form a functional IgH gene on either allele die via apoptosis (Fang *et al*., 1996). In contrast, formation of a functional VHDHJH exon permits expression of IgH protein (IgM isotype) in the cytoplasmic compartment (Igμ protein). In turn, Igμ associates with two surrogate light chains (λ5 and VPRE-B) and the signaling molecules Igα and Igβ to generate the pre-B cell receptor (pre-BCR) (Hombach *et al*., 1990; Melchers, 2005). The pre-BCR discharges signals that inhibit further VH→DHJH recombination and enforce allelic exclusion, which is critical for maintaining B cell monospecificity (Loffert *et al*., 1996). Several lines of evidence support an active feedback inhibition for maintenance of allelic exclusion by the pre-BCR, including (1) repression of endogenous VH→DHJH recombination in mice that express a functional IgH transgene (Manz *et al*., 1988), and (2) allelic inclusion in mice lacking a functional pre-BCR (Loffert *et al*., 1996).

In addition to allelic exclusion, the pre-BCR triggers a proliferative burst to expand the numbers of pro-B cells expressing IgH protein, which can then couple with distinct IgL chains (Young *et al*., 1994). RAG expression is downregulated during this proliferative burst at both the transcriptional and posttranscriptional levels (Hsu *et al*., 2003; Lin and Desiderio, 1994). These cells also extinguish expression of λ5, VPRE-B, and CD43 before they differentiate into resting pre-B cells (fraction C) (Hardy and Hayakawa, 2001). The $B220^{+}$/$CD43^{-}$ pre-B cells activate recombination of their IgL loci, again in a stepwise manner. The vast majority of pre-B cells first target the Igκ locus for Vκ→Jκ recombination (Ehlich *et al*., 1993). However, if both Igκ alleles are assembled out of frame, the pre-B cell clone retargets recombinase activity to the Igλ locus. Functional rearrangement at either IgL locus permits expression of a complete BCR. The emerging B cell terminates RAG expression, migrates to the spleen where it undergoes further differentiation to become a mature IgM^{low}/IgD^{hi} B lymphocyte, and then screens for foreign antigens (Hardy and Hayakawa, 2001).

Because V(D)J recombination randomly generates receptor diversity, there is a significant chance that the first BCR expressed by an emerging B cell clone will be autoreactive. In emerging B cells, stimulation of an autoreactive BCR by self-antigen signals for the persistent expression of RAG-1/2, which continues to target IgL loci for recombination. This process, termed receptor editing, permits the emerging lymphocyte to test several IgL chains with its heavy chain to eliminate autoreactivity (Li *et al*., 2001; Nemazee and Hogquist, 2003). Receptor editing can be achieved via several pathways to generate new antigen specificity. For example, rearrangement of a 5′Vκ with a 3′Jκ gene segments deletes the existing VκJκ join to create a new variable region exon. Alternatively, the autoreactive clone may target recombination to an RSS

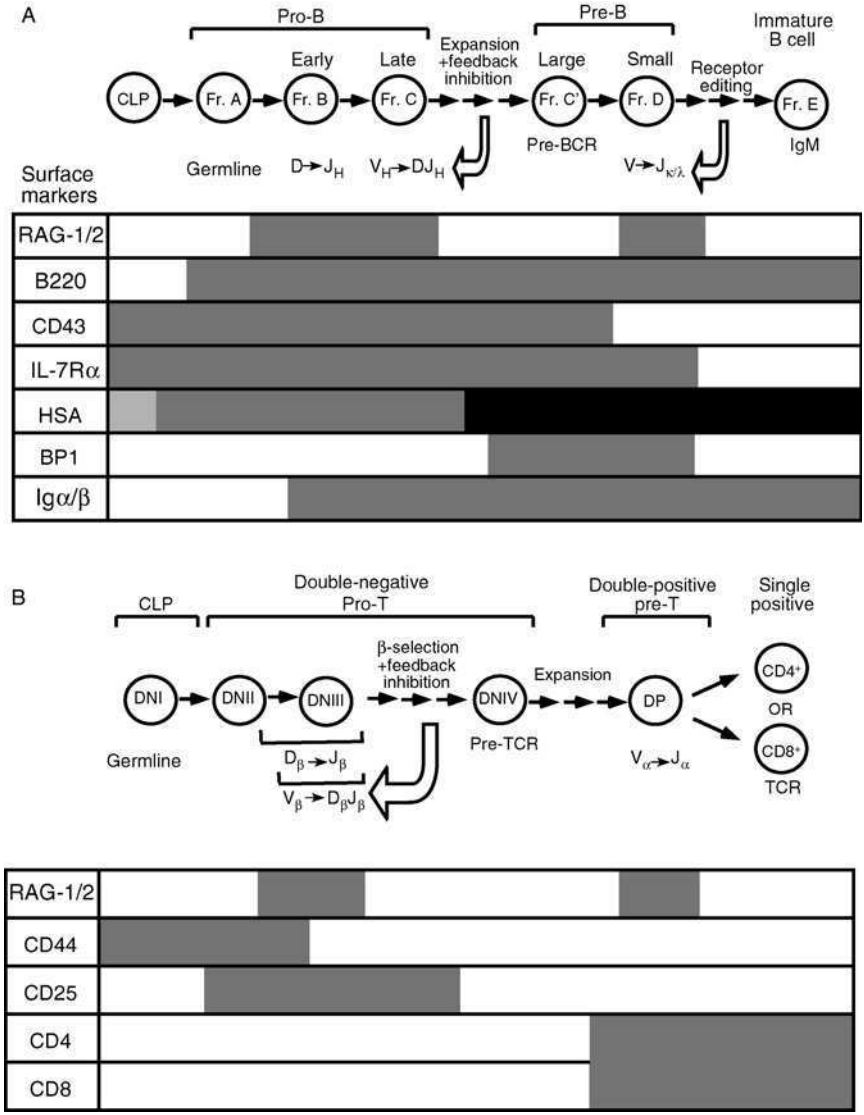

Figure 3 (A) Schematic representation of mouse B cell development. B lymphopoiesis in mouse bone marrow originates from a common lymphoid progenitor (CLP) and proceeds in a stepwise fashion through distinct subsets of precursor B cells (Fr. A to Fr. E as described in Hardy *et al.*, 1991). The stage-specific rearrangement of Ig genes and surface expression of the pre-BCR are indicated. The expression of characteristic cell surface markers is depicted and darker shading indicates a higher relative level of expression. (B) Diagram of mouse α/β T cell development. Following migration of progenitor cells to the thymus, development progresses through the

located downstream of Cκ (Fig. 2), inactivating the autoreactive allele and generating a new IgL specificity on either the second Igκ allele or an Igλ allele. However, if BCR engagement persists following receptor editing, the autoreactive clone will be eliminated by apoptosis (Goodnow *et al*., 2005).

The process of T cell development is analogous in many respects with that of B lymphopoiesis (Fig. 3B). The majority of precursors become α/β rather than γ/δ T cells, and lineage commitment appears to hinge on which set of genes first undergo productive rearrangements (Robey, 2005). On T lineage commitment, thymocytes lack expression of the CD4/CD8 coreceptors and are termed double negative (DN) pro-T cells. The DN population can be further categorized into the DNI–DNIV subsets based on CD44/CD25 expression (Rothenberg and Taghon, 2005). The DNII–DNIII subsets first target recombinase to the DβJβ clusters, followed by Vβ→DβJβ rearrangement. Assembly of a functional TCRβ gene leads to expression of a pre-TCR in DNIV cells, which consists of the TCRβ chain, the surrogate TCRα chain (pTα), and the CD3 coreceptor complex (von Boehmer, 2005). Expression of the pre-TCR inhibits further Vβ→DβJβ recombination but stimulates several other processes (collectively called β-selection), including (1) clonal expansion of $TCR\beta^+$ pro-T cells, (2) differentiation into $CD4^+/CD8^+$ double positive (DP) pre-T cell stage, and (3) activation of Vα→Jα recombination (Aifantis *et al*., 1997; Shinkai *et al*., 1993). T cell clones that express a functional TCRα gene undergo positive selection and differentiate into the CD4 helper or CD8 cytotoxic T cell lineage. Autoreactive clones are removed from the T cell repertoire by apoptosis during negative selection in the thymus. Similar to receptor editing at IgL loci, precursor T cells can undergo multiple rounds of Vα→Jα recombination until these cells express a TCR that progresses through both the positive- and negative-selection checkpoints (Hawwari *et al*., 2005; Huang *et al*., 2005).

5. Regulation of V(D)J Recombination: The Accessibility Hypothesis and Beyond

The stepwise, ordered assembly of antigen receptor genes requires targeting, then retargeting, of V(D)J recombinase to distinct regions within Ig and TCR loci at different stages of lymphocyte development. The numerous levels of regulation include: (1) tissue specificity, (2) ordered assembly within each locus

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CD4/CD8 double-negative (DNI-IV), double-positive, and single positive ($CD4^+$ or $CD8^+$) stages. The stage-specific rearrangement of TCR genes and surface expression of the pre-TCR are indicated. Pre-TCR expression triggers β-selection, a cascade of T cell differentiation and proliferation events, as well as feedback inhibition of further TCRβ gene rearrangement.

(D→J then V→DJ), (3) stage specificity (e.g., TCRβ in pro-T and TCRα in pre-T cells), and (4) allelic exclusion. The selectivity of these genomic rearrangements occurs despite the use of a common recombinase that is expressed at all stages of precursor B and T cell development (Yancopoulos *et al*., 1986). Moreover, the RSS substrates for V(D)J recombinase are virtually indistinguishable when comparing Ig and TCR loci.

A first clue to the mechanisms by which a common enzyme/substrate system differentially targets gene segments for recombination came from the discovery of "germline transcripts" by the Alt and Perry laboratories. Their studies revealed that transcription of germline gene segments is initiated in the cell types that target these segments for recombination (Van Ness *et al*., 1981; Yancopoulos and Alt, 1985). For example, unrearranged Vβ segments are transcribed in pro-T cells but not in pre-T or B lineage cells (Senoo and Shinkai, 1998). Since these initial observations, the general correlation between germline transcription and V(D)J recombination has been extended to all Ig and TCR gene segments and even artificial substrates (reviewed in Oltz, 2001; Sleckman *et al*., 1996). The link between transcription and recombination led to the hypothesis that each step in antigen receptor gene assembly is controlled by modulations in chromatin accessibility to the common recombinase complex. Specifically, recombinationally inert gene segments would be packaged into a chromatin configuration that is refractory to RAG binding and/or cleavage; whereas targeted gene segments would be packaged into an "open" chromatin configuration that is accessible to both RAG and RNA polymerase complexes. Further support for the accessibility hypothesis derived from studies showing that fibroblasts engineered to express RAG-1/2 could target actively expressed chromosomal substrates (open) for recombination while endogenous Ig and TCR loci remained both transcriptionally and recombinationally silent (Schatz *et al*., 1992). Subsequently, the accessibility hypothesis has been validated by numerous experimental approaches that directly or indirectly measure levels of chromatin accessibility at gene segments (see later).

Although changes in chromatin accessibility can account for the majority of regulatory processes governing V(D)J recombination, the RAG complex and its substrate RSSs can also influence rearrangement efficiencies. Mouse knockouts and cell model studies show that a truncated form of RAG-2 (lacking its C-terminus) encodes for its "core" enzymatic activity and can efficiently perform D→J but not V→DJ recombination (Akamatsu *et al*., 2003; Kirch *et al*., 1998; Liang *et al*., 2002). Cortes and colleagues reported that the C-terminus of RAG-2 binds directly to all four core histones (West *et al*., 2005). Specific mutations in the C-terminus that abolish its binding to histones also impair VH→DHJH but not DH→JH recombination in pro-B cell lines. One exciting

possibility is that the C-terminus of RAG-2 may serve as a bridge between chromatin and recombinase to facilitate the long-range synapsis of RSSs.

The precise sequence of RSSs also contributes to restrictions in the order and type of gene segments used at the TCRβ locus. Although the 12/23 rule permits direct joining between Vβ and Jβ gene segments, these recombination products are almost never observed *in vivo* (Bassing *et al*., 2000). Using an elegant series of knockout and substrate models, Alt and Sleckman have shown that an intrinsic property of the Jβ-RSS restricts its efficient usage to recombination with Dβ-RSSs but not with Vβ-RSSs. In contrast, the Vβ-RSSs are more compatible for recombination with the 5′Dβ-RSS (Bassing *et al*., 2000; Sleckman *et al*., 2000). The precise mechanisms involved in this beyond 12/23 control remain unknown. However, the specificity of gene segment selection at TCRβ does not rely on thymocyte-specific factors and likely reflects a more general feature of the recombinase itself, which may preferentially pair certain RSSs for coupled cleavage (Jung *et al*., 2003; Tillman *et al*., 2003). Consistent with this possibility, ordered Dβ→Jβ then Vβ→DβJβ recombination is not controlled by simple proximity of the Dβ and Jβ gene segments. This order is recapitulated at engineered TCRβ loci in which the Vβ cluster is positioned proximal to Dβ1 (Ferrier *et al*., 1990b; Senoo *et al*., 2003).

Notwithstanding these important but more specialized restrictions, it has become clear that chromatin accessibility is the primary determinant for establishing the recombination potential of gene segment clusters. As such, we will provide a brief overview of chromatin and the mechanisms that regulate its accessibility to nuclear factors, including the RAG proteins.

6. Chromatin Structure and Accessibility Control Mechanisms

A significant hurdle for the evolution of eukaryotes from prokaryotes was the packaging of ~2 meters of chromosomal DNA into nuclei that are several microns in size. Eukaryotes solved this problem by packaging genomic DNA into nucleosomes, the basic building block of chromatin. A single nucleosome consists of ~146 bp of DNA wrapped around an octamer of four histone pairs (H2A, H2B, H3, and H4) (Khorasanizadeh, 2004; Wolffe and Guschin, 2000). In most chromatin, nucleosomes are separated by ~20–60 bp of spacer DNA, which gives rise to a simple structure resembling "beads on a string." The histone protein, termed H1, can bind to linker DNA and is essential for the condensation of open chromatin into more compact forms (e.g., the 30-nm fiber) (Wolffe and Guschin, 2000). The mechanisms that give rise to even higher degrees of chromatin compaction remain vague.

Eukaryotes harbor three general types of chromatin in their nuclei (Fig. 4). The most highly compacted form, constitutive heterochromatin, is stained

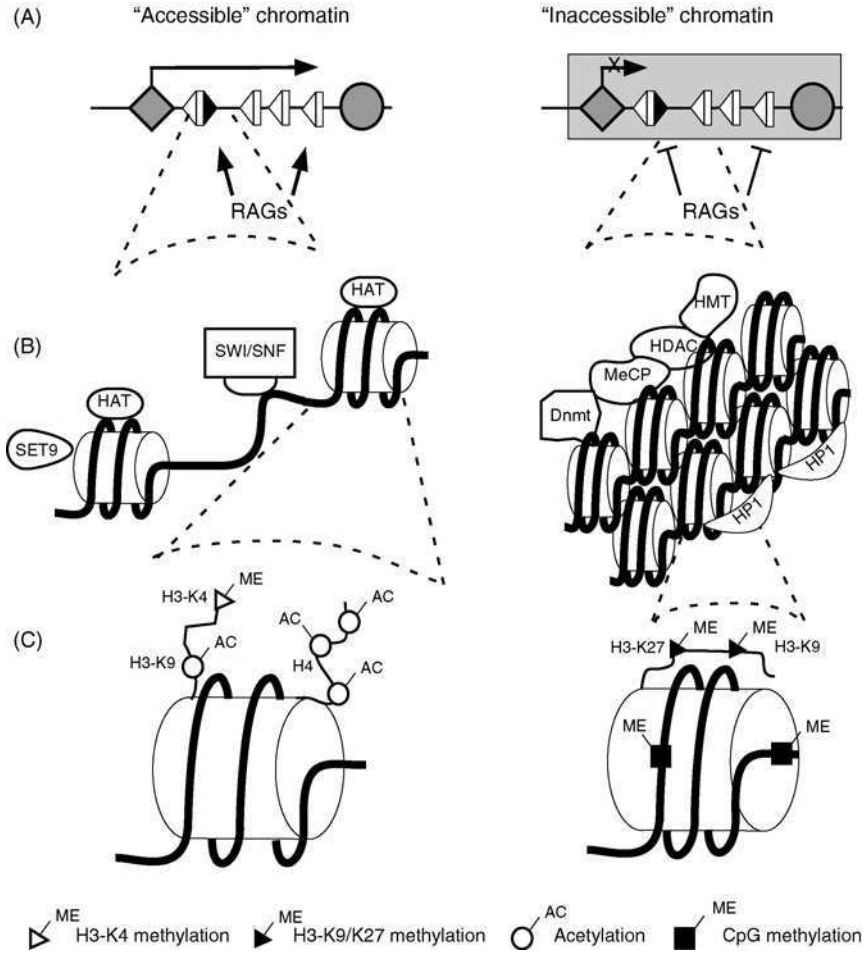

Figure 4 (A) Schematic representation of recombinase accessible (left) and inaccessible chromatin (right). Germline promoters and enhancers are depicted as diamonds and circles, respectively. The two types of chromatin are shown at increasing levels of resolution (top to bottom). (B) Nucleosomal DNA (dark spirals) wrapped around an octamer of four histones (H2A, H2B, H3, and H4), which is represented as a cylinder. Nucleosomes are loosely packed in accessible chromatin (left) and usually associate with activating TFs, HATs, and nucleosome-remodeling complexes (SWI/SNF). Inaccessible chromatin has more densely packed nucleosomal arrays (right) and associates with an interacting cascade of chromatin modifiers that usually includes DNA methyltransferases (Dnmt), methyl-CpG–binding proteins (MeCP), histone deacetylases (HDAC), histone methyltransferases, and the heterochromatin protein HP-1. (C) The general patterns of chromatin modifications at accessible (left) and inaccessible chromatin (right) are shown. A key for symbols representing each modification is given at the bottom.

intensely by DNA-specific dyes and represents the most inaccessible state. Accordingly, very few expressed genes are found in heterochromatic regions, which include pericentric repeats and the inactive X chromosome (Fahrner and Baylin, 2003). A second form of chromatin, termed euchromatin, is not highly stained by DNA dyes and represents an open state that contains most of the cell's expressed genes. Regions of euchromatin are generally more accessible to nuclear factors and more susceptible to attack by nucleases. A third configuration of chromatin, called facultative heterochromatin, is an intermediate form that exhibits many hallmarks of inactive chromatin but is not constitutively closed. Regions of facultative heterochromatin contain genetic loci that are silent but can be induced for expression given the proper cues and chromatin remodeling (Fahrner and Baylin, 2003). In addition to standard histones, eukaryotes express a panel of variants that perform specialized functions. These include: (1) macroH2A, which is a major component of constitutive heterochromatin (Chadwick *et al*., 2001), (2) H3.3, which replaces H3 at expressed genes and marks the locus for continued expression (McKittrick *et al*., 2004), and (3) H2AX, which is found in ~10% of nucleosomes and becomes phosphorylated at sites of DNA damage (Chen *et al*., 2000).

Although the nucleosomal structure of cellular DNA solves the basic packaging problem, it generally impedes interactions between DNA and most nonhistone proteins, including TFs and the basal transcription machinery (Geiman and Robertson, 2002). In this regard, numerous lines of evidence indicate that V(D)J recombinase can engage its target RSSs in nucleosomal DNA only after substrates become accessible. First, RAG cleavage of RSSs is blocked *in vitro* when substrates are packaged into mononucleosomes (Kwon *et al*., 1998). Second, antigen receptor loci undergoing rearrangement exhibit many hallmarks of accessible euchromatin, including hypersensitivity to nucleases, whereas recombinationally silent loci are largely refractory to nucleases (Chattopadhyay *et al*., 1998; Chowdhury and Sen, 2003). Third, Schlissel and colleagues have shown that recombinant RAG proteins cleave RSSs in nuclei from primary lymphocytes with the appropriate tissue-, stage-, and allele-specificity (Stanhope-Baker *et al*., 1996). Together, these studies suggest that most antigen receptor loci begin as facultative heterochromatin in CLPs. On lineage commitment, developmental cues signal for an opening of specific chromatin domains to render the appropriate gene segments accessible to recombinase.

Eukaryotes have developed a complex set of mechanisms to alter chromatin accessibility at both the local and long-range levels. Many of these mechanisms involve the recruitment of protein complexes that covalently modify either the histone or DNA components of chromatin (Berger, 2002; Richards and Elgin, 2002). A broad panel of TFs recruit protein complexes that acetylate,

methylate, phosphorylate, or ubiquitinate histones. These modifications enable nucleoprotein modules to recruit other coactivators including components of the core transcription machinery. These observations have led to the "histone code" hypothesis. According to this hypothesis, modifications in N-terminal tails of histones generate binding sites for additional chromatin remodeling complexes, which in turn control the transcriptional status of flanking genes (Fig. 4) (Jenuwein and Allis, 2001).

A well-recognized example of the histone code hypothesis is the modification of lysine-9 on histone H3 (H3-K9). This amino acid is targeted by a broad spectrum of histone acetyltransferases (HATs) and histone deacetylases (HDACs), which do not bind DNA directly but are recruited by TFs or repressor complexes (Emerson, 2002; Nakayama *et al*., 2001; Narlikar *et al*., 2002). Acetylation of H3-K9 leads to high-affinity interactions with bromodomains in other HAT or nucleosome-remodeling complexes (see later), which further augment chromatin accessibility (Peterson and Workman, 2000). Accordingly, expressed loci normally associate with nucleosomes bearing H3-K9 acetylation, whereas silent loci are characterized by hypoacetylated H3-K9 residues (Litt *et al*., 2001). Studies have extended these links to the process of V(D)J recombination. Acetylation of nucleosomes can partially relieve the inhibition of RAG-mediated cleavage at RSSs *in vitro* (Kwon *et al*., 2000). Moreover, antigen receptor loci that undergo active rearrangement are associated with hyperacetlyated histones *in vivo*, whereas inert gene clusters remain hypoacetylated (Chowdhury and Sen, 2003; McMurry *et al*., 1997; Morshead *et al*., 2003).

In contrast to acetylation, methylation at H3-K9 leads to the reduced expression of associated transcription units (Lachner *et al*., 2001). The degree of methylation at H3-K9 also influences the magnitude of gene repression and the formation of distinct chromatin configurations. Dimethylation at H3-K9 is found predominantly at repressed genes in euchromatin or facultative heterochromatin. This epigenetic mark is imprinted by two histone methyltransferases (HMTs) in mammals, called G9a and GLP (Peters *et al*., 2003; Tachibana *et al*., 2002, 2005). Trimethylation of H3-K9 is observed predominantly at constitutive or pericentric heterochromatin and is the enzymatic product of two redundant HMTs called Suv39h1 and Suv39h2 (Peters *et al*., 2001, 2003). Consistent with the histone code hypothesis, methylated H3-K9 recruits an entirely different set of remodeling complexes relative to its acetylated counterpart. This set of complexes feature the presence of a chromodomain and function to impair chromatin accessibility (Bannister *et al*., 2001; Lachner *et al*., 2001). H3-K9 methylation marks antigen receptor gene segments that are recombinationally inert and this modification can dominantly repress accessibility to V(D)J recombinase at chromosomal substrates (Johnson *et al*., 2004; Morshead *et al*., 2003; Osipovich *et al*., 2004).

In addition to acetylation/methylation of H3-K9, mammalian histones are marked by a constellation of covalent modifications (Cosgrove *et al*., 2004; Jenuwein and Allis, 2001). Several of these epigenetic marks have been studied extensively in the context of gene expression, and to some extent, for correlations with V(D)J recombination. These include: (1) histone H4 acetylation, which correlates with transcriptional activation at open chromatin (Jenuwein and Allis, 2001), (2) H3-K4 methylation, which is accomplished by SET1 and is characteristic of expressed genes (Santos-Rosa *et al*., 2002), (3) H3-K27 methylation, which is targeted by the Ezh2 component of polycomb complexes and serves as a long-term memory mark for silent chromatin (Cao *et al*., 2002; Kuzmichev *et al*., 2002), and (4) H3-K79 methylation by the DOT1 methyltransferase, which identifies active chromatin and prevents silencing in yeast (Ng *et al*., 2003). Collectively, these and other histone modifications comprise a flexible, yet highly complex code, which specifies numerous cellular processes including gene activation and V(D)J recombination. The exquisite specificity of the histone code is underscored by studies of the IFN-β regulatory region, which forms an enhanceosome structure on binding its cognate TFs. Acetylation of the enhanceosome-proximal nucleosome at H3-K9/K14 is required for the recruitment of TFIID via a pair of bromodomains (Agalioti *et al*., 2002). In contrast, the SWI/SNF nucleosome remodeling complex is recruited via interactions between its bromodomain and an acetylated lysine at H4-K8.

In addition to histone tail modifications, the DNA component of chromatin can be covalently marked by methylation at CpG dinucleotides (Bird, 2002). This reversible modification is mediated by a family of DNA methyltransferases (Dnmt) that exhibit distinct functions. In mammals, the Dnmt1 enzyme maintains CpG methylation following cellular replication, while Dnmt3 isoforms perform *de novo* methylation (Bestor, 2000; Chen *et al*., 2003). In general, CpG methylation is indicative of transcriptional repression whereas actively expressed genes are hypomethylated (Bird, 2002). Mounting evidence suggests a functional interplay between the H3-K9 and DNA methylation machineries. Nucleosomes methylated at H3-K9 present a docking site for heterochromatin-associated proteins, including isoforms of HP1 (Bannister *et al*., 2001; Lachner *et al*., 2001). The HP1α isoform interacts with Dnmt3, which may then target local CpG sites for methylation (Fuks *et al*., 2003a). Modified CpG sites interact with a specialized set of DNA-binding proteins (e.g., MeCP2) that form complexes with HDACs and HMTs (Fuks *et al*., 2003b; Jones *et al*., 1998). Thus, recruitment of G9a or Suv39h1/2 suppresses gene expression through a self-reinforcing mechanism that relies on extensive crosstalk between the histone and DNA methylation machineries. In the context of antigen receptor gene assembly, the vast majority of recombinationally active loci are hypomethylated on CpG dinucleotides, whereas recombinationally

inert loci exhibit CpG hypermethylation (Bergman *et al.*, 2003). Moreover, CpG methylation has been shown to directly suppress V(D)J recombination of ectopic or transgenic substrates (Demengeot *et al.*, 1995; Engler *et al.*, 1991; Hsieh *et al.*, 1992).

Chromatin modifications and TFs serve as binding platforms for ATP-dependent complexes that remodel neighboring nucleosomes and expose associated DNA (Kingston and Narlikar, 1999). Three major families of remodeling complexes have been characterized to date. Two of these families, termed ISWI and Mi-2, function mainly as transcriptional repressors (Emerson, 2002; Narlikar *et al.*, 2002). In contrast, members of the SWI/SNF family facilitate transcription of nucleosomal substrates and can interact with the activation domains of TFs (Peterson and Workman, 2000). In keeping with the histone code, components of the SWI/SNF complex possess bromodomains to enhance binding at acetylated regions within the chromatin of expressed loci (Hassan *et al.*, 2001b). Although the composition of SWI/SNF can vary, two general classes have been identified. These classes are functionally distinct and contain either Brg1 or Brm as their critical ATPase subunit (Kadam and Emerson, 2003). The precise mechanism of SWI/SNF action on nucleosome arrays has not been established. However, the functional outcome of SWI/SNF action is threefold: it alters the translational position of nucleosomes on DNA, modifies histone octamers to increase DNA accessibility, and loops out intervening DNA between nucleosome entry and exit sites (Kassabov *et al.*, 2003; Kingston and Narlikar, 1999). A link between nucleosome remodeling and V(D)J recombination is suggested by *in vitro* studies. Pretreatment of mononucleosome substrates with SWI/SNF partially rescues RSS cleavage by RAG proteins (Kwon *et al.*, 2000). Moreover, chromatin immunoprecipitation (ChIP) analyses revealed that Brg1 occupies regions within Ig and TCR loci that are recombinase accessible (Morshead *et al.*, 2003).

7. Regulation of Antigen Receptor Loci by Nuclear Topography

Changes in chromatin accessibility are regulated in large part by covalent modification of histones and genomic DNA. However, the complex nature of nuclear dynamics creates an opportunity for additional levels of gene regulation. These processes rely on interactions between a given genetic locus and distinct regions within its chromosome or within the nucleus (Smale and Fisher, 2002). Advances in fluorescence *in situ* hybridization (FISH) techniques permit unprecedented views of changes in subnuclear localization or the topography of large genetic loci. These studies demonstrate that silent genes generally reside in the nuclear periphery (Goldmit *et al.*, 2005; Kosak *et al.*, 2002; Roldan *et al.*, 2005). The mechanistic basis for this form of repression

remains unclear but may involve sequestration of regulatory elements away from TFs or association with nuclear lamina (Kosak *et al*., 2002). In contrast, expressed genes are more centrally located in nuclei, which presumably offer a more permissive environment for transcription. The transcriptional status of some genes also correlates with their colocalization at distinct domains in the chromosome. For example, transcriptional silencing of the TdT gene is accompanied by its relocalization to pericentromeric regions of heterochromatin (Brown *et al*., 1997).

FISH analyses of precursor B cells have produced astounding links between changes in subnuclear location and IgH gene regulation. In pioneering studies, Fisher and colleagues showed that, consistent with monoallelic expression of the IgH locus, mature B cells retain one IgH allele at the nuclear periphery while the second allele is more centrally located (Brown *et al*., 1997). In activated B lymphocytes, sequences that direct association of silenced IgH loci with the nuclear periphery are located in the 5′ portion of the VH cluster (Yang *et al*., 2005). Nuclear localization also correlates with the tissue-specific control of IgH locus activation. In non-B cells, both IgH alleles are perinuclear, whereas they migrate to a more central location in pro-B cells that are poised to undergo IgH recombination (Kosak *et al*., 2002).

In addition to nuclear localization, significant topological constraints may be placed on antigen receptor loci to mediate the synapsis of two RSSs separated by large distances. FISH analysis of pro-B cells revealed that IgH loci undergo a large-scale compaction via a looping mechanism (Kosak *et al*., 2002; Sayegh *et al*., 2005). This stage-specific compaction process brings the VH region into spatial proximity with its target JH cluster, which presumably facilitates RSS synapsis and VH→DHJH recombination. On productive IgH gene assembly, pre-BCR signaling leads to a decontraction of IgH loci in pre-B cells and relocalization of the nonfunctional IgH allele to pericentromeric heterochromatin (Roldan *et al*., 2005). Although causal relationships remain to be established between these nuclear events, locus decontraction, coupled with its relocation to a repressive chromosomal milieu, provides an attractive mechanistic model for the process of allelic exclusion.

Similar changes in location and topography are involved in the stage-specific regulation of the Igκ locus. Although both Igκ alleles are centrally located in the nucleus of pro-B cells, only a single allele adopts the hallmarks of accessible chromatin in pre-B cells (i.e., histone acetylation and CpG hypomethylation) (Goldmit *et al*., 2005). This active allele also undergoes large-scale contraction, which brings Vκ gene segments into proximity with their Jκ partners (Roldan *et al*., 2005). Consistent with monoallelic regulation of Igκ assembly, the second, inactive allele is recruited to repressive heterochromatin in pre-B cells (Goldmit *et al*., 2005). The generality of these correlations

between nuclear localization, association with centromeric regions, and locus compaction have not been established for TCR loci. An exciting goal for future studies will be to decipher the network of *cis*-acting elements that orchestrate compartmentalization of antigen receptor loci within the nucleus and identification of the nuclear factors that facilitate locus compaction.

8. Regulation of Chromatin Accessibility and V(D)J Recombination by *Cis*-Acting Elements

Gene expression programs are specified in large part by a collection of *cis*-acting elements that include transcriptional promoters, enhancers, locus control regions (LCRs), silencers, and boundary elements. A primary function of these regulatory motifs is to dock TFs that indirectly modulate the accessibility of neighboring chromatin. The observed link between germline transcription and recombination of gene segments suggests these two processes share common regulatory elements. Consistent with this possibility, transcriptional control elements are scattered throughout Ig and TCR loci. Promoters and enhancers within these loci are mostly arranged in a split configuration, which may afford modular control of transcription/recombination at distinct clusters of gene segments.

This regulatory model was confirmed in early studies using TCRβ or TCRδ transgenic substrates, which demonstrated a direct role for transcriptional enhancers in targeting their efficient recombination (Ferrier *et al.*, 1990a; Lauzurica and Krangel, 1994). Likewise, recombination of chromosomal substrates in cell models requires the inclusion of any active enhancer/promoter combination, even those of viral origin (Oltz *et al.*, 1993; Sikes *et al.*, 1999). Germline deletion of enhancers or promoters within antigen receptor loci consistently impairs rearrangement of linked gene segments (see later). Together, these studies demonstrate that the biologic action of V(D)J recombinase is tightly regulated by promoters and enhancers, which serve as ACEs to guide antigen receptor gene assembly and lymphocyte development. In this section, we review the role of ACEs in the regulation of recombinase accessibility, focusing on unique mechanistic insights garnered from studies of individual Ig and TCR loci.

8.1. Regulation of TCRβ Gene Assembly

The TCRβ locus provides an excellent model for *cis*-acting regulation because the DβJβ region contains only a single enhancer (Eβ) and one germline promoter in each DβJβ cluster (Fig. 2). Eβ function is T lineage specific and is activated at the earliest stage of thymocyte development (McDougall *et al.*,

1988). Accordingly, inclusion of Eβ in a transgenic TCRβ minilocus activates its recombination in DN thymocytes (Ferrier *et al*., 1990a). The ACE function of Eβ was confirmed by its targeted deletion in mice, which cripples recombination at both DβJβ clusters (Bories *et al*., 1996; Bouvier *et al*., 1996). The Eβ knockout also ablates germline transcription of both DβJβ clusters and converts their associated chromatin modifications into a heterochromatic pattern (e.g., H3/H4 hypoacetylation and CpG hypermethylation) (Mathieu *et al*., 2000; Spicuglia *et al*., 2002). The ACE function of Eβ can be replaced with a heterologous enhancer. In transgenic miniloci, the IgH enhancer, Eμ, can target Dβ→Jβ recombination in both B and T lineage cells (Ferrier *et al*., 1990a). However, targeted replacement of the endogenous Eβ element by Eμ permits TCRβ recombination in thymocytes but not in B lineage cells, where Eμ normally functions (Bories *et al*., 1996). These findings suggest that a negative regulatory element, which is missing from the transgenic substrate, may repress Eμ function in precursor B cells if the enhancer is positioned within the TCRβ locus.

A second reason that many studies of accessibility control have focused on the TCRβ locus is the extensive characterization of a germline promoter associated with the Dβ1 gene segment. This promoter, called PDβ1, is positioned directly 5′ of Dβ1, includes a consensus TATA sequence within the 5′Dβ-RSS, and directs germline transcription through the Dβ1Jβ cluster in pro-T cells (Doty *et al*., 1999; Sikes *et al*., 1998). Germline transcription analyses indicate the presence of an analogous promoter near Dβ2; however, the putative PDβ2 element remains to be characterized (Whitehurst *et al*., 1999).

The essential ACE function of PDβ1 in TCRβ gene assembly has been demonstrated at both the endogenous locus and in model substrates (Sikes *et al*., 1999; Whitehurst *et al*., 2000). Deletion of PDβ1 cripples transcription and rearrangement of the Dβ1Jβ but not the Dβ2Jβ cluster, suggesting that the promoter may influence chromatin accessibility over a limited range (Whitehurst *et al*., 1999). In this regard, Dβ→Jβ recombination in minilocus substrates is severely impaired by moving PDβ1 only 400 bp from its native location, even though the promoter remains transcriptionally active (Sikes *et al*., 2002). Using thymocytes from mice harboring targeted deletions of ACEs, we discovered that PDβ1 regulates chromatin accessibility in a highly localized manner (Oestreich *et al*., 2006). Deletion of the germline promoter inhibits accessibility over a region of less than 450 bp surrounding the Dβ1 gene segment. In contrast, chromatin accessibility at the Jβ1 segments is relatively unaffected by the promoter deletion. Thus, it appears that the enhancer has a PDβ-independent ACE function that opens chromatin throughout both DβJβ clusters, with the exception of the Dβ1 gene segment.

Recombinase accessibility and germline transcription of this segment require additional, highly localized chromatin remodeling that depends on PDβ1. The additional wave of remodeling likely occurs on formation of a stable holocomplex between the promoter and Eβ elements (Oestreich *et al.*, 2006).

In contrast to Dβ→Jβ rearrangement, much less is known about the *cis*-acting elements that regulate the second step of TCRβ gene assembly—Vβ→DβJβ recombination. Vβ gene segments are clearly active for germline transcription and exhibit hallmarks of active chromatin in DN cells (Jackson and Krangel, 2005; Senoo and Shinkai, 1998). However, neither of these features are altered in Eβ knockout thymocytes, suggesting this element does not control chromatin accessibility at Vβ segments (Mathieu *et al.*, 2000). The additional element(s) that controls Vβ accessibility likely is not located between the Vβ cluster and Dβ1 because germline deletion of this region has no effect on TCRβ gene assembly (Senoo *et al.*, 2003). Studies have demonstrated that Vβ promoters, which drive transcription of rearranged VβDβJβ exons, also contribute an ACE function for their recombination. Deletion of the Vβ13 promoter significantly inhibits its rearrangement in cis; however, allelic exclusion of the gene segment remained intact (Ryu *et al.*, 2004). Accessible chromatin is restricted to regions surrounding Vβ segments in DN cells rather than spread throughout the entire Vβ cluster (Jackson and Krangel, 2005). Together, these findings suggest that Vβ promoters may function as enhancer-independent ACEs to induce highly localized changes in chromatin and target Vβ gene segments for recombination. However, validation of this model awaits additional Vβ promoter knockouts and a more extensive characterization of chromatin in thymocytes from these animals.

8.2. Regulation of TCRα/δ Gene Assembly

Pro-T cells that functionally rearrange a TCRβ allele undergo clonal expansion and differentiate into pre-T cells via a process called β-selection (Krangel *et al.*, 2004). These DP thymocytes also initiate Vα→Jα rearrangements that primarily target the most 5′Jα gene segments (Krangel *et al.*, 2004). Because the RAG genes are not extinguished immediately on TCRα expression, many DP cells proceed with a stepwise process of secondary and tertiary Vα→Jα rearrangements (Guo *et al.*, 2002; Hawwari *et al.*, 2005). These additional recombination events utilize Vα/Jα gene segments that are located progressively further upstream or downstream from the preceding rearrangement (Hawwari *et al.*, 2005; Huang *et al.*, 2005). Accordingly, DP cells that are limited in their survival potential exhibit a skewed TCRα repertoire containing mostly 5′Jα gene segments (Guo *et al.*, 2002). One consequence of this receptor revision process is the potential for allelic inclusion at TCRα loci. A significant proportion of

peripheral T cells express two functional TCRα proteins in their cytoplasmic compartment (Padovan *et al*., 1993). The monospecificity of antigen recognition in these cells is primarily maintained at the level of preferential TCRα pairing with the expressed TCRβ protein.

An additional consequence of Vα→Jα recombination is deletion of all Dδ/Jδ gene segments. Because many of the V gene segments in the α/δ locus can participate in both Dδ and Jα joins, an outstanding question is how regional control of recombination is achieved at the clusters of interspersed TCRα and TCRδ segments (Krangel *et al*., 2004). Transcription within the TCRα/δ locus is controlled by distinct enhancers. The Eα element is positioned downstream of Cα, while Eδ is situated between the Vα and Jα clusters (Fig. 2). Targeted deletion of Eα results in a severe reduction of germline Jα transcription and Vα→Jα rearrangement in developing thymocytes (Sleckman *et al*., 1997). Studies have shown that Eα controls not only the Jα cluster but also affects germline transcription and chromatin modifications at the subset of proximal Vα gene segments that are used preferentially in DP cells (Hawwari and Krangel, 2005). Thus, the ACE function of Eα extends over an astounding range of at least 400 kb. Transcription and rearrangement of the more distally located Vα segments is unaffected by the Eα knockout, suggesting that additional ACEs regulate this portion of the locus.

Elimination of Eα did not significantly alter the level of TCRδ rearrangement but attenuated transcription of rearranged TCRδ genes (Sleckman *et al*., 1997). In contrast, germline deletion of Eδ severely impairs recombination of TCRδ gene segments but spares Vα→Jα rearrangement (Monroe *et al*., 1999). Regional control within the TCRα/δ locus by the two separate enhancers cannot be explained by enhancer location because replacement of Eα with Eδ fails to restore TCRα recombination (Bassing *et al*., 2003). Consistent with the developmental control of locus rearrangement, Eδ is active in DNIII cells and inactive in DP cells precisely as Eα becomes functional (Krangel *et al*., 2004). As such, stage-specific activation of these linked enhancers may be the primary determinant for compartmentalization of their ACE function within the locus.

The promoter elements that control transcription at the TCRα/δ locus have been studied in considerable detail. A germline promoter, termed T early α (TEA) is positioned upstream of the most 5′Jα (Jα61) gene segment. A localized ACE function for TEA was confirmed by its germline deletion, which abrogates both transcription and recombination, specifically of 5′Jα segments (Villey *et al*., 1996). Studies indicate that a series of at least four germline promoters control the accessibility of specific regions within the Jα cluster (Hawwari *et al*., 2005). Thus, similar to the TCRβ locus, TCRα germline promoters serve as highly localized ACEs to control rearrangement of only

the most proximal gene segments, whereas Eα has a long-range effect on locus accessibility. A hierarchy of function also exists among Jα germline promoters because TEA-driven transcription of 5′Jα segments predominates when all promoters are present. However, when TEA is absent, due to its targeted deletion or Vα→Jα recombination, germline transcription and rearrangement of 3′Jα segments is enhanced (Hawwari *et al.*, 2005). Collectively, the promoter/enhancer knockout studies suggest a "zipper model" for sequential rearrangement of TCRα gene segments. Eα initially activates the most 3′ set of Vα gene segments, which preferentially rearrange with the 5′Jα segments due to dominant local control by TEA. These primary Vα→Jα recombination events delete TEA and progressively activate downstream promoters, which sequentially target Jα segments via their localized ACE function (Hawwari *et al.*, 2005). Stepwise assembly leads to extensive revision of TCRα gene products in DP thymocytes and is a critical component of the developmental program that generates a fully functional TCR repertoire (Huang *et al.*, 2005).

A powerful germline promoter associated with the Dδ2 gene segment has been described (Carabana *et al.*, 2005). Functional analyses of TCRδ transgenes suggest this germline promoter may regulate recombinase accessibility at the Dδ2 segment in an Eδ-dependent manner. However, the Dδ2 promoter exhibits a measureable level of transcriptional activity in the absence of Eδ. This Eδ-independent activity may provide the missing ACE function that directs TCRδ recombination in $E\delta^{-/-}$ mice.

8.3. Regulation of TCRγ Gene Assembly

Published studies of TCRγ locus regulation have focused primarily on the VγJγ cluster located directly upstream of the Cγ1 coding region (Fig. 2). A single enhancer situated 3′ to Cγ1 has been identified in this region (Eγ1), and two analogous enhancers exist downstream of Cγ1 (Kappes *et al.*, 1991; Spencer *et al.*, 1991; Vernooij *et al.*, 1993). A second DNAse hypersensitive site (HsA) is positioned between Vγ5 and Vγ2. The HsA element possesses no enhancer function but is required for integration site-independent expression of TCRγ transgenes containing Eγ1 (Baker *et al.*, 1999). Surprisingly, Vγ→Jγ recombination is unaffected by single or dual deletions of HsA and Eγ1; however, the dual deletion attenuates transcription of assembled TCRγ genes (Xiong *et al.*, 2002). As such, the ACE responsible for initial activation of TCRγ gene assembly remains at large.

One reason for the intense interest in regulation of the Cγ1 region relates to developmental restrictions imposed on the usage of specific Vγ gene segments. Rearrangements involving Vγ3 and Vγ4 predominate in fetal thymocytes, whereas Vγ5 and Vγ2 are used in adult pro-T cells (Baker *et al.*, 1998).

Thus, recombination of Vγ segments is tightly controlled over a relatively short distance (~10 kb). The highly localized regulation of recombinase accessibility is reminiscent of promoter-directed control at the TCRβ and TCRα loci. Exchange of the Vγ2 and Vγ3 promoters within a transgenic substrate reverses their usage in adult thymocytes (Vγ3 predominates) (Baker *et al*., 1998). Remarkably, preferential usage of Vγ3 in fetal thymocytes remains unaltered in the "swapped" substrate. Selection between the two Vγ gene segments in fetal thymocytes appears to depend solely on their chromosomal locations. A swap of only the Vγ3 and Vγ2 gene segments reversed their usage in fetal but not adult thymocytes ($V\gamma2^{hi}/V\gamma3^{low}$ in both) (Xiong *et al*., 2004). This reversal is not simply related to chromatin accessibility because both swapped gene segments are transcribed and hyperacetylated in fetal thymocytes. Thus, developmental regulation of Vγ usage depends on two distinct mechanisms—promoter-directed control of local recombinase accessibility in adults and accessibility-independent control in the fetus, which relies only on chromosomal location.

8.4. Regulation of IgH Gene Assembly

The murine IgH locus contains two functional enhancer regions. One enhancer, termed Eμ, is located in the intron between JH segments and Cμ-coding exons (Fig. 2). The core Eμ element is composed of numerous sites for TF binding, including Oct, ETS, and E2A family members (Hesslein and Schatz, 2001). The core enhancer is flanked by two stretches of AT-rich sequences, termed nuclear matrix attachment regions (MARs), which have been implicated in enhanced transcriptional activation of *cis*-acting elements (Cockerill *et al*., 1987). The second enhancer/LCR is located downstream of Cα and is called the 3′ regulatory region (3′RR). The mouse 3′RR is a composed of at least four DNAse hypersensitive areas, each of which exhibit distinct enhancer functions (Birshtein *et al*., 1997; Dudley *et al*., 2005). *In vivo*, the most important functions of the 3′RR are to control isotype switching and direct elevated expression of functional IgH alleles in mature B cells (Cogne *et al*., 1994; Manis *et al*., 1998).

Because Eμ is located most proximal to the DHJH cluster, it is a prime candidate for the ACE that controls initial IgH gene assembly. In this regard, Eμ controls local chromatin accessibility at heterologous sequences in pro-B cells from transgenic mice (Jenuwein *et al*., 1993). Initial studies of knockout mice lacking Eμ afforded surprising results. Deletion of either the core Eμ or Eμ/MARs elements had little effect on DH→JH recombination but dramatically inhibited VH→DHJH recombination (Sakai *et al*., 1999; Serwe and Sablitzky, 1993). One complicating factor in these studies was that rearrangement levels

were measured in mature B cells from chimeric animals that were heterozygous for the mutant allele. Thus, continued rearrangement of the mutant allele in developing B cells could mask the full impact of the Eμ deletion on DH→JH recombination. Reexamination of IgH assembly in homozygous $E\mu^{-/-}$ animals indicates a more profound inhibition of DH→JH rearrangement in the pro-B cell compartment (5–10×) (Afshar *et al.*, 2006; Perlot *et al.*, 2005). Consistent with its ACE function, deletion of Eμ also abolished germline transcription throughout the JHCμ region and at upstream DH elements (Afshar *et al.*, 2006; Perlot *et al.*, 2005). Thus, the substantial block in VH→DHJH rearrangement at Eμ-deficient alleles likely results from a combination of defective DH→JH recombination in pro-B cells and enforcement of allelic exclusion in pre-B cells that undergo delayed DH→JH rearrangement at the mutant IgH allele.

Notwithstanding, deletion of Eμ produces an incomplete block in DH→JH recombination, suggesting the existence of compensatory elements. An extensive survey of the DHJH cluster for DNAse-hypersensitive regions revealed only two sites in pro-B cells—Eμ and a region directly 5′ of the JH-proximal DQ52 gene segment (Chowdhury and Sen, 2001). This region, called PDQ52, was shown to possess both promoter and enhancer activity in pro-B cell lines (Kottmann *et al.*, 1994). As such, PDQ52 was a likely candidate for the compensatory ACE in Eμ-deficient alleles. However, targeted deletion of this region had no effect on germline transcription of the DQ52/JH region or on overall DH→JH recombination (Afshar *et al.*, 2006; Nitschke *et al.*, 2001). Thus, additional promoter activity must lie upstream of the originally defined PDQ52 element. By analogy to PDβ1, removal of all germline promoter function from the DQ52 region (alone or in combination with Eμ) may produce a complete block in DH→JH rearrangement. Alternatively, an additional control element may contribute to DHJH accessibility in the absence of Eμ. One such candidate is the 3′RR that has been shown to exert its regulatory influence over a distance of at least 200 kb (Dudley *et al.*, 2005). A dual deletion of Eμ and the 3′RR should resolve this outstanding issue.

The ACEs that control VH→DHJH recombination remain a mystery. It also remains unclear whether the VH cluster is controlled in a highly localized manner or by master regulatory elements that exert long-range control over large clusters of VH segments. However, the chromosomal position of VH segments plays an important role in their recombination potential. The most DH-proximal gene segments (7183 family) are at least partially accessible in pro-B cells, coincident with recombination of the DHJH cluster (Chowdhury and Sen, 2001; Roldan *et al.*, 2005). The central portion of the VH locus becomes activated at a later time point in pro-B cell development, perhaps following DH→JH recombination (Chowdhury and Sen, 2001). The most distal

VH segments (J558 family) are activated in response to IL-7, a pro-B cell growth factor that activates the STAT-5 TF (Corcoran *et al*., 1998). All VH gene segments have a 5′ promoter that drives their transcription before and after rearrangement (Ballard and Bothwell, 1986; Yancopoulos and Alt, 1985). Studies suggest that these promoters may function as ACEs because interference with STAT-5 binding to distal VH promoters blocks their germline transcription and recombination (Bertolino *et al*., 2005). However, direct genetic evidence that any VH promoter controls local or regional accessibility to recombinase is currently lacking.

8.5. Regulation of Igκ Gene Assembly

Gene expression at the Igκ locus is controlled by a collection of *cis*-acting elements that include three enhancers: one in the Jκ/Cκ intron (iEκ), a second located 9 kb downstream of Cκ (3′Eκ), and a recently defined element, called Ed, positioned downstream of 3′Eκ (Fig. 2) (Gorman and Alt, 1998; Liu *et al*., 2002). To date, the ACE functions of only iEκ and 3′Eκ have been tested by germline deletions. Single deletions of either enhancer significantly impair Vκ→Jκ rearrangement (5–10× each), while a dual Eκ/3′Eκ deletion completely cripples Jκ transcription and recombination in cis (Gorman *et al*., 1996; Inlay *et al*., 2002; Takeda *et al*., 1993; Xu *et al*., 1996). Thus, the two most proximal Igκ enhancers cooperatively regulate recombinase accessibility at least at the Jκ cluster (Vκ transcription was not measured).

Germline transcription of the Jκ cluster is controlled by a pair of promoters located immediately 5′ and ~6 kb upstream of Jκ1 (Martin and van Ness, 1990). The ACE function of Jκ germline promoters or Vκ promoters in targeting Vκ→Jκ recombination have not been tested *in vivo*. However, studies have shown that all three of the known enhancers form a stable holocomplex with the promoter of a rearranged Vκ gene in plasma cells expressing high levels of mature Igκ transcripts (Liu and Garrard, 2005). It will be of great interest to see whether the germline Jκ promoters interact with all or only a subset of the enhancers in pre-B cells that are poised for Igκ gene assembly.

Changes in chromatin accessibility likely regulate the Igκ locus for stage-specific activation in pre-B cells and its monoallelic assembly. With regards to stage specificity, *in vivo* footprint analyses indicate that major changes occur in TF occupancy at 3′Eκ but not at iEκ during the pro→pre-B cell transition (Shaffer *et al*., 1997). Consistent with a primary role for 3′Eκ in this process, iEκ directs the activation of transgenes in pro-B cells (Pelanda *et al*., 1996). Thus, the chromosomal context of iEκ and its stage-specific cooperation with 3′Eκ appear to be important for ordered activation at Igκ.

The mechanisms that control allelic exclusion at Igκ are now beginning to emerge. Unlike the IgH locus, the two Igκ alleles are activated sequentially in pre-B cells as evidenced by the erasure of CpG methylation and asynchronous timing of locus replication (Mostoslavsky *et al.*, 1998, 2001). Analogous to their roles in Vκ→Jκ recombination, iEκ and 3′Eκ are both important for monoallelic activation of the Igκ locus (Mostoslavsky *et al.*, 1998). Mice harboring a GFP knock-in at the Jκ cluster exhibit monoallelic GFP expression in a surprising low percentage of pre-B cells (Liang *et al.*, 2004). These GFP^+ cells also contain the vast majority of Vκ→Jκ rearrangements. Together, these studies suggest a model in which iEκ and 3′Eκ are cooperatively activated in only a small percentage of pre-B cells. In turn, this cooperative interaction promotes chromatin accessibility, germline transcription, and recombination of the Jκ cluster. The inefficiency of this process and its monoallelic nature would essentially preclude simultaneous recombination of two Igκ alleles in a single pre-B cell.

8.6. Regulation of Igλ Gene Assembly

Pre-B cells that fail to generate an alloreactive Igκ allele proceed to rearrange their Igλ locus. The genetic and epigenetic mechanisms governing ordered IgL recombination remain unclear. Transcription of rearranged Igλ genes is regulated by at least two enhancer regions called Eλ2-4 and Eλ3-1 (Fig. 2). The Eλ3-1 element lies at the 3′ end of the Vλ1Jλ1 cluster, while Eλ2–4 is located at the 3′ end of the Vλ2Jλ2 region (Hagman *et al.*, 1990). Both enhancers are active in mature B cells, however, their role in the initial activation of Igλ germline transcription and recombinase accessibility has not been addressed.

The ACE function of Vλ promoters has been tested only in transgenic recombination substrates. These studies indicate that murine and human Vλ promoters can drive substrate Vλ→Jλ recombination but specific mutations that cripple promoter activity also inhibit substrate rearrangement (Lauster *et al.*, 1993; Stiernholm and Berinstein, 1995). All three Jλ gene segments are subject to germline transcription in pre-B cells but the precise location and functional architecture of relevant promoters have not been reported (Bendall *et al.*, 2001). Consistent with the emerging theme that promoters regulate accessibility to recombinase in a highly localized manner, targeted insertion of a neo-expression cassette upstream of Jλ1 dramatically increases its germline transcription and rearrangement (Sun and Storb, 2001). Thus, the strength of a promoter driving Jλ germline transcription, rather than the promoter's specific architecture, may determine the efficiency of recombination at Igλ.

9. Role of Transcription in Accessibility Control of V(D)J Recombination

Cis-acting elements regulate chromatin accessibility and recruit factors that facilitate efficient transcription of linked genes. Numerous studies established tight spatial and temporal correlations between transcription and changes in chromatin accessibility that render gene segments accessible to V(D)J recombinase. These studies suggest that transcription itself may regulate the recombination potential of gene segments. Alternatively, chromatin alterations that generate recombinase accessibility may coincidentally permit transcription as a byproduct of chromatin opening at promoters.

Many studies support a role for transcription in accessibility control mechanisms. Expression of transfected recombination substrates almost invariably correlates with their recombination efficiencies (Blackwell *et al*., 1986; Oltz *et al*., 1993). Targeted deletion of germline promoters that drive transcription through linked gene segments block their efficient rearrangement (Hawwari *et al*., 2005; Sikes *et al*., 2002; Villey *et al*., 1996; Whitehurst *et al*., 2000). Mice defective for IL-7 signaling exhibit a dramatic reduction in both transcription and recombination of distal VH gene segments (Corcoran *et al*., 1998). Finally, null mutation of the OcaB gene produces defects in the rearrangement of specific Vκ segments; the same subset of segments that is impaired for germline transcription (Casellas *et al*., 2002).

Despite these findings, mounting evidence suggests that promoters and enhancers function as ACEs via mechanisms that are, at least in part, independent of transcription. Numerous examples have been reported where transcription of gene segments is insufficient for their recombination (Okada *et al*., 1994; Tripathi *et al*., 2000). Certain VH segments are transcribed in wild-type or PAX-5-deficient pro-B cells but are not rearranged efficiently (Angelin-Duclos and Calame, 1998; Hesslein *et al*., 2003). Moreover, targeted insertion of Eα adjacent to the Vβ12 segment drives its transcription in DP thymocytes but fails to target it for rearrangement (Jackson *et al*., 2005). Conversely, several examples of transcription-independent recombination have been reported. Tethering of the glucocorticoid receptor to episomal substrates disrupts nucleosomal arrays at neighboring gene segments and leads to their recombination in the absence of detectable transcription (Cherry and Baltimore, 1999). Likewise, inversion of the PDβ1 promoter in chromosomal substrates cripples transcription through DβJβ gene segments but Dβ→Jβ rearrangement is unaffected (Sikes *et al*., 2002). Thus, a regulatory model has emerged in which transcriptional readthrough of gene segments is neither necessary nor sufficient for their recombination. Instead, the ACE function of promoters is necessary to induce localized changes in chromatin accessibility that facilitate recognition by the RAG complex. It remains likely, however, that

transcription can serve to either augment or to propagate recombinase accessibility beyond promoter-proximal regions.

Classic sterile transcripts initiate from either germline or V segment promoters and proceed in a sense direction through target gene segments and RSSs. However, a second form of germline transcription exists within the VH cluster. Corcoran and colleagues detected both genic and intergenic transcripts through the VH region in pro-B cells (Bolland *et al.*, 2004). These newly identified RNAs were expressed in an antisense orientation relative to the VH promoters and coding regions. Importantly, antisense transcription is developmentally regulated and correlates with the targeting of VH gene segments for recombination (i.e., activated subsequent to DH→JH recombination and extinguished following VH→DHJH rearrangement). Analogous to its function at the β-globin locus (Gribnau *et al.*, 2000), antisense transcription may play an important role in the initiation and/or propagation of remodeling events that extend chromatin accessibility over the broad VH region. These findings present several exciting avenues for future research including establishing the generality of antisense transcription at other antigen receptor loci, identification of the *cis*-acting elements that control antisense VH transcription, and establishing a causal relationship between antisense transcription and recombinase accessibility.

10. Transcription Factors Involved in Accessibility Control of Ig and TCR Loci

The *cis*-acting elements that regulate transcription and chromatin accessibility initially serve as conduits for TFs. In turn, the bound TFs recruit chromatin modifiers, nucleosome remodeling complexes, and the basal transcription machinery. A commonality exists in the mechanisms by which ACEs drive transcription and target recombination of antigen receptor genes. However, the regulation of these two processes also diverge because transcription, and in some cases local chromatin accessibility, is insufficient to target gene segments for recombination. These findings suggest that recombinase "accessibility" may require unique nuclear factors or topological constraints that are unimportant for gene expression. Although unique recombinase accessibility factors have yet to be identified, several TFs are clearly essential for widespread or regional accessibility within antigen receptor loci. The importance of these TFs in V(D)J recombination has been uncovered by numerous approaches, including classical molecular biology techniques, TF knockouts in mice, and knock-in mutation of TF sites. In this section, we focus on a limited panel of TFs that play fundamental roles in lymphocyte development and, in particular, the lineage- or stage-specific regulation of V(D)J recombination.

10.1. B Lymphopoiesis

A clear picture of the TF network that regulates pro-B cell commitment and subsequent development has emerged. The central players in this regulatory cascade are the TFs PU.1, E2A, EBF, PAX-5, and STAT5, the latter of which is activated by IL-7R signaling (Medina and Singh, 2005; Murre, 2005; O'Riordan and Grosschedl, 2000). The Ets family member PU.1 serves as the linchpin for B cell commitment. PU.1 is required to activate expression of the IL-7R receptor gene, which mediates pro-B cell survival (DeKoter *et al*., 2002). In turn, IL-7R signaling upregulates expression of E2A, which directly activates EBF transcription, perhaps in conjunction with PU.1 (Medina and Singh, 2005). The EBF and E2A factors cooperate to turn on expression of PAX-5, which simultaneously activates the gene expression program for B cells while repressing genes required for commitment to the T cell and myeloid lineages (Nutt *et al*., 1999). The TF components of this cascade coordinately regulate genes required for progression through pro- and pre-B stages of development, including RAG, Ig, surrogate light chain, and BCR coreceptors (Igα and Igβ) (Medina *et al*., 2004). One complication in establishing causal links between phenotypes in TF-knockout mice and accessibility control at Ig gene segments is the potential for indirect effects. For example, most of the essential TFs also regulate RAG expression. As such, observed defects in Ig gene assembly may simply reflect low or absent recombinase activity in the mutant pro-B cells. Nonetheless, substantial progress has been achieved on several fronts and we now provide a brief overview of each TF with emphasis on Ig locus regulation.

10.1.1. PU.1

The absolute level of PU.1 expression is a critical determinant of cellular commitment to either the myeloid or lymphoid lineage. Reconstitution of PU.1-null progenitors with low levels of PU.1 supports B cell differentiation, whereas clones expressing elevated levels commit preferentially to the myeloid lineage (DeKoter and Singh, 2000). The role of PU.1 in B cell specification is due, in part, to the upregulation of IL-7Rα. Retroviral transduction of the IL-7Rα gene is sufficient to overcome the B cell-differentiation block in PU.1-deficient progenitors (DeKoter *et al*., 2002). Mice harboring conditional knockout alleles have been used to demonstrate that PU.1 expression is dispensable for development beyond the pre-B cell stage, potentially due to functional redundancy with a second ETS family member called Spi-B (Polli *et al*., 2005).

To date, direct evidence is lacking for the involvement of PU.1 inaccessibility control of Ig loci because of its requirement for RAG expression.

However, PU.1 clearly binds to a cognate site in Eμ, which is essential for full transcriptional activation by the enhancer (Nelsen *et al*., 1993). Data indicate that PU.1 also enhances chromatin accessibility at proximal regions in chromosomal substrates (Marecki *et al*., 2004). Importantly, ectopic expression of PU.1 in non-B cells induced restriction enzyme sensitivity at Eμ as well as sterile transcription originating from within the enhancer (Nikolajczyk *et al*., 1999).

PU.1 also binds to 3′Eκ and regulates its function via recruitment of a coactivator, termed interferon responsive factor 4 (IRF4 or PIP-1) (Eisenbeis *et al*., 1995). PU.1 and IRF4 bind 3′Eκ only after the pro-B→pre-B cell transition, suggesting that this TF complex is a major determinant for stage-specific activation of Igκ (Pongubala *et al*., 1992; Shaffer *et al*., 1997). Similarly, binding of PU.1/IRF4 to a site in Eλ2–4 is required for expression of Igλ genes in terminally differentiated B cells (Eisenbeis *et al*., 1993). PU.1/Spi-B-deficient progenitors rescued by ectopic IL-7Rα expression exhibit normal levels of IgH and Igκ transcription (germline and rearranged) but are impaired for transcription/recombination at the Igλ locus (Schweitzer and DeKoter, 2004). Although these data are tantalizing, a direct function for PU.1 in accessibility control of Ig loci awaits targeted mutation of its cognate sites in enhancers and reconstitution of RAG expression in PU.1-deficient progenitors.

10.1.2. STAT-5 and IL-7R Signaling

The cytokine IL-7 was first identified as growth factor for pro-B cells (Lee *et al*., 1988). Accordingly, expression of its receptor, composed of the unique IL-7Rα subunit and the common gamma chain receptor, is restricted to the pro-B stage of development (Hardy and Hayakawa, 2001). Targeted deletion of the IL-7Rα gene imposes a block in B cell development precisely at the CLP→pro-B cell transition (Miller *et al*., 2002). When compared with wild-type cells, IL-7-deficient CLPs have markedly reduced levels of EBF and PAX-5 transcripts. The defect in PAX-5 expression and B cell specification can be rescued in these mutant cells following ectopic expression of EBF (Dias *et al*., 2005). Thus, the primary function of IL-7R signaling in B cell commitment likely reflects its requirement for EBF expression.

A role for IL-7R signaling in the process of IgH gene assembly was revealed by analyses of IL-7Rα$^{-/-}$ mice. In these animals, DH→JH recombination is normal but VH→DHJH recombination is inhibited in a position-dependent manner (Corcoran *et al*., 1998). The D-proximal VH segments are targeted for efficient rearrangement (7183 family), whereas germline transcription and recombination of distal VH segments (J558) is severely impaired in the IL-7Rα-deficient cells. Consistent with these genetic data, Bertolino *et al*. demonstrated

that the primary TF target of IL-7Rα signaling, STAT-5, associates with distal VH promoters in an IL-7-dependent manner. STAT-5 binding to these promoters recruits the Oct-1 TF and facilitates histone acetylation, germline transcription, and rearrangement of linked VH segments (Bertolino *et al*., 2005). Conversely, the stage-specific loss of IL-7R expression during the pro-B→pre-B cell transition plays an important role in the maintenance of allelic exclusion at IgH loci. Restoration of IL-7R signaling in mature B cells reactivates VH segments as judged by enhanced histone acetylation and chromatin accessibility (Chowdhury and Sen, 2003). Thus, the activation status of STAT-5 and IL-7R signaling regulates both the induction and repression of VH→DHJH recombination, at least for distal regions of this cluster.

10.1.3. E2A and EBF

The E2A gene encodes for two basic-helix-loop-helix (bHLH) TFs, called E47 and E12, via alternative mRNA splicing (Murre, 2005). EBF is an immediate downstream target of E2A gene products, and ablation of either E2A or EBF leads to a complete block at the earliest stage of B cell development, prior to the onset of DH→JH recombination (Bain *et al*., 1994; Lin and Grosschedl, 1995). In the context of IgH gene assembly, E2A knockouts exhibit a multifocal defect, including attenuated transcription of both RAG-1 and the germline DHJH cluster (Bain *et al*., 1994). A mechanistic basis for these phenotypes may be provided by the presence of several E2A sites (E-boxes) in Eμ and *cis*-acting elements that regulate RAG expression (Hsu *et al*., 2003; Nelsen *et al*., 1993).

In addition to its role in IgH germline transcription, several lines of evidence support an accessibility control function for E2A and EBF at Ig loci. The E2A sites in Eμ are essential for efficient rearrangement and germline transcription of transgenic recombination substrates (Fernex *et al*., 1995). Likewise, targeted mutagenesis of E2A sites in iEκ cripples Jk transcription and Vκ→Jκ rearrangement in pre-B cells (Inlay *et al*., 2004). Gain-of-function studies also reveal a critical function for E47 and EBF in activation of Ig gene assembly. Ectopic expression of E2A in pre-T cells and in human embryonic kidney (HEK) cells that express RAG-1/2 enhances DH→JH recombination (Romanow *et al*., 2000; Schlissel *et al*., 1991). In HEK cells, E2A and EBF also induce recombination at the Igκ and Igλ loci, respectively (Romanow *et al*., 2000). However, usage of Vκ and Vλ gene segments in these transfected cells is highly restricted. Neither germline transcription nor the presence of E2A/EBF sites in the V promoters appears to be sufficient for targeting recombination at a specific gene segment. Thus, induction of IgL gene assembly may be an indirect effect of E2A/EBF expression or may be mediated by sites not within the promoter

regions. A mechanistic link between E2A and chromatin accessibility is provided by its interaction with p300/CBP and the SAGA complex, both of which are potent histone acetyltransferases (Hesslein and Schatz, 2001; Murre, 2005).

10.1.4. PAX-5

Although E2A and EBF play crucial roles in B cell specification and Ig gene assembly, the paired box TF, PAX-5, is required for complete B lineage commitment. PAX-5 is a direct target of EBF, is exclusively expressed in B lineage cells (Barberis *et al.*, 1990; Medina and Singh, 2005), and its targeted deletion blocks B lymphopoiesis at the pro-B cell stage (Urbanek *et al.*, 1994). Bone marrow progenitors from PAX-5-deficient mice can differentiate into functional myeloid and T cells but not B lineage cells (Nutt *et al.*, 1999; Rolink *et al.*, 1999).

In addition to B cell specification, PAX-5 functions in the tissue-specific assembly of IgH genes. Enforced expression of PAX-5 in thymocytes activates rearrangement of proximal but not distal VH segments (Fuxa *et al.*, 2004; Hsu *et al.*, 2004). These gain-of-function data are completely consistent with studies from PAX-5 knockout mice, which exhibit normal levels of DH→JH and proximal VH→DHJH recombination in pro-B cells but a profound defect in the rearrangement of distal VH gene segments. Surprisingly, this block in distal VH rearrangement is uncoupled from histone hyperacetylation and germline transcription, which are unaffected in PAX-5$^{-/-}$ pro-B cells (Hesslein *et al.*, 2003). Instead, PAX-5 expression is required for large-scale compaction of the IgH locus, a process that presumably brings distal VH gene segments into proximity of DHJH joins (Fuxa *et al.*, 2004). Because PAX-5 expression in T cells fails to activate IgH locus contraction, Busslinger and colleagues have proposed that it must cooperate with a B cell-specific factor in this contraction process.

10.1.5. Pre-BCR Signaling

The pro-B→pre-B cell transition is characterized by a rapid cellular expansion, feedback inhibition of VH→DHJH recombination, and the initiation of Igκ gene rearrangements. These cellular and molecular processes are impaired on deletion of genes encoding any component of the pre-BCR (membrane IgM, Igα, Igβ, λ5, and VPRE-B) (Zhang *et al.*, 2004). The identity of endogenous ligand(s) for the pre-BCR, if any, remains unclear. In mice lacking surface IgM due to targeted deletion of its membrane exon (μMT mice), surface Igα/β expression is maintained and ligation of this coreceptor suppresses VH→DHJH and stimulates Vκ→Jκ recombination in the mutant pro-B cells (Maki *et al.*, 2000).

The full complement of downstream signaling molecules that mediate the pro-B→pre-B cell transition remains unclear; however, a series of three Src-related tyrosine kinases, called Blk, Lyn, and Fyn, have functionally redundant roles in this process (Saijo *et al*., 2003). Importantly, these kinases also mediate signals that differentially control assembly of the IgH and IgL genes. Expression of constitutively active Blk in μMT mice bypasses the pro-B cell developmental block, suppresses VH→DHJH recombination, and stimulates Igκ gene assembly (Tretter *et al*., 2003). A key issue for future studies will be to link these signaling pathways with the relevant TFs and chromatin modifiers that regulate recombinase accessibility at Ig loci.

10.1.6. Additional TFs

Compared with the IgH locus, much less is known regarding the TFs that activate recombination of IgL loci. Early studies identified consensus sites for the POU domain factors Oct-1 and -2 in nearly all VH and VL promoters (Ballard and Bothwell, 1986). An octamer site in Eμ is also critical for its full function in reporter gene assays (Staudt *et al*., 1986). The Oct-1 protein is expressed ubiquitously, while Oct-2 is restricted to the B cell lineage, suggesting that Oct-2 may play an important role in Ig gene regulation (Staudt *et al*., 1988). However, recruitment of Oct-1 by STAT-5 regulates activation of distal VH gene segments in response to IL-7R signaling (Bertolino *et al*., 2005). Transcriptional activation by either Oct factor requires an independent co-activator, called OcaB, which is expressed primarily in B lineage cells (Luo and Roeder, 1995). Despite these provocative links, transcription and recombination of the IgH locus are unaffected by dual deletion of the Oct-2 and OcaB genes (Corcoran *et al*., 1993; Schubart *et al*., 2001). In contrast, pre-B cells derived from OcaB-knockout mice are defective for germline transcription and recombination at a specific subset of Vκ gene segments (Casellas *et al*., 2002). These defects, however, do not stem directly from repression of general chromatin accessibility because the affected Vκ segments retain histone hyperacetylation and CpG hypomethylation. One possible explanation for this apparent paradox is that OcaB may recruit additional factors, which alter unique features of chromatin to further augment recombinase accessibility (e.g., a nucleosome-remodeling complex).

The inducible TF NF-κB was originally identified by its binding to a site in iEκ that is essential for its enhancer function in reporter gene assays (Lenardo *et al*., 1987; Sen and Baltimore, 1986). Subsequent studies revealed many forms of NF-κB, each of which are dimers of the Rel domain proteins NF-κ B1, NF-κB2, c-Rel, RelA, or RelB (Siebenlist *et al*., 2005; Sun and Ballard, 1999). These dimeric complexes regulate the expression of numerous genes

involved in lymphocyte development and inflammatory responses, including the RAG genes during IgL receptor editing (Verkoczy *et al.*, 2005). With regards to accessibility control, inhibition of NF-κB-transactivating subunits (RelA and c-Rel) blocks germline transcription and rearrangement of both IgL loci in pre-B cell models (Bendall *et al.*, 2001; Scherer *et al.*, 1996). However, assembly and transcription of Igκ genes is normal in pre-B cells harboring mutations at the κB site in iEκ or null mutations of the various Rel genes (Inlay *et al.*, 2004; Siebenlist *et al.*, 2005). Several explanations for this apparent paradox have been proposed. For example, an inherent difference may exist between the cell models and primary pre-B cells. In primary cells, activation by 3′Eκ may compensate for a crippled iEκ enhancer, but 3′Eκ is functionally inert in most cell models (Scherer *et al.*, 1996). Alternatively, the κB site may repress iEκ function in pro-B cells via its binding of NF-κB1/NF-κB2 dimers, which lack transactivation domains and recruit repressive HDACs (Sen, 2004). In the cell model experiment, only NF-κB1/2 are expressed in the nucleus on RelA/c-Rel repression. In normal pre-B cells, the transactivating subunits RelA and c-Rel predominate and may replace the NF-κB1/2 dimers to relieve repression (Shaffer *et al.*, 1997).

10.2. T Lymphopoiesis

Numerous TFs cooperate to guide T cell development via dynamic changes in gene expression and targeting of recombinase to regions within TCR loci. The molecular paradigm for T cell development overlaps, in part, with key features of B cell development. For instance, the E2A family of bHLH proteins are essential for the early stages of both B and T cell development (Murre, 2005). A distinctive feature of early T cell development is the requirement for signaling through the transmembrane receptor Notch 1, which is critical for T lineage specification from CLPs (Radtke *et al.*, 2004). Conditional deletion of the Notch 1 gene completely blocks T cell development at its earliest stages (Radtke *et al.*, 1999). Conversely, expression of a constitutively active form of Notch 1 in progenitors induced ectopic T cell development in the bone marrow while simultaneously blocking B lymphopoiesis (Pui *et al.*, 1999). Conditional inactivation of Notch 1 also produced a specific block in Vβ→DβJβ rearrangement (Wolfer *et al.*, 2002); however, the mechanistic basis for this recombinational defect remains undefined.

Unlike the linear cascade of TFs that regulate B cell development, the functional interactions between TFs involved in T cell differentiation and TCR gene assembly are more complex. Many of the important TFs have recurrent roles throughout T cell development or are members of TF families that have compensatory functions. In this section, we review our current

knowledge of the TFs that regulate ACE function within TCR loci and their role in accessibility control mechanisms.

10.2.1. E2A and HEB

Thymocytes predominantly express heterodimeric forms of bHLH factors, which are composed of E2A gene products and its related homologue HEB (Murre, 2005). A dominant negative form of HEB inhibits TCRβ gene assembly, specifically at the Vβ→DβJβ stage (Barndt *et al*., 2000). Whether this phenotype is related to accessibility control of Vβ gene segments by bHLH proteins remains to be determined. Studies indicate an important role for E2A in TCR repertoire development for γ/δ cells. Adult thymocytes from E2A knockout mice exhibit increased usage of "fetal" V gene segments (Vγ3 and Vδ1) and suppress rearrangement of segments normally used in adults (Vγ2 and Vδ5) (Bain *et al*., 1999). Parallel effects were observed for germline transcription at the relevant V segments. Moreover, coexpression of E2A or HEB with RAG-1/2 in HEK cells activates recombination at a restricted set of V, D, and J segments within the TCRγ and TCRδ loci (Ghosh *et al*., 2001). Taken together, these findings indicate that bHLH factors are functionally important for the regulation of recombinase accessibility during γ/δ T cell development.

10.2.2. STAT-5 and IL-7R Signaling

Germline deletion of the IL-7Rα gene severely impairs Vγ→Jγ recombination and transcription of Jγ gene segments (Maki *et al*., 1996). Subsequent studies have confirmed that the IL-7R/STAT-5 signaling axis directly affects chromatin accessibility of Jγ gene segments to recombinase. In addition to germline transcription, the IL-7Rα knockout leads to a profound reduction in histone acetylation and cleavage by recombinant RAG in nuclei from the mutant thymocytes (Huang *et al*., 2001; Schlissel *et al*., 2000; Ye *et al*., 2001). A potential mechanistic basis for these defects is the presence of essential STAT-5 sites in all Jγ promoters. The role of STAT-5 sites in 3′γ enhancer regions during TCRγ gene assembly has not been tested.

10.2.3. Signals and TFs that Mediate TCRβ Allelic Exclusion

On functional gene assembly, TCRβ proteins associate with the surrogate TCRα chain (pTα) and CD3 components, leading to surface expression of a pre-TCR on DN cells. The pre-TCR initiates signaling via its association with the tyrosine kinase p56lck, which activates the ZAP-70 kinase to target phosphorylation of the SLP-76 adaptor protein (reviewed in von Boehmer, 2005). This signaling cascade ultimately leads to the nuclear translocation of NFAT

and NF-κB TFs and induces differentiation into the DP compartment via β-selection. Pre-TCR signaling also regulates key aspects of TCR gene assembly, including allelic exclusion. Deletion of the pTα gene permits continued Vβ→DβJβ recombination even after a functional TCRβ allele is generated by a DN thymocyte (Aifantis *et al*., 1997).

Evidence supports the following model for regulation of allelic exclusion and β-selection by pre-TCR signaling. Prior to functional TCRβ gene assembly, p56lck is inactive, which permits ongoing Vβ→DβJβ rearrangement. Expression of a functionally joined TCRβ gene leads to surface expression of the pre-TCR, which activates p56lck and blocks further Vβ→DβJβ recombination. DN cells engineered to express a constitutively active form of p56lck fail to initiate Vβ→DβJβ recombination (Anderson *et al*., 1993). Subsequent phosphorylation of SLP-76 and activation of PKC also impinges upon allelic exclusion and proliferation of the DP subset (Aifantis *et al*., 1999; Michie *et al*., 2001). However, the signaling pathways for these two processes eventually bifurcate because downstream activation of Ras/Raf induces proliferation but does not affect allelic exclusion (Gartner *et al*., 1999).

The identities of TFs that mediate allelic exclusion have begun to emerge. Deletion of the Ets-1 gene breaks allelic exclusion mediated by the expression of a functional TCRβ transgene (Eyquem *et al*., 2004). The activity of functional E2A/HEB dimers decreases on pre-TCR signaling and, as noted in an earlier section, a dominant negative form of HEB blocks Vβ→DβJβ recombination (Barndt *et al*., 2000). Thus, pre-TCR-mediated reductions in E2A and increases in Ets-1 may block chromatin accessibility at Vβ gene segments during allelic exclusion.

10.2.4. Additional TFs

In contrast to studies in B cells, the targeted deletion of TF genes has generated limited information regarding the role of specific proteins in accessibility control at TCR loci. Our knowledge relies primarily on experiments involving transgenes or reporter substrates in which the TF sites have been mapped, mutated, and analyzed for functionality. One example of this approach is analysis of the germline promoter and enhancer elements that regulate accessibility of the DβJβ cluster. The PDβ1 germline promoter is completely enhancer dependent and possesses an array of binding sites for general TFs that cooperatively regulate its function (Doty *et al*., 1999; Sikes *et al*., 1998). Studies demonstrate that Eβ is required for loading a specific subset of these factors at PDβ1 (SP1 and ATF/CREB) (Spicuglia *et al*., 2002). The loading of other factors is either Eβ-independent or only modestly

enhanced by Eβ. In addition, Eβ directs the recruitment of specific chromatin modifiers, including HATs and nucleosome-remodeling complexes to the Dβ1 region (Spicuglia *et al*., 2002). These findings suggest that SP1 or ATF/CREB may be key TFs for switching PDβ from an off-state to an on-state and triggering Dβ→Jβ recombination.

In the context of transcriptional activation, the core Eβ consists of sites for GATA-3, bHLH TFs, and up to three sites for Runx1 (Takeda *et al*., 1990). Using a transgenic recombination substrate, Tripathi *et al*. have also defined the regions within Eβ that are essential for recombinase accessibility at linked gene segments. This recombinational activity is restricted to an ~100-bp region spanning two E-boxes and an ETS/Runx1 motif, the latter of which was dispensable for germline transcription (Tripathi *et al*., 2000). Using a similar approach, Krangel and colleagues defined the minimal Eα element required for V(D)J recombination of a TCRδ minilocus. This activity was restricted to a 116-bp fragment referred to as Tα1,2, which contains binding sites for TCF/LEF and ETS family members (Roberts *et al*., 1997). However, miniloci containing Tα1,2 rearrange earlier in ontogeny than those containing the full-length Eα element and Tα1,2 permits substrate assembly in both γ/δ and α/β T cells. Subsequent studies using the same approach defined the core Eα element (~275 bp), denoted Tα1–4, which is required for proper developmental regulation of substrate rearrangement (Balmelle *et al*., 2004). Comparison of Tα1,2 and Tα1–4 architecture suggests the involvement of SP1 and CREB members in this stage-specific regulation by Eα.

The ACE function of TCRδ *cis*-acting elements has been analyzed using transgenic miniloci. Mutational analyses of Eδ in these miniloci revealed a critical function for c-Myb and CBF/PEBP2 in substrate recombination. A subfragment of Eδ containing sites for CBF/PEBP2, GATA-3, and c-Myb is sufficient for transcriptional activation in reporter genes but insufficient to mediate recombination of miniloci (Hernandez-Munain *et al*., 1996). These findings indicate that c-Myb and CBF/PEBP2 must collaborate with additional factors bound to the Eδ region for establishing full accessibility to V(D)J recombinase (Lauzurica *et al*., 1997). In addition to Eδ, a strong transcriptional promoter is situated upstream of the Dδ2 gene segment (Carabana *et al*., 2005). By analogy with other germline promoters, this PDδ2 element may serve as an ACE to direct highly localized changes in chromatin accessibility. A functional dissection of the promoter revealed that the URF1, RUNX1, and c-Myb factors bind PDδ2 *in vivo* and are required for its maximal activity in reporter gene assays. In the absence of Eδ, the PDδ2 promoter retains low levels of transcriptional activation and may direct residual TCRδ recombination in Eδ-deficient mice (Carabana *et al*., 2005).

11. Control of Recombinase Accessibility by Chromatin Modifications and Remodeling

The biologic action of V(D)J recombinase at a given antigen receptor locus is tightly regulated by ACEs. These ACEs endow loci with an open configuration that facilitates formation of the requisite nucleoprotein complexes. One category of ACEs, enhancer elements, has a long-range effect on chromatin accessibility that can span entire clusters of gene segments (Oestreich *et al*., 2006). In contrast, the ACE function of germline promoters is more spatially restricted (Hawwari *et al*., 2005; Sikes *et al*., 1998). In antigen receptor loci, these two types of ACEs are separated by large distances and communicate through space via the formation of stable promoter/enhancer holocomplexes (Liu and Garrard, 2005; Oestreich *et al*., 2006). The ACE holocomplex may focus high concentrations of chromatin modifiers and remodeling complexes at regions containing target gene segments.

Although it is clear that accessibility to V(D)J recombinase requires chromatin remodeling, the epigenetic and biochemical mechanisms involved in this process are just beginning to emerge. Similar to studies of gene expression, numerous correlations now exist between chromatin modifications, nuclease sensitivity, and V(D)J recombination. Despite these links, causal relationships between many of these processes have not been established. Moreover, tantalizing new data suggest that RAG proteins play a more direct role in bridging chromatin and recombination because the C-terminus of RAG-2 binds directly to histones *in vitro* (West *et al*., 2005). This may translate *in vivo* to a regulatory scheme in which the RAG complex associates with higher affinity to histones bearing specific modifications, increasing the local concentration of recombinase at specific RSSs. In this section, we describe our current knowledge regarding the functional relationships between chromatin modifications, nucleosome remodeling, and V(D)J recombination.

11.1. CpG Methylation

Methylation of CpG dinucleotides is an important component of many mechanisms that enforce heritable silencing of genetic loci. Accordingly, hypermethylated regions within the genome generally adopt inaccessible chromatin configurations (Vermaak *et al*., 2003). The repressive nature of this DNA modification is likely due to the recruitment of methyl-CpG–binding proteins, such as MeCP2, which interact with HDAC activities and can recruit nucleosome-remodeling complexes that establish a repressive chromatin environment (Fuks *et al*., 2003b; Nan *et al*., 1998). Consistent with this model, gene segments located in regions of CpG hypermethylation are usually silent

with respect to V(D)J recombination (Bergman *et al*., 2003). Thus, erasure of CpG methylation is thought to be a prerequisite for the establishment of a recombinase accessible locus.

A primary function of ACEs may be to target demethylation or to protect gene segments from *de novo* methylation (Demengeot *et al*., 1995; Mostoslavsky *et al*., 1998). Deletion of either PDβ1 or Eβ from the endogenous TCRβ locus produces a dramatic increase in CpG methylation and a corresponding decrease in nuclease sensitivity within the DβJβ cluster (Mathieu *et al*., 2000; Whitehurst *et al*., 2000). In one reported instance, CpG methylation was shown to directly suppress V(D)J recombination. The 3′Dβ1-RSS contains a CpG dinucleotide. Analysis of joins at TCRβ loci lacking PDβ1 suggested that methylation at this dinucleotide is incompatible with RAG-mediated cleavage (Whitehurst *et al*., 2000). Subsequent *in vitro* studies confirmed this interpretation (Nakase *et al*., 2003). However, because the vast majority of RSSs lack a CpG motif, the primary effect of CpG methylation at antigen receptor loci likely is to inhibit chromatin accessibility of gene segments to recombinase.

Changes in DNA methylation play a dual regulatory role at the Igκ locus, ensuring its stage-specific activation while restricting functional rearrangement to a single Igκ allele (Bergman and Cedar, 2004). During the pro-B→pre-B cell transition, a single, randomly selected Igκ allele undergoes demethylation within the JκCκ region (Mostoslavsky *et al*., 1998). The demethylated allele exhibits numerous hallmarks of an accessible locus, including early replication, germline Jκ transcription, and histone hyperacetylation (Goldmit *et al*., 2005). In contrast, the remaining hypermethylated allele associates with repressive chromatin and is decorated with the heterochromatin protein, HP1. Monoallelic demethylation is enhancer dependent (iEκ and 3′Eκ are required) and the hypomethylated allele is targeted for the vast majority of Vκ→Jκ recombination (Mostoslavsky *et al*., 1998). Thus, ACE-mediated demethylation may be a primary mechanism for maintaining allelic exclusion at Igκ. An interesting goal for future studies is to understand the factors that govern monoallelic choice and how the second Igκ locus becomes activated if the first allele is nonproductive.

11.2. Histone Modifications

In general, genes segments within recombinationally active loci exhibit the same pattern of histone modifications that characterize expressed genes. For example, recombinogenic DβJβ clusters in pro-T cells possess high levels of H3-K9 and H4 acetylation, high levels of H3-K4 methylation, but low levels of H3-K9 methylation (Morshead *et al*., 2003; Tripathi *et al*., 2002). The opposite

pattern of histone modifications is seen at the DβJβ region in pro-B cells (Morshead *et al.*, 2003). These correlative data strongly suggest that the histone code is a primary determinant in controlling tissue-, stage-, and allele-specific changes in chromatin accessibility to the RAG complex.

11.2.1. Histone Acetylation

Numerous correlations have emerged between H3/H4 acetylation and the recombination potential of antigen receptor gene segments (Chowdhury and Sen, 2003; Espinoza and Feeney, 2005; McMurry and Krangel, 2000; Morshead *et al.*, 2003). These data suggest that histone hyperacetylation is a necessary component of recombinase accessibility; however, definitive cause/effect relationships between these two processes have not been established. A growing body of evidence indicates that histone hyperacetylation clearly is not sufficient for targeting rearrangements. Deletion of the PDβ1 germline promoter left histones hyperacetylated over most of the Dβ1Jβ cluster, but these gene segments fail to rearrange efficiently (Oestreich *et al.*, 2006; Whitehurst *et al.*, 2000). A similar disconnect between hyperacetylation and recombination was reported for distal VH gene segments in pro-B cells from PAX-5-deficient mice (Hesslein *et al.*, 2003).

Hypoacetylation of H3/H4 is clearly a feature of recombinationally inert gene segments. The conversion from a hyper- to a hypoacetylated status at H3-K9 appears to be an important component of allelic exclusion. During the pro-B→pre-B cell transition, the loss of IL-7R signaling leads to a simultaneous reduction in acetylation levels and chromatin accessibility at VH gene segments (Chowdhury and Sen, 2003). A similar reduction in acetylation is observed at Vβ segments during the DN→DP transition (Tripathi *et al.*, 2002). Little is known about the HATs and HDACs that mediate changes in the acetylation status of antigen receptor loci. However, deletion of Eβ perturbs the ratio of HAT complexes at the germline promoter region, leading to an increased occupancy by P300 at the expense of CBP and PCAF (Spicuglia *et al.*, 2002). The shifting balance of HAT complexes may alter the precise array of H3 and H4 lysine residues that are targeted for acetylation and thereby fail to present the proper docking platform for requisite chromatin-remodeling complexes (Agalioti *et al.*, 2002).

11.2.2. H3-K9 Methylation

Chromatin at recombinationally inert loci is invariably hypoacetylated at H3-K9 but is enriched for methylation on this histone residue (Johnson *et al.*, 2004; Morshead *et al.*, 2003). For example, VH segments display a tissue-specific difference in dimethyl H3-K9, with a hypermethylated status in thymocytes

and nonlymphoid cells versus a hypomethylated/hyperacetylated status in pro-B cells (Johnson *et al*., 2004). This tissue-specific erasure of H3-K9 methylation at VH segments requires the expression of PAX-5 in pro-B cells. Thus, PAX-5 potentially regulates VH→DHJH recombination at two distinct levels—IgH locus contraction and revision of chromatin modifications at the VH cluster (Fuxa *et al*., 2004; Johnson *et al*., 2004). It will be interesting to determine whether a mechanistic link exists between these two processes. In contrast to its tissue-specific regulation, H3-K9 dimethylation apparently is not involved in stage-specific control of VH→DHJH recombination because the VH cluster remains hypomethylated in pre-B cells after allelic exclusion inhibits their recombination (Johnson *et al*., 2004). However, the status of trimethyl H3-K9, a modification that has been implicated in more stable forms of gene repression, has not been examined at the VH or any other gene segment cluster.

Unlike other histone modifications, a direct cause/effect relationship between H3-K9 methylation and recombinase accessibility has been established using a TCRβ minilocus. Recruitment of the G9a histone methyltransferase (HMT) to active chromosomal substrates cripples both germline transcription and Dβ→Jβ recombination even when functional ACEs are present (Osipovich *et al*., 2004). The repressive effects of G9a recruitment on histone modifications and substrate accessibility are highly localized and reversible in nature. These features are reminiscent of the transient silencing induced at the TdT and RAG loci in DP thymocytes, where only small regions proximal to their promoters are reversibly methylated at H3-K9 (Su *et al*., 2004). In contrast, persistent and widespread H3-K9 methylation occurs during heritable silencing of these genes at the DP→SP transition. It remains possible that pro-B cells employ a similar strategy to rapidly establish inaccessible chromatin at VH segments for allelic exclusion (and perhaps pro-T cells for Vβ segments). This may occur by recruitment of an HMT to establish highly localized regions of H3-K9 dimethylation at VH segments, which would rapidly extinguish their accessibility to recombinase. A more stable form of repression may develop on differentiation to the pre-B cell stage via widespread distribution of trimethyl H3-K9 and CpG methylation throughout the entire VH cluster. A major goal for future studies will be to decipher the temporal dynamics of chromatin modifications in developing lymphocytes and identify the HMTs that target loci and silence inappropriate rearrangement of their composite gene segments.

11.2.3. H3-K27 Methylation

Methylation of H3-K27 normally associates with the stable repression of transcription units (Peters *et al*., 2003). The methyl-H3-K27 mark is imprinted by Polycomb group proteins, such as the Ezh2 methyltransferase, which is

a critical component of the PRC2 repressor complex (Cao *et al.*, 2002; Kuzmichev *et al.*, 2004). To date, there have been no reports of H3-K27 methylation status at recombinase accessible versus inaccessible antigen receptor loci. This may be due to the limited utility of available antibodies for ChIP assays. However, the Ezh2 gene has been deleted specifically in B lineage cells using a conditional knockout approach. Surprisingly, the Ezh2 deletion inhibits rearrangement of distal VH gene segments but has no effect on their germline transcription or histone acetylation (Su *et al.*, 2003). Ablation of Ezh2 reduces the overall levels of histone methylation at distal VH segments, but it remains unclear whether this decrease corresponds to methylation at the H3-K27 residue. Because Ezh2 and H3-K27 methylation are normally repressive, this unexpected finding may reflect an indirect rather than a direct effect of the HMT on recombinational control at the distal VH cluster.

11.3. Nucleosome Remodeling

In vitro studies have clearly established that positioned nucleosomes form potent barriers for RAG-mediated cleavage of substrates (Kwon *et al.*, 1998). This reductionist approach also revealed that the precise phasing of a nucleosome relative to an RSS profoundly influences the efficiency of RAG cleavage. Importantly, treatment of nucleosomal substrates with Brg1, the ATPase component of many SWI/SNF remodeling complexes, rescues RAG cleavage (Kwon *et al.*, 2000). These studies are even more exciting given the finding that many RSSs have an intrinsic nucleosome positioning function, which may provide an inherent protection from inappropriate recombination until the associated nucleosome is remodeled (Baumann *et al.*, 2003). Thus, recombinase accessibility at compatible RSSs almost certainly relies on the reorganization of resident nucleosomes via the action of ACEs.

In this regard, a subset of histone modifications (e.g., acetylation), as well as the basal transcription machinery itself, can recruit SWI/SNF complexes to sites of active transcription (Hassan *et al.*, 2001a). ChIP studies have revealed that the catalytic component of this remodeling complex, Brg1, is broadly associated with clusters of gene segments that are poised for recombination (Morshead *et al.*, 2003). Importantly, this association is enhancer dependent for the DβJβ cluster in pro-T cells (Spicuglia *et al.*, 2002). A medium resolution map of nucleosomes at the DβJβ cluster suggests that deletion of Eβ may increase nucleosome density at the recombinase inaccessible Dβ segment (Spicuglia *et al.*, 2002). Despite these advances, large gaps exist in our knowledge of the genetic and epigenetic requirements for recruitment of remodeling complexes to antigen receptor loci. Likewise, the ACE-dependent features

of nucleosome organization and reorganization that occurs at targeted gene segments needs to be addressed.

An independent readout for changes in nucleosome structure and accessibility is the sensitivity to digestion by restriction endonucleases (RE). However, this technique cannot distinguish between changes in nucleosomal positions and alterations to the structure of a fixed nucleosome, both of which lead to more accessible configurations. Notwithstanding, these assays have proven useful for judging the effects of ACEs on chromatin accessibility at specific sites within antigen receptor loci (Mathieu *et al*., 2000). For example, we have used RE sensitivity to demonstrate that in the absence of PDβ1 the Eβ enhancer has an intrinsic ACE function (Oestreich *et al*., 2006). This function generates a nearly full level of chromatin accessibility throughout both DβJβ clusters, with the striking exception of the Dβ1 gene segment, which remains inaccessible in promoterless loci. These findings suggest that one or two nucleosomes associated with the Dβ1 region uniquely require the ACE function of PDβ1 for additional remodeling. Thus, the entire process of TCRβ gene assembly may hinge on PDβ-directed remodeling of a single, perhaps fixed, nucleosome associated with the Dβ-RSS.

12. Concluding Remarks

V(D)J recombination is the only known mechanism for site-specific alteration of the genome in somatic cells. One reason for its unique status is the inherent danger of generating and repairing double-strand DNA breaks, especially in hematopoietic cells that naturally undergo rapid expansion. Thus, although V(D)J recombination is critical for the development of a fully functional immune repertoire, this process must be tightly regulated to avoid aberrations that give rise to chromosomal translocations and lymphoid tumors. In this regard, studies focused on understanding accessibility control of V(D)J recombination have experienced a reawakening in the past several years. This revival, in large part, was the result of technical advances that allowed researchers to characterize changes in chromatin modifications and nuclear architecture in much greater detail.

Together with gene targeting technology, these FISH and ChIP studies have produced a clearer picture of how various regions within Ig and TCR loci are targeted for assembly. In general, antigen receptor loci are associated with the nuclear periphery, a repressive environment for chromatin, in nonlymphoid cells and primitive progenitors. On lineage commitment, the appropriate locus becomes activated via its whole-scale movement to a more permissive environment near the center of the nucleus. In the context of V→DJ recombination, a higher level of topological control exists, in which the locus must undergo

compaction to bring the V clusters into spatial proximity with distal gene segments. A subsequent decontraction process distances the V segments and may be an integral component of allelic exclusion mechanisms. Coincidental with these large-scale changes, transcriptional promoters and enhancers are activated and serve as ACEs that function individually or synergistically. These split ACEs communicate through space to form stable holocomplexes that, in turn, regulate local accessibility of gene segments to recombinase. The mechanisms of ACE function clearly involve the revision of histone modifications either locally or in a long-range manner. These ACEs also must recruit chromatin remodeling complexes to loosen the grip of associated nucleosomes and thereby expose neighboring RSSs to the recombinase complex.

Despite these tremendous advances, many fundamental questions remain to be addressed. Studies by Krangel and colleagues demonstrate that, at least for allelic exclusion, V(D)J recombination is subject to additional constraints beyond general chromatin accessibility (Jackson *et al.*, 2005). The targeted insertion of Eα near a Vβ gene segment renders it transcriptionally active, hyperacetylated, and sensitive to RE digestion in DP cells; however, this gene segment remains refractory to recombinase activity. A potential explanation for this finding is that Eα cannot override the potent silencing of recombination from either nuclear relocalization or chromosomal decontraction. Similarly, it remains unknown whether RAG complexes are compartmentalized to any extent in the nucleus of precursor lymphocytes. Insertion of Eα may fail to block relocalization of the Vβ cluster away from such a RAG-rich area.

In a more general sense, the molecular mechanisms by which V segment clusters are activated and subsequently suppressed to maintain allelic exclusion remain a complete mystery. The processes of locus contraction/decontraction likely regulate the efficiency of recombination at these more distal segments but the next daunting challenge is to establish cause/effect relationships. A great deal of work also remains on the genetic and epigenetic fronts of recombinational control, especially for V segment clusters. Another major goal of future studies will be to decipher the histone code for recombinase accessibility and the TFs/chromatin modifiers involved in laying this code. This will certainly be a long-term effort because the specific factors are likely different for each locus or even clusters within a locus. A related goal is to map nucleosome positions and the effects of ACEs on nucleosomes associated with target RSSs. Finally, we need to generate a clearer understanding of the mechanisms for long-range communication between ACEs and identify the ACE-binding factors that bridge holocomplex formation.

By analogy to the explosion of knowledge over the past several years, new insights will likely rely on technical advances. In this regard, two important

advances would be: (1) RNAi-mediated knockdown of gene expression in primary lymphocytes for studies of protein function, and (2) the advent of microarrays spanning the mouse genome for use in "ChIP-on-chip" studies of histone modifications and factor binding. The momentum in the area of accessibility control bodes well for answering many of these fundamental questions in the near future.

Acknowledgments

We are grateful to numerous colleagues for their valuable discussions over the years and apologize to any colleagues whose work may not have been cited. This work was supported by the following grants from the National Institutes of Health: P01 HL68744 and CA100905 to E.M.O., T32 HL69765 to R.M.C., T32 CA09385 to K.J.O., and a Cancer Center Support Grant (P30 CA68485) to the Vanderbilt-Ingram Cancer Center.

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Targeting Integrin Structure and Function in Disease

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Abstract

Initially linked to the pathogenesis of inflammatory and hematologic diseases, integrins have become validated drug targets with the approval of five drugs. Moreover, there are several promising drug candidates in preclinical and clinical stages of development for multiple clinical indications. Integrins are attractive drug targets as their antagonism can block several steps in disease progression or maintenance. Integrin inhibitors can block the proliferation, migration, or tissue localization of inflammatory, angiogenic, and tumor cells, as well as signaling and gene expression contributing to disease. There has been a rapid increase in the elucidation of integrin structure, their allosteric mechanisms of bidirectional signaling, and the structure of complexes with drugs. This information brings greater focus to how integrins support various cellular functions and how they have been and may be targeted to develop novel drugs. Here we review conformational switches, including an internal ligand, which allosterically regulate the transition from low- to high-affinity ligand binding. We address some of the successes, disappointments, and challenges in targeting competitive or allosteric sites to develop therapeutics. We also discuss new opportunities, including a structure-based approach to discover novel drugs to treat inflammatory and other diseases. This approach

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ADVANCES IN IMMUNOLOGY, VOL. 91 0065-2776/06 $35.00

DOI: 10.1016/S0065-2776(06)91003-7

targets structural relatives of the von Willebrand factor A-domain present in integrins and many functionally diverse proteins.

1. Introduction

Integrins are a large family of complex, multidomain cell adhesion receptors. They are heterodimeric receptors, which through the various pairing of 18 α- and 8 β-subunits, comprise a family of 24 members (Table 1) (Hynes, 1992). The family is extended through alternative splicing of certain members (von der Mark *et al.*, 2002; Yeh *et al.*, 2003). They possess 12 or 13 tertiary structure ectodomains and can be structurally divided as those that do (9 integrins) or do not possess an α-subunit A- or I-domain. The overall structure of integrins initially visualized by electron microscopy (EM) is a globular head domain presented on two long stalks (Nermut *et al.*, 1988).

Integrins may be loosely grouped as those that bind cell surface or different classes of extracellular matrix (ECM) proteins (Table 1). Certain integrins, however, can bind a large repertoire of ligands of different classes including soluble ligands. In response to cell activation expression of cell surface ligands are induced, for example, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1. Cryptic integrin-binding sites in ECM are exposed through protease activity during processes such as remodeling. Integrin ligands generally form a multivalent substrate that drives integrin clustering stimulating signaling responses and "adhesion strengthening" (Miyamoto *et al.*,

Table 1 Representative Integrin Ligands Are Grouped by Ligand Class

Basal ECM	Provisional ECM	Cell surface CAMs
$\alpha1\beta_1$ Col, LN	$\alpha11b\beta_3$ Fg, vWF	$\alpha L\beta_2$ ICAM-1, -2, -3, RAGE, JAM-1
$\alpha2\beta_1$ Col, LN	$\alpha5\beta_1$ FN	$\alpha M\beta_2$ ICAM-1, JAM, Fg, iC3b
$\alpha10\beta_1$ Col	$\alpha8\beta_1$ FN, TN	$\alpha X\beta_2$ ICAM-1, JAM, Fg, iC3b
$\alpha11\beta_1$ Col	$\alpha v\beta_1$ FN, TN	$\alpha D\beta_2$ VCAM-1, ICAM-3
$\alpha3\beta_1$ LN, Tsp	$\alpha v\beta_3$ FN, TN, Tsp, vWF, (Col)	$\alpha4\beta_1$ VCAM-1, FN
$\alpha6\beta_1$ LN	$\alpha v\beta_5$ VN, Del-1	$\alpha4\beta_7$ VCAM-1, MadCAM-1, FN
$\alpha6\beta_4$ LN	$\alpha v\beta_6$ FN, TN	$\alpha E\beta_7$ E-cadherin
$\alpha7\beta_1$ LN	$\alpha v\beta_8$ FN, Col, LN	
$\alpha9\beta_1$ Col, LN, TN		

Col, collagen; LN, laminin; Tsp, thrombospondin; TN, tenasin; JAM, junctional adhesion molecule; ICAM, intercellular adhesion molecule; Fg, fibrinogen; FN, fibronectin; VCAM, vascular cell adhesion molecule; MadCAM, mucosal addressin cell adhesion molecule; vWF, von Willebrand factor; (Col), cryptic collagen site.

1995). ECM, cell surface ligands, and snake venom disintegrins possess integrin-binding site motifs with an essential acidic residue, for example, RGD, IEL, or LDV (Ruoslahti, 1996; Staunton *et al.*, 1990; Vonderheide *et al.*, 1994). These motifs have been exploited for structure-based drug design.

Differentially expressed on various cell types, integrins can be restricted to a single cell type such as platelet αIIbβ3. They can be cell lineage restricted such as the four leukocyte integrins that share a common β2 or CD18-subunit, αLβ2, αMβ2, αXβ2, and αDβ2. Others may have broad cell type distribution such as α5β1. The expression of some integrins and their ligands are modulated by cellular activation during normal or disease states. For example, αVβ3 and α4β1 are induced *de novo* in endothelial cells during angiogenesis and the expression of lymphocyte α1β1 and α2β1 is induced as they migrate into inflamed tissues.

Functions supported by integrins include immune response, hemostasis, wound healing, and tissue remodeling. Underlying these functions is their capacity to mediate firm or stable cell adhesion, rolling adhesion under shear stress, migration, and cellular signaling leading to regulation of gene expression, cell survival, or proliferation (Hynes, 1992). These different activities involve different states of integrin affinity that are regulated allosterically and bidirectionally.

Integrin-dependent binding requires modulation of its activation state from low to intermediate to high affinity. Regulation of leukocyte trafficking and hemostasis are quintessential examples of this dynamic integrin ligand-binding activity. Rapid triggering of the high-affinity state is essential for appropriate localization of platelets or leukocytes at endothelial sites of injury or inflammation. Induction of a high-affinity state, that is "inside-out" signaling, results from agonists signaling through various receptors such as chemokine G-protein–coupled receptors (GPCR). Occupation of only 10–100 IL-8 receptors is sufficient for a rapid conversion of 20,000 integrin heterodimers from low- to high-affinity state (Lum *et al.*, 2002). In a subsecond time frame, cytoplasmic activators, such as talin, bind to the integrin tails and induce a shift from a low- to a high-affinity ectodomain conformation (Kinashi, 2005; Whittaker and Hynes, 2002). The increase in affinity appears to be primarily a function of an approximate tenfold or greater decrease in dissociation rates with a relatively small change in association rates (Jun *et al.*, 2001; Lupher *et al.*, 2001; Zwartz *et al.*, 2004).

Affinity modulation is the primary event initiating firm adhesion. The upregulation of leukocyte integrin affinity is an early event, independent of actin rearrangement and preceding multivalent ligand-driven integrin clustering or adhesion strengthening (Beals *et al.*, 2001; Kim *et al.*, 2003). High affinity reverts back to low affinity through several mechanisms including clearance or decreased production of agonists and production of antagonists like TGFβ. (Smith *et al.*, 1996). In migrating cells, mechanisms for modulating integrin

binding must regulate nascent adhesion occurring at the leading edge lamellipodia and release at the uropod (Ridley *et al.*, 2003). Thus, integrin-ligand binding at cell contacts is dynamic and exchange occurs with integrins recruited from outside the contact site. Some integrins, including αLβ2, α4β1, and α7β1, demonstrate an intermediate-affinity state that appears to limit their potential ligand repertoire to their highest affinity ligands and allow certain functions such as "rolling" adhesion under shear stress (Alon *et al.*, 1995; Berlin *et al.*, 1995; Tang *et al.*, 2005; Yeh *et al.*, 2003). Moreover, integrin inside-out signaling through a connection with cellular contractile processes mediates remodeling of the ECM (Bokel and Brown, 2002; Ridley, 2004).

Ligand binding induces conformational changes in integrins and intracellular signaling, referred to as "outside-in" signaling. Outside-in signaling supports cell survival, proliferation and more specifically, immune responses. With the exception of β4, integrins possess short cytoplasmic tails that bind directly or indirectly to a large number of cytoplasmic kinases, scaffolds, and cytoskeletal proteins that support signals to and from integrins (Liu *et al.*, 2000; Yamada and Miyamoto, 1995). Prominent are tyrosine kinases such as focal adhesion kinase (FAK) and Src family members. FAK can serve as both a scaffold and kinase and initiate signaling cascades including the mitogen-activated kinase (MAPK) pathway. Different Src family members bind to various β-subunit tails. C-Src binds constitutively to the β3 integrin tail, and on integrin clustering outside-in activation is initiated via Src *trans*-autophosphorylation (Arias-Salgado *et al.*, 2003).

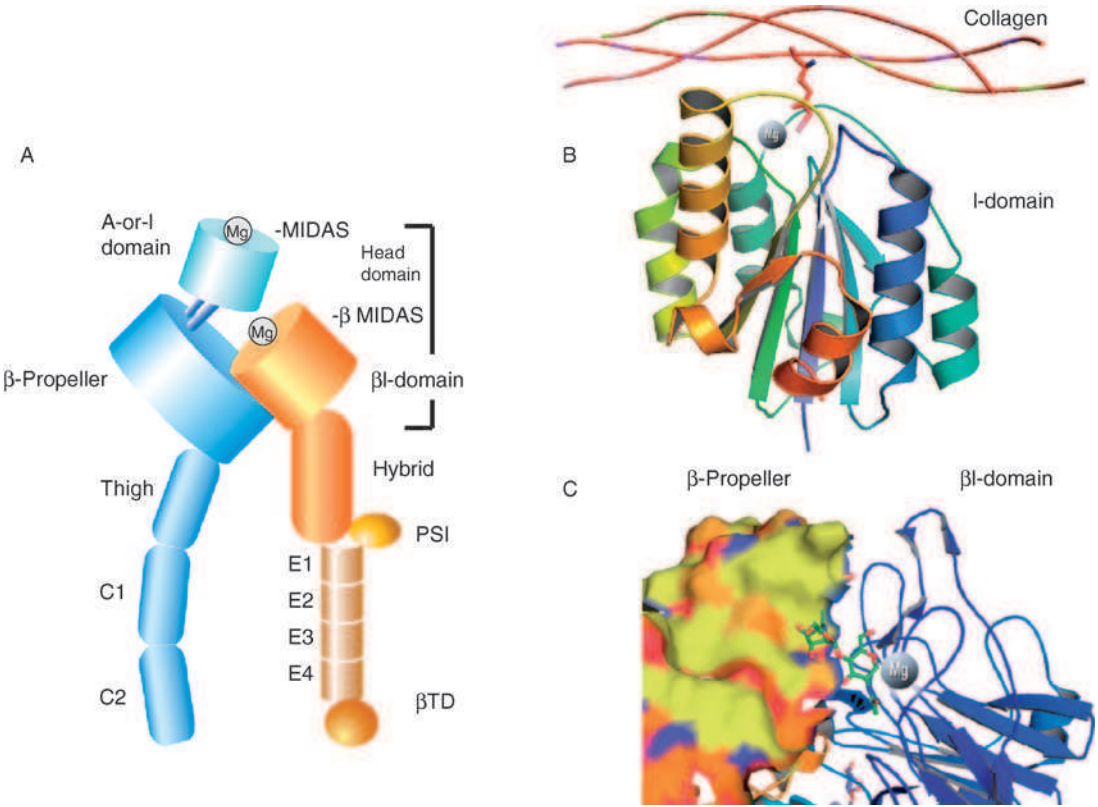
Integrin-dependent cell survival is induced through signaling pathways such as PI3K/AKT/Bcl-2. Conversely, release from an integrin substrate can result in de-adhesion-induced apoptosis or anoikis (Valentijn *et al.*, 2004). Integrin-dependent survival signaling protects cells from stresses such as lack of growth factors (GF). In this manner, blocking α4β1 increases the sensitivity of tumor cells to cytotoxic drugs (Matsunaga *et al.*, 2003). Likewise, antagonism of αVβ3 confers sensitivity of quiescent colon tumor cells to apoptosis induced by a MAPK pathway inhibitor (Burbridge *et al.*, 2003). One mechanism for tumor cell resistance to cytotoxic drugs, such as cisplatin, may involve increases in integrin-mediated adhesion and signaling (Nakahara *et al.*, 2003).

Integrin-dependent signaling in response to changes in ECM may facilitate cellular proliferation and transformation. Integrins act as mechanosensors such that stress applied to an integrin induces changes in cytoskeletal organization and nuclear form (Walker *et al.*, 2005). Consequently, nuclear transport of transcription factors is altered, cotranscription factors can be released from G actin, enter the nucleus, and activate immediate early genes and cell cycle entry. During chronic inflammation or tissue remodeling, there is matrix metalloproteinase (MMP) expression, degradation of basal matrix, and deposition of provisional matrix proteins. In this environment integrin ligands and

utilization changes, epithelial cells can depolarize and there are increases in Rho activity, Rac activity, mitochondrial superoxide, genomic instability, and response to epidermal growth factor (EGF) (Comoglio and Trusolino, 2005; Coussens *et al.*, 2002; Paszek *et al.*, 2005; Radisky *et al.*, 2005). Thus, integrin-sensing changes in the ECM appears to contribute to transformation. Engagement of certain integrins increases GF responses, which is consistent with hyperproliferation of keratinocytes, smooth muscle cells, and myofibroblasts in psoriasis, rheumatoid arthritis, and fibrotic diseases.

Central to an immune response is the formation of a highly organized signaling complex, the immune synapse, at the contact between antigen-presenting cells and T cells (Bromley *et al.*, 2001; Monks *et al.*, 1998). In the process of forming this complex, αLβ2 binds its cell surface ligands early and promotes maturation of the synapse. T cell receptor (TCR) signaling increases αLβ2 affinity or avidity and an integrin-rich peripheral ring, the p-SMAC, forms around the TCR-signaling complex. Engagement of αLβ2 enhances a TCR-mediated proliferation and cytokine production (Fan *et al.*, 1993; Wang *et al.*, 2006). Engagement of αLβ2 activates Src family tyrosine kinases, GTP exchange factors, GTPases, such as VAV and Rac-1, p44/42 MAPK, and PI3K (Perez *et al.*, 2004; Sanchez-Martin *et al.*, 2004).

Integrin-mediated signaling is cooperative with many associated plasma membrane proteins. Several integrins associate with one or more GF receptors (Brown, 2002). These associations can result in GF-dependent enhancement or GF-independent activation of GF receptors (Comoglio *et al.*, 2003). Several integrins can signal through two pathways, activating ERK or Jun terminal kinase (JNK), to facilitate cellular activation and proliferation by GF receptor tyrosine kinases (Giancotti and Ruoslahti, 1999; Lee and Juliano, 2004; Walker *et al.*, 2005). GF are relatively weak JNK activators and, thus, the contribution by integrins may provide sufficient activation resulting in AP1 formation and immediate early gene regulation for cell cycle progression. In addition to GF receptors, the activity of integrins and many associated membrane proteins are bidirectionally regulated. Associated proteins include ion channels, proteases, protease-activator receptors (uPAR), amino acid transporters, IgG Fc receptors, and tetraspanins (Brown, 2002; Cherubini *et al.*, 2005). Integrin conformation and ligand binding activity as well as site of ligand interaction may be directly regulated by association with a tetraspanin or uPAR (Nishiuchi *et al.*, 2005; Wei *et al*., 2005). Integrin signaling or cell surface recycling is facilitated through localization to different membrane-signaling sites such as lipid rafts and/or tetraspanin webs (Fabbri *et al.*, 2005; Hakomori, 2004; Hogg *et al.*, 2003). Thus, through several routes integrins influence cellular, mechanical, and chemical responses that converge on regulation of gene expression and cell cycle regulation.


A
A-or-I domain
Mg
-MIDAS
Head domain
-β MIDAS
Mg
β-Propeller
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C2
βTD
B
Collagen
Mg
I-domain
C
β-Propeller
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In vascular, inflammatory, autoimmune, or hyperproliferative diseases, integrin function can become dysregulated and contribute to pathogenesis. Integrin binding supports the development of tumor vasculature, tumor growth, and metastasis. They support the recruitment, activation, and effector functions of inflammatory leukocytes. How certain integrins function in the pathogenesis of diseases and how they have been targeted in the context of structure-function elucidation is discussed later. There have been several excellent reviews of integrin structure and integrin-targeted therapeutics (Arnaout *et al.*, 2005; Shimaoka and Springer, 2003; Simmons, 2005; Takagi and Springer, 2002; Yonekawa and Harlan, 2005). Here we review structure determinations that explain allosteric regulation and drug mechanisms, with emphasis on the A- or I-domain. We review clinical progress, emerging drug targets and describe an extension of drug discovery into structurally related targets and related clinical indications.

2. Structure, Ligand-Binding Sites, and Competitive Antagonists

The first integrin domain structure determination was that of the α-subunit A- or I-(inserted) domain including αMβ2 and αLβ2 (Lee *et al.*, 1995a,b; Qu and Leahy, 1995). These A-domains possess a Rosmann-like or dinucleotide fold, similar to von Willebrand factor A (VWA)-domains, with a central twisted β-sheet surrounded by amphipathic α-helices (Fig. 1B). The carboxy and amino termini of the I-domain are proximal to one another at the surface distal to the ligand-binding surface. The I-domain is inserted through short linkers, between the second and third repeats of the β-propeller (Fig. 1) (Larson *et al.*, 1989). Ligands bind to the metal ion–dependent adhesion site (MIDAS) where metal is

Figure 1 Integrin structure and ligand-binding sites. (A) Schematic of the noncovalently associated integrin heterodimer that, depending on whether they possess an α-subunit I-domain, are composed of 12 or 13 tertiary structure ectodomains (Xiong *et al.*, 2001). The α-subunit I-domain is inserted through linkers between repeats two and three of the seven repeat β-propeller. The α-subunit also contains immunoglobulin (Ig)-like thigh and calf domains, C1 and C2. The β-subunit contains an I-like domain (βI-domain) inserted in the Ig-like hybrid domain which in turn is inserted in the C-terminal loop of the plexins-semaphorins-integrins (PSI)-domain, four EGF-like domains, and a β-terminal domain (βTD). Both α- and β-subunits possess a transmembrane domain and a short cytoplasmic domain (with the exception of β4). In integrins that contain an αI-domain, there are nine cation-binding sites; one in ligand-binding MIDAS, four on the bottom surface of blades four to seven in the β-propeller, three sites in the βI-domain; β MIDAS, ADMIDAS, and LIMBS, and one in the α-subunit genu. (B) Ribbon model from crystal structure of α2β1. I-domain bound to collagen peptide (Emsley *et al.*, 2000). The collagen glutamate is shown bound to the MIDAS metal. (C) Ribbon model of αVβ3 β propeller and βI-domain bound to RGD peptide (Xiong *et al.*, 2002).

coordinated by residues from three loops, βA-α1, α3-α4, and βD-α5 (Emsley *et al.*, 2000). The residues of the MIDAS motif, DxSxS, T, and D, are conserved in these loops (Bhattacharya *et al.*, 2004; Lee *et al.*, 1995b). A ligand's acidic residue carboxyl completes metal coordination at this site (Emsley *et al.*, 2000).

The first integrin heterodimer crystal structure provided many important and some unexpected insights into integrin structure–functional relationships (Xiong *et al.*, 2001). This structure of αVβ3, which lacks an α-subunit I-domain, consists of 12 domains (Fig. 1 structure minus I-domain). The ligand-binding head domain is formed by an α-subunit seven bladed β-propeller and a β-subunit I-like (βI) domain. The structures and interface between the β-propeller and the βI-domain are very similar to that between α- and β-subunits of heterotrimeric G-protein. Ligands of integrins lacking an αI-domain bind a β-propeller cap subdomain composed of loops in repeats two and three, and the specificity-determining loop (SDL), and MIDAS of the βI-domain (Fig. 1) (Xiao *et al.*, 2004; Xiong *et al.*, 2002).

The βI-domain contains a MIDAS (β MIDAS) centered between two other metal-binding sites, the adjacent MIDAS, ADMIDAS, and the ligand-induced metal-binding site, LIMBS (Xiong *et al.*, 2001). Integrin-binding activity is regulated by differential metal occupancy. Occupation of the ADMIDAS in high Ca^{2+} conditions decreases ligand binding, whereas replacement by competing Mn^{2+} activates ligand binding. Low Ca^{2+} concentration, with Ca^{2+} occupancy at the LIMBS, may synergize with Mg^{2+} to support ligand binding (Chen *et al.*, 2003; Mould *et al.*, 2003). The rolling and high-affinity binding of α4β7 requires a functional ADMIDAS or LIMBS, respectively (Chen *et al.*, 2003).

The head domain is supported by two legs or stalks. The α-subunit stalk consists of Ig-like thigh and calf domains, C1 and C2. The β-subunit stalk consists of Ig-like hybrid, EGF-like, and β-terminal domains (βTD).

Competitive small molecule inhibitors have been based on binding motifs of ECM proteins and snake disintegrins. Based on binding to one of three sites, integrin inhibitors have been categorized as α/β I-like competitive, α/β I-like allosteric, and α allosteric antagonists (Shimaoka and Springer, 2003).

The costructure of integrins that lack an I-domain, with a bound ligand, has been determined. The first such costructure was the competitive RGD-based inhibitor, Celengitide, bound to αVβ3 (Table 2) (Xiong *et al.*, 2002). This costructure identifies salt bridges between the arginine guanidinium and the β-propeller and a bond between the aspartic carboxyl and metal in the βI-domain MIDAS (Xiong *et al.*, 2002). The glycine has few bonds and serves as a spacer between the two domains. High-affinity αIIbβ3 and αvβ3 competitive antagonists can stabilize α- and β-subunit association making it resistant to SDS (Zolotarjova *et al.*, 2001). The carboxyl group, which was identified as

essential in the structure–activity relationship (SAR) assessment during optimization of competitive inhibitors, is present in αVβ3, αIIbβ3, and α4β1 antagonists. The spacing between the basic and acidic groups, which confers specificity, results from differential bonding in various integrins (Xiao *et al.*, 2004).

3. Large Conformational Changes and Affinity States

There are several sites that appear to restrain integrin activation. These sites effectively regulate the bidirectional signal transmission from ligand binding leading to cellular signaling responses or vice versa, intracellular signaling leading to integrin activation.

Surprisingly, the αVβ3 crystal structure displays a 135° bend in the mid leg region such that the head domain and proximal leg domains (the headpiece) would face downward toward the cell surface (Fig. 2). This bent conformation was therefore proposed to play a key role in integrin regulation (Xiong *et al.*, 2001). A subpopulation of EM-imaged structures also displays this V shape configuration, indicating that the bend can occur in solution (Nermut *et al.*, 1988).

Three overall integrin conformations have been correlated with affinity; bent low affinity, extended with closed headpiece intermediate affinity, and ligand bound extended with open headpiece high affinity (Fig. 2). Several conformations were determined from electron microscope images using truncated integrins lacking transmembrane and cytoplasmic domains. In these studies, about 90% of αIIbβ3 and αVβ3 molecules are bent in Ca^{2+} buffer conditions that support low affinity (Takagi *et al.*, 2002). In contrast, activation by Mn^{2+} or RGD ligand results in extension in the vast majority of heterodimers. When the bent low-affinity conformation is stabilized by introducing a disulfide bridge between the α-subunit headpiece and the β-subunit tailpiece, αIIbβ3 and αVβ3 can not be induced by Mn^{2+} to bind soluble ligands (Takagi *et al.*, 2002). Reduction of the disulfide rescues wild-type Mn^{2+} induction of the high-affinity state and ligand binding. Conversely, an open headpiece high-affinity state was stabilized by introduction of a glycosylation site inside the interface between the βI and hybrid domains of α4β7. This converted conditions that supported rolling adhesion to those that resulted in firm adhesion (Chen *et al.*, 2004). In addition, Mn^{2+} induces cell surface integrin extension as determined by fluorescence resonance energy transfer (FRET) (Larson *et al.*, 2005a).

A bent conformation may not be equated with low affinity binding in all situations. The association of uPAR with α5β1 may stabilize a bent conformation and result in binding to an RGD-independent fibronectin binding site (Wei *et al.*, 2005).

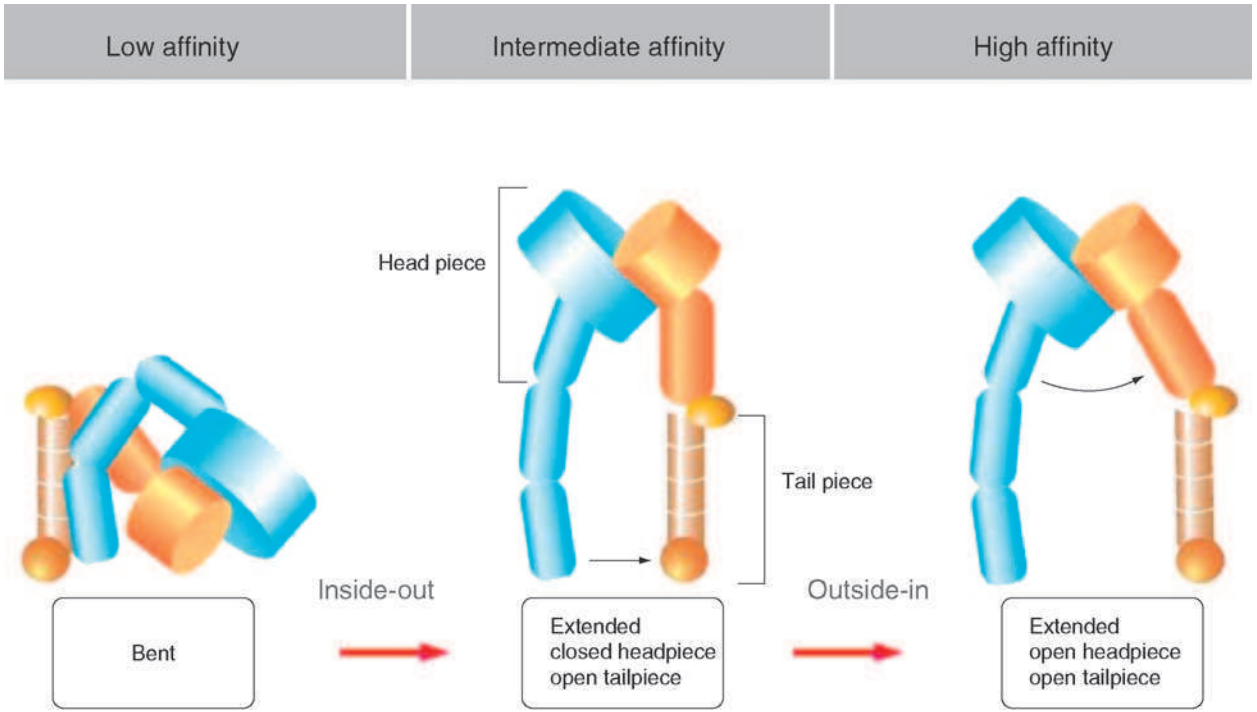


Figure 2 Schematic of integrin conformations correlated with activation states. Integrin structure–function relationships were determined through characterization of wild-type and mutant heterodimers. The bent αVβ3 structure when "locked" by a disulfide bridge and expressed in transfectants demonstrates low affinity (Takagi *et al.*, 2002). EM images indicate that cations or ligands, which induce an intermediate- or high-affinity state, induce an extended state (Takagi *et al.*, 2002). Low- and intermediate-affinity states may be in dynamic equilibrium, which is shifted by ligand binding. The high-affinity ligand bound state displays an open headpiece and tailpiece. Separation of α- and β-subunits of the tailpiece occurs with inside-out signaling as determined by fluorescence resonance energy transfer (FRET) (Kim, Carman and Springer, 2003).

These different conformation states have been correlated with activation state–dependent monoclonal antibody (mAb) epitopes for several integrins (Beals *et al.*, 2001; Humphries, 2004; Xie *et al.*, 2004). For example, the αLβ2 antibodies, NKI-L16 and A03, bind to an epitope in the α-subunit thigh domain that is exposed on extension (Xie *et al.*, 2004). Whereas KIM127 and 330E bind to the extended β2 leg region (Beals *et al.*, 2001; Beglova *et al.*, 2002). Epitopes not present in phorbol ester (PMA) induced αLβ2 extended configuration but present in Mn^{2+} induced extended integrins may report an open head domain (Xie *et al.*, 2004).

Competitive inhibitors, similar to natural ligands, can induce an extended active state and neoepitopes or ligand-induced binding sites (LIBS). LIBS are conformation-dependent epitopes detected by the binding of antibodies. They have been described for αIIbβ3 over a decade ago (Kouns *et al.*, 1990). The number of induced LIBS is variable, and a small molecule αIIbβ3 inhibitor has been reported that does not induce LIBS (Aga *et al.*, 2004).

4. I-Domain Allosteric Site and Antagonists

Among the first I-domain structures solved, one αM structure differed markedly in the position of MIDAS residues and the C-terminal helix (Lee *et al.*, 1995a,b). In this "open" conformation the MIDAS metal coordination was completed by a C-terminal acidic residue from an adjacent I-domain, suggesting that this might represent a pseudoligated high-affinity state. In addition, the C-terminal α7-helix was shifted 10Å downward from the MIDAS indicating that a large conformational shift might be linked to ligand binding.

A conformational change occurring between the ligand-bound and unbound state was demonstrated with α2 I-domain (Emsley *et al.*, 2000). Changes similar to the two αM structures were evident in the metal-coordinating MIDAS loops and in the α7-helix between unbound and collagen peptide–bound domains (Fig. 3). The MIDAS metal coordination was completed by a collagen glutamate residue. Movement of loop 1 and helix 1 forces the α7-helix out and down.

Evidence for an I-domain allosteric site (IDAS) that regulates binding at the αLβ2. MIDAS was demonstrated by NMR spectroscopy. ICAM-1 binding was found to profoundly affect two sites, the MIDAS and the α7-helix (Huth *et al.*, 2000). Substitution mutations located at the second site around the α7-helix resulted in activation or inhibition of ICAM-1 binding and were proposed to stabilize the I-domain in open (high-affinity) or closed (low-affinity) states, respectively. This IDAS, distal to the MIDAS, involves several α7-helix interactions with hydrophobic core β-sheet residues. Key contacts include the β-strand isoleucine 235 (I235) and the conserved α7-helix isoleucine 316

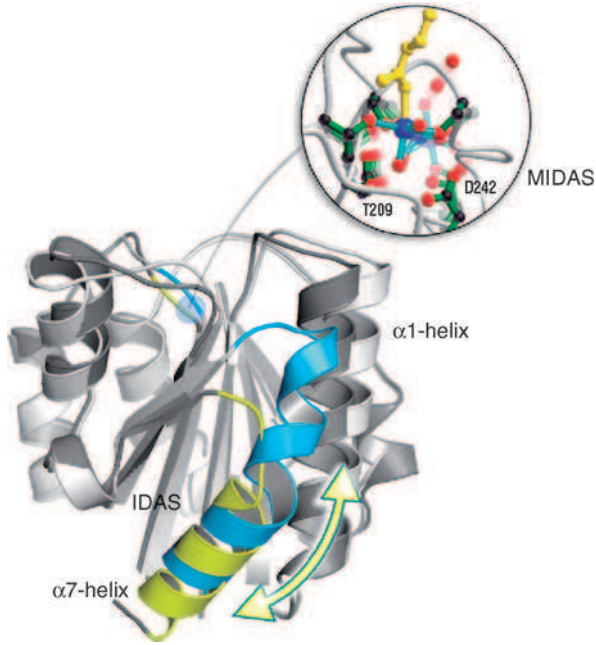


Figure 3 Model of the I-domain conformational shift. Crystal structures of ligated and non-ligated I-domains indicate that metal coordination in the MIDAS changes (see insert) as ligand binds and side chains undergo a conformational shift with the transition from the closed to the open high-affinity state. Linked to the recoordination of metal at the MIDAS is a large 10Å downward shift in the α7-helix. Introduction of disulfide bonds in αLβ2. I-domain that constrain the α7-helix position in open (yellow), closed (gray), and an intermediate (blue) conformation resulted in high, low, and intermediate affinity (Shimaoka *et al.*, 2000; Shimaoka and Springer, 2003).

(I316). In αM, mutation of I316 activates ligand binding through removing its binding to a conserved hydrophobic socket for isoleucine, SILEN (Xiong *et al.*, 2000). In addition, differential binding of an αMβ2 activation reporter mAb indicated that the N-terminal MIDAS proximal region of α7-helix was also altered by this conformational switch (Oxvig *et al.*, 1999).

Studies with mutant αLβ2. I-domains, constrained through introduction of cysteines that formed disulfide bonds, indicate that locking the position of the C-terminal helix in open, closed, and intermediate conformations resulted in high, low, and intermediate affinity (Shimaoka and Springer, 2003; Shimaoka *et al.*, 2000).

Crystal structure of an ICAM-1 dimer bound to two αLβ2. I-domains confirmed a switch to an open conformation on ligand binding, similar to that in α2 and αM (Shimaoka and Springer, 2003). The MIDAS metal coordination

was completed by the glutamate in the ICAM-1 amino-terminal domain-binding motif (Staunton *et al.*, 1990). In addition, the complex structure demonstrates an orientation that is optimized for an integrin heterodimer dimer interaction with ligand.

Thus, all I-domains may possess an allosteric site that undergoes a large conformational shift on ligand binding. Moreover, this shift from a closed to an open state is linked to the formation of a critical bond between the MIDAS metal and an acidic ligand residue.

Small molecule inhibitors that bind at the IDAS have been identified (Fig. 4). Crystal and NMR structures for allosteric inhibitors bound to the αLβ2. I-domain have been reported (Crump *et al.*, 2004; Gadek *et al.*, 2002; Kallen *et al.*, 1999; Kelly *et al.*, 1999; Last-Barney *et al.*, 2001; Wattanasin *et al.*, 2005; Weitz-Schmidt *et al.*, 2001; Winn *et al.*, 2001). Compounds that bind to this site have been referred to as α-allosteric antagonists (Shimaoka and Springer, 2003). The costructures indicate that several compounds with distinct scaffolds can occupy the IDAS. These structures show compounds bound to the closed inactive form of the I-domain indicating that they antagonize ligand binding by stabilizing the low-affinity I-domain conformation (Fig. 4). The hydantoin inhibitor, BIRT377, and statin inhibitor, LFA703, may also stabilize the bent conformation as determined by binding of extension-dependent reporter mAb and blocking of intermediate affinity-dependent rolling adhesion (Shimaoka and Springer, 2003). This function, however,

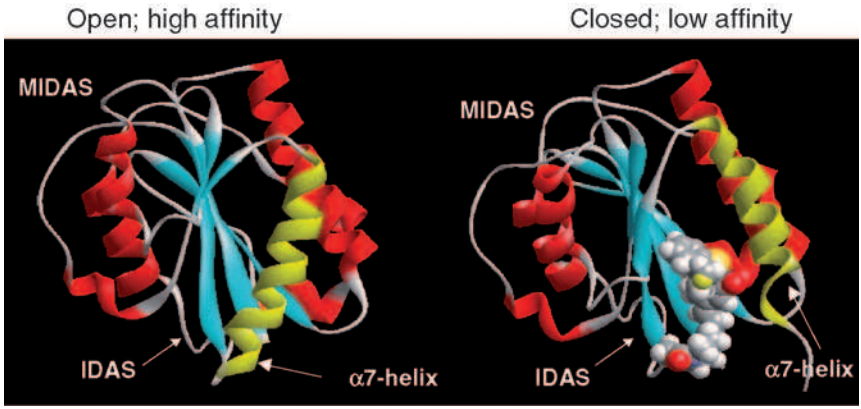


Figure 4 Binding of an allosteric inhibitor to the IDAS stabilizes a closed conformation. The NMR structure of a diarylsulfide inhibitor bound to αLβ2. I-domain demonstrates that the inhibitor is bound to the low-affinity closed conformation with the α7-helix in the "up" position (Liu *et al.*, 2000; Winn *et al.*, 2001). Thus, at least certain analogues of these inhibitors appear to stabilize the low-affinity state.

does not appear to be a universal property for inhibitors that bind to this site. For example, one diarylsulfide inhibitor analogue, IC487475, can stabilize rolling adhesion (Sarantos *et al.*, 2005).

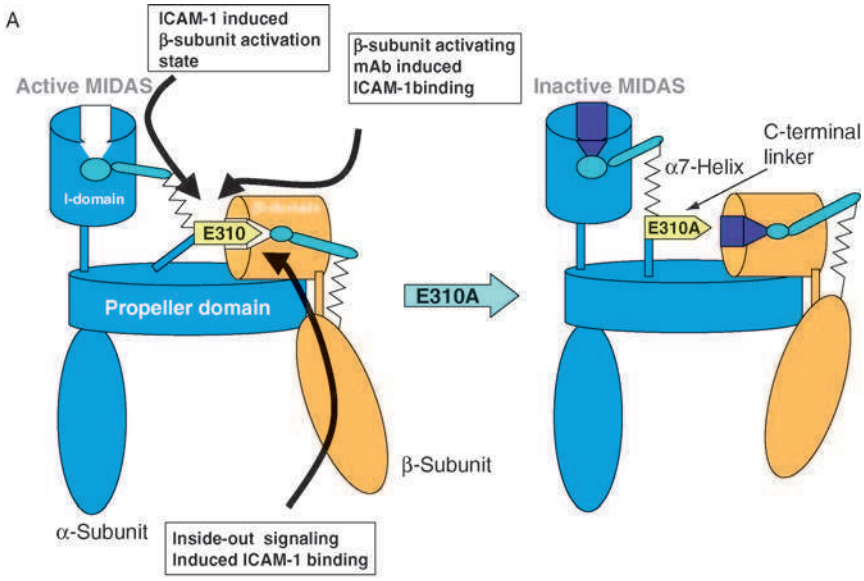
5. An Internal Ligand

Integrins containing an α-subunit I-domain possess a unique mechanism of bidirectional signal transmission between the α- and β-subunits. Several studies indicate that an αI-domain linker functions as an internal β MIDAS ligand that mediates signal transduction.

We have demonstrated that for αLβ2, the ability of a β-subunit activating mAb to induce α-subunit binding to ICAM-1 is blocked by substitution of an I-domain C-terminal linker glutamic acid, E310 (Fig. 5A) (Huth *et al.*, 2000). Conversely, the ability of ICAM-1 binding to the α-subunit to induce a β-subunit activation state, detected by antibody 327C, is also completely blocked by the substitution mutant E310A (Lupher *et al.*, 2001). Moreover, inside-out signaling leading to ICAM-1 binding is also blocked by E310A. An additional substitution of the proximal C-terminal linker residue tyrosine 307 (Y307) also resulted in markedly decreased ligand binding. The data indicate that the C-terminal I-domain linker residues play a critical role in regulating signal transmission between the α- and β-subunits. Mutation of this conserved glutamic linker residue in α1β1, α2β2, αMβ2, and αEβ7 also blocked signaling between α- and β-subunits (Fig. 5B) (DS and ML, unpublished data). This signal transmission was suggested to occur through binding of the C-terminal linker residue E310 to a proximal β-subunit site (Huth *et al.*, 2000). The corresponding conserved glutamate in αMβ2 was reported to play a critical role in ligand binding and proposed to interact directly with the proximal βI-domain MIDAS (Alonso *et al.*, 2002).

Compelling evidence for a critical contact between the conserved C-terminal linker glutamate and the β MIDAS was provided by substituting both the glutamate and β MIDAS residues with cysteines (Yang *et al.*, 2004b). The resulting disulfide bridge conferred constitutive activation of ligand binding. Inhibition of the double mutant with an inhibitor that binds at the IDAS, an α allosteric antagonist, suggests elasticity in the α7-helix linker region being pulled and, thus, a "pull spring" model of activation.

In this fashion, the C-terminal linker may function as an internal β MIDAS ligand and, thus, an additional allosteric site unique to integrins that possess an αI-domain. Thus, just as a conserved acidic residue in ligands bind α or β MIDAS, the C-terminal I-domain linker glutamate binds the β MIDAS to regulate ligand binding and signaling. Therefore, the β MIDAS serves a regulatory function in these integrins. The proximal Y307, which also contributes to regulation of ligand binding, may bind to a site adjacent to the



B

C-terminal linkers

AlphaL	**Y**	V	I	**E**310	G	T
AlphaM	F	A	I	**E**	G	T
AlphaX	F	A	I	E	G	T
AlphaD	Y	A	D	E	G	T
Alpha1	F	A	L	**E**	A	T
Alpha2	F	S	I	**E**	G	T
Alpha10	F	G	L	E	G	S
Alpha11	F	S	L	E	G	T
AlphaE	I	S	M	**E**	G	T
ICAM-1			I	E34	T	P
ICAM-2			L	E	T	S
ICAM-3			L	E	T	S

Figure 5 Model of an internal ligand. The I-domain C-terminal linker glutamate (E310) is critical for integrin bidirectional signaling by functioning as a ligand of the βI MIDAS. (A) Signal transmission between α- and β-subunits is blocked by substitution of E310. Alanine substitution of αLβ. E310 blocks the ability of an activating β2-specific mAb, 240Q, to induce α-subunit binding to ICAM-1 (Huth *et al.*, 2000). Conversely, the ability of ICAM-1 binding to the α-subunit to induce a β-subunit activation state, detected by 327C mAb, is also completely blocked by E310A (Lupher *et al.* 2001). Inside out signaling leading to ICAM-1 binding is also blocked by E310A. The corresponding glutamate mutation in α2, α1, αM, and αE was determined to block integrin signaling (DS and ML, unpublished data). E310 was proposed to bind proximally to β2 and specifically the β2. MIDAS (Alonso *et al.*, 2002; Huth *et al.*, 2000). Studies supporting this internal ligand interaction suggest a pull spring model (Yang *et al.*, 2004b). (B) The I-domain C-terminal linker glutamate, which appears to bind the βI MIDAS, is conserved in all integrin I-domains. Residues that blocked signaling when substituted with alanine are bold. Y307 also functions in regulation of ligand binding and is conserved in most linkers (Huth *et al.*, 2000). Y307 may bind the βI-domain to stabilize high- and/or intermediate-affinity states.

β MIDAS. The function of Y307 may be to contribute to stabilizing the high- and potentially the intermediate-affinity states. This may occur through different linker interactions with the βI-domain.

An amino-terminal I-domain linker residue substitution can also inhibit ligand binding (Huth *et al.*, 2000). The N-terminal linker may function to support reorientation of the I-domain with activation. This quaternary change might facilitate the transition to an open conformation and C-terminal linker binding with the β MIDAS. An αLβ2. I-domain agonistic mAb, MEM83, may have similar activity (Lu *et al.*, 2004).

Small molecule integrin inhibitors, the α/β I-like allosteric antagonists, have been identified, which appear to bind to the β-propeller and βI-domain MIDAS of αI-domain-containing integrins (Shimaoka and Springer, 2003) (ML and DS, unpublished data). Like the α-competitive inhibitors, they possess an essential carboxyl in their SAR. This essential carboxyl is not a feature of the α-allosteric antagonists that bind at the IDAS. These α/β I-like allosteric antagonists share several key features with the non-I-domain integrin α/β-competitive inhibitors. They can induce LIBS and an extended conformation. High-affinity αLβ2 and α1β1 inhibitors of this class can stabilize α- and β-subunit association (Shimaoka and Springer, 2003) (ML and DS, unpublished data). These compounds may therefore bind to the β-propeller and βI-domain in a manner similar to non-I-domain competitive inhibitors. As such, they would be competitive inhibitors of the internal I-domain linker binding to the βI-domain MIDAS.

6. βI-Domain Allosteric Regulation

The βI-domain is a key allosteric regulator of the β-subunit activation state. The structural similarity between α and βI-domains poses the possibility that both may undergo similar conformational shifts and allosterically regulate ligand binding. To mimic the I-domain downward α7-helix shift and pull on β6-α7-loop, two types of mutant βI-domains were created. One-turn α7-helix deletions were engineered into the βI-domain of β2 and β7 resulting in constitutive high-affinity ligand binding to α4β7 and αLβ2. (Yang *et al.*, 2004a). Introduction of disulfide bonds that locked the α7-helix of the β3 βI-domain in the open and closed conformations conferred high- and low-affinity states, respectively (Luo *et al.*, 2004).

Both α and βI-domains can undergo a similar conformational change between low- and high-affinity states. Crystal structures of ligand bound αIIbβ3 indicate that, relative to the structure of low-affinity state αVβ3, the βI-domain α7-helix has dropped downward forcing the hybrid and plexins-semaphorins-integrins (PSI)-domain to swing out by 62° (the open headpiece in Fig. 2) (Xiao *et al.*, 2004). The β leg swing out would destabilize interfaces between the α and β legs, as well as between the headpiece and legs and lead to

extension. The β and α I-domain MIDAS loops demonstrate similar movement between the inactive and active states (Xiao *et al.*, 2004). No small molecule antagonists that bind to the βI-domain allosteric site have been reported.

7. The PSI- and EGF-Domain Restraints

The PSI- and EGF-like domains have been implicated as a site of activation regulation. The PSI structure determination revealed that the hybrid domain is an insertion in the last loop of the PSI-domain (Xiong *et al.*, 2004). The "long-range" disulfide bridge constrains the PSI-domain to the N- and C-terminus of the hybrid domain. In addition, the PSI-domain would form putative contacts with the N-terminal EGF-domains. Activation reporter mAb have been identified that bind to the PSI- or EGF-domains of β3, β1, and β2 (Honda *et al.*, 1995; Ni *et al.*, 1998; Zang and Springer, 2001). Substitution of Cys in PSI- and EGF domains including the long-range PSI disulfide bond Cys5–Cys435 results in constitutive ligand binding and activation reporter mAb binding (Sun *et al.*, 2002; Zang and Springer, 2001). Mutation of many single cysteines in the EGF-domains of αIIbβ3 was able to activate ligand binding (Chen *et al.*, 2001; Kamata *et al.*, 2004). Regulation in this region could explain the activation of αIIbβ3 or β1 by mild reducing agents (Ni *et al.*, 1998; Zucker and Masiello, 1984). Both αIIbβ3 and αVβ3 have been reported to possess endogenous thiol isomerase activity (O'Neill *et al.*, 2000). The protein-disulfide isomerase inhibitor, bacitracin, blocks integrin-dependent adhesion suggesting that disulfide exchange may occur during adhesion (Lahav *et al.*, 2000, 2002). However, while α4β1-dependent adhesion induced by reducing agents can be blocked by bacitracin, inside-out induced adhesion is insensitive (Chigaev *et al.*, 2004). The mechanism by which EGF disulfides might maintain an inactive state may be through stabilizing contacts between the β and α stalks and between the stalk and head domains.

Extracellular membrane proximal calf-2, EGF4, and β tail domains have been implicated in regulation of integrin activation (Kamata *et al.*, 2005). αVβ3 ligand binding is markedly induced by Mn^{2+} whereas αIIbβ3 is not. Through domain swaps and substitution mutants, Mn^{2+}-induced binding was localized to the calf-2 domain, which forms an interface with EGF4 and the βTD (Kamata *et al.*, 2005).

8. The Cytoplasmic Domain Restraint

Interaction between the α and β cytoplasmic tails restricts integrin activation (Hughes *et al.*, 1996). Talin is a cytoplasmic protein that connects integrins to the actin cytoskeleton contributing to the focal adhesion signaling complex and

bidirectional signaling. Talin binds to β-1, -2 and -3 integrin tails (Liu *et al.*, 2000). The N-terminal head domain of talin activates αIIbβ3 and αLβ2 ligand binding (Calderwood *et al.*, 1999; Kim *et al.*, 2003; Vinogradova *et al.*, 2002). Conversely, ligand binding leads to cytoplasmic tail separation as determined by EM and FRET between fluorescently labeled tails (Kim *et al.*, 2003). To solve the binding interface structure, talin-β tail chimeras were used. This structure provided a molecular model of integrin activation through talin binding (Garcia-Alvarez *et al.*, 2003). The F3 subdomain of talin's N-terminal head domain, a variant of the phosphotyrosine (PTB)-binding domain, may initially bind to an accessible centrally located conserved NPxY motif. Binding may then progress to include additional more membrane proximal residues forcing the separation of the tails and ultimately separation of α and β stalks.

Thus, several sites restrain integrin activation and undergo a conformational shift between low- and high-affinity states. The level of restraint at each of these positions could vary between integrins, and this might result in different responses to inside-out signaling (Vorup-Jensen *et al.*, 2003). Certain sites, the MIDAS, β MIDAS, and IDAS have been targeted in drug development. Certain members of each class of inhibitors can stabilize different global conformations. In addition, both competitive and allosteric integrin inhibitor mAb have been identified (Beals *et al.*, 2001; Lu *et al.*, 2004). The marketed antibodies, which target αIIbβ3 (ReoPro) and αLβ2. (Raptiva), are competitive inhibitors that binds to the SDL of the βI-domain or to the I-domain, respectively (Artoni *et al.*, 2004; Edwards *et al.*, 1995).

9. Therapeutics

9.1. αIIbβ3 Antagonists

The first integrin-specific drugs targeted the platelet integrin, αIIbβ3. Central to hemostasis, αIIbβ3 binds to fibrinogen, von Willebrand factor (vWF), and CD40 ligand (CD40L, expressed on platelets) to mediate a critical final step in platelet adhesion and thrombus growth (Andre *et al.*, 2002; Shattil and Newman, 2004). Its role is underscored by its loss, resulting in severe bleeding problems in Glanzman's thrombocytopenia. Contributing to pathogenesis, αIIbβ3-dependent platelet adhesion supports ischemic events subsequent to thrombi and emboli growth.

Binding of αIIbβ3 may also function in an inflammatory response. Neutrophils can be recruited to sites of injured vasculature as they adhere efficiently to fibrinogen presented by αIIbβ3 on platelets immobilized on exposed ECM through αIIbβ3 and α2β1. (Kuijper *et al.*, 1996; Weber and Springer, 1997). In this manner, αIIbβ3 can increase neutrophil-mediated tissue

damage. Localization of platelets via αIIbβ3 also increases platelet-derived inflammatory mediators. For example, with activation, platelets produce CD40L, which when shed contributes to an inflammatory cascade (Henn *et al.*, 1998; Schonbeck *et al.*, 2000).

The first FDA-approved αIIbβ3 antagonists, antibody-based ReoPro (approved 1997), and the peptide or peptidomimetics, such as Integrilin (approved 1998) and Aggrastat (approved 1998), have been marketed for acute coronary syndromes and prevention of MI following percutaneous coronary intervention (PCI) (Table 2) (Hanson *et al.*, 2004). Although there is proven benefit for these αIIbβ3 antagonists, the development of these inhibitors and their use presented several substantial challenges.

One challenge in the development of αIIbβ3 inhibitors has been the optimization of their pharmacokinetic (PK) profile. Integrilin and Aggrastat are based on the ECM ligand RGD motif or related snake disintegrin, KGD motif. The specificity of these competitive antagonists, relative to other integrins that bind RGD, has been achieved through medicinal chemistry efforts. The structural basis for specificity includes the distance between the basic and acidic groups (Xiao *et al.*, 2004). This class of peptide or peptidomimetic inhibitors typically exhibit poor PK profiles; rapid plasma clearance, rapid metabolism, poor oral bioavailability, and a large variation in plasma levels, that is, peak/trough ratios. Decreasing off rates can partially compensate for these liabilities (Doherty *et al.*, 2003). The intravenously administered drugs used for short-term treatment are dosed to achieve the targeted optimum receptor occupancy of around 80–90%, needed for a maximal therapeutic index. Poor oral bioavailability and large peak/trough ratios contributing to a partial agonism and platelet aggregation, observed at low trough levels, has impeded the development of oral inhibitors (Cox, 2004).

A major challenge in developing αIIbβ3 antagonists is that as a key contributor to hemostasis treatment results in a variable frequency of bleeding. Antagonists of αIIbβ3 may induce thrombocytopenia. It was determined that some of these inhibitors, including Integrilin and Aggrastat, induce ligand-binding site (LIBS) epitopes (Aga *et al.*, 2004; Kouns *et al.*, 1990). Thus, the antagonists induced an active state conformation associated with ligand binding even though their binding function was blocked. This effect was also subsequently demonstrated for some inhibitors of leukocyte integrins (discussed later). Antagonist-induced conformational changes can result in the binding of drug-dependent antibodies (DDAB), which could contribute to thrombocytopenia (Brassard *et al.*, 2002). Preexisting DDAB was detected in 1–4% of humans.

The induction of an activation state conformation by certain inhibitors might potentially contribute to platelet activation in the absence of ligand. Signaling

Table 2 Integrin Targeted Drugs[a]

Integrin	Drug/Developmental product	Clinical indication	Company	Reference
αIIbβ3	ReoPro™, Abciximab (Fab)	Acute coronary syndrome, MI	Centocor & Johnson & Johnson	Hanson *et al*., 2004; http://www.centocor.com
	Integrilin™, Epifibatide (c.p.)	Acute coronary syndrome, MI	Millennium & Schering-Plough	Curran and Keating, 2005; http://www.mlnm.com
	Aggrastat™, Triofiban (s.m.)	Acute coronary syndrome, MI	Merck	Kumar and Herrnaann, 1997; http://www.merck.com
β2(CD18)	LeukArrest, Rovelizumab (hmAb)	Stroke, MI, shock, MS	ICOS Corporation	Faxon, 2000
	Erlizumab (hmAb)	MI, shock	Genentech	Rhee *et al*., 2000
αLβ2	Raptiva™, Efalizumab (hmAb)	Psoriasis	Genentech, & Xoma	Leonardi *et al*., 2005; Gordon *et al*., 2003
	IC747 (s.m.)	Psoriasis	ICOS Corporation	http://www.icos.com
α4β1	Tysabri™, Natalizumab (hmAb)	Multiple sclerosis	Biogen, Idec & Elan	Ghosh *et al*., 2003; http://www.biogen.com
	R411	Asthma (phase I)	Hoffmann-LaRoche	Hijazi *et al*., 2006
αVβ3	Vitaxin 1 and 2 (hmAb)	Melanoma (phase II) prostate cancer, RA (phase I)	Medimmune	http://www.medimmune.com
	Celengitide (pep)	Pancreatic cancer (phase II)	Merck	Burke *et al*., 2002
	L-000845704 (s.m.)	Postmenopausal osteoporosis (phase II)	Merck	Murphy *et al*., 2005
αVβ3/β5	CNTO 95 (hmAb)	Solid tumors (phase I)	Centocor	Jayson *et al*., 2004
	ATN-161 (pep)	Solid tumors (phase I)	Attenuon	http://www.attenuon.com
α4β7	MLN02 (hmAb)	Ulcerative colitis (phase II)	Miennium	Feagan *et al*., 2005
α5β1	M200 Volociximab (hmAb)	Solid tumors (phase II)	PDL & Biogen-Idec	http://www.pdl.com

[a]Abbreviations: s.m., small molecule; hmAb, humanized monoclonal antibody; pep, peptide.

is generally considered to be dependent on integrin clustering driven by multivalent ligands. Monovalent small molecules and Fab antagonists would not be expected to serve this function. In the presence of other stimuli, however, these inhibitors might cooperate in signaling. In particular, inhibitors that induce both α- and β-subunit LIBS, together with thrombin stimulation, induced thromboxane A2 production and Ca^{2+} signaling (Honda *et al.*, 1998). Furthermore, ReoPro, in conjunction with ADP signaling, stimulates P-selectin expression resulting in platelet-leukocyte aggregates (Schneider *et al.*, 2000). These inhibitor effects are consistent with integrin signaling cooperating with many other cell surface receptors. Thus, with other characteristics being equal, these competitive antagonists that induce an active, extended conformation may not provide the same level of efficacy or therapeutic index as those that stabilize an inactive conformation. An αIIbβ3 inhibitor has been reported that does not induce LIBS and, thus, would not be predicted to possess this potential liability (Aga *et al.*, 2004).

9.2. β2 Antagonists

The degree of contribution of a specific integrin to leukocyte function can vary depending on the vascular site of activation, cell type, and nature of activation. Thus, the contribution of the β2 (CD18), β1, and β7 integrins to leukocyte trafficking and posttrafficking activities varies in different settings (Bromley *et al.*, 2001; Shamri *et al.*, 2005; Simon and Green, 2005).

During leukocyte trafficking between blood and tissues, adhesion receptors are utilized sequentially. Leukocyte selectins, selectin ligands, and certain integrins, for example, α4β1 or α4β7, will tether and support leukocyte rolling adhesion on endothelium of lymphoid or other tissues. This allows membrane-bound agonists, such as chemokines, to activate integrin high-affinity binding and arrest via inside-out signaling. Chemokine induction of high affinity αLβ2 can also occur in a subsecond time frame (Shamri *et al.*, 2005). The β2 and β1 integrins can then mediate transendothelial and subendothelial migration. β2 integrins may play a predominant role on neutrophils where another major contributor to trafficking, α4β1, is not expressed. β2-dependent leukocyte trafficking is supported by multiple endothelial cell–expressed ligands including, ICAM-1, ICAM-2, RAGE, junctional adhesion molecule (JAM)-1, and JAM-2 (Chavakis *et al.*, 2003; Muller, 2003). β2 interaction with endothelial cells provides a signal for polarization and transmigration (Green *et al.*, 2006). The major contribution of β2 to leukocyte trafficking was demonstrated when loss of its expression or function was linked to leukocyte adhesion deficiencies (LAD), with persistent leukocytosis and recurrent bacterial infections (Anderson and Springer, 1987). Although β2 integrins play a dominant

role in the localization of leukocytes to certain tissues, such as the dermis, β2-independent neutrophil localization to lung has been reported (Mizgerd *et al.*, 1997). For localization in tissue parenchyma and effector cell activity, integrins that bind ECM, such as α1β1 and α2β1, may be dominant contributors (Andreasen *et al.*, 2003; de Fougerolles *et al.*, 2000).

Posttrafficking, β2 integrins mediate cell conjugate formation and signaling. They support antigen-specific T helper cell activation in lymphoid tissue, reactivation in inflamed tissues, and effector cell cytotoxic activity. Engagement of β2 integrins results in the production of cytokines that contribute to pathogenesis such as TNF-α (Wang *et al.*, 2006). The signaling complex formed between antigen-presenting cells and T lymphocytes, the immune synapse, is critically dependent on αLβ2. (Grakoui *et al.*, 1999). Blocking an integrin's costimulatory signal can result in T cell anergy or apoptosis. Neutrophil survival or apoptosis is also dependent on the function of β2 integrin–ligand interactions and the presence or absence of TNF-α (Mayadas and Cullere, 2005). The β2 integrins also play a key role in bacterial clearance. Both αMβ2 and αXβ2, also known as complement receptors 3(CR3) and 4 (CR4), bind iC3b-coated particles and mediate phagocytosis of microbial pathogens.

Antagonists of β2 integrins have been developed to target leukocyte functions in inflammatory or autoimmune diseases. Ischemia induces a proinflammatory state. On reperfusion, leukocyte localization and release of toxic mediators results in tissue damage. This ischemia-reperfusion injury may contribute to the pathogenesis of stroke, MI, and traumatic shock (Harlan and Winn, 2002). In animal models, leukocyte localization and subsequent tissue damage can be profoundly diminished by treatment with β2-specific antibodies (Thiagarajan *et al.*, 1997; Vedder *et al.*, 1990). However, a challenge for broad β2 inhibitors is to achieve an acceptable therapeutic index in the context of impaired bacterial clearance, which has been observed in preclinical studies (Talbott *et al.*, 1998).

In short-term clinical trials for MI, stroke, and traumatic shock, treatment with β2 antibodies, although providing some improvement in secondary endpoints, failed to meet primary endpoints (Table 2) (Harlan and Winn, 2002; Yonekawa and Harlan, 2005). In these trials, LeukArrest (Rovelizumab) was administered in a single dose. For example, either 0.3 mg/kg or 1 mg/kg was administered within 6 hours of chest pain and just prior to angioplasty in the MI trial, HALT-MI (Faxon *et al.*, 2002). A slight increase in minor infections occurred in the 1 mg/kg group.

Aspects of certain trial designs or the role of β2 in ischemia-reperfusion injury in humans may account for the failure of these trials to achieve their primary endpoints. In the shock trial, mortality was below expectation and the study populations were, thus, too small, that is, not adequately powered for

statistical evaluation. The dose, although adequate for dampening leukocyte trafficking, may not have been sufficient to block postextravasation leukocyte-mediated pathology. In addition, β2 may not be a predominant contributor to reperfusion injury in humans. It may be that leukocyte-independent damage, especially with longer duration of ischemia, is more crucial in man as opposed to reperfusion-mediated inflammation (Jolly *et al.*, 1986).

A β2 antibody also failed to demonstrate efficacy in longer term phase II trials of an autoimmune disease, multiple sclerosis (MS; see α4β1 antagonists and Table 2). Preclinical studies targeting a subset of β2 integrins αLβ2 and αMβ2 had demonstrated efficacy in animal models of MS, experimental autoimmune encephalomyelitis (EAE) (Gordon *et al.*, 1995). In a phase II trial with 45 patients receiving four weekly doses, there was no benefit as determined by changes in the Scripps Scale. There was, however, a significant reduction in a secondary endpoint, the formation of new lesions. The inflammation occurring during relapse in MS appears to be mediated by autoreactive T cells and, thus, treatment with a β2 mAb alone may not have provided benefit as T cell α4β1 may play a predominant role in human MS (Rice *et al.*, 2005). Consistent with β2 function in bacterial clearance, in phase 1 a single dose of 4 mg/kg dose resulted in a high rate of gingivitis. In longer term trials a 2 mg/kg dose of β2 antibody was well tolerated. This indicates that infection could restrict any attempt to completely inhibit β2 in long-term therapies.

Although broadly targeting all β2 integrins failed in these clinical trials, an αLβ2 specific antagonist has provided benefit in psoriasis with no substantial infections (discussed later). Thus, beneficial treatment of certain clinical indications can be achieved through antagonism of a specific β2 integrin with greater safety.

9.3. αLβ2 Antagonists

The leukocyte integrin αLβ2 is an important contributor to lymphocyte localization to inflamed tissues, the formation of an immune synapse, and effector cell conjugates. Thus, αLβ2 plays an important role in T cell activation, T helper and effector cell functions. In preclinical studies, an αLβ2 mAb has shown efficacy in a model of arthritis that demonstrates hallmarks of inflammation including leukocyte accumulation in synovial tissue and fluid (Watts *et al.*, 2005). In this model, disease induction and maintenance appears to be dependent on αLβ2, with participation by three endothelial ligands, ICAM-1, ICAM-2, and JAM-1.

Psoriasis is a chronic autoimmune disease of the skin affecting 2.6% of the US population (Lebwohl *et al.*, 2003). Hallmarks of the typical skin lesion include hyperproliferation of keratinocytes, hyperkeratosis, angiogenesis and

infiltration of leukocytes including neutrophils, monocytes, macrophages, T cells, natural killer (NK) and dendritic cells (Krueger and Bowcock, 2005). The mechanism underlying the initiation of psoriasis is not clear and viral, bacterial, and autoantigens have been proposed (Bos, 2005). Th1 T cells appear to be dominant, and there is clear evidence for an innate immune response contributing significantly to the pathogenesis. Thus, the response may involve αLβ2 initially during dendritic cell activation of naïve T cells, trafficking of T cells expressing the dermal homing marker, cutaneous lymphocyte antigen (CLA), and reactivation in psoriatic plaques. αLβ2 may support effector functions including TNF-α production contributing to keratinocyte hyperproliferation (Krueger, 2002; Nickoloff, 1999).

A humanized αLβ2 mAb, Efalizumab (Raptiva), that binds the I-domain was developed to treat psoriasis (Edwards *et al.*, 1995; Jordan, 2005). Phase III trials with 12 weeks of a weekly subcutaneous administration of Raptiva resulted in ~30% of patients achieving a 75% or better reduction in the psoriasis area and severity index (PASI-75) (Gordon *et al.*, 1995; Leonardi *et al.*, 2005). With extension to 24 weeks an additional 20% of patients achieved a PASI-75. Only around 4% of the placebo group demonstrated a similar reduction. Raptiva treatment has not resulted in severe adverse effects including increased bacterial infections, and Raptiva was FDA approved in 2003 to treat moderate to severe psoriasis.

Ex vivo studies indicate that Raptiva treatment decreases predominantly T lymphocytes in plaques by 40% (Mortensen *et al.*, 2005). Conversely, treatment increases peripheral blood lymphocyte counts by three to fourfold (Vugmeyster *et al.*, 2004). There were increases in $CD4^+$, $CD8^+$, and Th1 helper cells with the greatest increase in the memory $CD8^+$ population. This relative leukocytosis would be expected from blockade of αLβ2-dependent lymphocyte localization to inflamed tissue and overall decreased lymphocyte trafficking to lymphoid tissues. Memory lymphocytes express higher levels of αLβ2 and appear to be more dependent on αLβ2 in trafficking.

Raptiva treatment decreased αLβ2 surface expression to ~20% of baseline. This is likely the result of antibody-mediated internalization. Surprisingly, levels of other cell surface receptors, including αMβ2 and β7 integrins, are decreased (Vugmeyster *et al.*, 2004). Although significant, their decrease was less than that of αLβ2. The β7 integrins, α4β7 and αEβ7, function in lymphocyte homing to the gut but are also present in psoriatic plaques. These other receptors may be colocalized with αLβ2 and internalized in lipid rafts (Leitinger and Hogg, 2002). The potential contribution of decreased αMβ2, β7, or other membrane receptors to Raptiva's efficacy remains to be determined. In contrast to psoriasis, the outcome of treating psoriatic arthritis patients with Raptiva was not statistically significant (Mease, 2005).

Small molecule diarylsulfide inhibitors that bind αLβ2 IDAS, α allosteric antagonists, were also developed for the treatment of psoriasis (Fig. 5A) (Liu *et al.*, 2000; Winn *et al.*, 2001). In preclinical pulmonary and airpouch inflammation models, these inhibitors significantly decreased recruitment of eosinophils or neutrophils to inflamed tissues (Winn *et al.*, 2001). In phase II trials an inhibitor, IC747, did not achieve primary endpoints and higher potency inhibitors are being developed (http://www.icos.com). Other small molecule αLβ2 inhibitors that bind to the IDAS, statin and hydantoin compounds, as well as diaminopropionic compounds that apparently bind to the α/β allosteric site have been developed. Their entry into clinical trials has not been reported (Crump *et al.*, 2004; Gadek *et al.*, 2002; Kallen *et al.*, 1999; Kelly *et al.*, 1999; Last-Barney *et al.*, 2001; Wattanasin *et al.*, 2005; Weitz-Schmidt *et al.*, 2001). As discussed earlier, certain analogues of these α allosteric antagonists may stabilize a low- or intermediate-affinity αLβ2 conformation. Inhibitors that support a low-affinity conformation are not expected to contribute to undesired signaling, even in an inflammatory setting.

9.4. α4β1 Antagonists

In leukocyte trafficking α4β1, expressed on lymphocytes and monocytes but not neutrophils, may bind VCAM-1 on inflamed endothelium and fibronectin in tissue parenchyma. The binding of these ligands to α4β1 can support lymphocyte activation, proliferation, and survival and antagonism can induce apoptosis (Davis *et al.*, 1990; Leussink *et al.*, 2002; Nojima *et al.*, 1990; Shimizu *et al.*, 1990). Antagonists of α4β1 demonstrate efficacy in several animal models of inflammation or autoimmunity (von Andrian and Engelhardt, 2003).

MS is an autoimmune demyelinating disease. In EAE, an animal model of MS, myelin basic protein (MBP) in adjuvant, is used to induce T cell activation and an antibody response. T cells localize in the brain and recruit additional lymphocytes and monocytes. VCAM-1 is expressed on inflamed brain endothelium and on glial cells near lesions (Cannella and Raine, 1995). In one seminal study, disease was induced by injecting animals with a T cell clone specific for MBP (Yednock *et al.*, 1992). In this study, α4β1 mAb blocked >95% of monocytic cell adhesion to EAE vessels and provided clear benefit in a dose-dependent manner. Subsequent studies indicate that α4β1, and not α4β7, appears to contribute to disease development (Kanwar *et al.*, 2000a,b). Small molecule α4β1 antagonists, such as R411, CDP323 and SB683699, are in phase 1 and phase IIb trials for MS (Table 2). Treatment results in *ex vivo* inhibition of leukocyte binding to VCAM-1 (CDP323; UCB) and leukocytosis (SB683699; GlaxoSmithKline), a surrogate pharmacodynamic marker.

Crohn's disease is a chronic relapsing idiopathic inflammation that most commonly affects the ileum and colon but can occur at other sites throughout the gastrointestinal tract. Affecting approximately one half million individuals in the United States, it is thought to be initiated by an abnormal response to commensal intestinal bacteria (Bouma and Strober, 2003; Kelsall and Leon, 2005). Gut-associated mesenteric lymph node dendritic cells are induced to produce IL-12, promoting a Th1 response. Th1 production of INF-γ and TNF-α triggers an inflammatory cascade resulting in tissue damage. $CD4^+$ T cell trafficking from gut-associated lymphoid tissue to the lamina propria, the site of inflammation, is supported by $\alpha4\beta1$ and $\alpha4\beta7$ (Rivera-Nieves *et al.*, 2005). Blocking $\alpha4$ integrins or their ligands, such as mucosal addressin cell adhesion molecule (MadCAM)-1, in several animal models of colitis indicate that targeting $\alpha4$-dependent lymphocyte trafficking may have therapeutic value (Kato *et al.*, 2000; Podolsky *et al.*, 1993; Shigematsu *et al.*, 2001; Soriano *et al.*, 2000).

Approximately 3000 patients have been treated with a humanized IgG4$\alpha4$ integrin antagonist, Tysabri (Natalizumab), in clinical trials for MS or Crohn's disease (Ghosh *et al.*, 2003; Rice *et al.*, 2005). The results from phase II and two phase III trials AFFIRM and SENTINEL demonstrated clear benefit and supported an accelerated approval for relapsing MS (Miller *et al.*, 2003; Rice *et al.*, 2005). In the phase III AFFIRM trial, Tysabri significantly reduced the number of lesions as well as reduced relapses by 66% after 1 year. In the SENTINEL trial, a combination treatment with INF-$\beta1\alpha$, there was a 54% reduction in relapses over INF-$\beta1\alpha$ alone. In a large 10-week phase III Crohn's trial, ENACT-1, significant benefit was observed when the patient groups that had been actively treated previously or had high C-reactive protein were analyzed separately (Van Assche and Rutgeerts, 2004).

Tysabri was FDA approved in January 2005 for treatment of MS. However, 3 months later the drug was withdrawn from the market after two patients developed progressive multifocal leukoencephalopathy (PML) (Kleinschmidt-DeMasters and Tyler, 2005; Steinman, 2005). PML is a lethal opportunistic infection of oligodendrocytes. It is hypothesized that the immunosupression resulting from blockade of $\alpha4\beta1$ allows for a productive reactivation of the causative agent, *JC polyomavirus*. PML arising in patients treated with Tysabri has raised concerns about other therapeutics that block lymphocyte trafficking. It is possible that prospective studies that monitor JC viremia may allow for a treatment strategy for JC virus. A similar approach has been successful for the related *BK polyomavirus*, which reactivates in immunosuppressed kidney transplant patients (Brennan *et al.*, 2005). In mid-2006 the FDA allowed Tysabri to re-enter the market.

Antagonists of $\alpha4\beta1$ may benefit other diseases such as fibrosis (see Section 9.8).

9.5. α4β7 Antagonists

The integrin α4β7 is expressed on a subpopulation of lymphocytes that home to gut lymphoid tissue where its endothelial cell ligand MadCAM-1 is expressed on postcapillary venules of lamina propria, high endothelial venules of peyers patches, and mucosal lymph nodes. MadCAM-1 expression is upregulated in the small and large intestine in inflammatory bowel disease (Arihiro *et al.*, 2002).

Ulcerative colitis is a chronic relapsing inflammation that affects the colon inflicting one half million individuals in the United States. The inflammatory response in ulcerative colitis is mediated by Th2 T cells. Studies targeting α4β7 or MadCAM-1 in animal models of colitis indicate that inhibition of a4β7-dependent lymphocyte trafficking may be an effective treatment for colitis (Kato *et al.*, 2000; Shigematsu *et al.*, 2001; Soriano *et al.*, 2000). In a clinical trial, patients with active ulcerative colitis were treated with a humanized α4β7-specific antibody, MLN02, intravenously on days 1 and 29 (Feagan *et al.*, 2005). At week 6, clinical remission rate was 33% for the treatment group receiving 0.5 mg of MLN02 and 14% for placebo. Approximately 66% of patients improved by at least three points on the clinical scoring scale, versus 33% for placebo, and 28% had endoscopically evident remission, versus 8% for the placebo group. By week 8, 44% of MLN02-treated patients developed a human anti-human antibody (HAHA) response. The 24% that had titers >1:125 demonstrated a clinical remission frequency equivalent to placebo, whereas the remainder achieved 42% remission. Thus, the HAHA response to MLN02 appears to have limited its effectiveness.

9.6. αVβ3 Antagonists

The integrin αVβ3 is expressed on monocytes, osteoclasts, and tumor cells and is induced, *de novo*, on endothelial cells during angiogenesis (Stupack and Cheresh, 2004).

Several αV integrins, α5β1 and α4β1 have been implicated in supporting endothelial cell growth, migration, and survival during angiogenesis. These integrins may function in overlapping or distinct capacities, functioning as sensors of the rapid degradation of basal matrix and deposition of provisional matrix, that is, remodeling. Together with associated GF signaling, these integrins regulate angiogenesis.

During angiogenesis, αVβ3 binds to provisional ECM proteins, fibronectin, fibrinogen, vitronectin, as well as protease-exposed cryptic sites in the basal ECM proteins, collagen type IV, and laminin. Adhesion via αVβ3 may support endothelial cell survival through several mechanisms such as suppression of

the p53 apoptotic pathway (Burbridge *et al.*, 2003; Stromblad *et al.*, 1996). In contrast, soluble αVβ3 ligands promote activation of caspase 8 and apoptosis (Brooks *et al.*, 1995; Kim *et al.*, 2000a). As expression of αVβ3 can increase on both tumor vasculature and tumor cells, supporting angiogenesis and tumor survival, αVβ3 has been recognized as a promising cancer drug target. In preclinical models of angiogenesis and tumor growth, antagonists of αVβ3 and αVβ5 are potent inhibitors of angiogenesis (Allman *et al.*, 2000; Brooks *et al.*, 1995). The peptide-based αVβ3 inhibitor, Celengitide, in combination with radioimmunotherapy increased the cure rate in a human breast xenograft cancer model (Burke *et al.*, 2002).

Clinical trials are ongoing for a humanized αVβ3-specific mAb, Vitaxin, and peptide-based αVβ3 and αVβ5 inhibitors (Table 2). In a phase I trial, Vitaxin was administered to 17 patients who had progressive stage IV colon, breast, ovarian, and other malignancies that failed to respond to other treatments (Gutheil *et al.*, 2000). Vitaxin was administered weekly over 6 weeks intravenously with increasing doses, 0.1 to 4 mg/kg. Vitaxin stabilized approximately half of the 14 evaluable patients and one patient with end-stage sarcoma remained on Vitaxin with stable disease for 2 years. Preliminary data from a phase II trial has been reported in which 112 stage IV metastatic melanoma patients were treated weekly with 8 mg of Vitaxin per kg administered IV, with ($n = 55$) or without ($n = 57$) Dacarbazine (DTIC) every 3 weeks. Vitaxin alone resulted in a median survival time of 12.7 months, with 52% survival at 1 year as opposed to a median of 9.4 months and 42% survival at 1 year for patients treated with Vitaxin and DTIC (http://www.medimmune.com). In a separate phase III trial with DTIC alone, there was a 7.9 month median survival and 33% survival at 1 year. Vitaxin is also being evaluated in a clinical trial for treatment of prostate cancer (http://www.ClinicalTrials.gov). Vitaxin is well tolerated with no significant adverse effects reported. In addition a humanized antibody that binds to αV integrins, CNTO 95, is in an ongoing phase I trial for solid tumors (Table 2). Moreover, Celengitide in combination with Gemcitabine arrested head and neck squamous cell carcinoma growth for 12 months in patients who had previously experienced short periods of remission following surgery and other treatments.

An antagonist of αVβ3 is also in clinical trials for postmenopausal osteoporosis (Murphy *et al.*, 2005). Estrogen deficiency results in bone loss due to increased osteoclast-mediated bone resorption. The most abundant osteoclast integrin αVβ3 supports the interaction of osteoclasts with bone ECM, RGD-containing proteins. In preclinical models of osteoporosis, αVβ3 antagonists decreased bone resorption (Engelman *et al.*, 1997; Yamamoto *et al.*, 1998). In phase I trials, a nonpeptide orally active small molecule αVβ3 inhibitor L-000845704 (Table 2) decreased a marker of resorption, urinary

N-telopeptide cross links, by 40%. In a 12-month study, 227 women treated twice daily with 200 mg demonstrated a significant increase in lumbar, hip, and femoral neck bone mineral density (Murphy *et al.*, 2005).

9.7. α5β1 Antagonists

The fibronectin receptor α5β1 is expressed in smooth muscle, fibroblasts, hematopoetic, and endothelial cell types. The expression of α5β1 is upregulated in tumor vasculature. α5β1 supports basic fibroblast growth factor-induced endothelial cell proliferation, distinguishing it from αVβ5, which supports vascular endothelial growth factor-induced proliferation (Kim *et al.*, 2000a).

In tumor vasculature, α5β1 is present and accessible to antibodies on the luminal surface (Magnussen *et al.*, 2005; Parsons-Wingerter *et al.*, 2005). In a preclinical setting, an α5β1 antibody blocked angiogenesis and tumor growth (Kim *et al.*, 2000b). An α5β1 mAb, M200 (Volociximab), is in a phase II trials for late stage melanoma, metastatic renal cell carcinoma and metastatic adenocarcinoma of the pancreas (Table 2). Interim results indicate that M200 appears to be well tolerated.

9.8. Emerging Targets

There are numerous *in vitro* or preclinical studies implementing integrins in the pathogenesis of diseases not discussed earlier. Fibrosis is an example of a large unmet medical need that might be addressed with integrin antagonists.

Fibrotic diseases of liver, lung, kidney, and other organs possess inflammatory and wound-healing components (Lupher *et al.*, 2006). Myofibroblasts are the primary producers of collagen in fibrotic diseases. Th2 T cells and M2 macrophages drive myofibroblast differentiation, proliferation, and collagen production. Macrophage-secreted TGF-β1 induction of myofibroblast collagen synthesis becomes dysregulated during the remodeling process that occurs in physiologic response to tissue injury. Consequently, tissue architecture and function is disrupted.

α1β1 integrin (also known as very late antigen-1, VLA-1) is one member of a family of four β1 integrin molecules (α1β1, α2β1, α10β1, and α11β1) that have been shown to bind to the ECM proteins collagen and laminin (Hemler and Lobb, 1995). α1β1 signals via Shc into the MAP kinase pathway and, thus, can regulate cell proliferation following collagen ligation (Pozzi *et al.*, 1998). Furthermore, β1 signaling may be required for TGF-β-mediated activation of the MAP kinase pathway leading to epithelial to mesenchymal transition (EMT) (Bhowmick *et al.*, 2001).

α1β1 is expressed on several cell populations relevant to fibrotic disease, including microvascular endothelial cells, fibroblasts, and myofibroblasts

(Racine-Samson *et al.*, 1997). It is also expressed on certain activated cells of the immune system, including T cells, macrophages, and NK cells, but not on normal peripheral blood mononuclear cells (PBMC) (de Fougerolles *et al.*, 2000).

α1β1 may play a major role in liver and kidney fibrosis. For example, *in vitro* an α1 mAb blocks liver and kidney myofibroblast adhesion to collagen, and endothelin stimulation of myofibroblast contraction of collagen lattices (reviewed in Lupher 2006). In addition, α1β1 is the primary integrin expressed by myofibroblasts *in vivo* and can regulate MMP expression thereby affecting collagen degradation. α1β1 is also required for myofibroblast migration induced by TGF-β1, EGF, or collagen I. Finally, blocking mAb to α1β1 significantly decreased mesangial cell–mediated collagen deposition, collagen gel contraction, production of serum creatinine, and increased survival, even when administered after the onset of measurable interstitial fibrosis.

Collectively, these data are consistent with the concept that α1β1 may impact fibrotic disease progression at multiple stages. α1β1 expression on macrophages and T cells may influence M1 and M2 macrophage retention in the peripheral tissue, or subsequent expression of cytokines. α1β1 expression on myofibroblasts appears to play a critical role in their contractile activity *in vitro* and may represent their main collagen receptor for mediating collagen contraction *in vivo*, thereby impacting tissue architecture and local vascular tone. Finally, α1β1 regulation of MMP expression could impact the collagen-remodeling ability of myofibroblasts and macrophages at sites of active fibrogenesis.

αVβ6 is another integrin strongly implicated in the development and control of fibrotic disease (Sheppard, 2000). αVβ6 expression is restricted to epithelial cells and it functions in binding to the ECM proteins fibronectin, tenascin-C, and vitronectin through the linear tripeptide sequence Arg-Gly-Asp (RGD) recognized by all αV integrins. Significant interest in the role of αVβ6 in fibrotic disease stemmed from the unexpected phenotype in α6 null mice of significant inflammation in the lungs and skin (Huang *et al.*, 1996), but a complete lack of pulmonary fibrosis in these animals on challenge with bleomycin (Munger *et al.*, 1998). Although the phenotype of the α6 null mice was reminiscent of the enhanced inflammation observed in TGF-β1 null animals (Shull *et al.*, 1992), no difference in expression of TGF-β was observed between wild type and α6 null animals after bleomycin treatment nor at baseline (Munger *et al.*, 1998). Instead, Munger *et al.* determined that similar to other αV integrins, αVβ6 bound to the RGD sequence within the TGF-β-inactivating LAP protein. Unlike αVβ3 or αVβ5 binding, αVβ6 binding was a strong activator of TGF-β function in cell culture. However, the conformational change in the TGF-β/LAP complex induced by αVβ6 binding does not

appear to release free active TGF-β, thus, providing a means for spatially restricted presentation of active TGF-β (Munger *et al.*, 1990).

Just as integrins are implicated in controlling activation and propagation of the fibrotic process, they may also play a role in fibrosis resolution. Spontaneous resolution of liver fibrosis in rats coincides with decreased TIMP-1 expression, enhanced MMP activity, and apoptosis of the activated myofibroblasts (Iredale *et al.*, 1998; Issa *et al.*, 2001), all of which may be regulated to a degree by integrin engagement. Although the natural trigger for hepatic myofibroblast apoptosis is currently unknown, endothelial cell survival *in vitro* and *in vivo* requires ligation of the integrin αVβ3. A study demonstrated that αVβ3 is expressed by rat and human liver myofibroblasts *in vitro* (Zhou *et al.*, 2004). Blockade of αVβ3 function in these cells resulted in decreased proliferation, increased apoptosis, decreased TIMP-1 expression, and increased MMP-9 expression (Zhou *et al.*, 2004). Therefore, these data suggest that αVβ3 may play a sensory role in recognizing degradation of ECM during resolution, thus, triggering apoptosis of myofibroblasts.

As discussed earlier α4β1 can play a dominant role in monocytic cell recruitment and signaling in inflamed tissues. In a mouse model of pulmonary fibrosis, an α4β1 antibody reduced the numbers of myofibroblasts and fibrotic lesions (Wang *et al.*, 2000).

9.9. IDAS-Based Drug Discovery

Structural relatives of the integrin I-domain are present in many functionally diverse proteins. Integrin I-domains represent a subfamily of Rossmann folds or VWA-domains that differ in certain properties, including size of loop insertions and β-strand orientation (Emsley *et al.*, 2000; Lee *et al.*, 1995b; Qu and Leahy, 1995; Whittaker and Hynes, 2002). In the human proteome there are over 140 VWA-domains. These VWA-domains are present in secreted, cell membrane, or intercellular proteins including ECM, ion channels, protease regulators, anthrax toxin receptors, complement proteins, ATPases, and transcriptional regulators. They are often present in multimolecular complexes and as in integrins are predicted to mediate protein–protein interactions. For example, characterization of a voltage-gated Ca^{2+} channel associated subunit, α2δ-2 mutant, indicates that the MIDAS is important to trafficking of the channel to the plasma membrane (Canti *et al.*, 2005). Additional domains have an imperfect MIDAS, for example, lacking one of the conserved residues but may still coordinate metal and require metal for binding (Tomsig and Creutz, 2000; Whittaker and Hynes, 2002). However, not all VWA-domains bind metal or undergo a major conformational shift on ligand binding. One example is of vWF binding to collagen (Romijn *et al.*, 2001).

Integrin I-domains possess ~200 residues and share 18–59% amino acid identity. Although they share a common tertiary structure, most I-domains share less than 25% amino acid identity with non-integrin VWA-domains. IDAS residues possess a broad range of homology between I and VWA-domains, around 9–46%.

Targeting VWA-domains with antagonists or agonists identified from a library consisting of analogues of compounds that bind to the IDAS is an efficient approach to identifying modulators for many integrins and non-integrins (DS, unpublished data). These residues form a pocket that appears to accommodate certain hydrophobic analogues from IDAS inhibitor focused libraries developed around the αLβ2 diarylsulfide antagonists. From these focused compound libraries, antagonists and agonists have been identified for many I and VWA-domains, including αXβ2, αMβ2, VLA-1, VLA-2, and complement factor B, as well as, bacterial proteins (DS and ML, unpublished data).

The β2 integrin αXβ2 is expressed on dendritic cells, monocytes, NK cells, granulocytes, and subpopulations of T cells. It can support phagocytosis and ligation of αXβ2 can induce production of inflammatory mediators. Blocking αXβ2 mAb significantly inhibit delayed-type hypersensitivity and contact hypersensitivity responses in mice (DS, unpublished data). Inhibitors of αXβ2 binding to ICAM-1 were identified from the IDAS inhibitor focused libraries with IC50 values <10 μM. These selective inhibitors were determined to bind to the αXβ2 1-domain. Through a virtual screening approach, additional classes of αXβ2 inhibitors have been identified through a virtual screen of a modeled ligand-bound αXβ2 IDAS (Fig. 6).

Complement proteins function in the innate immune system to provide protection from microorganisms. With excessive or inappropriate activation, they also can contribute to the pathogenesis of inflammatory diseases such as rheumatoid arthritis and dermatitis. The complement protein, factor B, plays a central role in the alternative complement pathway. Factor B possess an VWA-domain linked to a serine protease in a default inactive conformation. The VWA-domain binds C3b on the surface of pathogens in a Mg^{2+}-dependent manner and results in activation of the serine protease and the formation of C3 convertase. We have determined the X-ray crystal structure of factor B VWA-domain (Bhattacharya *et al.*, 2004). This structure adopts an open conformation due to a fortuitous crystal contact that completes the metal coordination at the MIDAS. Modeling indicates that the factor B VWA-domain could also form a closed conformation without steric hindrance. We have identified diarylsulfide IDAS inhibitor analogues that antagonize C3 convertase activity (ML and DS, unpublished data). This supports the notion that non-integrin I-domains appear also to be able to convert from a closed to open structure

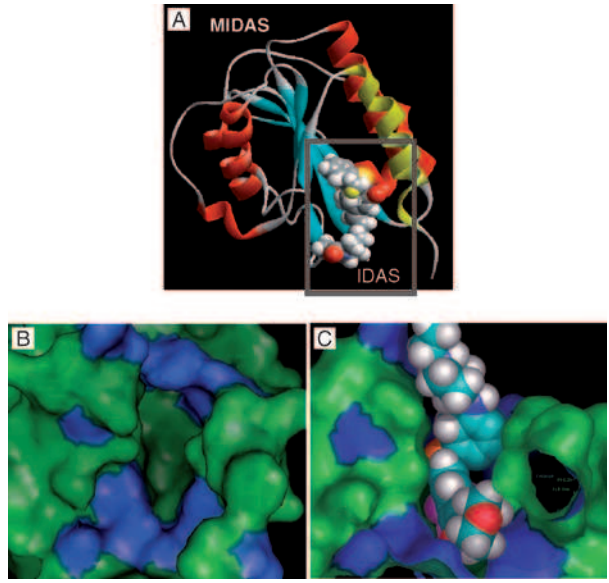


Figure 6 Identification of αXβ2 antagonists through a virtual screen of a modeled I-domain. (A) Location of the αLβ2 IDAS between the α7-helix and β-sheet. (B) The ligand bound conformation of αLβ2 IDAS. Residues in αLβ2 IDAS (green) that differed with αXβ2 were converted to αXβ2 residues (blue) in silico. The modeled ligand bound conformation of the αXβ. IDAS was then screened for potential inhibitors using a modification of DOCK. (C) Model of the complex with a docking hit, an indole compound that was determined to block αXβ2-dependent adhesion.

similar to integrin I-domains. This conformational switch is predicted to be directly linked to activation of the serine protease.

Thus, analogues of I-domain inhibitors that bind to the IDAS can be identified that antagonize different I-domains and non-integrin VWA-domains. Synthesis of additional analogues based on these screening hits has produced more potent and selective inhibitors. These inhibitors may be developed to treat inflammatory diseases, cancer, and bacterial infections.

10. Concluding Remarks

There have been several successes in targeting integrins for drug development. Antibody-based and small molecule inhibitors of αIIbβ3 benefit patients undergoing percutaneous coronary intervention. The αLβ2 antibody, Raptiva, is

an effective treatment for psoriasis and α4β1 antagonism demonstrated remarkable efficacy in phase III MS trials.

The development of integrin therapeutics faces common drug development challenges and expected attrition. Both competitive and α/β allosteric antagonists possess a critical carboxyl and tend to possess liabilities associated with many peptidomimetics. The failure of broad β2 inhibitors to demonstrate efficacy in ischemia-reperfusion injury may reflect the fact that many animal models are poor correlates for human disease.

Toxicities resulting from target-specific mechanism of action present distinct challenges. These include bleeding in the case of αIIbβ3 inhibitors, an increased infection rate with broad β2 antagonists, and development of PML in patients treated with the α4β1 antibody, Tysabri.

Despite the stated limitations, there are additional opportunities for novel integrin antagonists. Promising inhibitors are in clinical trials, including those that target αV and α5β1. Given their distinct function, inhibition of certain integrins, such as αVβ6, may benefit fibrosis or other unmet medical needs. Preclinical studies suggest new indications for integrin antagonists including infectious diseases. (Larson *et al.*, 2005b; Triantafilou *et al.*, 2001). Preclinical studies also indicate combination therapy with integrin antagonists to enhance the efficacy of cancer therapeutics (Burbridge *et al.*, 2003; Matsunaga *et al.*, 2003).

New classes of inhibitors may emerge from recent and ongoing drug development and structural studies. The allosteric sites that regulate integrin activation states offer additional opportunities to circumvent the limitations of peptidomimetic antagonists. Drugs that stabilize the interface between the headpiece and stalks might be developed with improved PK properties, efficacy, and decreased drug-dependent antibody responses. Novel drugs may also be developed that antagonize the interaction with associated proteins, which can block integrin as well as associated protein functions (Silletti *et al.*, 2001). There are also opportunities to develop novel integrin and non-integrin drugs that target the IDAS for autoimmune, cancer, and microbial diseases.

Acknowledgments

We would like to thank Kosi Gramatikoff and Rosemay Vazeux for their assistance with the Figures. We would also like to thank Chanchal Sadhu for αXβ2 related data, Brian Shoichet and J. Irwin for docking studies, and Ama Sikorski and Scott Simon for helpful comments.

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Endogenous TLR Ligands and Autoimmunity

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Abstract

Based on an evolutionary conserved repertoire Toll-like-receptors (TLRs) donate specificity to innate immune cells. Therefore, TLRs are considered as paradigmatic for "self" versus "non-self" discrimination. This view, however, needs to be modified since TLR's also appear to recognise "endogeneous", that is host-derived ligands, examples being host-derived DNA and –RNA. Here I discuss physiological and pathophysiological consequences of endogeneous ligand-recognition by TLRs. I conclude that endogeneous ligand recognition by TLRs drives sterile inflammation sustained by innate immune cells in certain autoimmune disorders.

1. Introduction (Short Overview on TLR Immunobiology)

Toll-like receptors (TLRs) recognize invariant pathogen-specific molecular patterns (PAMPS) derived from bacterial and viral species (Akira and Takeda, 2004; Beutler, 2004; Medzhitov, 2001; Wagner, 2004). TLRs are germline encoded, nonrearranging receptors primarily expressed by innate immune cells such as macrophages and dendritic cells (DCs). Based on an evolutionary conserved repertoire (Hoffmann and Reichhart, 2002), TLRs donate specificity to innate immune cells since they discriminate "foreign" from "self." Among 11 reported TLRs, TLRs 2 (1, 6), 4, 5, and 11 are expressed at the cell membrane and recognize pathogen-derived lipoproteins, lipopolysaccharide (LPS), flagellin, and propellin, respectively (Fig. 1). On the other hand, the TLR family members recognizing pathogen-derived nucleotides are expressed in endosomal/lysosomal compartments (Ahmad-Nejad *et al.*, 2002; Heil *et al.*, 2003; Matsumoto *et al.*, 2003), presumably on translocation from the endoplasmatic

ADVANCES IN IMMUNOLOGY, VOL. 91 0065-2776/06 $35.00

DOI: 10.1016/S0065-2776(06)91004-9

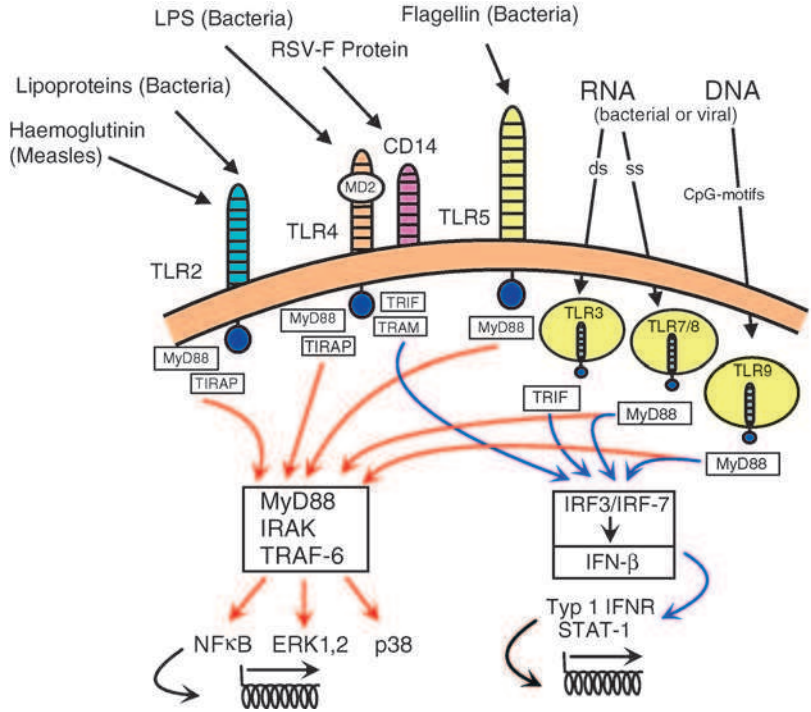

Figure 1 TLR-mediated signal pathways for induction of proinflammatory cytokines or type 1 interferons (IFN). Innate immune cells express TLRs 2 (1/6), 4, 5, and 11 (not shown) at the cell membrane, while TLRs 3, 7 (8), and 9 initiate signaling within endosomal/lysosomal compartments. Thus the efficacy of endosomal ligand translocation represents a bottleneck for the stimulatory potential of the respective ligands. In the case of TLRs 7 (8) and 9, both induction of NFκB-dependent proinflammatory molecules and that of type 1 IFN is controlled by the adaptor molecule MyD88, while TLRs 3 and 4 use the adaptor molecule TRIF for induction of type 1 IFN. IRF-3 and IRF-7 play key roles for type 1 *IFN* gene activation. On IFNβ induction, the type 1 IFN receptor amplifies via STAT signaling the induction of *IFN-α* gene family members (blue arrows). Activated MyD88 recruits IRAK family members and TRAF-6 and thus activates NFκB and the kinases ERK1, 2, and p38 (red lines).

reticulum (ER) (Latz *et al*., 2004). It follows that the ligands for TLR3, that is, double-stranded (ds) RNA, for TLRs 7 and 8, that is, single-stranded (ss) RNA (TLR8 is not operative in mice; Heil *et al*., 2003), and for TLR9, that is, CpG-DNA, first need to translocate to endosomal compartments in order to become functional. Translocation occurs either directly via receptor-mediated endocytosis (Wagner, 2004) or during processes associated with cell infection by pathogens. Thus, the efficacy of endosomal translocation might represent a major determinant for ligand-driven TLRs 3, 7–9 signaling (vide infra).

One of the hallmarks of TLR signaling is the induction of costimulatory molecules on antigen-presenting cells (APCs) and the induction of chemokines and cytokines including type I interferons (IFN) and Interleukin 12 (IL-12). The latter two cytokines are key for Th1-polarized T cell responses, since they promote both clonal expansion and differentiation into effector cells by acting directly on CD8 T cells (Kolumam *et al*., 2005; Marrack and Kappler, 2004). In most cases, TLR triggered-induction of chemokines and cytokines depends on a common molecular pathway that is anchored by an adaptor protein termed MyD88. In the case of TLRs 2 and 4, MyD88 heterodimerizes with TIR domain-containing adaptor protein (TIRAP) while in the case of TLRs 7–9, 5, and 11, MyD88 acts as homodimer. TLR3 uses the adaptor molecule TIR-domain-containing adaptor protein inducing IFN-β (TRIF), and not MyD88, as homodimer, while in the case of TLR4, TRIF heterodimerizes with TRIF-related adaptor molecule (TRAM) (Fig. 1). MyD88 contains a structural region termed death domain, which allows association with molecules that further transmit signals (Akira and Takeda, 2004). These MyD88-interacting molecules include IL-1 receptor–associated kinase (IRAK-1), IRAK-4, the TNF receptor–associated factor (TRAF) 6 as well as the IFN regulatory factor (IRF)-5 (Taniguchi *et al*., 2001). As complex, these signaling molecules are critical for the activation of the transcription factors NFκB and AP-1, which in turn control induction of proinflammatory cytokines. The complexity of the MyD88-signaling pathways is further underscored by the TLR-dependent induction of type IFNs. Not only TLRs 3 and 4 trigger type I IFN production (via TRIF and TRIF/TRAM, respectively) but also the cytoplasmatic MyD88 transductional-transcriptional "processor." For example, IRF-7 has been shown to interact with MyD88 (Honda *et al*., 2004; Kawai *et al*., 2004) and TRAF3 has been defined as component that controls activation of type I IFN responses (via both the TRIF and the MyD88 signal pathway), without affecting induction of NFκB-dependent proinflammatory cytokines (Häcker *et al*., 2005).

Analysis of cellular expression of TLRs has been hampered by discrepancies of mRNA expression and responsiveness to TLR agonists as well as lack of reliable antibodies (Ab) to TLRs. For example, human neutrophils express mRNA for TLRs 1–10, yet respond only to LPS (TLR4 agonist) and to zymosan (TLR2 agonist) (Neufert *et al*., 2001). Human natural killer (NK) cells respond only to CpG-DNA when primed by IL-12 (Sivori *et al*., 2004), and eosinophils preferentially respond to the TLR7 agonist R848, even though they express mRNA for TLRs 1, 4, 9, and 10 (Nagase *et al*., 2003). Of note, freshly isolated human or murine plasmacytoid (p) DCs express only functional TLRs 7 and 9, whereas human myeloid DCs express functional TLRs 1, 2, 3–6 but not TLR9 (reviewed in Krieg, 2002; Wagner, 2004). This contrasts with expression pattern observed in human common (c) DCs. All splenic murine but not human c-DC subsets express functional TLRs 7 and 9. Yet mouse pDCs lack TLR3 and

murine CD8α$^+$ DCs do not express TLR5 and are poor in TLR7 (Edwards *et al*., 2003). Somehow unusual is the situation with cells of the adaptive immune system such as B and T cells. B cells express both TLRs and clonally rearranged antigen receptors. For example, immunostimulatory CpG-DNA motifs have originally been unraveled via their mitogenicity toward murine B cells (reviewed in Krieg, 2002; Wagner, 2004). In contrast, naive human B cells fail to respond to CpG-DNA unless costimulated via CD40, while human memory B cells respond to CpG-DNA by producing "their memory" antibodies (Bernasconi *et al*., 2002). Of note, activated human and murine T cells express TLR2, and synthetic TLR2 agonists reportedly costimulate antigen-driven primary T cell activation, while memory T cells respond directly to TLR2 agonists (Komai-Koma *et al*., 2004; Sobek *et al*., 2004). Surprisingly, regulatory CD4$^+$ and CD25$^+$ murine T cells (Treg) reportedly express functional TLR4 (Caramalho *et al*., 2003), and TLR8-mediated signaling in human CD4 Treg ablates their regulatory (reg) T cell function (Peng *et al*., 2005).

It is striking that the subset of TLRs-recognizing molecular ligands unique to microbial and perhaps fungal cells (TLRs 1, 2, 4, 5, 6, and 11) are cell surface bound, while TLRs that recognize bacterial and viral nucleotides (TLRs 3, 7, 8, and 9) are found intracellularily in vesicular, endosomal compartments (Ahmad-Nejad *et al*., 2002; Bernasconi *et al*., 2002; Latz *et al*., 2004), presumably on translocation from the ER (Latz *et al*., 2004). At present, it is assumed that cell surface–bound TLRs discriminate self from "nonself" by primarily recognizing pathogen-derived foreign ligands. However, a number of studies indicate that TLRs 2 and 4 might also respond to endogeneous, that is, host-derived ligands, including fibrinogen, heat shock proteins, or β-defensins (Biragyn *et al*., 2002; Smiley *et al*., 2001). While the caveat of potential contamination of such *in vitro*-defined endogeneous ligands has to "withstand" time, endogeneous ligands for TLRs 2 and 4 may at least in part, explain diseases associated with "sterile" inflammation, perhaps including at least in part rheumatoid arthritis and arteriosclerosis (Andreakos *et al*., 2004). On the other hand, it has become clear that endogeneous (host-derived) RNA and DNA trigger TLRs 7 (8) and 9, respectively, DNA-CpG methylation not withstanding. Perhaps it is the endosomal localization of TLRs 3, 7, 8, and 9, which helps to distinguish foreign from self nucleotides (vide infra).

It is important to discriminate between "primary" and "secondary" cytokines triggered upon TLR activation. Cytokines, such as IL-12 or TNF-α, are produced by innate immune cells (DCs, macrophages) in direct response to ligand-driven TLR activation. Yet such primary cytokines can amplify inflammatory responses, an example being TNF-α known to activate cellular responses via TNF receptor 1- or 2-expressing immune cells (Beutler, 2004). Perhaps, the efficacy of TNF-α blockade in treating rheumatoid arthritis (Elliott *et al*., 1994)

or Crohn's disease (van Dullemen *et al*., 1995) is due to blockade of such "second-wave" cell activation by inhibiting TNF-α-mediated secondary NFκB activation. Primary cytokines, such as IL-12, can also prime cells for the production of secondary cytokines, an example being interferon-γ (IFN-γ) production by IL-12-primed NK cells challenged with CpG-DNA (Sivori *et al*., 2004).

2. The Innate: Adaptive Immune Connection

The thymus anlage continuously produces thymocytes expressing newly, at random-assembled TCRs out of which high-affinity autoreactive thymocytes become deleted on encountering self-major histocompatibility complex (MHC) peptides (Marrack and Kappler, 1997; Sprent and Kishimoto, 2002). Since thymic (central) purging of autoreactive T cells is insufficient, peripheral tolerance mechanisms operate in addition, an example being induction/maintenance of reg T cells (Bluestone and Abbas, 2003; Sakaguchi *et al*., 2001). In addition, there is compelling evidence that immature DCs also play an important role in peripheral tolerance (Steinman *et al*., 2003). For example, antigen (Ag) targeted to nonactivated, homeostatic DCs leads to Ag-specific tolerance provided the DCs remain immature (Bonifaz *et al*., 2002). Such Ag-presenting immature DCs provide signal 1 for T cell activation associated with a small burst of Ag-specific T cell proliferation followed by their deletion, via apoptosis. In contrast, when Ag-presenting DCs concomitantly become activated by CD40 or TLR ligands they trigger a productive T cell immune response, as mature DCs display a large number of costimulatory molecules (providing signal 2) as well as Th1- or Th2-polarizing cytokines (providing signal 3) (Bonifaz *et al*., 2002; Steinman, 1991). It follows that maturation of Ag-presenting DCs is not only key for the induction of robust immune responses to foreign antigens, but also that "inappropriate" activation of what should otherwise be an immature tolerogenic DC might break peripheral tolerance to self-Ag.

3. TLR-Mediated Immunostimulatory Potential of Self-RNA and -DNA

Earlier experiments implied that unmethylated CpG-DNA motifs stimulate immune cells expressing TLR9, while methylation of cytosine or inversion of CG → GC dinocleotides ablates their immunostimulatory potential (Krieg *et al*., 1995; Wagner, 1999). These results offered an explanation for the failure of vertebrate DNA to activate innate immune cells in a TLR9-dependent fashion since vertebrate DNA is heavily methylated. However, as the TLR9 subfamily members are expressed at subcellular endosomal compartments, it is also possible that vertebrate DNA fails to stimulate because it poorly accesses TLR9-expressing endosomes. Today several lines of evidence support the latter contention. First, sera of patients with systemic lupus erythematodes

(SLE) contain immune complexes (ICs) consisting of antibodies and self-DNA and such ICs trigger IFN-α/β production in human pDCs, presumably on Fc-receptor-mediated endocytosis (Vallin *et al*., 1999a,b). Second, genetic studies by Marshak-Rothstein *et al*. established the ability of chromatin-containing ICs to activate murine rheumatoid factor (RF) B cells via sequential engagement of BCR and TLR9 (Leadbetter *et al*., 2002; Viglianti *et al*., 2003). In this system BCR "helps" to translocate ICs into endosomes. On translocation, DNA (within ICs) triggers TLR9 signaling to costimulate B cell activation. Similarly, chromatin ICs translocated via FcRIII (mouse) and FcγRIIa (human)-mediated endocytosis trigger DC activation in both TLR9-dependent and -independent manners (Bave *et al*., 2003; Boule *et al*., 2004; Means *et al*., 2005). To show that vertebrate DNA can activate TLR9, we used the cationic lipid DOTAP® to translocate vertebrate DNA into endosomes of DC. We noted that it is the efficacy of endosomal translocation that represents a major restriction point for the immunostimulatory potential of ds vertebrate DNA and of non-canonical ssDNA (Yasuda *et al*., 2005). We also noted that unlike phosphorothioated CpG-oligonucleotides (ODN), canonical and noncanonical phosphorodiester CpG-DNA sequences activate DCs in a TLR9-dependent fashion (Yasuda *et al*., 2005). Altogether, these studies implicate that endogeneous (host) DNA displays immunostimulatory potential, provided it is efficiently translocated to endosomes of DCs or B cells. Alternatively, host-DNA displays its immunostimulatory potential when its endosomal degradation is impaired, as shown for cells derived from DNAse-II-deficient mice (Nagata, 2005).

Studies revealed that uridine-rich ssRNA sequences represent a natural ligand for murine TLR7 and human TLRs 7 and 8 (Diebold *et al*., 2004; Heil *et al*., 2004). Uridine-rich ssRNA is not only found in viral RNA, such as influenza virus (Diebold *et al*., 2004) or HIV (Heil *et al*., 2004), but also characterize small U1 nuclear ribonucleoproteins (snRNPs) (Achsel *et al*., 2001; McClain *et al*., 2002). In SLE, snRNPs are present in surface membrane "blebs" of cells undergoing apoptosis. We, as others, found that such snRNPs drive pDCs to produce IFN-α/β in a TLR7-dependent fashion (Savarese *et al*., 2005; A. Krieg and A. Marshak-Rothstein, personal communication). The latter data imply that the BCR/TLR9 "two receptor" paradigm established for chromatin DNA-mediated (co)activation of autoreactive B cells can be extended to a BCR/TLR7 paradigm for snRNPs (RNA), against which autoantibodies are prevailing in a subset of SLE patients.

4. Function of IFN-α/β

The IFN-α/β family members consist of many *IFN-α/β* gene products and single genes encoding for IFN-β, IFN-ω, and IFN-λ (Taniguchi and Takaoka, 2002;

Theofilopoulos *et al*., 2005). Stimulation of many cell types results in IFN-α/β production (Theofilopoulos *et al*., 2005). pDCs (also termed "natural IFN-α/β-producing cells"), however, display the unique capacity to secrete large amounts of IFN-α/β on infection with RNA virus (TLR7 dependent) (Diebold *et al*., 2004), DNA virus (TLR9 dependent) (Diebold *et al*., 2004; Hochrein *et al*., 2004; Krug *et al*., 2004; Lund *et al*., 2003), or in response to CpG type-A ODNs (TLR9 dependent) (Krug *et al*., 2001). CpG type-A ODNs are characterized by a central phosphodiester palindrome (containing a CpG motif) and phosphothioated poly-Gs at the 5′ and 3′ end, thereby forming G-tetrad-linked nanoparticles almost in the size of viruses (Kerkmann *et al*., 2004). pDCs induce plasma (B) cell differentiation via IFN-α/β (Jego *et al*., 2003) and modulate reg T cells (Gilliet and Liu, 2002). As far as known, TLR2 (1, 6) fails to trigger IFN-α/β production, while both TLR4 and the endosomally expressed TLRs 3, 7–9 do so. As mentioned, TLRs 7 (8)- and 9-driven IFN-α/β production is MyD88/IRF-7 dependent (Kawai *et al*., 2004), while TLR4 drives IFN-α secretion via TRIF/TRAM → IRF-3 and TLR3 via TRIF → IRF-3 (Fig. 1).

Although the functions of IFN-α/β first have been delineated via their antiviral activities, they effectively modulate adaptive immune responses. For example, IFN-α/β promotes clonal burst of primary activated T cells, proliferation of memory T cells, and prevents T cell apoptosis (Kolumam *et al*., 2005; Marrack and Kappler, 2004; Tough *et al*., 1999). IFN-α/β facilitate γ-IFN production via STAT 4 activation (Nguyen *et al*., 2002), is essential for cross-presentation (Le Bon *et al*., 2003), activate NK cells (Biron *et al*., 1999), and augment MHC class I expression, thus allowing autoreactive CD8 T cells to destroy their target cells as exemplified for insulin-producing pancreas islet cells (Lang *et al*., 2005).

5. Cytokine-Driven DC Activation: A Portal for Autoimmunity?

Immune cells communicate with each other via cytokines. Yet, there appears to exist a connection between certain cytokines and autoimmune diseases. For example, TNF-α is regarded as determining factor in the pathogenesis of rheumatoid arthritis since anti-TNF-α therapy profoundly ameliorates disease progression (Feldmann and Maini, 2001). Another example represents SLE that displays hallmarks of an IFN-α/β-driven disease (Banchereau *et al*., 2004; Vallin *et al*., 1999b). Given that under homeostatic conditions self-Ag presenting immature DCs are one of the determinants controlling peripheral tolerance to self-Ag (Steinman *et al*., 2003), autoimmunity might be viewed as a system driven by functionally opposite cytokines, examples being IFN-α/β and TNF-α, known to control each other (Banchereau *et al*., 2004). According to this scenario autoimmunity reflects a dynamic system influenced by two

opposing vectors, that is, TNF versus IFN-α/β and IL-4 versus IFN-γ. If one of the vectors prevails beyond a certain threshold, immunopathology emerges as consequence. For example when IFN-α/β prevails, then IFN-autoimmunity ensues (SLE, thyreoiditis, diabetes) while TNF-α drives TNF-autoimmunity, such as rheumatoid arthritis, IL-4 triggers allergic diseases and IFN-γ sustains inflammation (reviewed in Banchereau *et al*., 2004).

6. TLRs and Autoimmunity

Autoimmune diseases are complex multigenic and chronic disorders. Given their poorly understood complexity, attempts to define specific roles of TLRs for initiation and maintenance of autoimmune diseases are certainly premature. Here I will not discuss risk factors for their development, such as individual haplotypes (Holmdahl, 1998), polymorphism in genes involved in immunregulation (Pitkanen and Peterson, 2003), or potential mimicry of self components by infectious agents (von Herrath, 2000). Instead, I focus on data implicating a role of TLRs in SLE and speculate on a possible role of TLRs in rheumatoid arthritis.

Although under homeostatic conditions TLRs discriminate between self and nonself, translocation of host-DNA and small nuclear RNAs (snRNAs) (within ICs) into TLRs 7- and 9-expressing endosomes of B cells or DCs causes TLR-dependent cellular activation. In the case of pDCs this triggers robust IFN-α/β secretion, DNA-CpG methylation not withstanding. This conclusion is based on the pioneering work of Alm and Ronnblom (Lovgren *et al*., 2004; Ronnblom and Alm, 2001; Ronnblom *et al*., 2003), on work of Marshak-Rothstein *et al*. who unraveled the BCR/TLR9 two receptor paradigm for activation of autoreactive B cells (Leadbetter *et al*., 2002; Viglianti *et al*., 2003), the stimulatory effects of FcRIII (mouse)- and FcγRIIa (human)-mediated translocation of chromatin IC into DCs (Boule *et al*., 2004; Means *et al*., 2005), and our own work (Savarese *et al*., 2005; Yasuda *et al*., 2005). We used DOTAP® or ICs for endosomal translocation of either vertebrate DNA or snRNAs in pDCs and observed high IFN-α production. Altogether, these data clearly demonstrate TLR-dependent immunostimulatory potential of endosomally translocated self-DNA or self-RNA. Under pathophysiological conditions, this translocation requires either IgG antibodies (Boule *et al*., 2004; Lovgren *et al*., 2004; Savarese *et al*., 2005) or the BCR of RF-B cells, while under homeostatic conditions resistance to DNA-induced B cell autoimmunity prevails via ERK inhibitory pathways and selective desensitization of BCRs from mitogenic calcineurin signaling (Rui *et al*., 2003). This leads to the question whether TLRs 7–9 signaling plays a causative (primary) or simply an enhancing (secondary) role in precipitating autoimmunity. Given a

specific genetic background, we assume that certain virus infections are first to precipitate TLRs 7–9 driven autoimmune reactions.

Increased levels of IFN-α/β in the sera of SLE patients were first reported 25 years ago (reviewed in Ronnblom and Alm, 2001), and therapeutical IFN-α treatment can induce clinical autoimmune disease (Gota and Calabrese, 2003). In addition, microarray studies revealed in PMBC of SLE patients an IFN-α "signature" (Peterson *et al*., 2004) as if IFN-α/β is driving SLE (reviewed in Banchereau *et al*., 2004). Within this scenario, interferonic self-DNA triggers pDCs to produce large amounts of IFN-α/β, which in turn triggers differentiation/maturation of monocytes into IFN-α/β–producing DCs (Blanco *et al*., 2001). Therefore, TLRs 9 and 7 activation by self-DNA and self-RNA, respectively, might be instrumental to sustain serum-borne IFN-α/β levels in SLE.

While in SLE host DNA and host RNA can cause TLRs 7 (8)- and 9- driven α-IFN production by pDCs, TLR-mediated activation of APCs might break self-tolerance and thus trigger development of autoimmunity. For example, T cell receptor TLR transgenic B10.S mice, specific for an Ag-causing experimental autoimmune encephalomyelitis (EAE) in other mouse strains normally do not develop EAE because their APCs (DCs) remain immature under homeostatic conditions. Yet tolerance can be broken via TLR4 (LPS)- and TLR9 (CpG-DNA)-mediated DC activation (Waldner *et al*., 2004). Since infection with certain pathogens has often been associated with autoimmune manifestation, normally quiescent self-reactive T cells might be triggered by TLR-induced APC activation and subsequently cause immunopathology.

So far TLR expression on innate immune cells and B cells has been the focus of this chapter. However, T cells should not be left out. There are two complementing reports on TLR2 expression on human and murine T cells (Komai-Koma *et al*., 2004; Sobek *et al*., 2004), one using as TLR2 ligand *outer membrane protein* (OMP) of Lyme arthritis–inducing Borrelia bacteria (Sobek *et al*., 2004), the other a synthetic tripalmylated lipopeptide (Komai-Koma *et al*., 2004). Since Lyme disease–associated arthritis has some features in common with rheumatoid arthritis, there is a need to explore a possible role of pathogen-derived TLR2 ligands in costimulating collagen-specific T cells in rheumatoid arthritis. Although speculative, both pathogen-derived "exogeneous" as well as host-derived "endogeneous" TLR2 ligands, including Hsp might come into play in rheumatoid arthritis.

7. TLRs and "Innate Autoimmunity"

Chronic inflammation and disordered lipid metabolism are hallmarks of arteriosclerosis. Independent of whether driven by pathogen-derived exogeneous or by host-derived endogeneous TLR ligands (the latter causing "sterile

inflammation"), proinflammatory pathways of innate immune cells foster the development of arterial placques (reviewed in Michelsen *et al*., 2004a). Interestingly, at least two groups have now provided the first *in vivo* demonstration that in mouse MyD88 signaling plays a role in innate immune cell pathology leading to arteriosclerosis (Bjorkbacka *et al*., 2004; Michelsen *et al*., 2004b), one study (Michelsen *et al*., 2004b) in addition implicating TLR4. In parallel, a population-based epidemiologic study arrived at the conclusion that certain TLR4 polymorphisms are associated with low risk for carotid artery artheriosclerosis (Kiechl *et al*., 2002), a finding debated by others (Yang *et al*., 2003). Intriguingly, the proinflammatory and proartherogenic lipoprotein "minimal" modified LDL (low-density lipoprotein) binds *in vitro* to CD14/TLR4 (Miller *et al*., 2003) as well as to the scavenger receptor CD36 (Aitman *et al*., 1999), shown to act as coreceptor for TLR2 (6)-mediated recognition of bacterial lipopeptides (Hoebe *et al*., 2005). Thus, increasing evidence points to a possible connection between endogeneous ligands for TLRs 2 (6), 4, 7–9 and what is termed sterile inflammation. Therefore, one might view sterile inflammation as immunopathology of innate "autoimmunity" as driven by endogeneous ligands of TLRs.

Acknowledgments

Thanks to S. Bauer, G. Häcker, F. Schmitz, and all members of the laboratory for helpful discussions. The work cited was supported by the Deutsche Forschungsgemeinschaft, FORIMMUN and Coley Pharmaceuticals GmbH.

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Genetic Analysis of Innate Immunity

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Abstract

The inflammatory response to microbes—and host perception of microbes in general—is largely initiated by a single class of receptors, named for their similarity to the prototypic Toll receptor of Drosophila. *The mammalian Toll-like receptors (TLRs) are ultimately responsible for most phenomena associated with infection. This includes both "good" effects of infection (e.g., the induction of lasting specific immunity to an infectious agent) and "bad" effects of infection (systemic inflammation and shock). Although they are essential for host defense, no other endogenous proteins can match their lethal potential. The TLR complexes transduce the toxicity of lipopolysaccharide (LPS), cysteinyl lipopeptides, and many other molecules of microbial origin. The identification of the TLRs as the key conduit to host awareness of microbial infection was a victory for reductionism, proving that the complexity of infectious inflammation as a phenomenon belies the simplicity of its origins. It was achieved by a classical genetic approach, proceeding from phenotype to gene. Further analysis of the signaling pathways activated by the TLRs has depended on both classical and reverse genetic methods. Additional work will ultimately disclose the extent to which sterile inflammatory diseases are mediated by aberrations in these pathways.*

ADVANCES IN IMMUNOLOGY, VOL. 91 0065-2776/06 $35.00

DOI: 10.1016/S0065-2776(06)91005-0

1. Introduction

Innate immunity contains and eradicates microbes in the immediate aftermath of inoculation and is so efficient that most infections are resolved without any need for an adaptive immune response. Perhaps if it were but slightly more effective, we would not be aware of its existence at all. Microbes would be known to us only as saprophytes or commensals, and it would be taken for granted that complex eukaryotic organisms did not support microbial invasion or growth. There would be no concept of pathogens, and perhaps no understanding of why we resist infection. Implicit in this discussion is the fact that awareness of phenomena often grows from their occasional failure. And the science of genetics is based on such exceptions.

Innate immunity normally operates on a microscopic scale to prevent infection from spreading, but its macroscopic hallmark is inflammation. When activated on a large scale (e.g., in the context of a widely disseminated infection), innate immunity can be counterproductive and can actually cause the death of the host (Fig. 1). All immune responses are expensive, in terms of the energy they require, their potential for harm to normal tissues, and the specialized cellular machinery that must exist to serve them. Probably for this reason, many defensive mechanisms are inducible rather than constitutive, and as such *an immune system must be capable of sensing the presence of infection.* An important qualification applies here, one that is fairly obvious. While no system is perfect and immune responses do damage healthy tissues, *a mechanism for discrimination between self and nonself* must be operative. Finally, an immune system must also have *an effector arm*, capable of ridding the host of infection or at least containing infection. Innate immunity developed these fundamental requirements shortly after the rise of metazoans, and although it is sometimes portrayed as "primitive" compared to the adaptive immune system, it is actually highly refined, having existed longer than the adaptive immune system.

The understanding that mammals are endowed with an innate ability to sense fungi, bacteria, viruses, and other microbes, recognizing them as nonself and mounting an aggressive response, is nothing new. Rather, it followed closely on the identification of microbes as the causal agents of infectious disease. In the 1890s, the description of "endotoxin" as a heat-stable and highly toxic component of the cholera vibrio (Pfeiffer, 1892) set the stage for the identification of molecules that initiate the innate immune response. By the 1940s the lipopolysaccharide (LPS) character of endotoxin was known and by the 1970s the structure of the toxic "lipid A" moiety of endotoxin had been solved (Luderitz *et al.*, 1973; Takayama *et al.*, 1983). Within a decade thereafter, active lipid A molecules had been synthesized artificially (Galanos *et al.*, 1985; Imoto *et al.*,

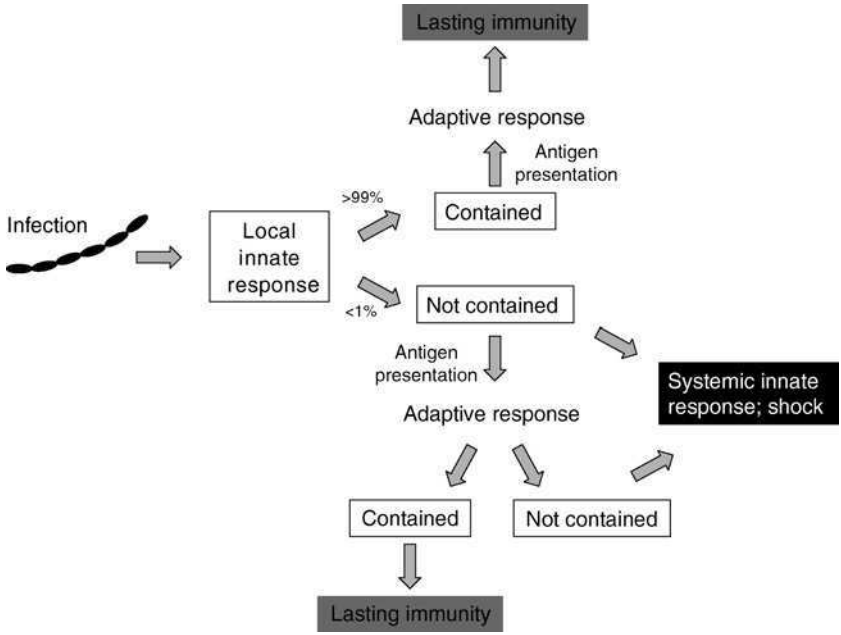

Figure 1 Immune system "decision tree." The great majority of infections are swiftly resolved by the innate immune system, and while they may still eventuate an adaptive response, pose no threat to the host. On occasion, infections are not resolved at an early stage, and become more widespread, triggering a systemic innate response as well as an adaptive response. Such infections do pose a threat to the host, almost always trigger an adaptive response, and may or may not be contained.

1984). Concurrently, the inflammatory and immunoadjuvant character of other conserved molecules of microbial origin (e.g., double-stranded RNA or dsRNA, unmethylated DNA, bacterial lipopeptides, and β-glucans) was described by many authors (Bloksma *et al.*, 1983; Bultmann *et al.*, 1975; Clark, 1979; Haranaka *et al.*, 1984; Urushizaki *et al.*, 1984).

The existence of specific receptors for LPS and other immunostimulatory molecules was at all times assumed, given contemporary knowledge of biological sensing and how it generally worked. Such receptors were widely discussed from the 1970s onward (Adye *et al.*, 1973; Bright *et al.*, 1990; Chaby *et al.*, 1984; Goodman and Morrison, 1985; Jacobs and Eldridge, 1984; Kirikae *et al.*, 1991; Lei and Morrison, 1988; Nygren *et al.*, 1979; Russo and Lutton, 1977; Springer and Adye, 1975; Springer *et al.*, 1973, 1974; Washida, 1978a,b), and the appellation "pattern recognition receptors" (Janeway, 1989) added little to the field, save perhaps a convenient acronym. The identity of the receptors themselves remained mysterious.

CD14, a glycosylphosphoinositol (GPI)-tethered protein expressed on myeloid cells and B cell lines, was identified as one molecular component of the LPS receptor (Wright *et al.*, 1990). The availability of a B cell line that had lost sensitivity to LPS (Mains and Sibley, 1983) supported the concept that CD14 helped to concentrate the LPS signal (Kirkland *et al.*, 1990; Lee *et al.*, 1992, 1993), and in 1996, targeting of the gene encoding CD14 confirmed the importance of this molecule as an LPS sensor *in vivo* (Haziot *et al.*, 1996). However, it was not until 1998 that the signaling "core" of the LPS receptor could be found (Poltorak *et al.*, 1998). This was accomplished through exercise of the classical germline genetic approach, beginning with phenotype and ending with the identification of a molecule. The ultimate revelation was remarkable because the LPS receptor, when found, proved to be one member of a small family of molecules, dedicated to the detection of stimuli that typify essentially all microbes.

The decipherment of the molecular-sensing mechanism by which mammals detect infection was a signal advance in immunology, although much work remains to be done. For those who wish to learn about it right away, the story continues in Section 2. But sensing, as already mentioned, is only one aspect of immunity, and it would be best to begin with the broadest possible perspective. Moreover, it is important to see how classical genetic tools are applied to analysis of immunity, and a more detailed consideration of these issues follows immediately below.

1.1. View of the Evolutionary Biologist: The Key Characteristics of an Immune System

Bacteria and fungi have devised chemical weaponry (e.g., antimicrobial peptides) to promote their survival in the face of competition from other microbes. Bacteria have also evolved restriction endonucleases to guard against the predations of bacteriophage. Multicellular organisms have similarly evolved to survive in the microbial milieu that surrounds them. But they find themselves cast less in the role of competitors, and more in the role of a nutrient source. Many bacteria, fungi, and viruses have developed active programs for the destruction and consumption of multicellular life forms. This is to say that they have evolved as pathogens.

Multicellular species are disadvantaged by their long generation time, which limits the speed at which they may evolve to counter the innovations of pathogens. One mechanism that emerged to cope with this was the development of sensors that could detect signature molecules of microbes that are subject to strong evolutionary constraint. A second countermeasure was the development of a recombinatorial immune system capable of recognizing

virtually all molecules, including those that are not subject to evolutionary constraint and even those that do not presently exist. Recombinatorial immune systems have arisen at least twice in evolution (Pancer *et al.*, 2004) and may have arisen on other occasions yet unknown. In principle, other mechanisms might also have been applied to the generation of highly diverse receptors (e.g., alternative splicing), but no examples of this have come to light.

In general, it may be said that multicellular organisms seize on almost every opportunity in order to protect themselves. A few examples illustrate this point.

1.1.1. Innate Immunity in the Eye of the Beholder

Hemoglobin is a tetrameric protein with two alpha and two beta chains, and its essential function is the transport of oxygen within the blood. However, a mutant form of hemoglobin in which the beta chains are modified by a point mutation (Glu6Val; HbS) offers humans semidominant protection against falciparum malaria. Although the mutation is strongly deleterious in homozygous form, exceptional individuals tolerate it very well; a fact that probably reflects mitigating effects of other mutations (Steinberg, 1989, 1996, 2005). It is likely that HbS would ultimately be driven to fixation, provided that the selective pressure of *Plasmodium falciparum* were strong enough. [A null allele of the gene encoding the *P. vivax* receptor *was* driven to fixation in some parts of the world (Horuk *et al.*, 1993), notwithstanding the fact that this receptor appears to be quite dispensable on erythrocytes]. At that point, hemoglobin would rightly be considered as a protein with two functions essential for life. Its role in oxygen transport would presumably be undiminished. But its role as an effector protein of the innate immune system, required for containment of an otherwise virulent pathogen, would be evident as well. At present, it must be considered that hemoglobin is an evolutionary work in progress.

The Toll protein of *Drosophila melanogaster*, discussed further later, provides a different sort of example, essentially opposite to the first. In all likelihood, this protein evolved with a primary function in immunity since the TIR (*T*oll/*i*nterleukin-1 receptor/*r*esistance) domain is involved in immunity throughout the phylogenetic tree. But the Toll protein was also co-opted to serve embryonic development in the absence of infection, and in this case the mutations that permitted this adaptation were driven to fixation. Flies cannot develop normally without Toll, which functions as a maternal effect gene (Anderson *et al.*, 1985; Schupbach and Wieschaus, 1986). In fact, its immunological function was discovered long after its developmental function was known (Lemaitre *et al.*, 1996).

In mammals, many mutations of the Hermansky–Pudlak Syndrome (HPS) constellation affect hair and skin color by virtue of their effects on proteins involved in melanosome transport (Li *et al.*, 2004). A number of the same mutations also affect neutrophil, platelet, and/or natural killer (NK) cell function by altering the transport or exocytosis of lysosomes and other granules, which are structural analogues of melanosomes. The HPS mutations thereby compromise coagulation and innate immunity as well as pigment deposition. Famous among mutations of this type (though not formally a member of the HPS group) is the *beige* mutation of mice, equivalent to the Chediak–Higashi Syndrome (CHS) mutation in humans and also known by different names in other mammalian species (Barbosa *et al.*, 1996). Did melanosome/lysosome transport develop primarily to serve pigmentation or to serve immunity or coagulation?

The central lesson in each of the examples above is that entirely different biological processes are built on common structural substrata. Hence, many innate immune functions have been built on biological systems with different functions. In other cases, proteins with primary immunological functions have been appropriated to serve nonimmune functions. Innate immunity may be seen as a capability that develops over eons, in many individuals and at many times. But viewing it as a snapshot as we must, the classical geneticist takes a more acute view of the situation and seeks to define its essential components as they stand. The tools of genetics may also identify proteins that might potentially serve an innate immune function. But that is not how the art is usually practiced.

1.2. View of the Classical Geneticist

Biology begins with phenomena, and the key phenomenon at issue in the science of innate immunity is the evident fact that metazoans are hardwired to resist infection. Even in species that lack any semblance of adaptive immunity, robust resistance is apparent. Although the mammalian host may be inoculated with microbes on thousands of occasions, in most cases the microbes are promptly killed and may never come to conscious attention or even provoke a measurable adaptive immune response.

Many biologists are inclined to probe phenomena with hypotheses, seeking support for their hypothesis through experimentation. By contrast, geneticists refrain from doing so, at least in the beginning. Instead, they seek to develop alternative states of phenomena that have an ascribable genetic basis, that is, phenotypes (Fig. 2). From this point, the geneticist defines the molecules that participate in a given phenomenon. Only then do hypotheses follow. The genetic approach is the most powerful in biology, and more than any other,

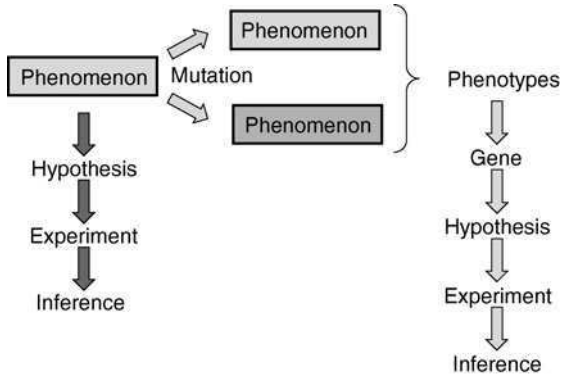

Figure 2 A phenomenon may be directly approached by hypothesis, or analyzed genetically, which entails the use of phenotype. The latter approach is slower in the sense that it entails an added step (the creation of phenotype). But it assures that at least some molecular components that support the phenomenon will be discovered, where immediate resort to hypothesis does not. Moreover, at times, phenomena may be inapparent in the absence of a phenotype, that is, mutations reveal the existence of a biological process that was previously unknown.

it has brought us to our current level of understanding about how biological systems operate.

Classical genetics offers many advantages over other forms of investigation, but two of these stand out. First and foremost, the classical genetic approach is unbiased. It does not invite false inferences in the way that hypothesis-driven research may. There is no vested interest in validating a hypothesis. Rather, there is only the phenotype and its cause. The geneticist does not propose explanations for a phenotype, but searches until he or she finds them. Second, the classical genetic approach entails exploration, and exploration can produce discoveries that are beyond imagining. While the validation of a hypothesis may make a scientist feel clever, it cannot be said to produce surprise, for the scientist has already proposed the hypothesis. In fact, hypothesis-driven research most commonly yields surprises when hypotheses are overturned; a circumstance that is not uncommon, but one which few scientists strive to achieve.

The geneticist is surprised quite regularly, for example, whenever a phenotype is traced to a protein that previously had no known function. And much of a geneticist's effort is directed toward the achievement of surprise. The classical geneticist works, often through mutagenesis, to produce exceptions to the norm, then works to solve the puzzles that he or she has produced. In the realm of immunity, the classical genetic approach may so far be credited with the discovery of the key proteins mediating microbial perception (disclosed by

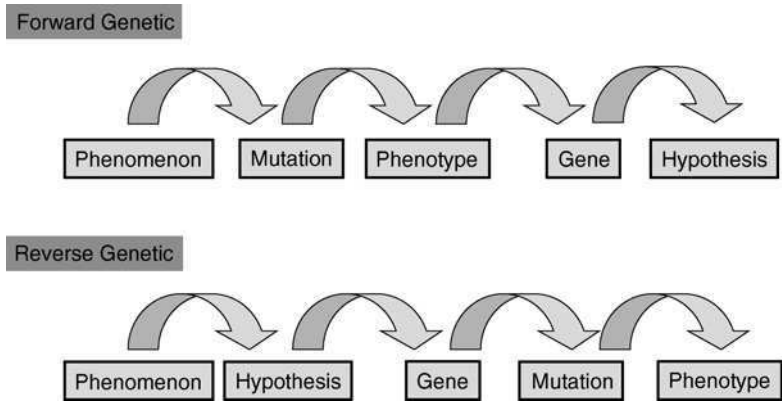

Figure 3 Comparison of the "forward" (classical) and "reverse" genetic approaches. Both begin with phenomena, but in the reverse genetic method hypotheses lead directly to the analysis of gene function, usually through the creation of targeted mutations, while hypotheses are the outcome of the forward genetic method.

the *Lps* phenotype, discussed at length in this chapter), proteins mediating tolerance [disclosed by the *scurfy* (Brunkow *et al.*, 2001), *itchy* (Perry *et al.*, 1998), APECED (Nagamine *et al.*, 1997; The Finnish-German APECED Consortium, 1997), and *roquin* (Vinuesa *et al.*, 2005) phenotypes, among many], and genes mediating effector function [e.g., the *CGD* phenotype (Royer-Pokora *et al.*, 1986)]. In each case, positional cloning was used to identify the cause of phenotypic variation. This process entails genetic mapping of a phenotype to a circumscribed region of the genome (the "critical region") and progressive exclusion of candidate genes until the culpable mutation is found. Reverse genetics (Fig. 3) compliments the classical approach and was so named on the premise that gene (or protein) function could be guessed from gene (or protein) structure. It is a hypothesis-driven pursuit. But as noted later, it can be more efficient than forward genetics, once the latter has opened the door to further discovery.

1.3. Beginning with Phenotype: Sources and Species

A great deal of phenotypic variation is seen in human populations, but mapping the causal mutation(s) is a difficult proposition. In the absence of a large family with many affected individuals, or an extended population in which a phenotype is likely to have arisen from a single mutational event, the identification of causal mutations can prove daunting. If there is a single proband, the question of causality usually remains mute. Nongenetic methods

are instead required to understand the phenotype, and occasionally hypothesis-driven approaches suffice to achieve this goal.

In model organisms, including mice, mapping presents little difficulty. Rather, phenotype is in short supply. More accurately, phenotype appropriate for genetic analysis is a precious commodity. When a phenotypic difference is observed between relatively distant strains of mice (e.g., between the average two *Mus musculus* strains, which can be distinguished at more than one million sites across the genome), it may be difficult to work with. The phenotype may be caused by the combined effect of genetic differences at multiple loci (a *complex trait*), or it may largely result from a difference at a single locus (a *quantitative trait locus* or QTL). In the event that a QTL is responsible for the phenotype, it is mapped and identified by positional cloning, but it is likely that many genetic differences will need to be parsed in order to prove the identity of the culpable mutation, since between distant strains many (and perhaps most) genes are distinguishable, either with regard to the structures of the proteins they encode or with regard to the levels at which they are expressed.

A still more fortunate circumstance arises occasionally when very closely related strains of mice are seen to differ phenotypically. Such was the case with the Lps^d mutation, which arose on the C3H/HeJ background (extremely similar to C3H/HeN and C3H/OuJ, from which C3H/HeJ had been separated only a few years earlier) (Heppner and Weiss, 1965). It was also the case with the LPS-unresponsive phenotype that arose on a C57BL/10 background (Coutinho *et al.*, 1977). In this setting once a mutation has been mapped to a critical region, it will likely be the only mutation that exists to distinguish the two strains.

Regrettably, such mutations are rare, and it does not pay to wait for them to arise. A proactive approach has been adopted in many laboratories, wherein phenotype is deliberately created using germline mutagens and sought by screening. Once a monogenic phenotype is at hand, it may inexorably be found through positional cloning. Many different mutagens have been used over the years to produce phenotype, but systematic analyses have led to the conclusion that the alkylating agent *N*-ethyl-*N*-nitrosourea (ENU) is the mutagen of choice in mice, rendering a mutagenic efficiency close to the maximum that can be tolerated by that species.

1.3.1. ENU as a Supermutagen and Its Potential

Administered to male mice (designated the G0 population) in three doses at weekly intervals, ENU creates ~3000 point mutations (usually A→T transversions or A→G transitions) across the haploid genome (Concepcion *et al.*, 2004;

Justice *et al.*, 1999). These mutations are transmitted to G1 animals by breeding, and a fraction of the mutations are brought to homozygosity in each of numerous G3 mice by one of several optional inbreeding strategies. G1 animals may be screened for dominant phenotype; G3 animals may be screened for both dominant and recessive phenotype.

ENU causes transient sterility and eliminates most spermatogonia in the G0 animals to which it is administered. Between 10 and 100 precursors repopulate the testis over a period of 12 weeks following mutagenesis. ENU dosing is limited by the induction of sterility in G0 males, which is irreversible if too much of the mutagen is injected. However, even if fertility were maintained, it is clear that the mutation load induced by ENU is such that substantial attrition occurs among the G3 population. It may be calculated from the data of Kile *et al.* (2003), who trapped recessive lethal mutations within a circumscribed region of the genome using balancer chromosomes, that ~20% of G3 mice die before weaning as a result of recessive lethal mutations induced by ENU. A tenfold higher rate of mutation would eliminate all but about 14% of G3 animals.

A large body of evidence indicates that ENU-induced phenotype results almost entirely from coding change (including splicing errors) rather than from changes in regulatory regions within the genome. ENU creates about 30 coding changes across the genome in each G1 mouse, and about 4 coding changes are transmitted to homozygosity in each G3 mouse. As just mentioned, homozgosity for 4 random coding changes leads to about 20% attrition of the G3 population, and homozygosity for 40 random coding changes would cause about 90% attrition. The impact of ENU on immune function is less immediately evident than it is on viability because the *genomic footprint* of immunity (i.e., the sum of all nucleotides that are "at risk" to create a detectable immunological phenotype when changed) is smaller than the genomic footprint of viability. However, the size of the immunity footprint is substantial and may be calculated from ENU mutagenesis as detailed later.

Since ENU induces only about four homozygous coding changes across the genome, and since it is usually possible to exclude more than 99.9% of the genome by genetic mapping, one may be relatively confident that a coding change found within a critical region is in fact responsible for the phenotype that is sought. Final confirmation of causality may depend on transgenesis or transfection studies.

Among many advantages of ENU as a mutagen, it is often found that point mutations yield viable hypomorphic, neomorphic, or antimorphic alleles of genes where knockout alleles are nonviable or, in some cases, produce no phenotype. The allelic series created by ENU may therefore offer biological insight where knockouts may not. Further, the mutagenic power of ENU

could, in principle, be harnessed to create functionally null alleles of all genes more quickly and cheaply than gene targeting. In a population of 100,000 G1 mice, premature stop codons could be identified in the great majority of genes.

1.3.1.1. Concept of Phenotypic Saturation A certain set of nucleotides, scattered across the genome, comprise the genomic footprint of a phenotype that is studied with ENU. The footprint is saturated gradually when mutagenesis is applied. Each nucleotide is changed with a probability of 10^{-6} in each G1 mouse. Each mutation is transmitted to homozygosity with a likelihood of 1/8 per G3 mouse (if pedigrees are based on backcrosses). In each G3 mouse examined, the chance of a homozygous change in a given nucleotide is, therefore, 1.25×10^{-7}. If the genomic footprint contains 60,000 bp (as seems to be the case for some rather large footprints), one G3 mouse in 134 will show evidence of the phenotype. But saturation of the footprint is a gradual process and will not be closely approached until many millions of mice have been examined.

The genomic footprint is parceled into an unknown number of genes and long before the footprint is saturated, every gene in the footprint will likely be struck. Nonetheless, some genes may contain only a very few base pairs from the footprint, perhaps two or three. These genes, then, might escape detection for a very long time. All in all, it is wisest to consider ENU mutagenesis as a tool of discovery best applied to "break open" a phenotype. Diminishing return is experienced in its application, and particularly where ENU reveals the function of the first member of a family of paralogous genes, the function of the other paralogues can most rapidly be found by other methods.

1.3.1.2. Source of Phenotype in Natural Populations The "common disease, common variant" hypothesis (Cargill *et al.*, 1999; Chakravarti, 1999; Lander, 1996) holds that disease phenotypes result from a high frequency of mutations that are adaptive in their native environment but maladaptive in the present setting. HbS would be one well-validated monogenic example with immunological significance. Type II diabetes is often proffered as a more speculative polygenic example (without immunological implications). Autoimmune diseases are considered as still more speculative instances of diseases in which a trade-off has been made between optimal immune efficacy and optimal tolerance, favoring the former. The countervailing "multiequivalence" model of Wright *et al.* (1999) holds that rare codominant mutations are responsible for much of human phenotype (including immunological phenotype).

The concept that recent, rare mutations are responsible for much human phenovariance has gained ground, as rare coding variants of *TLR4* have been shown to be abundant in patients with meningococcal sepsis (Smirnova *et al.*,

2003), and rare mutations in genes that are known to influence HDL levels (*ABCA1*, *APOA1*, and *LCAT*) have been shown to be abundant in individuals with markedly aberrant HDL levels (Cohen *et al.*, 2004). As just noted, ENU creates only about 30 heterozygous coding changes per G1 mouse. It has been calculated that about one to two *de novo* coding changes occur per generation in humans (Nachman and Crowell, 2000). Such changes are subject to minimal selective pressure in the heterozygous state and are likely to be transmitted and maintained in the population for long periods of time. Within 600 years (~30 human generations), a *de novo* mutational load comparable to that induced by a single cycle of ENU mutagenesis may accumulate in a human population. Hence, a great deal of relatively recent genetic variation exists among humans populations and may account for much of the phenovariance that is witnessed in every day life.

1.3.2. Other Methods: Haplotype Mapping and Its Potential

Complex genetics diseases in humans (including immunological diseases such as systemic lupus erythematosus or systemic lupus erythematosus (SLE) and type I diabetes) have been approached through haplotype mapping, on the premise that the causative mutations occurred on a traceable genetic background, and occurred long ago. While it must be granted that not enough time has passed to permit a full assessment of the potential of high-density haplotype mapping, it is already clear that it will miss a great deal. The mutational burden of the last few thousand years will escape detection in genome-wide linkage analyses founded on more ancient haplotype anchors, and most useful anchors are more ancient than this.

Whole genome mapping analyses may be seen as the "poor man's sequencing," in that one nucleotide is examined for every few kilobases of genomic sequence in an effort to infer whether ancient mutations lie within genes of interest. But if numerous recent—rather than ancient—mutations are causal, they will be missed. This would include many of the mutations responsible for "obvious" monogenic diseases, for example, most of the thallasemias. On the other hand, no mutations would be missed by whole genome sequencing. It might even be imagined that the advent of extremely cheap, ultrahigh throughput sequencing might spell the "end of genetics," in that only phenotyping work would remain to be done.

1.3.2.1. The (Not Too Distant) Horizon: Sequencing Whole Genomes in Minutes for Pennies It is roundly estimated that by the end of the present decade, genomic sequencing will be performed at a rate of 1 Mb/s for relatively low cost (perhaps less than $1000 per genome). It is not yet certain that this

prediction will come to pass, but it may be guessed with confidence that at some point exceptionally fast and inexpensive sequencing will be possible. At that time, it might be feasible to establish immediate correlations between phenotype and genotype. In some instances, given large enough sample populations, even complex phenotypes might be resolved. In one imaginary scenario, type I diabetes might prove to be caused by 12 different combinations of mutations occurring within a population of 17 target genes.

On the other hand, such a solution might very likely remain elusive for type I diabetes and for many other complex phenotypes, in that they may often be caused by rare combinations of mutations that will escape detection even in large samples of affected individuals. Many phenotypes will also undoubtedly be subject to environmental and/or epigenetic influence.

2. How Do We Detect Infection?

In mammals, perception of microbes is chiefly, though not exclusively, mediated by the Toll-like receptors (TLRs), a family of molecules known since the early 1990s (Nomura *et al.*, 1994; Taguchi *et al.*, 1996) for their structural similarity to Toll, a protein with both developmental (Anderson *et al.*, 1985) and immune-related (Lemaitre *et al.*, 1996) functions in *Drosophila melanogaster*. Toll and the TLRs are single-spanning plasma membrane receptors of variable size, with leucine-rich repeats (LRR) comprising the bulk of the extracellular domain in every family member. A characteristic TIR motif, conserved in defensive proteins found in vertebrates, invertebrates, and even plants, occupies the bulk of the cytoplasmic domain of every family member.

The first mammalian TLR to be identified was called "TIL" to denote its "Toll/IL-1 receptor–like" structure (in present nomenclature TLR1) (Taguchi *et al.*, 1996). Mapped to a chromosome in 1996, it was guessed to be involved in development, since that was the known function of its *Drosophila* homologues (Anderson *et al.*, 1985). In 1997, Janeway and colleagues cloned a second mammalian TLR, naming it "h-Toll" (in present nomenclature TLR4). Based on the fact that Toll was by that time known to have an immunological role in *Drosophila* (Lemaitre *et al.*, 1996), and based on the demonstration that h-Toll could activate NF-κB when forced to multimerize on the surface of mammalian cells, they surmised that the molecule could "activate adaptive immunity" (Medzhitov *et al.*, 1997).

At the time, the following objections to the conclusion that TLRs activate adaptive immunity might have been offered: (1) NF-κB has both immunological and nonimmunological roles, hence, the fact of NF-κB activation by h-Toll ligation did not prove an immunological function; (2) many other host cytokine receptor molecules are also capable of activating NF-κB, yet none is regarded

as the purveyor of a requisite second signal; (3) NF-κB activation was not known to be essential for the upregulation of B7.1 and B7.2, costimulatory molecules that Janeway and colleagues took to be equivalent to adaptive immune activation, and in fact, as subsequent work showed, the type I interferons upregulate costimulatory molecule expression when produced in response to microbial ligands (Hoebe *et al.*, 2003b; Le Bon and Tough, 2002; Le Bon *et al.*, 2001); and (4) most important of all, it was not clear whether the mammalian TLR paralogues recognized endogenous ligands (as is the case in *Drosophila*) or microbial ligands, or for that matter any ligands at all. Nor was it known whether h-Toll or other TLR paralogues were actually involved in primary responses to microbes. In the case of *Drosophila*, only Toll has an immunological function while eight other Toll paralogues do not.

In recent years, it has become clear that TLR signaling is *not* required for a robust adaptive immune response (Section 4.1). To the present time, there is no evidence of a nonredundant second signal for adaptive immune activation. The microbe-sensing function of the TLRs was not deduced by a comprehensive search for receptors that mediated microbial adjuvanticity. Rather, it emerged from genetic analysis of a longstanding phenotype, unresponsiveness to LPS.

2.1. Discovery of the Sensing Role of Mammalian TLRs Was Based on Genetics

The TLRs were first clearly shown to act as innate immune sensors through a pure genetic approach. The mice in question, C3H/HeJ (control strain C3H/HeN) and C57BL/10ScCr (control strain C57BL/10ScSn), had been known to be LPS resistant for several decades. The resistance phenotype was first noted in C3H/HeJ in 1965 (Heppner and Weiss, 1965). An independent mutation was identified in C57BL/10ScCr mice in 1977, and proved to be allelic with the C3H/HeJ defect (Coutinho and Meo, 1978; Coutinho *et al.*, 1977). The Lps^d allele of C3H/HeJ mice was mapped to mouse chromosome 4 (Watson *et al.*, 1978) and, well in advance of positional cloning, was known to be deleterious in the sense that gram-negative infections [e.g., with *Salmonella typhimurium* (O'Brien *et al.*, 1980; Rosenstreich *et al.*, 1982) or *Escherichia coli* (Hagberg *et al.*, 1984)] were poorly contained in mutant mice. This was a solid link between sensing and innate immunity per se. Failure to recognize LPS led to a situation in which the infection would grow out of control. The fact that a single mutation entirely abolished LPS signaling foretold the existence of a single pathway for LPS sensing long before the sensor was identified.

In 1998, the *Lps* locus was positionally cloned and LPS resistance in C3H/HeJ and C57BL/10ScCr mice was concretely ascribed to separate mutations of

Tlr4. In C3H/HeJ mice, a point mutation (P712H, later referred to as the BB loop mutation) abolished LPS signaling (Poltorak *et al.*, 1998); in C57BL/10ScCr mice, the *Tlr4* locus was entirely removed by a deletion, the limits of which were later defined (Poltorak *et al.*, 2000b). Hence, TLR4 appeared to be an indispensable component of the long-elusive receptor for LPS.

Earlier, biochemical and cell-based genetic work had established that CD14, a GPI-anchored LRR protein expressed on the plasma membrane of myeloid cells (later rediscovered by the *Heedless* mutation as described later) was a separate component of the LPS-sensing complex (Lee *et al.*, 1992; Wright *et al.*, 1990). This finding was validated by gene knockout studies (Haziot *et al.*, 1996), and can now be understood in part on a structural basis (Jiang *et al.*, 2005; Kim *et al.*, 2005). However, prior to the identification of TLR4 as the core component of the LPS receptor, it was not clear how the LPS signal could traverse the plasma membrane. Later, a small secreted protein called MD-2 was identified as a third part of the LPS-signaling complex (Nagai *et al.*, 2002; Shimazu *et al.*, 1999). Other components may still be unknown.

2.2. Small Family of Paralogous Proteins Senses Most of the Microbial World

LPS is a structural component of the vast majority of gram-negative microbes and, hence, is represented on about half of all eubacterial species. The evolutionary strategy inherent in sensing LPS is immediately obvious: it is an indispensable signature of gram-negative infection and is distinguishable from all molecules of the host. Hence, with a single receptor complex, encoded by a limited number of genes, most gram-negative bacteria are readily detected, and escape through mutation is difficult given the structural role of LPS in those organisms. It should be noted that although most vertebrates are endowed with a TLR4 orthologue, LPS sensing seems to be a "mammals only" phenomenon among vertebrates. No mechanism for LPS sensing exists in *Drosophila*, nor in most other animal species, such exceptions as horseshoe crabs of the genus *Limulus* (Tsuji and Harrison, 1979) and the giant African snail *Achatina fulica* (Biswas and Mandal, 1999) notwithstanding. In these species, the sensing mechanism does not involve TLR4, or at least is not known to. The fact that mammals and not other vertebrate lines utilized TLR4 for LPS sensing points to the fact that innate sensing mechanisms can evolve quite rapidly. In its ancestral embodiment, TLR4 may very well have served a viral-sensing function, which it retains to the present day in addition to its LPS-sensing function (Georgel *et al.*, unpublished data).

It was immediately supposed that paralogous members of the TLR family might detect other conserved molecules of microbes that initiate inflammatory

responses similar to those driven by LPS, although usually less intense. From 1999 onward, knockout mutations in the remaining TLRs established that each member of the family is endowed with nonredundant recognition specificity. Each TLR detects conserved molecules of microbial origin. It appears likely that all microbes can be detected via TLRs, leading to host awareness of infection. Although other systems for the detection of microbes exist, none is more broadly active or more essential for host survival.

2.3. Evolution of the TLRs and Related Receptors

The TLRs range in size between ~80 kD and 120 kD and are based on recurrent LRR motifs in their ectodomains. All TLRs have a characteristic TIR motif as the major element within the cytoplasmic domain. This is the most conserved part of the molecule and is the part used in most studies of TLR evolution. It is found in some form in plants and in bacteria, and in the latter may serve as a decoy molecule, inserted into the cytoplasm of host cells via a secretion system, where it interferes with host TLR signal transduction.

In the mouse there are 12 TLRs, in the human 10; in both species together 13 (Tabeta *et al.*, 2004). Human TLR10 is not represented in the mouse, and mouse TLRs 11, 12, and 13 are not represented in humans. Other TLRs may exist in other mammals, but no other orthologues have yet been identified. In other vertebrate lines, some of the TLRs have been lost and others duplicated (viz. two nearly identical copies of TLR2 in chickens and in alligators). The *Drosophila* genome encodes nine Tolls (so called to distinguish them from the mammalian TLRs) and only one of these has any known immunological function. When phylogenetic trees are generated using any of a number of methods, *Drosophila* Toll-9 is revealed as a relatively close relative of the mammalian TLRs, suggesting that its precursor antedated the divergence of the last common ancestor of flies and humans, while other modern Tolls of the fly arose subsequently.

The TLRs and Tolls, and the IL-1 and IL-18 receptor subunits, as well as the SIGIRR and ST2 receptors, all share homology within the cytoplasmic domain. However, only the TLRs and Tolls have ectodomains with LRRs. LRRs are the structural building blocks of the recombinatorial receptors represented on lymphoid cells in the hagfish and lamprey (Pancer *et al.*, 2004). These are GPI-tethered proteins that lack cytoplasmic-signaling domains. By way of analogy with CD14 and TLR4, it is conceivable (although not yet established) that these recombining receptors once did (and perhaps still do) signal by way of interaction with conventional membrane-spanning TLRs. Evidently, LRR proteins have been utilized under many circumstances for their ability to tightly

bind target molecules, a propensity first noted in the case of ribonuclease inhibitor, with its exceedingly high affinity for ribonuclease (Kobe and Deisenhofer, 1993, 1995). Other LRR receptors in mammals include RP105, first cloned as a "radioprotective antigen" (Miura *et al.*, 1996; Miyake *et al.*, 1995) and now believed to contribute to LPS sensing by B cells (Ogata *et al.*, 2000), and CD14, which as already mentioned is a component of the macrophage LPS receptor complex.

The CD14 protein (Kim *et al.*, 2005) and the TLR3 ectodomain (Bell *et al.*, 2005; Choe *et al.*, 2005) have both been crystallized and their structures solved. Like ribonuclease inhibitor, both are horseshoe-shaped molecules. TLR3 appears to be dimeric (Bell *et al.*, 2005; Choe *et al.*, 2005), and it is thought that all of the TLRs are probably dimeric. The TLRs are now seen as core elements of receptor complexes, and it is entirely possible that different accessory components of TLRs are installed on different cell types, befitting different sensing requirements. These, in turn, may deliver different signals.

It might have been guessed that the TLR ectodomains, driven by the pressure of a rapidly changing microbial environment, would themselves undergo rapid (diversifying) selection within populations. However, while the TLR ectodomain is the most rapidly changing part of the molecule within populations (Smirnova *et al.*, 2001) and between species (Smirnova *et al.*, 2000), it is clearly subject to weak purifying selection (Smirnova *et al.*, 2001). In this respect, it differs from the class I and class II MHC proteins, which also have direct contact with nonself and are driven to enormous diversity. It may be pointed out that the diversity of molecules recognized by the MHC proteins is far greater than that recognized by the TLRs, which after all detect relatively conserved molecules of microbial origin.

Also interesting from an evolutionary point of view is the fact that some TLRs are lost and others retained by vertebrate genera. Even where TLRs are retained for long periods of time, their sensing function may abruptly change. The best example of this is provided by TLR4, which seems to have acquired LPS-sensing potential only in the mammalian line (already mentioned in Section 2.2).

2.4. Magnitude and Consequences of TLR Signaling

The TLRs ignite the cytokine response that occurs during infection and, to a very large extent, shape the whole of the inflammatory response with all of its consequences, both beneficial and harmful. The TLR-signaling pathways, as we presently understand them, are "hourglass-shaped" in that hundreds of chemically distinguishable ligands impinge on a much smaller number of TLRs

(12 in the mouse or 10 in the human) and signal via a total of only four adapter proteins (Akira and Takeda, 2004; Beutler, 2004; Beutler *et al.*, 2006). These adapters signal via a smaller number of primary kinase molecules [IL-1 receptor–associated kinase (IRAK)-1 and IRAK-4 for MyD88-dependent signaling; Tank binding kinase 1(TBK1) and/or IKKi for MyD88-independent signaling]. Thereafter, a much larger number of kinases are activated via recruitment of TNF receptor–associated factor (TRAF)-6 and the transcription factors NF-κB, AP-1, interferon responsive factor (IRF)-3, and IRF-7 are activated, leading ultimately to the transcriptional modulation of hundreds or thousands of target genes that impel and sustain the inflammatory response (Fig. 4).

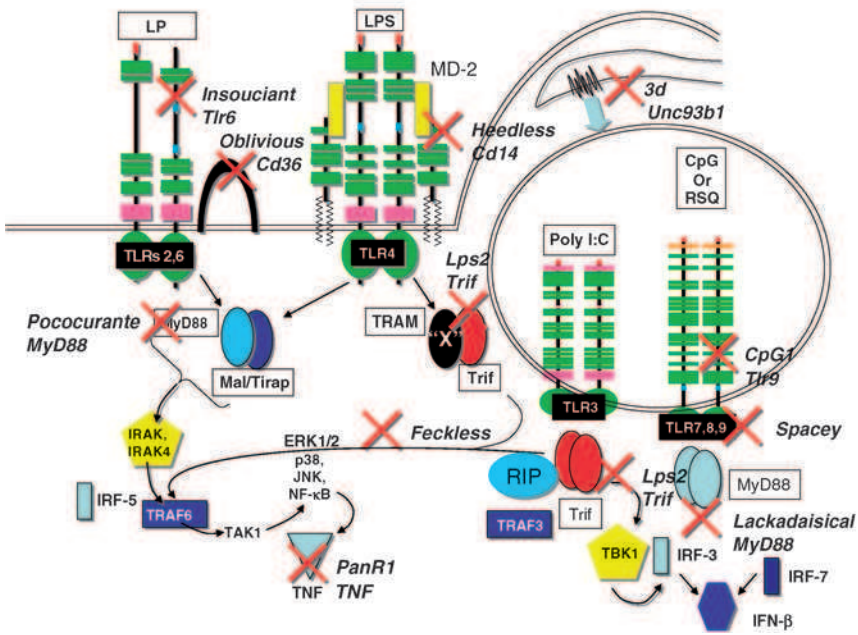

Figure 4 ENU-induced mutations and what they have shown about the TLR-signaling apparatus. Each ENU-induced mutation recovered so far is indicated by a red "X." Phenotypic names are presented together with molecular identifications where established. Other molecular components have been established by gene targeting and/or other methods. The pathway map that emerges has been validated by examining the effects of combined mutations, epistasis experiments, or protein: protein interaction studies. Note that TLRs 3, 7, and 9 are endosomal proteins, while TLRs 2, 6, and 4 (shown) as well as TLRs 1 and 5 (not shown) are expressed on the plasma membrane. UNC-93B is an endoplasmic reticulum protein that influences endosome function.

The magnitude of the response is remarkable given the low abundance of receptors per cell (estimated at ~1000 per macrophage). Given that the lethal effect of LPS is mediated by hematopoietic cells (Michalek *et al.*, 1980), and given that only a fraction of the hematopoietic descendants actually respond to LPS, it may be estimated that only about 100 ng of TLR4 protein transduces the full effect of a lethal dose of LPS. No other endogenous protein is known to be capable of such destructive effects. The level at which signal amplification occurs is not entirely clear.

Acting directly or indirectly via cytokines, the TLRs augment the recruitment and function of professional innate immune responders such as neutrophils, monocytes, NK cells, and NKT cells. They also mediate the adjuvant effect of microbes, favoring a strong adaptive response to alloantigens presented in the course of an infection. The origin of these exceptionally complex biological processes, once nebulous, is now understood at a fundamental level. They are traceable to a discrete collection of receptors, and a relatively small number of microbial ligands. The ligand specificity of the TLRs is, therefore, briefly summarized here.

2.5. What the TLRs Each Detect

TLR2, acting as a heteromer with TLR1 or TLR6 or as a homodimer, is responsible for the detection of microbial lipopeptides (LP) (Buwitt-Beckmann *et al.*, 2005; Morr *et al.*, 2002; Takeuchi *et al.*, 2000, 2001, 2002), lipoteichoic acid (LTA) (Schroder *et al.*, 2003), and zymosan (Gantner *et al.*, 2003; Sato *et al.*, 2003; Underhill *et al.*, 1999). The TLR2/TLR1 heterodimer detects triacyl lipopeptides, such as PAM_3CSK_4, while the TLR2/TLR6 heterodimer detects diacyl lipopeptides, such as macrophage-activating lipopeptide-2 (MALP-2) of *Mycoplasma pneumoniae*. TLR2 also detects diacyl lipopeptides, such as PAM_2CSK_4, acting either as a homodimer or in conjuction with another TLR not yet recognized. It is possible, though not yet certain, that lipopeptides are the sole ligands detected by TLR2 complexes and that zymosan and LTA preparations activate TLR2 complexes because they are contaminated by lipopeptides.

TLR4, as already mentioned, detects LPS (Georgel *et al.*, 2006; Lien *et al.*, 2000; Poltorak *et al.*, 1998, 2000a) as well as certain proteins of viral origin (Jiang *et al.*, 2005; Jude *et al.*, 2003; Kurt-Jones *et al.*, 2000), and it may originally have evolved primarily as a viral sensor because many LPS-insensitive species (e.g., reptiles, amphibians, and fish) have retained TLR4, perhaps to detect rhabdoviral proteins (Georgel *et al.*, 2006). TLR5 detects flagellin, a protein represented in certain gram-negative and gram-positive bacteria (Hayashi *et al.*, 2001; Smith *et al.*, 2003). TLR3 detects dsRNA, which is produced in the course of

virtually all viral infections (Alexopoulou *et al.*, 2001). TLR7 (in mice) and TLR8 (in humans) detect single-stranded RNA (ssRNA) (Diebold *et al.*, 2004; Heil *et al.*, 2004; Lund *et al.*, 2004) or small mimetic drugs, such as imiquimod, resiquimod, and loxoribine (Heil *et al.*, 2003; Hemmi *et al.*, 2002; Jurk *et al.*, 2002), and TLR9 detects DNA bearing unmethylated CpG motifs (Hemmi *et al.*, 2000). It can, under some conditions, detect host DNA as well and, as such, may contribute to the development of autoimmune processes (Leadbetter *et al.*, 2002, 2003; Viglianti *et al.*, 2003). TLR11 detects profilin of *Toxoplasma gondii* (Yarovinsky *et al.*, 2005). In the sense that it recognizes a protein target, it is similar to TLR5, which detects flagellin of both gram-positive and gram-negative organisms (Hayashi *et al.*, 2001). The function of TLRs 12 and 13, which like TLR11 exist in mice but not humans (Tabeta *et al.*, 2004), remains unknown.

The IL-1 and IL-18 receptors also belong to the TLR superfamily, in that they have substantial cytoplasmic domain homology to the TLRs, although they lack the LRR ectodomain structure that all TLRs possess. Their relationship to Toll was discovered even before the mammalian TLRs were identified (Gay and Keith, 1991). The IL-1 and IL-18 receptors activate many of the same signaling events as the TLRs. Their ligands are endogenous rather than microbial but produced in response to TLR-derived signals. IL-1, IL-18, and their receptors may be viewed as components of a system for signal amplification invoked when the host is infected. Other components of the TLR superfamily include ST2, SIGIRR, and TIGIRR, proteins that, like the IL-1 and IL-18 receptors, have immunoglobulin-type motifs in their extracellular domains. ST2 (Brint *et al.*, 2004) and SIGIRR (Wald *et al.*, 2003) appear to exert inhibitory effects on signaling by other members of the family, although the IL-1-like IL-33 has been identified as a ligand for ST2, suggesting that its primary function is not in modulation of other TIR domain receptors (Schmitz *et al.*, 2005). Rather, it probably transduces an inflammatory signal.

2.6. Subcellular Location of the TLRs and Its Significance

While TLRs 1, 2, 4, 5, and 6 are detectable on the surface of cells, the nucleic acid–sensing TLRs (3, 7, 8, and 9) are located within endosomes and do not appear on the plasmalemma. Endosomal location is presumably responsible, in large part, for their ability to discriminate between nucleic acids of host origin versus microbial origin (Ahmad-Nejad *et al.*, 2002; De Bouteiller *et al.*, 2005; Matsumoto *et al.*, 2003; Nishiya and DeFranco, 2004; Nishiya *et al.*, 2005; Rutz *et al.*, 2004).

The TLRs are not only expressed by professional defensive cells but also, at least in many cases, by other cells as well. Expression anatomy databases give

some clues concerning tissue distribution of the TLRs. However, the information has not yet been shown to be predictive of function. Although the TLRs are subject to variable regulation by inflammatory stimuli (sometimes up, sometimes down), and although the promoter regions of some TLRs have been studied in detail (Rehli *et al.*, 2000), it is clear that the TLRs are expressed constitutively on a certain collection of cells and are thus ready to detect microbes should infection occur. It is also clear that the TLRs operate as parts of multiprotein complexes. Other proteins—some with structural resemblance to the TLRs and some without—contribute to TLR-mediated microbial sensing. These accessory proteins may markedly influence whether and how cells perceive microbial infections.

3. Forward and Reverse Genetic Analyses of TLR Signaling

The signal transduction pathways utilized by the TLRs has been elucidated in large part through the use of both forward and reverse genetic methods. In the former approach, the germline is mutated at random using the chemical mutagen ENU, and animals with aberrations of TLR signaling are identified by isolating peritoneal macrophages from the germline mutants and screening diminished production of tumor necrosis factor (TNF) in response to TLR stimulation. To date, more than 21,000 G3 animals have been so examined, with the result that 11 mutations have been found. These mutations have been shown to reside in 10 genes, and 8 of the genes have been identified. It has been surmised that about 23% phenotypic saturation has been achieved; hence, about 45 proteins serve the signaling pathways linking 7 of the TLRs to the production of bioactive TNF (Beutler, 2005).

In the reverse genetic approach, genes encoding presumptive components of the TLR-signaling pathway are knocked out and the effect on TLR signaling is then measured. In specific instances, entirely new components of the signaling pathway have been deduced by hypothesis or biochemical methods and confirmed with reverse genetics. Examples of receptor interacting protein (RIP) this include TRAF-3 (Hacker *et al.*, 2006), IRF-5 (Takaoka *et al.*, 2005), and receptor interacting protein (RIP) (Meylan *et al.*, 2004), none of which was obviously involved but each of which was ultimately proved to be involved in the TLR-signaling pathways by reverse genetic experimentation.

Forward genetic analysis is less efficient than reverse genetic analysis in the sense that it does not rapidly disclose the function of homologous members of a protein family. On the other hand, forward genetic analysis permits the identification of proteins that could not otherwise be guessed to participate in a given biological process. Examples include CD36 and UNC-93B, as discussed later. Random germline mutagenesis has also permitted a reasoned

estimate of the complexity of the TLR-signaling pathways, that is, how many proteins make nonredundant contributions to signaling. We have calculated that in all, about 40–50 proteins are required for 7 of the TLRs to activate the biosynthesis and secretion of TNF (Fig. 4), although not required for life (Beutler, 2005; Beutler *et al.*, 2006).

3.1. Elucidation of the Adapter Proteins That Carry TLR Signals into the Cytosol

The TLRs were guessed to signal by way of cytoplasmic adapter proteins with domain similarity to the receptors themselves, since two such adapters had been recognized prior to the discovery of TLR function. These were MyD88 (Lord *et al.*, 1990; Wesche *et al.*, 1997) and MAL (Fitzgerald *et al.*, 2001), also known as Tirap (Horng *et al.*, 2001). MyD88 was ultimately shown to be required for signaling via most TLRs, except TLR3 (Hoebe *et al.*, 2003a), and for signaling via IL-1 and IL-18 receptors (Adachi *et al.*, 1998). MAL (Yamamoto *et al.*, 2002a) was shown to be required for signaling via TLRs 2 and 4. It was recognized by the year 2001 that adapter proteins other than MyD88 must serve approximately half of responses elicited via TLR4 and essentially all responses elicited via TLR3 (Kawai *et al.*, 2001). Knockout of both MyD88 and MAL was achieved by the year 2002, whereon it was realized that MAL was not the "missing adapter" as initially stated (Horng *et al.*, 2001).

The structural basis of MyD88-independent signaling was first elucidated when an ENU-induced mutation called *Lps2* was found to disrupt this pathway selectively, positionally cloned, and shown to affect a distantly related TIR adapter protein (Hoebe *et al.*, 2003a), elsewhere identified by two-hybrid system analysis and called Toll-interleukin-1 receptor domain-containing adapter molecule (TICAM)-1 (Oshiumi *et al.*, 2003a) or by homology searches and called TIR domain containing adapter inducing interferon β (TRIF) (Yamamoto *et al.*, 2002b). The *Lps2* mutation and the targeted deletion of the *Trif* (*Ticam1*) gene (Yamamoto *et al.*, 2003a) disclosed that both TLRs 3 and 4 signaling depend on TRIF-related adapter molecule (TRAM). Moreover, mice lacking both TRIF and MyD88 are severely immunocompromised and unable to respond to most TLR ligands.

A fourth adapter, known as adapter X (Hoebe *et al.*, 2003a), TICAM-2 (Oshiumi *et al.*, 2003b), or TRIF-related adapter molecule (TRAM) (Yamamoto *et al.*, 2003b), was also identified in the course of these studies. TRAM was suggested (Hoebe *et al.*, 2003a) and later definitively shown (Yamamoto *et al.*, 2003b) to mediate responses to the TLR4 ligand LPS but not the TLR3 ligand dsRNA. TRAM can, by itself, mediate responses to a newly discovered TLR4

ligand, the G-glycoprotein (gpG) encoded by vesicular stomatitis virus (VSV) (Georgel *et al.*, 2006). A fifth putative adapter protein known as sterile alpha motif/armadillo motif protein (SARM) has so far not been shown to participate in TLR signaling (Mink *et al.*, 2001).

In general, the TIR adapter proteins are depicted as functional pairs (dimers), and there is good reason to believe that they may behave as dimers during signaling, despite the fact that crystallographic evidence of dimerization has remained somewhat confusing, in that multiple modes of interaction between subunits have been observed (Tao *et al.*, 2002). Docking studies, coupled with analysis of ENU-induced or site-directed mutations in MyD88, give a different picture of TIR interactions, as discussed later.

MyD88 is believed to form a homodimer (to signal from TLRs 5, 7, 8, and 9) or a heterodimer with MAL (to signal from the TLR2 complexes and from TLR4). TRIF may form a homodimer (to signal from TLR3) or a heterodimer with TRAM (to signal from TLR4). TRAM may also form a homodimer to signal from TLR4. Some macrophages are "TRIF independent" and are able to signal via the TRAM homodimer alone instead of the usual TRIF/TRAM heteromer (Hoebe *et al.*, 2003a). All macrophages utilize the TRAM homodimer to respond to gpG (Georgel *et al.*, 2006).

The ability of different TLRs to direct the formation of different adapter combinations is of key importance to the downstream signaling effects that are rendered. For example, the MyD88/MAL adapter combination is evidently incapable of stimulating activation of either IRF-3 or IRF-7 and, therefore, cannot activate type I IFN synthesis. On the other hand, the TRIF homodimer can activate IRF-3, as can the TRIF/TRAM heterodimer. The MyD88 homodimer can activate IRF-7. On this basis, TLRs 3, 4, 7, 8, and 9 can activate type I IFN production whereas TLR2 cannot do so. However, some puzzles persist. When gpG stimulates TRAM recruitment, it causes abundant production of type I IFN. But it does not activate NF-κB (Georgel, unpublished data). Yet the TRAM homodimer is postulated to drive TNF production in the absence of TRIF, a process that does require NF-κB activation.

3.1.1. Receptor-Selective Mutations in MyD88, and the Nature of the Receptor:Adapter Interface

Two phenovariants, called *Lackadaisical* (*Lkd*) and *Pococurante* (*Poc*), were ascribed to missense errors of MyD88, which as detailed earlier carries signals from most of the TLRs. Lkd largely (although incompletely) prevents signaling via TLRs 7 and 9. Poc prevents signaling via all MyD88-dependent receptors except the TLR2/TLR6 heterodimer, and is specific in its effect, in that only diacyl lipopeptide sensing is spared (i.e., signaling induced by MALP-2 and/or

PAM_2CSK_4 is preserved, while signaling induced by zymosan and LTA are not). In fact, PAM_2CSK_4 signaling may be mediated by both TLR2/TLR1 and TLR2/TLR6 heteromers, insofar as it is not abolished by mutations in TLR6 alone but is abolished by a knockout mutation of TLR2. *Poc* selectively abolishes TLR2/TLR1 signaling (e.g., sensing of PAM_3CSK_4).

The *Lkd* and *Poc* mutations exert receptor-selective effects, and *de facto* it can be assumed that they participate in the receptor:adapter interface. The *Lkd* mutation is located between the death domain and the TIR domain of MyD88, and closer to the former. This region of the molecule has not been studied structurally, and no comment may be made about the interaction between the *Lkd* residue and the receptor itself. The *Poc* mutation is located within the TIR domain and affects a residue that contributes to a flat spot on the surface of the TIR domain.

Remarkably, the BB loop mutation, once believed to abolish signaling function when engrafted into any of the TLRs or into MyD88, behaves in the same way as the Poc site mutation. When the BB loop mutation is engrafted into MyD88 (P200H), the modified adapter supports signaling from the TLR2/TLR6 heterodimer (but not other receptors). When the BB loop or Poc site mutations are engrafted into TLR2, both mutant receptors support detection of diacyl lipopeptides (but not other TLR2/TLR6 ligands). When engrafted into TLRs 4 or 9, both mutations entirely abolish sensing of LPS.

The reciprocal relationship between the mutations in MyD88 and TLR2 suggest that the interaction between MyD88 and TLR2 is different than that between MyD88 and the other TIR domain receptors. However, it should be noted that introducing both BB loop and Poc site mutations into either TLR2 or MyD88 completely ablates signaling function. Taken together, these findings strongly suggest that: (1) the Poc site and the BB loop both contribute to the receptor:adapter interface, (2) the binding of MyD88 to the TLR2/TLR6 heteromer is qualitatively different than the binding of MyD88 to other TLRs.

Independent data, from docking studies using the program SurfDock (Norledge *et al.*, 2003), indicate that the major form of interaction between MyD88 and TLR2 is one in which the BB loops and Poc sites both interact across the interface in a homotypic fashion (Jiang *et al.*, 2006). More than 650 $Å^2$ of surface are buried in the interaction, consistent with a high degree of stability. This most probably reflects the true association between receptor and adapter, the shape of the nexus through which all TLR signals are channeled.

The next most probable docking state is one in which the C-terminal αE helices of TIR domains interact in an antiparallel fashion. As described in work performed by Li and colleagues (Li *et al.*, 2005), and by our own laboratory (Jiang *et al.*, 2006), the αE helix of a TIR domain seems to be involved in TIR

multimerization (either on the part of receptors or adapters). It is at this site that different TIR domains combine homotypically or heterotypically to trigger downstream effects.

The existence of receptor-selective mutations, such as *Lkd* and *Poc*, suggests the likelihood that drugs might be used to selectively target TIR domain interactions. At present, peptide inhibitors based on the structure of the BB loop have shown some promise in their potential to disrupt IL-1R:MyD88 interaction, although they do not disrupt TLR4:MyD88 interaction (Bartfai *et al.*, 2003).

3.2. Cofactors for TLR Signaling

Some (and conceivably all) of the TLRs depend on accessory molecules in order to signal properly and as such may be regarded as core elements in receptor complexes that sense conserved microbial determinants (Fig. 5). CD14 and CD36 are particularly important examples, and each was phenotypically tagged by a premature stop codon induced by ENU. UNC-93B, an intrinsic protein of the endoplasmic reticulum (ER), provides another example, albeit probably an example of a very different kind.

3.2.1. TLR4 Signaling Depends on MD-2 and CD14

Where TLR4 signaling is concerned, one of the accessory components is MD-2, a small secreted protein that is required for all LPS responses (Nagai *et al.*, 2002; Shimazu *et al.*, 1999) and is believed to directly engage LPS

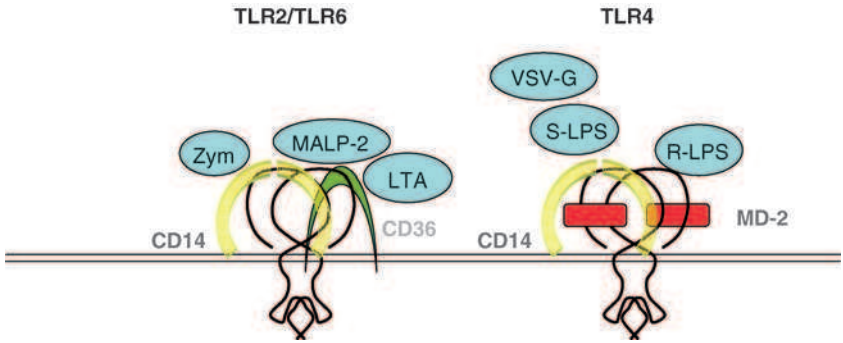

Figure 5 TLRs function as the core elements of receptor complexes with shared and unique elements. Germline mutations have proved the participation of CD14, CD36, and MD-2 in signaling by TLR2 and TLR4 complexes. Coreceptors broaden the specificity of the receptor complexes and in some cases influence the choice of adapters that are recruited. MALP-2 and LTA require CD36 for full signaling efficacy and zymosan does not. CD14 is partly required by all TLR2/TLR6 agonists, fully required by smooth LPS (S-LPS), and partly required by rough LPS (R-LPS). VSV-G, vesicular stomatitis virus glycoprotein G.

(Akashi *et al.*, 2003; Rioux *et al.*, 2001) as does TLR4 itself (Lien *et al.*, 2000; Poltorak *et al.*, 2000a).

A second component of the TLR4 receptor complex is CD14, which is required for the detection of smooth LPS (but not rough LPS or lipid A). CD14 is a LRR protein like TLR4 itself, with a "half horseshoe" shape (Kim *et al.*, 2005), and exists in both GPI-anchored and soluble forms (Frey *et al.*, 1992). Its role in LPS signaling was deduced from cDNA expression studies as early as 1990, but its selectivity as an adjunct to the detection of smooth (rather than rough) LPS and its permissive effect on MyD88-independent signaling were determined from studies of the *Heedless* mutation (Jiang *et al.*, 2005). In the absence of CD14, only MyD88-dependent signaling occurs. On this basis, it has been proposed that CD14 exerts a steric effect on the receptor complex, permitting recruitment of either MyD88 and MAL alone or all four adapter proteins (Jiang *et al.*, 2005). Moreover, in the absence of CD14, the unconventional TLR4 ligand gpG cannot elicit a response (Georgel *et al.*, 2006; Jiang *et al.*, 2005). It is likely that the organizing effect of CD14, therefore, permits the accommodation of some ligands within the receptor ectodomain complex, but that it is not required for all ligands.

Some cells, such as mast cells and B cells, fail to express CD14, and as a result do not respond to smooth LPS (Huber *et al.*, 2006). CD14 can therefore be viewed as a qualitative regulator of TLR4 signaling, which determines exactly what type of response to LPS any particular cell will experience. CD14 also contributes to TLR2-mediated responses (Jiang *et al.*, 2005; Manukyan *et al.*, 2005; Schroder *et al.*, 2003; Yauch *et al.*, 2004), evidently acting as a coreceptor as it does for the TLR4 receptor complex.

3.2.2. TLR2 Signaling Depends on CD14 and CD36

TLR2 signals in conjunction with TLRs 1 or 6, and very likely with TLR10 in humans since TLR10 is a close phylogenetic relative of TLRs 1 and 6. It is widely believed that TLR2 also signals as a homodimer, since some ligands (e.g., the lipopeptide PAM_2CSK_4) are TLR2 dependent but not TLR1 or TLR6 dependent (Morr *et al.*, 2002). It is possible that other TLRs, with less homology to TLRs 1 and 6, also participate in the TLR2 complex. Unpublished studies in our own laboratory have suggested that TLR11 of mice augments TLR2-dependent lipopeptide-induced signaling. The TLR2 complex seems to detect diverse ligands with little structural similarity to one another (e.g., LTA, zymosan, and di- and triacyl cysteinyl lipopeptides). For this reason alone, it might be imagined that many components of the receptor complex have yet to be determined. One added component, required for sensing diacylglycerides via the TLR2/TLR6 heterodimer, is CD36. A double-spanning plasma membrane protein

CD36 had previously been identified as a receptor for anionic phospholipids (Rigotti *et al.*, 1995), oxidized low-density lipoprotein (Endemann *et al.*, 1993; Kunjathoor *et al.*, 2002) and other classes of lipoprotein (Calvo *et al.*, 1998), and for the uptake of fatty acids (the "fatty acid translocator," or FAT) from plasma (Abumrad *et al.*, 1993; Bonen *et al.*, 2002; Brinkmann *et al.*, 2002; Glazier *et al.*, 2002; Ibrahimi *et al.*, 1996). It has also been identified as a receptor for thrombospondin (Asch *et al.*, 1987; Silverstein *et al.*, 1989; Tuszynski *et al.*, 1989), and for the β-amyloid fragment derived from amyloid precursor protein (APP) (Bamberger *et al.*, 2003; El Khoury *et al.*, 2003; Moore *et al.*, 2002). While it is believed to activate tyrosine kinases, such as Syk, in a ligand-dependent manner (El Khoury *et al.*, 2003), CD36 has very few cytoplasmic amino acids (less than five at either the N-terminal or C-terminal end of the molecule) and it is difficult to imagine that they form a domain of sufficient size or complexity to achieve this directly. Hence, it is reasonable to assume that CD36, like CD14, might signal indirectly.

Earlier shown to achieve close proximity with CD14 in response to ceramide stimulation of cells (Pfeiffer *et al.*, 2001), CD36 was recognized as a coreceptor for TLR2/TLR6 signaling by positional cloning of the ENU-induced mutation *oblivious*, which caused diminished sensing of MALP-2 (a diacyl lipopeptide) and LTA. *Oblivious* was ascribed to a premature stop codon in CD36, and subsequently found to cause susceptibility to gram-positive bacterial infections (Hoebe *et al.*, 2005). The *oblivious* mutation was further observed to block NKT cell activation by gangliosides, such as α-galactosylceramide (unpublished data), and also inhibits the priming of CD4 cells by a specific antigen administered in the context of a cell that has been induced to undergo apoptosis (Janssen *et al.*, in press). CD36 thus seems to make an important contribution to several different immunological processes, occurring in diverse cell types. Notably, CD36 is one of three class B scavenger receptors. The other two paralogues have been denoted SCARB1 and SCARB2 (Calvo *et al.*, 1995). SCARB1 knockout mice show a similar defect of NKT cell function but no defect of TLR2/TLR6 signaling (Hoebe *et al.*, unpublished data). SCARB2 knockout mice die postnatally with hydronephrosis and multiple neurological disorders (Gamp *et al.*, 2003).

CD36 is relatively polymorphic in human populations (Aitman *et al.*, 2000; Kashiwagi *et al.*, 1994, 1995a,b, 1996; Nozaki *et al.*, 1995; Yamamoto *et al.*, 1994) and it has been suggested that individuals with CD36 mutations are hypersusceptible to cerebral malaria (Aitman *et al.*, 2000). Both at a population level and at a cellular level, it is likely that the presence or absence of CD36 influences TLR2/TLR6 signaling, and it is possible that other members of the CD36 family are also influential in TLR signaling. The *Pococurante* mutation (Section 3.1.1) has shown, among other things, that TLR2/TLR6 signaling

provides substantial protection to the host by itself (Jiang *et al.*, 2006), and CD36 is required for full integrity of TLR2/TLR6 signaling. Particularly within tissue environments in which TLR signaling pathways other than the TLR2/TLR6 pathway are minimally active or absent, CD36 may have a very prominent role in host defense.

3.2.2.1. The Modular Character of the TLR2/TLR6 Receptor Complex From the foregoing discussion, it may be deduced that the TLRs generally function as multisubunit protein complexes. The TLR2/TLR6 receptor is particularly elaborate and most likely contains, so far as we know to date, a total of at least six proteins. These include the receptor components TLR2, TLR6, CD14, CD36, and the adapter components MyD88 (which may engage TLR2 in at least two conformationally distinguishable ways) and MAL. Dectin-1 a type II transmembrane molecule with C-type lectin domains (Ariizumi *et al.*, 2000) may represent yet another accessory molecule that acts to enhance TLR2/TLR6 signaling induced by β-glucans such as zymosan (Brown *et al.*, 2003; Gantner *et al.*, 2003, 2005), although its role as such has not been confirmed by a germline mutation as yet.

The steric relationship between these proteins is not yet clear, despite crystallographic advances that have led to much better understanding of at least one TLR (TLR3) (Bell *et al.*, 2005; Choe *et al.*, 2005) and TIR domains themselves (Khan *et al.*, 2004; Tao *et al.*, 2002; Xu *et al.*, 2000). If we begin with the assumption that the "core" of the complex is a heterodimer of TLRs 2 and 6, we may first note that some TLR2/TLR6-dependent signaling events (e.g., zymosan detection vs diacyl lipopeptide detection) seem to depend selectively on different regions of the TLR2 ectodomain (Meng *et al.*, 2003). Second, some TLR2/TLR6-signaling events clearly depend on the presence or absence of CD36 (again, zymosan signaling is CD36 independent while diacyl lipopeptide and LTA detection require CD36) (Hoebe *et al.*, 2005). All TLR2/TLR6 signaling events depend on CD14, but only in part (Hoebe *et al.*, 2005). And the *Pococurante* mutation (described in Section 3.1.1) entirely abolishes zymosan and LTA signaling but not lipopeptide signaling (Jiang *et al.*, 2006). The differential effects of mutations in different accessory proteins can be taken to imply that signaling is not "all or none." Rather, the core receptor can function when some components of the complex are missing. But signaling is qualitatively different under this circumstance.

3.2.3. TLRs 3, 7, and 9 Signaling Depend on UNC-93B

The nucleic acid sensors TLRs 3, 7, and 9 are located within endosomes, and acidification of the endosomal compartment is required for signaling from any

of these TLRs to occur (Ahmad-Nejad *et al.*, 2002; De Bouteiller *et al.*, 2005; Hacker *et al.*, 1998; Lee *et al.*, 2003; MacFarlane and Manzel, 1998; Manzel *et al.*, 1999; Rutz *et al.*, 2004; Sioud, 2005). Other properties of the endosome, still poorly understood, are also of key importance, since a single mutation known as *3d* can abolish signaling from TLRs 3, 7, and 9, although it does not affect any of these proteins directly, neither their expression levels nor their subcellular location (Tabeta *et al.*, 2006). Mice homozygous for the *3d* mutation are immunocompromised, in that they do not mount adequate responses to mouse cytomegalovirus (MCMV) or *Staphylococcus aureus* infections. In the former case, the infectious process is acute, and defective TLR signaling is almost certainly responsible for the enhanced virulence of the microbe (Tabeta *et al.*, 2004, 2006). In the latter case, the infection model is chronic, and a more complex problem may be at issue. The *3d* mutation markedly impairs exogenous antigen presentation, which like TLR3/7/9 signaling, depends on events that occur in the endosome. A failure of adaptive immunity may prevent the host from clearing the pathogen.

The *3d* mutation is a missense error within the *Unc93b1* gene, which encodes UNC-93B, a 12-spanning component of the ER. The protein cannot be identified within any category of the endosomes, a fact that suggests the likelihood of physical contact or signaling between the ER and the endosomes mediated by UNC-93B itself. In one scenario, UNC-93B might act as a mediator of fusion between the ER and endosomes, permitting the former to contribute proteins essential for the function of the latter. UNC-93B is particularly abundant in antigen-presenting cells such as myeloid and plasmacytoid dendritic cells and B cells. It is far less abundant in other tissues, and this might diminish nucleic acid sensing via TLRs 3, 7, and 9.

In *Caenorhabditis elegans*, the prototypic UNC-93 protein is believed to be a regulatory subunit of a tripartite two-pore potassium channel. The other components of the channel are Sup9 and Sup10. The former is homologous to the mammalian acid-sensitive potassium channel (TASK) proteins. The latter has no known mammalian homologue. Mammalian UNC-93B is not believed to be associated with potassium transport because mutant mice neither have abnormal potassium concentration within any of the endocytic vesicles nor within the ER nor do they fail to acidify endosomes (Tabeta *et al.*, 2006). It is possible, although uncertain, that various paralogues of the same family of proteins might serve a channel function in mammals. Two paralogues, UNC-93A and UNC-93C, have been identified by our group. UNC-93A, UNC-93B, and UNC-93C each bear a DUF895 motif (DUF, domain of unknown function) that is a member of the major facilitator sequence (MFS) clan. The DUF895 motif may have functional importance within each of the UNC-93 proteins. One testable possibility is that it represents an interaction domain.

Alternatively, it may direct the traffic of other molecules that are required for normal endosome function.

3.2.4. Other Mutations Disclosed by ENU Mutagenesis

In the nature of the classical genetic approach, some mutations are found within familiar components of the system under study. *CpG1*, a mutation in TLR9, *Insouciant* (*Int*), a mutation in TLR6, and *PanR1*, a mutation in TNF (the endpoint of the assay used in screening for mutations), are in this category. Each was, nonetheless, informative. The *CpG1* mutation disclosed for the first time that TLR9 plays an essential role in defense against MCMV infection (Tabeta *et al.*, 2004). The *PanR1* mutation pointed to a surface residue involved in TNF receptor engagement, one with dominant properties that might potentially be used in anti-TNF therapy (Rutschmann *et al.*, 2006). It also showed that the development of germinal centers and secondary lymphoid organs may not actually depend on TNF (a conclusion that was based on studies with the TNF knockout). The *Int* mutation was informative in the analysis of TLR2/TLR6 signaling (Jiang *et al.*, 2006).

3.2.5. Present Understanding of Tissue-Specificity and Inhibition of TLR Signaling

It has been noted that TLR4 is expressed on colonic epithelium (Ortega-Cava *et al.*, 2003), but it may be argued that there is little reason for it to be expressed there, since the presence of high concentrations of LPS may be taken for granted in the colon. In fact, many of the TLRs are expressed on many different cell types that are not ordinarily regarded as "professional" immune cells, and which may or may not actually signal the presence of microbes in the way that macrophages do. Obviously, there are many ways such specificity might be enforced. The target genes induced by TLR signal transduction (e.g., encoding inflammatory cytokines) might be genetically accessible in some cells but silent in others. Components of the transducing pathways might be expressed in some cells but not others. Components of the transducing pathways might be translated to form different products as a result of alternative splicing in different cell types or might be posttranslationally modified in different cellular environments. Repressors of signaling [including suppressor of cytokine signaling (SOCS)-1 (Kinjyo *et al.*, 2002), ST2 (Brint *et al.*, 2004), SIGIRR (Wald *et al.*, 2003), or IRAK-M (Kobayashi *et al.*, 2002)] might be differentially expressed, limiting signaling in some cells but not others. And signals elicited by other cytokines and/or hormones can exert a strong inhibitory influence on specific components of the TLR-signaling pathway.

Examples of each type of phenomenon are known. For example, the TNF gene is inaccessible in HeLa cells and that the highly accessible TNF locus of RAW 264.7 macrophages is extinguished on fusion of RAW 264.7 cells and HeLa cells so that LPS is no longer able to induce it. The sequences responsible for silencing are located in close proximity to the coding region itself (Kruys *et al.*, 1993). The MyD88 gene can be spliced to form two mature transcripts (Burns *et al.*, 2003; Janssens *et al.*, 2003), one incorporating exons 1–5, and one lacking exon 2. The latter encodes a shortened isoform of the protein, MyD88S. MyD88S contains both the N-terminal death domain and the C-terminal TIR domain characteristic of the long form of MyD88 but lacks a substantial portion of the intervening polypeptide chain. It appears to inhibit all TLR signaling, perhaps by a competitive mechanism, but is selective in doing so. Where IL-1R signaling is concerned, MyD88S prevents NF-κB activation but not AP-1 activation (Janssens *et al.*, 2003). The *Lackadaisical* mutation (Section 3.1.1) resides within the region that is missing in MyD88S, and prevents productive signaling via TLRs 7 and 9, but not other TLRs (Jiang *et al.*, 2006). MyD88S is expressed in spleen and is induced by LPS activation of macrophage cell lines (Burns *et al.*, 2003). Glucocorticosteroid hormones, which impede TLR signaling (Beutler *et al.*, 1986), operate by preventing the formation of p65/IRF-3 complexes within responsive cells (Ogawa *et al.*, 2005).

4. TLR-Independent Detection of Microbes

Not all microbial perception depends on TLRs. Some of the molecules that trigger TLR responses are detected by alternative systems as well. For example, the genetically defined dsRNA1 pathway (Hoebe *et al.*, 2003b) revealed the presence of dsRNA sensors independent of TLR3 and TRIF, the TLR sensor of dsRNA and its sole adapter protein. The molecular basis of nucleic acid sensing in the cytosol has now been approached using reverse genetic methods, and at least two RNA helicases (dubbed RIG-I and MDA-5) also bearing CARD domains seem to detect viral RNA molecules, triggering activation of TBK1 by way of an intermediate CARD domain protein called IPS-1, MAVS, VISA, or Cardif (Kawai *et al.*, 2005; Meylan *et al.*, 2005; Seth *et al.*, 2005; Xu *et al.*, 2005). Lgp2, another CARD/helicase protein, may have an inhibitory role in signaling (Rothenfusser *et al.*, 2005; Yoneyama *et al.*, 2005). DNA can also be detected through a TLR-independent mechanism, details of which remain mysterious at present (Ishii *et al.*, 2006; Okabe *et al.*, 2005). Both TLR-dependent and TLR-independent pathways for sensing nucleic acids have been reviewed elsewhere in detail (Kawai and Akira, 2006).

Among intracellular sensors of microbial infections, a family of proteins with LRR motifs, nucleotide-binding domains, and CARD domains has also

received frequent mention in recent times. In particular, the nucleotide-binding oligomerization domain (NOD) proteins NOD1 and NOD2 have been identified as sensors of peptidoglycan (Inohara *et al.*, 2005), and NOD2 mutations have been causally associated with Crohn's disease through positional genetic methods, although with low penetrance (Hugot *et al.*, 1996, 2001; Ogura *et al.*, 2001). One plausible interpretation holds that Crohn's disease is a form of immunodeficiency in which failure to detect intracellular microbes permits their proliferation within cells of the mucosa, and ultimately to a strong inflammatory response mediated by other sensors such as TLRs. In fact, in a mouse model, deficiency of NOD2 causes enhanced susceptibility to orally administered *Listeria monocytogenes* (Kobayashi *et al.*, 2005).

4.1. TLR-Independent Activation of Adaptive Immunity

From the standpoint that molecules such as LPS, poly I:C, and unmethylated DNA have long known to have adjuvant properties, the fact that TLRs can augment an adaptive response is unsurprising. They are, of course, the receptors for these ligands. They mediate perception of molecules required for adjuvanticity, including, for example, the active principles in Freund's adjuvant, or LPS, which was long known to be an adjuvant (Condie *et al.*, 1955), dependent on the product of the *Lps* locus (Skidmore *et al.*, 1975).

However, TLR signaling is in no sense "required" to activate an adaptive immune response. The misperception that TLRs are required for this purpose grew from the pronouncement that h-Toll could "activate adaptive immunity" with the attendant suggestion that TLRs provided an essential second signal for adaptive immune responses (Medzhitov *et al.*, 1997). To the contrary, it is now abundantly clear that strong adaptive immune responses develop in the absence of TLR signaling, for example, in mice lacking MyD88 and TRIF activity. Neither T cell nor B cell activation depends on TLR signaling. Allografts are rejected by these mice, and normal levels of serum immunoglobulins are observed in their blood. They show normal lymphoid organ size and architecture. The notion that two signals are required for an adaptive immune response to an antigen, and that one of these signals must be delivered by TLRs, is incorrect.

4.1.1. Adaptive Immune Activation in the Context of Cell Death

While apoptosis is often perceived as a "bland" process that eventuates no immune response, evolutionary reasoning suggests that cell death, elicited by microbial infection, ought to be detected in and of itself. This view has been expressed as the "danger hypothesis," although it might be commented that "danger" is a rather nonspecific descriptor (Matzinger, 2001a,b, 2002). In this

context, a cell death–driven pathway, initiated by a definable population of lymphoid cells that acquire antigenic material from targets that have been induced to undergo apoptosis and present it to cross-prime $CD8^+$ T cells, has been described (Janssen *et al.*, 2006). Antigens administered to mice within cells that have been irradiated or induced to undergo apoptosis by Fas ligation are readily detected by means of this pathway. Notably, cells killed by freeze-thawing or heating do not serve as adjuvants.

Whatever their effects on restricted populations of dendritic cells *in vitro*, apoptotic cells do not elicit tolerance *in vivo* but instead stimulate a powerful $CD8^+$ T cell response to foreign proteins. By a wide margin, the death-driven pathway is the most potent means of inducing a $CD8^+$ T cell response to antigens, in that picogram quantities of antigen, presented in the context of apoptosis, will drive cytotoxic T-lymphocyte (CTL) development (Fig. 6).

The death-driven immunoadjuvant pathway, like all known adaptive immune response pathways, depends on the upregulation of costimulatory proteins that drive T cell division. A number of different laboratories have demonstrated the importance of type I IFN in the upregulation of costimulatory proteins (Hoebe *et al.*, 2003b; Le Bon and Tough, 2002; Le Bon *et al.*, 2001), and type I IFN production is required for the response to apoptotic cells as well. The death-driven pathway exists within a lymphocyte-like population of antigen-presenting cells (APCs), noted for their $CD8^-$, $B220^-$ status, and is fully operational in the absence of TLR signaling. However, both the UNC-93B protein and the type I IFN receptor are essential for cross-priming to occur via this pathway (Janssen *et al.*, 2006). The nature of the receptors that sense cell death, and the nature of the molecules that are detected on dying cells, remain to be established.

Since cell death occurs commonly *in vivo*, but does not trigger autoimmune responses under normal circumstances, it follows that the death pathway must be highly regulated and that it must be capable of distinguishing physiologic from pathologic cell death. This, in turn, would imply the ability to recognize specific molecules that mark the latter process as pathologic. Given that next to nothing is known about the mechanism of detection and the signals that arise from it, a classical genetic approach is highly appropriate and has already been initiated to study the phenomenon (Hoebe, unpublished data).

5. Global Resistome

So far, a tightly focused forward genetic screening effort has been described, one aimed at elucidation of components of the TLR-signaling pathways. But the larger question of which genes (and how many genes) are required for host defense in general can be approached using germline mutagenesis as well.

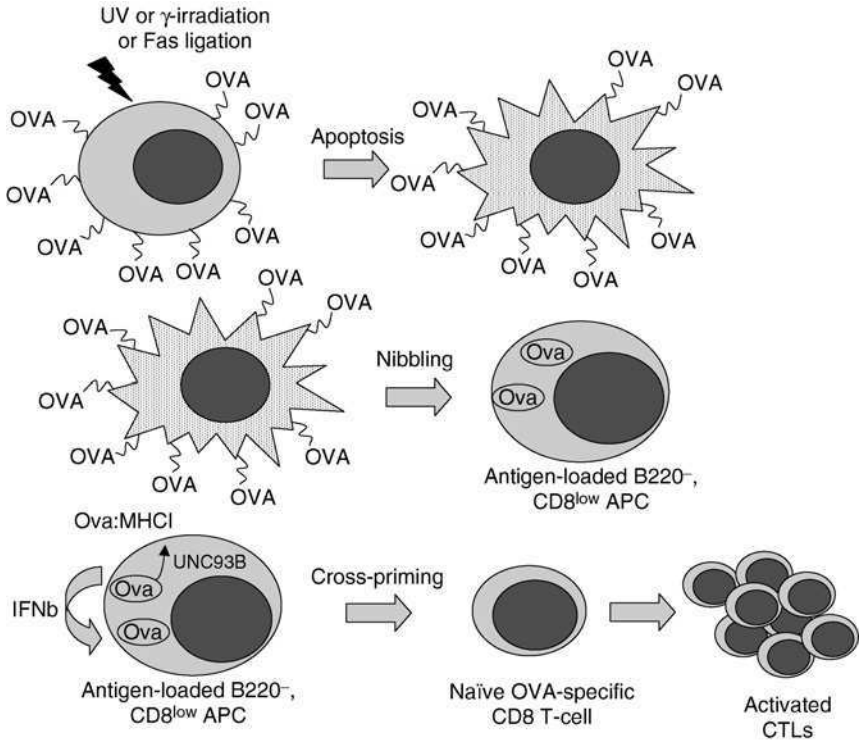

Figure 6 An immunoadjuvant pathway driven by programmed cell death. The pathway does not depend on TLR signaling, and the triggering ligands induced by programmed cell death are unknown. The *3d* mutation of Unc93b1 prevents the APC from presenting exogenous antigen so that cross-priming is inhibited. Type I interferon production and action (presumably autocrine) is not dependent on UNC-93B but is required for cross-priming to occur.

Using a specific pathogen as a probe to test host resistance, it is possible to identify all genes with nonredundant function in that phenomenon. MCMV is an excellent screening tool, since it has exceptionally sharp dose-lethality characteristics. While C57BL/6 mice never succumb to an inoculum of 10^5 PFU of Smith strain MCMV, they never survive an inoculum of 10^6 PFU. In an initial study of 3500 G3 mice (Crozat *et al.*, 2006), a total of eight recessive mutations were identified. One of these (*Domino*) was positionally cloned, and found to represent a missense allele of *Stat1*, causing complete inactivation of the STAT1 protein. STAT1 is well known to be required for both type I and type II IFN signal transduction. The other mutations are presently being mapped and cloned in turn.

Remarkably, while the *Domino* mutation leads to death within 4 days following MCMV inoculation, other mutations are far more severe in their effect on survival. Other mutations known as *Goodnight*, *Solitaire*, and *Slumber* (each yet to be identified) cause death within 1.5 to 3 days following inoculation (Crozat *et al.*, 2006). This would suggest that some components of the resistome are far more important to survival of the host than any part of the IFN system.

From the fact that eight mutations were observed among 3500 mice, it was deduced that 1 pedigree in 33 carried a recessive mutation causing immunocompromise in the face of MCMV infection. Further, ~30,000 bp comprise the genomic footprint of the MCMV susceptibility phenotype, and at least 1% of all genes play a nonredundant role in resistance to MCMV, defined as the ability to survive for 6 days following inoculation of the virus. This defined window of time largely excludes the contribution of adaptive immunity, which develops subsequently. If 1% of all genes serve nonredundant functions in resistance to MCMV, we might next inquire into the size of the "global resistome": that collection of genes that confers resistance to all microbes (Fig. 7).

It is repeatedly observed that the innate immune system is degenerate, in the sense that many different microbial species are managed through a common set of host defense molecules. For example, TLR4 contributes to the recognition of numerous gram-negative organisms and some viral pathogens. MyD88 is important for the response to *most* organisms. So too are many distal executors of the innate immune response, for example, cytokines such as type I and type II IFN and TNF. For this reason, it may be anticipated that the global resistome might not be much larger than the MCMV resistome, perhaps encompassing a small percentage of all genes.

6. Endogenous Activators of TLR Signaling?

In the immediate aftermath of the discovery that TLRs could sense LPS and other molecules of microbial origin, a plethora of publications suggested that endogenous proteins [heat shock protein 70 (HSP70), antimicrobial peptides, fibrinogen, fibronectin, and others] lipids, and nucleic acids served as ligands for TLR activation as well. While some of these reports undoubtedly reflect the impurity of the preparations used and the impressive sensitivity of TLRs as detectors of microbial molecules, the question remains: can TLRs sense endogenous molecules of the host? And if so, are they important factors in sterile inflammatory diseases?

We might begin with a consideration of rheumatoid arthritis, ankylosing spondylitis, psoriasis, and Crohn's disease. At least the first three of these four

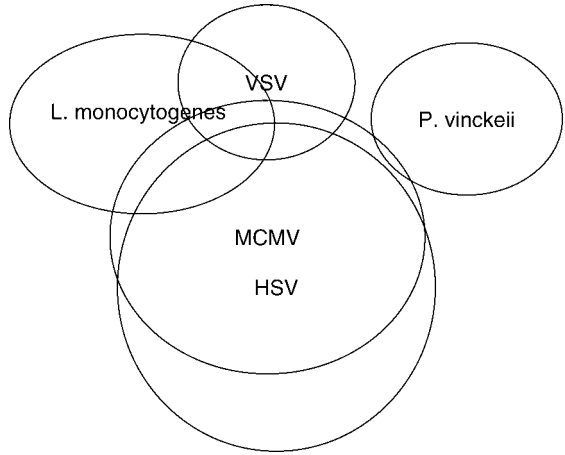

Figure 7 Different equipment is required for protection against different microbes. For each microbe, a resistome may be defined, that set of genes with nonredundant function in host resistance. When pathogens are closely related (e.g., Mouse cytomegalovirus, MCMV, and herpes simplex virus, HSV), resistomes may be expected to overlap extensively, whereas for more distantly related pathogens, far less overlap of resistomes is expected. For some pathogens, a relatively small resistome may apply, whereas for others it will prove to be large, reflecting the complexity of the pathogen and its prevalence over an evolutionary timescale. In many instances, it is found that genes confer resistance to a wide variety of different microbes (consistent with the inherent degeneracy of the innate immune system). Examples would include MyD88, IFNg, and others. While the MCMV resistome is believed to comprise ~1% of the genome, the universal resistome might not be many times larger.

diseases would generally be considered "sterile" inflammatory diseases, that is, diseases in which microbial flora or pathogens play no direct inciting role. If autoimmune, they are not classically so, in the sense that autoreactive T cells are not thought to play a primary role in pathogenesis. The terms "innate autoimmunity" applies here, in that the innate immune system is clearly capable of damaging host tissues through chronic, inappropriate inflammation (Beutler, 2004).

It is well established that TNF is an important mediator in the pathogenesis of each of these four diseases, insofar as marked amelioration is observed when TNF activity is blocked (Elliott *et al.*, 1994a,b). It may also be said that NF-κB activation is a minimum condition for TNF synthesis to occur (Shakhov *et al.*, 1990), although a nonredundant requirement posttranscriptional events (Han *et al.*, 1990) have also been established. What, then, induces NF-κB activation?

Several pathways might conceivably be implicated, including the TGF-β-signaling pathway, which stimulates activation of TGF-β-activated kinase 1 (TAK-1), and thence, potentially, to the activation of the signalosome

(Ninomiya-Tsuji *et al.*, 1999; Takaesu *et al.*, 2003). The TLR-signaling pathways are also suspect, and it is possible that endogenous molecules may, at times, trigger TLR responses. For example, hyaluronic acid has been shown to activate TLR4 (Termeer *et al.*, 2002), and hyaluronic acid is known to be released in the course of many inflammatory processes. Mammalian DNA can also trigger activation of TLR9 (Leadbetter *et al.*, 2002; Viglianti *et al.*, 2003). In this connection, it has been pointed out that anti-chromatin antibodies bring mammalian DNA into the mammalian endocytic pathway, where by triggering TLR9 it may induce the expansion of B cell clones that are specific for chromatin or chromatin:IgG complexes. This type of autoamplification loop may drive inflammation in authentic autoimmune diseases like SLE (Leadbetter *et al.*, 2003). Finally, endogenous lipids might activate TLR signaling via the CD36/TLR2/TLR6 complex (Hoebe *et al.*, 2005). In this connection, TLR signaling appears to contribute to atherosclerosis, currently regarded as a chronic inflammatory disorder (Bjorkbacka *et al.*, 2004).

The potential to block inflammatory disease by inhibiting TLR signaling is particularly appealing, first because the TLRs are probably the most powerful proinflammatory receptors in existence, and second since it is clear from mutations in mice that the blockade of individual TLRs is relatively well tolerated. Most microbes are detected when they elicit responses from multiple TLRs. In effect, the TLRs cover for one another, although each shows unique specificity. Although global blockade of TLR signaling causes relatively severe immunocompromise, it too might be contemplated as a therapeutic strategy if blockade were partial or short term. Since the TLRs detect small molecular agonists, and since there is precedent for the development of TLR-specific antagonists (Hawkins *et al.*, 2002; Kawata *et al.*, 1999), it is probable that each of the TLRs might be blocked pharmacologically.

7. Conclusions About Innate Immunity and the Classical Genetic Approach

The impulse to experiment is one that geneticists resist. They try, instead, to ascertain the key molecular participants in a biological phenomenon before asking how these molecules work. They deliberately attack phenomena with mutations, seeking to make them fail, then map the mutations to understand why they failed. On many occasions, well-known but recalcitrant phenomena may be understood through the classical genetic approach; more rarely, mutations may even expose biological phenomena that were not previously known to exist. In the realm of innate immunity, genetic analyses have revealed how the mammalian host becomes aware of infection and have shed much light how the key receptors of the innate immune system signal. From this point, new exceptions have been sought. While the TLRs unquestionably mediate

adjuvant effects, they are not required for adaptive immune responses. What molecules are? How does programmed cell death drive CTL activation? What proteins are essential for the antiviral response, and among these how many support immune sensing, how many support immune effector function, and how many represent dedicated cellular restriction factors that impede viral proliferation? Germline genetic methods have opened many fields and, in the near term, may be expected to offer unique insight into each of these questions.

Acknowledgments

This work was support by the National Institute of Health (AI054523).

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TIM Family of Genes in Immunity and Tolerance

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Abstract

T cells on activation differentiate into different subsets (Th1 or Th2) with distinct effector functions. These T cell subsets are primarily differentiated on the basis of the cytokines that they produce, however, we have identified a novel gene family called TIM *(T cell, immunoglobulin, mucin domain–containing molecules), whose members are differentially expressed on Th1 and Th2 cells. Three of the family members (Tim-1, Tim-3, and Tim-4) are conserved between mouse and man. Genomic association of the TIM family and polymorphisms in both Tim-1 and Tim-3 in different immune-mediated diseases suggest that the family may have an important role in regulating immunity, both in terms of normal immune responses and in diseases like autoimmunity and asthma.*

1. Introduction

On activation with antigen: major histocompatibility complex (MHC) and costimulatory molecules, T cells differentiate into subsets with distinct cytokine profiles and effector functions. In 1986, Mosmann, Coffman, and colleagues described two different subsets of $CD4^+$ effector T cells based on

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ADVANCES IN IMMUNOLOGY, VOL. 91 0065-2776/06 $35.00

DOI: 10.1016/S0065-2776(06)91006-2

cytokine production, namely Th1 and Th2 (Mosmann *et al.*, 1986). These cells are not distinct lineages, but rather arise from a common naïve $CD4^+$ T cell precursor in response to different stimuli and the cytokine milieu (Abbas *et al.*, 1996). However, once they are committed to a lineage, they maintain a heritable pattern of cytokine production that cannot be changed (Glimcher and Murphy, 2000; Murphy *et al.*, 1996). Since the original identification of Th1 and Th2 cells, additional T cell subsets have been identified, including Th3, Tr1, and Th-IL-17 cells, which again are each induced by different cytokines, produce different effector cytokines, and have distinct effector functions (Chen *et al.*, 1994; Groux *et al.*, 1997; Langrish *et al.*, 2005).

Th1 cells produce the prototypic cytokine IFN-γ, which is predominantly involved in the clearance of intracellular pathogens through the activation of macrophages and the induction of immunoglobulin class switching to antibodies that fix complement and assist in pathogen clearance (Abbas *et al.*, 1996; Boehm *et al.*, 1997). Th2 cells produce IL-4 and assist in the elimination of extracellular pathogens by activating B cells and inducing immunoglobulin class switching to IgE and IgG1, leading to mast cell activation (Abbas *et al.*, 1996; Nelms *et al.*, 1999). However, generation of a Th1 response to self antigens has been shown to induce autoimmune diseases, and an excessive Th2 response to environmental antigens has been shown to induce allergies, atopy, and asthma (Abbas *et al.*, 1996). Th1 and Th2 cells crossregulate the functions of one another through antagonistic activities of cytokines and transcription factors (Mowen and Glimcher, 2004; Szabo *et al.*, 2003). Although Th1 cells are known to mediate organ-specific autoimmunity, another subset of T cells, which are induced by IL-23 and produce IL-17 (Th-IL-17) have been shown to induce tissue injury and autoimmune disease (Langrish *et al.*, 2005). Since IL-23 shares a subunit with IL-12, which induces Th1 cells, it has been suggested that Th1 cells and Th-IL-17 cells may be related in their phenotype and function (Bettelli and Kuchroo, 2005), although some data suggest that the two subsets may be distinct (Harrington *et al.*, 2005; Park *et al.*, 2005).

T-bet was identified as the master regulator of Th1 differentiation (Szabo *et al.*, 2000). Induction of this transcription factor during differentiation is vital for Th1 development and IFN-γ production, and expression of T-bet *in vitro* can reverse Th2 polarization and induce IFN-γ production even in committed Th2 cells (Szabo *et al.*, 2000, 2002). T-bet upregulates IL-12Rβ2, and engagement of the IL-12 receptor by IL-12 induces Stat4 phosphorylation and leads to transactivation of the IFN-γ gene (Afkarian *et al.*, 2002; Szabo *et al.*, 2000). A positive feedback loop then ensues, wherein the increased IFN-γ further upregulates T-bet and thus ensures Th1 commitment (Lighvani *et al.*, 2001; Szabo *et al.*, 2002). GATA-3 has been demonstrated to be the key transcription

factor specifically expressed in Th2 cells, and its expression is sufficient to direct T cell differentiation to the Th2 pathway (Mowen and Glimcher, 2004). Stat6, another member of the Stat family of transcription factors, is specifically activated by IL-4R (Wurster *et al.*, 2000). Activated Stat6 binds to the IL-4 promoter and 3′ enhancer, thus stimulating IL-4 production and establishing a positive feedback loop to augment Th2 differentiation and effector functions (Kotanides and Reich, 1996; Mowen and Glimcher, 2004). Previous data suggested that Th1 and Th2 cells crossregulate the expansion and function of one another, however, no molecular mechanism for this crossregulation had been identified. Analysis of the key Th1:Th2 transcription factors T-bet and GATA-3 has demonstrated that, during Th1 differentiation, Itk-mediated tyrosine phosphorylation of T-bet allows T-bet to bind to the N-terminal region of GATA-3 and prevent GATA-3 from upregulating gene programs involved in Th2 differentiation (Hwang *et al.*, 2005). This demonstration that the master regulator of Th1 differentiation can directly interact with and inhibit the master regulator of Th2 differentiation indicates that T cell differentiation can be crossregulated and controlled once a T cell has committed to a specific path.

Since Th1 and Th2 cells play such important roles in the induction of many immunopathological conditions, we and others have been searching for cell surface molecules that can phenotypically differentiate Th1 cells from Th2 cells, so that specific T cell subtypes can be identified and isolated in a pathological condition *in vivo*. Despite the extensive study of Th1 and Th2 cells over the past two decades, these cells have traditionally been distinguished by their patterns of cytokine production, as very few cell surface markers could reliably distinguish between them. Several molecules were reported as Th1-specific (CXCR3, CCR5, and Chandra) or Th2-specific (CCR3, CCR4, CXCR4, CCR8, ICOS, and T1/ST2), but the differences in expression of these molecules was generally quantitative rather than qualitative, additionally, several of these molecules are also present on naïve T cells and therefore are not useful markers for identifying terminally differentiated T helper subsets in an immunopathological condition *in vivo* (Bonecchi *et al.*, 1998; Lohning *et al.*, 1998; Venkataraman *et al.*, 2000). In addition to assisting in T helper subset identification, cell surface molecules specifically expressed on these subsets would be expected to play a role in regulating their effector functions and could allow manipulation of these functions.

To identify Th1-specific cell surface molecules, we generated a panel of monoclonal antibodies against mouse Th1 cells and found four monoclonal antibodies specific for Th1 but not Th2 cells (Dardalhon *et al.*, 2005; Meyers *et al.*, 2002; Monney *et al.*, 2002). Using expression cloning, we found that two of these antibodies recognized Tim (*T* cell, *i*mmunoglobulin, and *m*ucin

domain-containing molecule)-3, a novel molecule expressed on the surface of Th1 cells (Monney *et al.*, 2002).

2. *TIM* Family of Genes

Analysis of the chromosomal region surrounding *Tim-3* identified a family of similar *Tim* genes consisting of eight members (*Tims 1–8*) on mouse chromosome 11B1.1 and three members (*TIM-1*, *-3*, and *-4*) on human chromosome 5q33.2 (Fig. 1A). Within the mouse locus, the only other intervening genes are two ribosomal proteins (Xiao, S., and Kuchroo, V. unpublished observations), although several *TIM*-like pseudogenes are also present (Kuchroo *et al.*, 2003). The family members that are conserved between mouse and man, *Tim-1*, *-3*, and *-4*, have received the most attention to date. Since mouse *Tim-2* has high homology with mouse *Tim-1*, it has also been considered as a potential orthologue of human *TIM-1*. Each of the *Tim* genes is predicted to encode a type I membrane protein with a similar structure, consisting of a signal sequence followed by an IgV-like domain, a mucin-like domain, a transmembrane region, and an intracellular tail (Fig. 1B). Mouse Tim-1, -2, and -3 have a conserved predicted tyrosine phosphorylation motif in their intracellular tails, and each also contain other tyrosine residues that could be involved in signaling processes (Kuchroo *et al.*, 2003).

The first Tim protein to be identified was discovered as the cellular receptor for Hepatitis A virus (HAV) in African green monkeys (Kaplan *et al.*, 1996) and humans (Feigelstock *et al.*, 1998), and these studies demonstrated that TIM-1/HAV cellular receptor (HAVcr) transcripts were broadly expressed in all tissues examined, including liver, spleen, and kidney. Subsequent studies of renal ischemia-reperfusion injury in the rat found that Tim-1, referred to as KIM-1 (kidney injury molecule-1), is upregulated in tubular epithelial cells of kidneys during injury and tubular repair (Ichimura *et al.*, 1998). These authors noted the structural homology of KIM-1 and MAdCAM-1, and postulated its function as a kidney epithelial cell adhesion molecule (Ichimura *et al.*, 1998).

The *TIM* family is located in a genomic interval (mouse chromosome 11 and human chromosome 5q23–35) that has been repeatedly linked to autoimmune disease and asthma in both mouse and man. This region contains the IL-4 gene cluster (which includes the genes encoding IL-4, IL-5, IL-13, and GM-CSF) and several other immune-related genes, such as IL-12 p40, CD14, and ITK. In mice, this chromosome 11 region has been linked with susceptibility to type 1 diabetes (*Idd4*) and to experimental autoimmune encephalomyelitis (EAE) (*Eae6*) (Encinas and Kuchroo, 2000). In humans, genome wide scans have linked chromosome 5q23–35 with susceptibility to rheumatoid arthritis, autoimmune thyroid disease, type 1 diabetes, Crohn's disease, asthma, and allergic

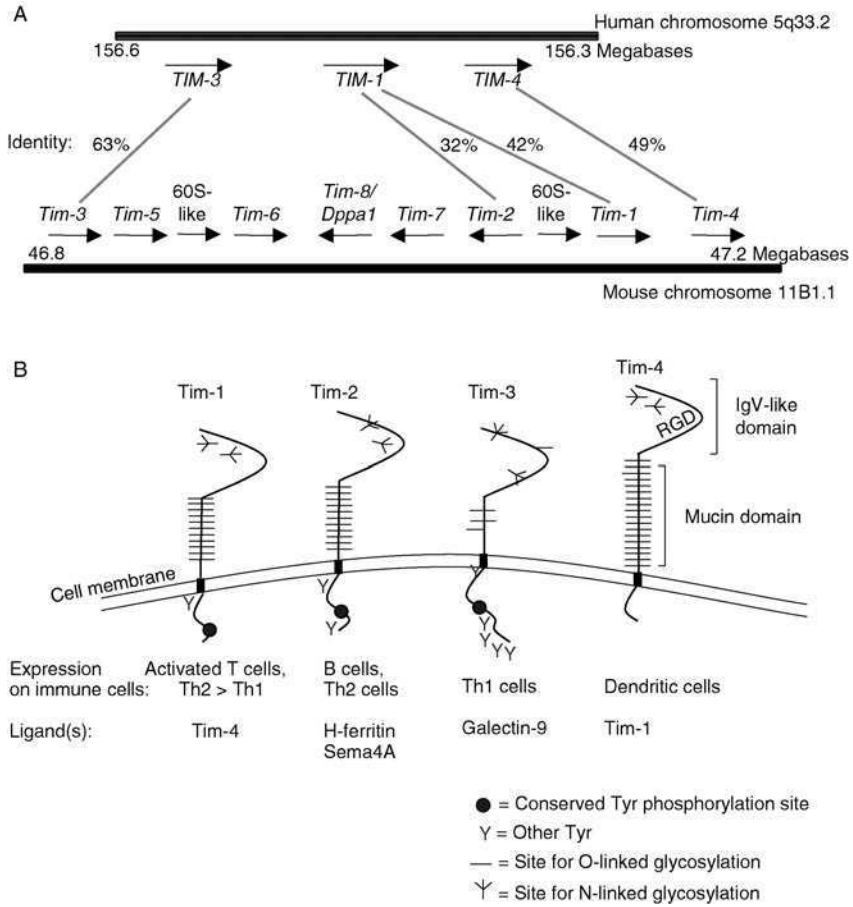

Figure 1 The *TIM* gene family. (A) The *TIM* locus. Arrangement of the human *TIM* and mouse *Tim* genes on chromosomes 5 and 11, respectively, are depicted. Arrows indicate the direction of messenger RNA (mRNA) transcription. Mouse *Tims 5–7* are predicted genes. *Tim-8* was previously identified as the developmental gene *Dppa1* (Bortvin *et al.*, 2003). The only intervening genes found within the *Tim* gene cluster are genes similar to ribosomal 60S proteins, indicated as "60S-like" in the figure. Percentages of protein sequence identity between the predicted mouse and human orthologues are indicated. Either or both mouse *Tim-1* and -2 may serve as the orthologue of human *TIM-1*. (B) Structures of the mouse Tim proteins. Predicted structures of the mouse Tim molecules studied to date (i.e., those that are conserved with human) are depicted. All are Type I membrane proteins predicted to be heavily glycosylated. The immune cell types identified to express each Tim protein, and the Tim ligands identified to date are indicated. The predicted tyrosine phosphorylation motif conserved between Tim family members is represented by a circle, while other tyrosine residues present in the intracellular tails are represented by "Y"s. Tim-4, although it contains no Tyr residues in its intracellular tail, has an RGD or integrin-binding motif in its IgV-like domain.

disease (Walley *et al.*, 2001; Yamada and Ymamoto, 2005). These susceptibility traits were previously assumed to be due to polymorphisms in the IL-4 gene cluster. However, because each of these diseases is a complex trait, involving multiple genes interacting with each other and with the environment, the identification of specific susceptibility genes within this lengthy chromosomal region has been extremely difficult.

To simplify the genetic analysis of the region, a congenic inbred mouse model of asthma was developed by introgressing DBA/2 genetic intervals into the BALB/c background, which greatly reduced the number of interacting genes. The congenic strain called C.D2 (HBA), which differed from BALB/c mice only at the chromosome 11 region syntenic to human chromosome 5q31–33, was found to be highly resistant to allergen-induced airway hyperreactivity (AHR), and also produced low levels of IL-4 (McIntire *et al.*, 2001). By genotyping and phenotyping 3000 progeny of these congenic mice, the *Tapr* (T cell airway phenotype regulator) locus was positionally cloned. Within this locus, the *Tim* gene family, which encoded distinct genetic variants of *Tim-1* and *-3* associated with the development of Th2-biased immune responses and the development of allergen-induced AHR, was identified (McIntire *et al.*, 2001). In conjunction with the discovery of Tim-3 as a molecule preferentially expressed on Th1 cells, these genetic studies suggest that polymorphisms in the *TIM* family might be responsible for the linkage of allergy and asthma and some forms of autoimmunity to this chromosomal region.

3. Tim-3 and Its Ligand Galectin-9 Inhibit Th1 Responses

Tim-3 was first identified to be a molecule expressed on terminally differentiated Th1 cells, although the gene (but not the protein) was also shown to be expressed in $CD11b^+$ cells (Monney *et al.*, 2002). In mice, the *Tim-3* gene also encodes a splice variant that is a soluble isoform of the 281 amino acid full-length, membrane-bound form of the protein (flTim-3), containing only the IgV domain fused to the Tim-3 intracellular tail (sTim-3) (Sabatos *et al.*, 2003). This creation of a soluble isoform by alternative splicing is in contrast to the cleavage mechanism that releases the soluble form of Tim-1 (Bailly *et al.*, 2002). Th1 cells express Tim-3 after two to three rounds of *in vitro* activation under Th1-polarizing conditions (Monney *et al.*, 2002). However, *in vivo*, Tim-3 can be readily detected on the surface of effector/memory cells (Zhu *et al.*, 2005). Human TIM-3 is 302 residues long and shares 63% identity with mouse Tim-3 (Monney *et al.*, 2002). Human TIM-3 has also been shown to be preferentially expressed in human Th1 cells as well as $CD8^+$ Tc1 cells, although much of this data is based on messenger RNA (mRNA) and not protein expression (Khademi *et al.*, 2004). TIM-3 is also expressed on

non-T cells including macrophages, DCs, and NK cells (Anderson *et al*., unpublished data).

Functional characterization of Tim-3 has identified its role in regulating the Th1 immune response. Administration of anti-Tim-3 antibody during the course of the autoimmune disease EAE significantly exacerbated disease, leading to increased mortality and an atypical, acute form of disease characterized histologically by an increased infiltration of activated macrophages actively phagocytosing myelin fragments in the CNS (Monney *et al*., 2002). Splenocytes from the immunized, antibody-treated mice showed a marked increase in basal proliferation in the absence of *in vitro* restimulation by specific antigen. Furthermore, this basal proliferation in the anti-Tim-3-treated mice was associated with increased expansion and activation of macrophages (Monney *et al*., 2002).

To further understand the mechanism by which Tim-3 operates *in vivo* and to identify the Tim-3 ligand (Tim-3L), fusion proteins containing either the full-length or soluble extracellular forms of Tim-3 fused to a human IgG1 Fc tail were generated. These fusion proteins (Tim-3.Ig) bound a variety of cell types, including macrophages and DCs, but the highest expression level was consistently found on naïve $CD4^+$ T cells, indicating that the Tim-3:Tim-3L interaction may be a T:T or T:APC (antigen-presenting cells) interaction (Sabatos *et al*., 2003; Sanchez-Fueyo *et al*., 2003). Administration of Tim-3.Ig to immunized mice demonstrated a similar increased basal proliferative response to that seen with administration of anti-Tim-3 antibody. Administration of Tim-3.Ig also resulted in the production of massive quantities of the Th1 cytokines IL-2 and IFN-γ, and these effects were almost exclusively mediated by spontaneously hyperproliferating Th1 cells (Sabatos *et al*., 2003). Tim-3 blockade by Tim-3.Ig treatment has similarly been shown to accelerate autoimmune diabetes progression in nonobese diabetic (NOD) mice (Sanchez-Fueyo *et al*., 2003).

The Tim-3:Tim-3L pathway has also been shown to have a crucial role in the induction of peripheral tolerance. High-dose tolerance, induced by the administration of aqueous antigen, was abrogated by administration of Tim-3.Ig in mice treated with high-dose soluble peptide prior to active immunization (Sabatos *et al*., 2003). Similarly, Tim-$3^{-/-}$ mice on the BALB/c background were not only resistant to the development of high-dose tolerance but also demonstrated significantly higher proliferation and IL-2 production than wild-type controls when tolerized with high-dose soluble antigen (Sabatos *et al*., 2003). Tim-3.Ig treatment or the use of Tim-3-deficient mice similarly prevented tolerance induction to MHC-mismatched allografts (Sanchez-Fueyo *et al*., 2003). This suggests that the interaction of Tim-3 with its ligand is crucial in regulating T cell expansion and peripheral tolerance.

The mechanism by which Tim-3 is involved in T cell expansion and regulating peripheral tolerance is not known. Blockade of the pathway via treatment with anti-Tim-3 antibody or Tim-3.Ig fusion proteins led to increased Th1 cell proliferation and cytokine responses, as well as loss of tolerance induction. Based on this data, we have hypothesized that the Tim-3:Tim-3L pathway could serve to downregulate effector Th1 responses. The role of sTim-3 in regulating Th1 responses is not known, but we hypothesize that sTim-3 may bind Tim-3L during the initial stages of Th1 differentiation and thus prevent it from binding the membrane-anchored form of Tim-3 on developing Th1 cells, allowing for the expansion and effector function of Th1 cells prior to their downregulation (Fig. 2). The role of macrophage regulation as seen by increased activation after anti-Tim-3 antibody treatment also remains unclear. It is possible that a small population of macrophages expressing Tim-3 was expanded by antibody treatment, or the macrophage activation observed may have been a secondary consequence of disrupting the interaction between Tim-3 and Tim-3L in T cells, which leads to the production of massive amounts of IFN-γ.

These data point to the critical importance of Tim-3L in regulating T cell expansion and tolerance, lending importance to this molecule's identification. Tim-3.Ig was shown to bind to T cells and also to a small proportion of macrophages and DCs (Sabatos *et al.*, 2003). Tim-3.Ig also stained $CD4^+$ $CD25^+$ regulatory T cells, interestingly, on activation the expression of Tim-3L was downregulated on naïve T cells but was retained or even increased on regulatory $CD4^+CD25^+$ T cells (Sanchez-Fueyo *et al.*, 2003). If the Tim-3:Tim-3L interaction is an inhibitory interaction, the expression of Tim-3L on the surface of

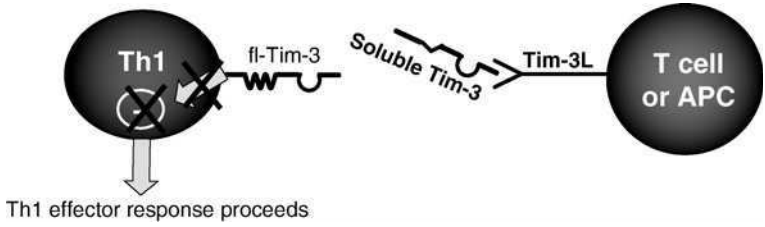

Figure 2 Mechanisms by which Tim-3 may modulate immune function. The differentiation of naïve $CD4^+$ T cells into Th1 cells induces the upregulation of both soluble and membrane-bound full-length (fl) Tim-3. Soluble Tim-3 may occupy Tim-3L, thus preventing the interaction between fl-Tim-3 and Tim-3L, and consequently also preventing the negative signal that this interaction delivers into Th1 cells. The uninhibited Th1 cell can thus proliferate and carry out its effector functions. When the Th1 cells are terminally differentiated, soluble Tim-3 production may be decreased, so fl-Tim-3 on the surface of Th1 cells can interact with its ligand on various immune cells and APCs in the target tissue to transduce an inhibitory signal into the Th1 cell, terminating the response.

$CD4^+$ $CD25^+$ regulatory T cells may play a crucial role in regulating T cell expansion and tolerance (Fig. 3A).

A search for sources of Tim-3L identified a number of tumor cell lines, including TK-1, that expressed high levels of Tim-3L as ascertained by the Tim-3.Ig binding (Zhu *et al.*, 2005). Using Tim-3.Ig, a molecule of about 35 kDa was immunoprecipitated from the surface of the TK-1 cells, and its

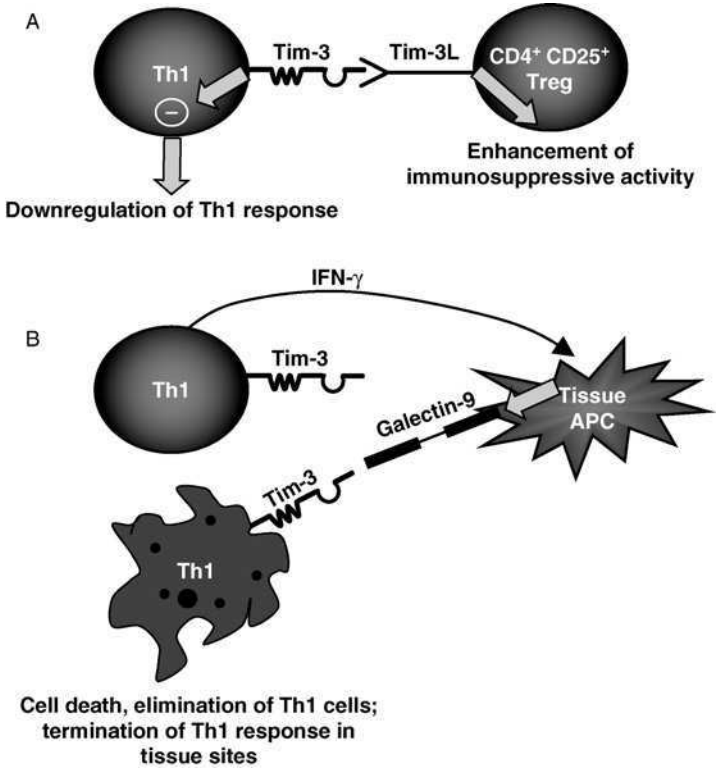

Figure 3 (A) An interaction between Tim-3 on Th1 cells and its ligand on $CD4^+$ $CD25^+$ regulatory T cells may act to downregulate immune responses, both by inducing an inhibitory signal into Th1 cells via Tim-3 and by signaling into the Tim-3L-expressing Tregs. Signals transduced into the Treg may serve to enhance the immunosuppressive phenotype of the $CD4^+$ $CD25^+$ regulatory T cells. (B) An interaction between Tim-3 on Th1 cells and its ligand, galectin-9, leads to Th1 apoptosis and the termination of a productive Th1 response. IFN-γ produced by Th1 cells induces the upregulation of galectin-9 by tissue antigen-presenting cells. Galectin-9, which may exist in both soluble and membrane-bound (via attachment to cell surface carbohydrates) forms, binds to Tim-3 on the surface of Th1 cells and kills them. This interaction provides a mechanism by which a productive Th1 response can be terminated in tissue sites.

identity was established as galectin-9 by Maldi-Tof analysis of the immunoprecipitates using mass spectrometry (Zhu *et al*., 2005). Galectin-9 expression by transient transfection was used to confirm binding of this molecule to Tim-3.Ig, supporting that galectin-9 is indeed the ligand for Tim-3 (Zhu *et al*., 2005). Galectin-9 is produced as a soluble molecule, which attaches to the cell surface via membrane-bound carbohydrates (Wada and Kanwar, 1997), so the effect of soluble recombinant galectin-9 on T cell responses was analyzed *in vitro* and *in vivo*. Administration of galectin-9 *in vivo* during an ongoing immune response did not affect T cell proliferation but severely decreased IFN-γ, both in terms of quantity of the cytokine and number of Th1 cells producing it (Zhu *et al*., 2005). We found that galectin-9 mediated this effect by inducing cell death in Th1 but not Th2 cells. Galectin-9 induced calcium flux in Th1 cells, and adherence/clumping of the Th1 cells preceded cell death, which peaked at about 4 hours after galectin-9 treatment *in vitro* (Zhu *et al*., 2005). Galectin-9 is expressed in the immune system at the steady state level, and its expression is downregulated on naïve T cells upon activation (Zhu *et al*., 2005), consistent with previous observations of Tim-3.Ig binding. However, its expression is upregulated in tissue sites by IFN-γ (Asakura *et al*., 2002; Imaizumi *et al*., 2002), thus providing a self-reliant system by which Th1 cells are regulated in peripheral tissues. Thus, production of IFN-γ by Th1 cells on their reactivation in tissue sites, in addition to inducing inflammation, will result in the induction of galectin-9 on local antigen-presenting cells such as endothelial cells, fibroblasts, and astrocytes. This introduces an interesting paradigm whereby IFN-γ, which induces tissue inflammation, also induces an inhibitory ligand (galectin-9) in the target tissue that acts to delete Th1 cells and thereby prevent protracted inflammation in the target organ (Fig. 3B). Overall, the layer of regulation for Th1 effector cells imposed by the Tim-3: galectin-9 pathway may have specifically evolved to ensure the termination of a productive Th1 immune response, as uncontrolled and protracted proinflammatory Th1 responses will have severe pathological consequences for the host.

4. Tim-2 and Its Ligands

Mouse Tim-2 shares greatest identity with mouse Tim-1 and is suggested to be an orthologue of human TIM-1. Tim-2 is expressed on all splenic B cells and in nonimmune tissues, such as liver, especially in the epithelial cells of the bile duct and in renal tubular epithelial cells (Chen *et al*., 2005). Alhough it is not expressed on naïve T cells, Tim-2 is expressed moderately on activated T cells, and initial mRNA analysis suggests that it is preferentially upregulated in Th2 cells during Th2 differentiation (Chakravarti *et al*., 2005). The preferential expression of Tim-2 on Th2 cells has not been confirmed by protein expression as yet.

The tyrosine phosphorylation motif in the cytoplasmic tail of Tim-2 is phosphorylated upon T cell activation (Kumanogoh *et al*., 2002), supporting the hypothesis that Tim-2, and possibly Tim-1 and -3, signal via tyrosine phosphorylation. The use of a soluble Tim-2 fusion protein (Tim-2.Ig) to identify cell populations expressing Tim-2 ligand(s) (Tim-2L) has indicated that Tim-2L are expressed on activated APCs (Chakravarti *et al*., 2005). Sema4A, which is expressed on activated macrophages, B cells, and DCs, has been identified as one of the ligands for Tim-2 (Kumanogoh *et al*., 2002). In addition, Tim-2 has been shown to interact with H-Ferritin, leading to an intracellular signal and H-ferritin endocytosis (Chen *et al*., 2005). H-ferritin is a subunit of ferritin, which is both secreted and localized intracellularly and acts to buffer reactive oxygen species and induce immunosuppression (Harada *et al*., 1987; Pham *et al*., 2004; Torti and Torti, 2002). H-ferritin is specifically upregulated in inflammatory settings, and has previously been shown to bind to human B and T cells (Fargion *et al*., 1991), but Tim-2 is the first receptor identified for this molecule. The expression of Tim-2 in nonimmune tissues like liver and kidney may therefore be important for the specific uptake of H-ferritin and subsequent modulation of inflammation (Chen *et al*., 2005).

The role of Tim-2 and its ligands in regulating immune responses is only beginning to be understood. Studies employing Tim-2.Ig suggested a specific role for Tim-2 in the regulation of a Th2 response. Administration of Tim-2.Ig during the initiation and early development of an immune response resulted in the preferential induction of the Th2 cytokines IL-4 and IL-10 and inhibition of the Th1 cytokine IFN-γ (Chakravarti *et al*., 2005). Given the expansion of a Th2 response following the administration of Tim-2.Ig, the role of Tim-2 in the development of EAE was addressed. Soluble Tim-2.Ig fusion protein administered during the induction phase of EAE resulted in a delay in disease progression and a reduction in severity of disease as judged by clinical signs and lesion numbers in the brain and spinal cord (Chakravarti *et al*., 2005). Moreover, treatment with antibodies against Sema4A, the putative ligand of Tim-2, also inhibited the development of EAE (Kumanogoh *et al*., 2002). Consistent with a role for an interaction between Sema4A and Tim-2 in regulating the balance of effector Th cells, mice lacking Sema4A showed dysregulated T helper differentiation and impaired Th1 responses (Kumanogoh *et al*., 2005).

Similar to the mechanistic paradigm proposed earlier for Tim-3, Tim-2 expressed on T cells may have a role in inhibiting Th2 responses. This hypothesis is supported by the data that both loss of the Tim-2L Sema4A in the Sema4A-deficient mice and blockade of the Tim-2:Tim-2L interaction by *in vivo* administration of Tim-2.Ig resulted in the enhancement of Th2 responses and inhibition of Th1 responses. However, further studies are required to conclusively prove that Tim-2 is indeed an inhibitory molecule, as alternate

interpretations of the data from both Tim-2.Ig treatment and the Sema4A-deficient mice are also possible and could suggest that Tim-2 induces a positive signal into Th2 cells.

5. Tim-1, Th2 Cells, and Asthma

In mouse tissues using quantitative RT-PCR, the Tim-1 transcripts are present in high abundance in lymph node and kidney and low abundance in spleen, lung, and thymus, and absent in heart and liver tissue. Using a Tim-1-specific monoclonal antibody, Tim-1 was found to be detectable on $CD4^+$ T cells but not on $CD8^+$ T cells of unmanipulated BALB/c mice (Umetsu *et al.*, 2005). In addition, the splenic activated/memory population $CD4^+$ T cells ($CD62L^-$, $CD25^+$) from unmanipulated mice expressed higher Tim-1 levels than the naïve $CD62L^+$, $CD25^-$ population. $CD19^+$ B cells and $CD11c^+$ bone marrow–derived dendritic cells (DCs) expressed low levels of Tim-1 (Umetsu *et al.*, 2005), as did freshly isolated $CD8\alpha^+$ $CD11c^+$ splenic DCs (Sells *et al.*, unpublished data).

Following activation with specific antigen and DCs or with anti-CD3 and -CD28, purified T cells showed upregulation of Tim-1, peaking at 48–72 hours postactivation (Umetsu *et al.*, 2005). In contrast, Tim-1 expression on $CD8^+$ T cells did not increase following stimulation with anti-CD3 and -CD28 (Sells *et al.*, unpublished data). $CD4^+$ T cells cultured under Th1- or Th2-polarizing conditions showed that Th2 polarized (cultured with IL-4 and anti-IL-12) cells express uniformly high levels of Tim-1. In contrast, Tim-1 expression was low on T cells cultured and restimulated under Th1-polarizing conditions (with IL-12 and anti-IL-4) over the same time course (Umetsu *et al.*, 2005).

5.1. Anti-Tim-1 Enhances T Cell Expansion and Inhibits Respiratory Tolerance

The functional role of Tim-1 in immune cells was examined by culturing $CD4^+$ T cells with specific antigen and low numbers of bone marrow–derived DC as APC with anti-Tim-1 antibody. Proliferation of T cells was greatly increased in the presence of anti-Tim-1 monoclonal antibodies (mAb) compared with control mAb (Umetsu *et al.*, 2005). To determine if anti-Tim-1 mAb enhanced T cell proliferation by cross-linking Tim-1 and delivering a positive signal, or by blocking the interactions with a ligand that delivers a negative signal, T cells were cultured with monomeric Fab fragments of Tim-1. These experiments clearly demonstrated enhanced T cell proliferation in the presence of intact anti-Tim-1 mAb 3B3, but no enhancement in the presence of monovalent Fab fragments of anti-Tim-1 mAb, strongly suggesting that cross-linking of Tim-1

delivers a signal resulting in the activation and expansion of T cells (Umetsu *et al*., 2005). Whether this signal is due to a direct cross-linking of Tim-1 on T cells or DCs remains to be determined.

Since Tim-1 enhanced $CD4^+$ T cell responses *in vitro*, the effect of anti-Tim-1 mAb on T cell expansion was also examined *in vivo*. T cells purified from immunized and anti-Tim-1 mAb–treated mice showed greatly enhanced proliferation and production of IFN-γ, IL-4, and IL-10 as compared with mice treated with control mAb (Umetsu *et al*., 2005). These results demonstrated that administration of anti-Tim-1 mAb *in vivo* at the time of immunization enhanced $CD4^+$ T cell responses as characterized by increased levels of proliferation and cytokine production.

In addition to expanding T cells, anti-Tim-1 antibody was able to inhibit respiratory tolerance. Whereas intranasal administration of antigen induces respiratory tolerance, administration of anti-Tim-1 mAb prior and after exposure to intranasal antigen administration reversed the induction of tolerance and restored a vigorous response to the antigen. T cells from mice exposed to intranasal tolerogen and subsequently immunized with the specific antigen proliferated poorly to the immunizing antigen *in vitro* with a significant reduction in IL-4 and IL-10 production. Administration of anti-Tim-1 abrogated these tolerizing effects of intranasal antigen such that splenocytes from mice treated with anti-Tim-1 produced levels of IFN-γ, IL-4, and IL-10 which were comparable to or greater than those of nontolerized mice, indicating that anti-Tim-1 treatment abrogated respiratory tolerance induction (Umetsu *et al*., 2005). In the respiratory tolerance model, the development of respiratory tolerance also prevents the subsequent development of AHR and prevents airway inflammation. However, treatment of the mice with anti-Tim-1 mAb during the initial respiratory exposure to the tolerizing antigen prevented tolerance induction, resulting in severe AHR, which was accompanied by marked airway inflammation (Umetsu *et al*., 2005).

The molecular pathways by which Tim-1 costimulates T cell proliferation and cytokine production are beginning to be unraveled. Ectopic expression of Tim-1 by retroviral gene transduction in T cells of C57BL/6 mice during T cell activation and differentiation resulted in a significant increase in the number of cells producing IL-4 but not IFN-γ (de Souza *et al*., 2005). Furthermore, the Th2 clone D10 transfected with Tim-1 showed increased transcription from the IL-4 promoter, along with spontaneous activation of nuclear factor of activated T cells/activating protein 1 (NFAT/AP1) elements (de Souza *et al*., 2005). Moreover, ectopic expression of Tim-1 results in tyrosine phosphorylation of the intracellular tail of Tim-1, and Tim-1-mediated costimulation requires its cytoplasmic tail and the conserved tyrosine (Y276) within that domain (de Souza *et al*., 2005). The autophosphorylation of Tim-1 on forced

expression and transactivation of the IL-4 promoter by Tim-1 suggests that Tim-1 may have an important role in the regulation of Th2 responses.

Although Tim-1 and -2 are closely related, signaling pathways utilized by Tim-2 have not yet been elucidated. Cross-linking of Tim-2 on T cells resulted in phosphorylation of the Tim-2 tail, but whether Tim-2 phosphorylation also transactivates the IL-4 promoter has not been addressed and the downstream signals and transcriptional elements activated by Tim-2 cross-linking have not been elucidated. Although mouse Tim-2 is closely related to mouse Tim-1, initial data suggest that the Tim-2:Tim-2L interaction may be inhibitory (Chakravarti *et al*., 2005). The intracellular tails of Tim-1 and -2 are significantly different, sharing only 56% identity. Tim-2 contains six more amino acids than Tim-1 in the COOH-terminal end of its tail, and also contains an additional possible tyrosine phosphorylation motif. If Tim-1 and -2 differ functionally, this may be partly due to differences in the intracellular tails.

6. Tim-4, a Natural Ligand for Tim-1

Another Tim family member that is conserved between human and mouse is Tim-4. Expression analysis by quantitative real-time PCR showed that Tim-4 mRNA was expressed in spleen and lymph node, and at low levels in lung, liver, and thymus (Meyers et al., 2005). Tim-4 mRNA was not detected in resting T cells or in differentiated Th1 or Th2 cells, while splenic $CD11b^+$ and $CD11c^+$ cells (representing mainly macrophages and DCs, respectively) showed high-Tim-4 mRNA expression, with low levels present in the $B220^+$ subset (Meyers *et al*., 2005). Further analysis of DCs isolated from Flt3L-treated mice showed that Tim-4 expression was highest in the most mature, lymphoid-type DCs, which express CD11c and CD8 (Meyers *et al*., 2005). Thus, in contrast to other Tim family members, Tim-4 is expressed predominantly on APCs, but not on T cells. Shakov *et al*. (2004) demonstrated by gene expression profiling that Tim-4 expression is selectively downregulated in spleens of LTα- or LTβ-deficient mice. In this study, the level of Tim-4 expression in the spleen was found to correlate with the degree of disruption of splenic microarchitecture. Specifically, the presence of the splenic marginal zone was required for Tim-4 expression (Shakhov *et al*., 2004).

A soluble Ig fusion protein consisting of the extracellular IgV and mucin domains of Tim-4 fused to a human IgG1 Fc tail (Tim-4.Ig) was used to identify the expression pattern of a potential Tim-4 ligand (Tim-4L) by flow cytometry. In unfractionated splenocytes, Tim-4L was detected on most lipopolysaccharide (LPS)- and IFN-γ–activated B cells and on most ConA-activated T cells (Meyers *et al*., 2005). Based on the distinct expression pattern of Tim-4 on APCs as compared to the other T cell-expressed Tim proteins,

whether Tim-4 and another Tim molecule could form a receptor-ligand pair was examined. Tim-4.Ig was found to bind to CHO cells transfected with Tim-1 but not with Tim-2, -3, or -4 (Meyers *et al*., 2005). Conversely, Tim-1.Ig bound to Tim-4 transfectants but not to Tim-1, -2, or -3 transfectants. Anti-Tim-1 mAb could block these interactions, demonstrating the specificity of Tim-1:Tim-4 binding (Meyers *et al*., 2005). An interaction between Tim-4 and Tim-1 could also be observed on natural cells. Tim-4.Ig bound to activated but not unactivated T cells, which directly correlated with their expression of Tim-1, and Tim-1.Ig bound to activated $CD11b^+$ and $CD11c^+$ splenic cells, correlating with Tim-4 expression on these cells (Meyers *et al*., 2005). Additionally, Tim-4.Ig stained slightly higher proportions of activated Th2 than Th1 cells, which again correlated with Tim-1 expression (Meyers *et al*., 2005). Together, these studies confirmed the Tim-4:Tim-1 interaction on *in vivo*-derived cells.

The functional roles of the Tim-1:Tim-4 interaction in regulating immune responses were investigated through administration of Tim-1.Ig or Tim-4.Ig during an *in vivo* Th1-biased immune response. Administration of Tim-4.Ig, which binds to Tim-1, induced high levels of basal splenocyte proliferation and IL-2 and IFN-γ production *in vitro* without antigen restimulation. Analysis of purified cell populations demonstrated that the spontaneous hyperproliferation was entirely due to an effect of TIM-4.Ig on T cells (Meyers *et al*., 2005). These data suggested that Tim-4.Ig, like anti-Tim-1 mAb, costimulated T cells via cross-linking of Tim-1. This idea was further supported by *in vitro* experiments in which Tim-4.Ig could massively augment anti-CD3/anti-CD28-mediated stimulation of purified T cells. Qualitatively different outcomes were obtained depending on the concentration of Tim-4.Ig used, such that high concentrations enhanced proliferation, while low concentrations resulted in inhibition of T cell proliferation (Meyers *et al*., 2005). These differences indicated that the Tim-4:Tim-1 interaction could potentially costimulate T cell proliferation, but whether the inhibition of T cell proliferation by low doses of Tim-4.Ig was due to binding to Tim-1 or to another high-affinity inhibitory ligand was not clear (Fig. 4).

Similar to effects observed with Tim-4.Ig administration, spleen cells from mice immunized and treated with Tim-1.Ig showed significant basal proliferation in the absence of *in vitro* antigen restimulation. However, in contrast to the results with Tim-4.Ig treatment, spleen cells from mice treated with Tim-1.Ig secreted both Th1 and Th2 cytokines spontaneously in the absence of antigenic restimulation. *In vitro* reactivation of these cells with antigen resulted in inhibition of Th1 responses and enhancement of Th2 responses (Meyers *et al*., 2005). As the intracellular tail of Tim-4 lacks putative signaling motifs, Tim-1.Ig would be expected to specifically bind to Tim-4 on the

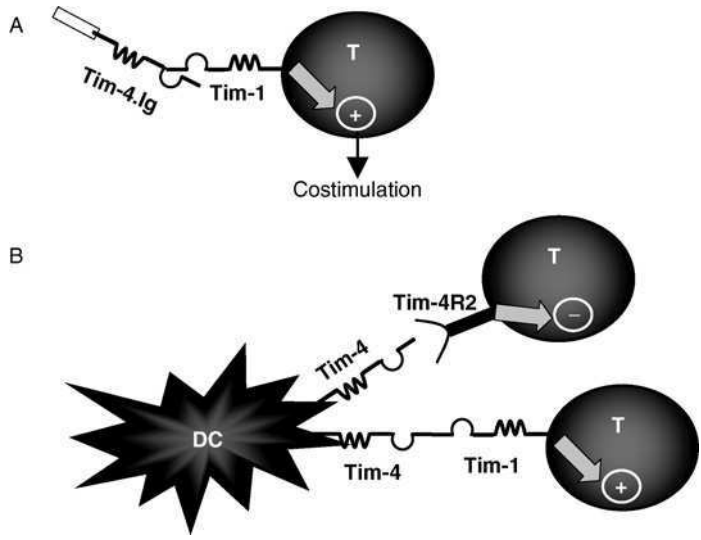

Figure 4 Possible mechanisms of Tim-4.Ig-mediated costimulation of T cells. (A) Tim-4.Ig cross-links Tim-1 on the surface of T cells and induces T cell expansion. (B) A second, inhibitory ligand for Tim-4 may exist on T cells. Since low doses of Tim-4.Ig can inhibit T cell activation, we propose that Tim-4 could have a high-affinity ligand on T cells (Tim-4R2) that inhibits T cell expansion when Tim-4 is present at low levels, while at higher concentrations, it would engage its lower-affinity ligand, Tim-1, and promote T cell expansion.

APCs and either cluster Tim-4 and enhance the signal delivered to T cells via Tim-1 or block the interaction between Tim-1 and Tim-4. However, studies with anti-Tim-1 antibody (Umetsu *et al.*, 2005) suggest that these simple expectations are unlikely to completely explain the effects observed with Tim-4.Ig. In light of the data describing signaling through Tim-1 into T cells (de Souza *et al.*, 2005), new interpretations of Tim-1:Tim-4 functions are possible, which can reconcile the differing data. If Tim-1, in the absence of an interaction with Tim-4, is able to deliver an IL-4-inducing signal into T cells, then Tim-4 and anti- Tim-1 mAb may induce a different signaling pathway that costimulates general T cell responses without inducing the Th2 preference seen with Tim-1 expression alone.

7. TIM Molecules in Health and Disease

Expression of TIM proteins in human T cells and the potential linkage of genetic polymorphisms in the human *TIM* genes with susceptibility to human autoimmune and allergic diseases is being actively studied. An analysis of

human Th1 and Th2 lines by real-time PCR by Khademi *et al*. demonstrated that Th1 lines expressed higher *TIM-3* mRNA, while Th2 lines were characterized by higher *TIM-1* expression. *TIM-1* mRNA was upregulated in cerebrospinal fluid (CSF) mononuclear cells of patients with MS, and the increased expression of *TIM-1* was associated with remission. While there was higher IFN-γ mRNA expression among CSF-derived mononuclear cells in multiple sclerosis (MS) patients relative to controls, levels of *TIM-3* were comparable between the two groups despite a significant correlation between mRNA expression of IFN-γ and TNF-α in CSF-mononuclear cells (Khademi *et al.*, 2004). The source of TIM-3 and IFN-γ among the CSF-mononuclear cells could not unambiguously be identified, complicating interpretation of these data. These data suggest that TIM molecules are also differentially expressed on human Th1 and Th2 cells and may be functionally involved in the course of human autoimmune disease. From the experimental animal data, it is clear that Tim-3 plays a crucial role in the regulation of effector Th1 cells and in mediating peripheral tolerance. Since interaction of mouse Tim-3 with its ligand results in the deletion of effector Th1 cells, we propose that the Tim-3:Tim-3L interaction has evolved to terminate Th1 responses in the target organ and resolve inflammation. Based on this proposition, one can postulate that loss of Tim-3 or polymorphisms in the *Tim-3* gene, which would alter Tim-3 signaling, will result in the escape of autoreactive Th1 cells from tolerance and the induction of protracted and chronic inflammation in the target organ. Systemic analysis of human TIM-3 expression on Th1 cells in the peripheral immune system and in infiltrating T cells in the target organ will address the issue of whether TIM-3 plays a crucial role in regulating the survival of pathogenic T cells in the target organ in a chronic autoimmune disease setting. Preliminary data suggest that Th1 cells obtained from the CSF of MS patients, although they produce high levels of IFN-γ, express relatively lower levels of TIM-3 when compared to Th1 cells obtained from peripheral blood of the same patients. Whether lowering the TIM-3 expression on Th1 cells in the CNS of the MS patients provides a mechanism by which T cells escape deletion and promote protracted autoimmune inflammation in the CNS remains to be determined (Anderson and Hafler, personal communication). Furthermore, whether loss of TIM-3 in Th1 cells in MS patients is due to a genetic polymorphism or selection of lower TIM-3-expressing T cells in the CNS of these patients remains to be determined. An association analysis of genotype and allele frequencies in exon 4 of the *TIM-1* gene in rheumatoid arthritis patients and controls suggested that variations in *TIM-1* exon 4 might be associated with susceptibility to rheumatoid arthritis (Chae *et al*., 2004). Thus, polymorphisms in human *TIM* genes may modulate susceptibility to chronic autoimmune diseases.

8. TIM-1, Hepatitis A Virus Cellular Receptor, and Linkage to Human Asthma

The human homologue of the gene encoding murine *Tim-1* was previously identified as the human HAV cellular receptor, hHAVcr1 (Feigelstock *et al.*, 1998; Kaplan *et al.*, 1996). Epidemiological studies demonstrated an inverse association between development of atopic diseases (asthma and allergy) and infection with HAV (Matricardi *et al.*, 2002), suggesting that HAV infection regulates development of asthma and alopy. Since TIM-1 is the receptor for HAV, this observation is of major importance. Like the murine *Tim-1*, the human *TIM-1* gene is highly polymorphic, and includes a six amino acid insertion polymorphism, 157insMTTTVP, as well as two single amino acid changes (McIntire *et al.*, 2003). In HAV seropositive subjects, there is a significant *inverse* association of the 157insMTTTVP insertion allele and atopy. Individuals who are homozygous or heterozygous for the insertion are much less likely to be atopic than individuals with no copies of the insertion allele ($p < 0.0005$). In HAV-negative individuals however, the insertion provided no protection against atopy, indicating that *TIM-1* is a very significant atopy susceptibility gene but that the 157insMTTTVP insertion protects against atopy only in HAV-exposed individuals (McIntire *et al.*, 2003). Because infection with HAV has been previously associated with protection against atopy (Matricardi *et al.*, 2002), it is possible that HAV directly prevents the development of atopy by binding to TIM-1, its receptor, and altering T cell differentiation. Since 157insMTTTVP is located in the center of the mucin-like sequence that is required for viral entry,

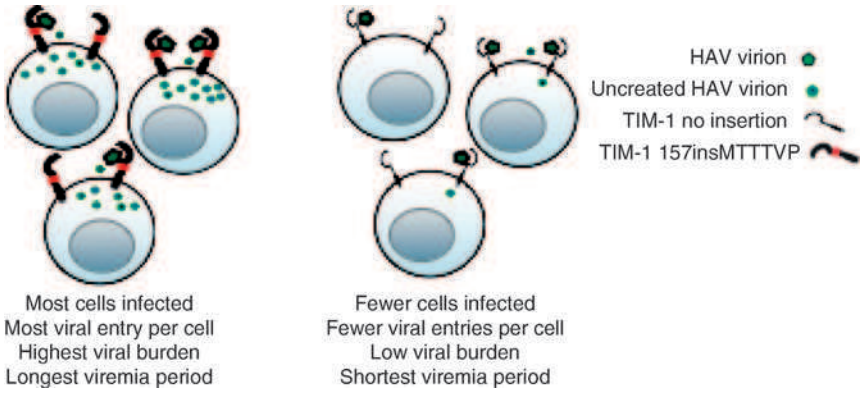

Figure 5 Possible mechanisms of TIM-1:HAV effect on atopy. 157insMTTTVP may alter the virus–receptor interaction at the mucin domain of TIM-1 that leads to HAV viral uncoating. The 157insMTTTVP insertion may increase viral entry and infection of effector lymphoid cells and thus regulate their function.

and because this polymorphism lengthens this critical region by 12–14%, the efficiency of viral entry may be substantially altered by this variation (Fig. 5). Moreover, because the incidence of HAV infection has dropped significantly over the past 20 years, these studies provide a molecular mechanism for the Hygiene Hypothesis, in that the protective effect of HAV infection in individuals carrying the 157insMTTTVP polymorphism is much less common today than 20 years ago. The role of *TIM-1* as an atopy susceptibility gene has been confirmed by several studies. Several independent studies found a very strong linkage between atopy and chromosome 5q33 at markers very close to *TIM-1* (Shek *et al*., 2001) or within *TIM-1* (Gao *et al*., 2005; Graves *et al*., 2005) or TIM-3 (Graves *et al*., 2005). In contrast, examination of a Japanese population demonstrated no association between TIM-1 and atopy (Noguchi *et al*., 2003). However, this may be attributable to the absence of HAV exposure in that population.

In conclusion, the *Tim* family of genes expressed on T cells and APCs appear to play very important roles in regulating effector T cell responses, such that allelic variations in these genes may influence the development of many immune-mediated diseases. Thus, modulation of the functions of TIM molecules may in the future prove effective in regulating many immune-mediated diseases.

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Inhibition of Inflammatory Responses by Leukocyte Ig-Like Receptors

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Abstract

The immune system must effectively regulate the balance between beneficial and detrimental inflammation. This process is achieved in part through cell surface receptors that rapidly integrate activating and inhibitory signals. The inhibitory members of the leukocyte Ig-like receptor (LILR) family, termed LILRBs, are broadly distributed among cell populations in the immune system and potently counterregulate cell activation induced by stimuli of innate and adaptive immune responses. Studies in mice and humans indicate that LILRBs appreciably downregulate harmful inflammatory responses induced by microbial, allergic, and cytotoxic mechanisms. Hence, the LILRBs likely play significant roles in regulating the incidence and severity of many inflammatory diseases, making them potential targets for therapeutic interventions.

1. Introduction

The ability of cell surface receptors to counterregulate innate and adaptive components of inflammatory processes is a relatively new concept in the field of inflammation. In recent years, our view of inflammation has expanded from one in which the presence and amounts of proinflammatory stimuli dictated outcomes, to one in which the ability of cells to downregulate their responses to those activation signals via cell surface receptors is also a key component. This conceptual evolution has tremendous implications for understanding regulation of the balance between beneficial inflammation that protects against

ADVANCES IN IMMUNOLOGY, VOL. 91 0065-2776/06 $35.00

DOI: 10.1016/S0065-2776(06)91007-4

microbial assault and detrimental inflammation that damages host tissue. In addition, that understanding is expected to provide new therapeutic approaches to boosting deficient responses and tempering overly exuberant responses, each of which can be life threatening in certain contexts.

Over the past 10 years, a large number of inhibitory receptors expressed on cells of the immune system have been identified. An element common to most of these receptors is the presence of the immunoreceptor tyrosine–based inhibitory motif (ITIM) in their cytoplasmic regions. The extracellular domains of these receptors typically belong to either the Immunoglobulin (Ig) or C-lectin superfamilies, but the mechanistic bases for how the receptors inhibit cell activation and inflammation emanate from their ITIMs.

A central family of ITIM receptors pertaining to counterregulation of inflammation consists of the inhibitory members of the leukocyte Ig-like receptors (LILRs), termed LILRBs. Most cells of the innate and adaptive immune systems express at least one LILRB. The human LILRBs are encoded within the leukocyte receptor complex (LRC) located on chromosome 19q13.4. The LRC also includes genes encoding the killer cell Ig-like receptors (KIRs) that are expressed primarily on natural killer (NK) cells and certain T cell populations, and which include ITIM-bearing receptors that principally downregulate cytotoxicity responses to certain virally-infected and transformed cells. However, as described later, the LILRBs are more broadly expressed on cell populations, are activated by a more diverse set of ligands, and as shown in animal studies, are clearly key negative regulators of inflammation *in vivo*. Hence, the focus of this chapter is on the biochemistry and immunobiology of the LILRBs.

2. LILRB1

cDNA encoding LILRB1, which is also termed leukocyte Ig-like Receptor 1 and Ig-like transcript 2 (see Table 1 for synonyms), was initially cloned from NK and B cell lines using oligonucleotides encoding consensus sequences of

Table 1 Nomenclatures Used for Members of the LILRB Family

LILRB	LIR[a]	ILT[b]
1	1	2
2	2	4
3	3	5
4	5	3
5	8	–

[a]Leukocyte Ig-like receptor.
[b]Ig-like transcript.

C2-type Ig-like domains (Samaridis and Colonna, 1997). LILRB1 was subsequently identified as a ligand for UL18, an MHC class I homologue encoded by human cytomegalovirus (Cosman *et al.*, 1997). Shortly thereafter, it was also defined by a mAb that restored the lytic capability of an NK cell line that was otherwise inhibited when target cells were transfected to express certain MHC class I alleles (Colonna *et al.*, 1997). LILRB1 has four Ig-like domains (Fig. 1), and in addition to binding UL18, LILRB1 binds a broad range of MHC class I molecules, including representatives of the HLA-A, HLA-B, HLA-C, HLA-F, and HLA-G loci (Allen *et al.*, 2001; Chapman *et al.*, 1999; Colonna et al., 1997, 1998; Lepin *et al.*, 2000; Navarro *et al.*, 1999; Shiroishi *et al.*, 2003). The broad recognition of MHC class I by LILRB1 is attributed to the binding of amino acids in its extracellular D1 and D2 Ig-like domains to residues in the α3 domain and β2μ of MHC class I, respectively, that are largely conserved among MHC class I molecules and also present in UL18 (Chapman *et al.*, 1999, 2000; Willcox *et al.*, 2003). In contrast, the KIRs recognize polymorphic residues in the α1 and α2 domains of MHC class I, leading to more restricted ligand recognition by each KIR (Moretta *et al.*, 1996). The broad recognition of MHC class I by LILRB1 is reminiscent of the binding of CD8 to MHC class I, and LILRB1 and CD8 compete for binding to MHC class I, raising the possibility that, in addition to the ITIM-based mechanism described later, LILRB1 may also inhibit cell activation by hindering the binding of CD8 to MHC class I (Shiroishi *et al*., 2003).

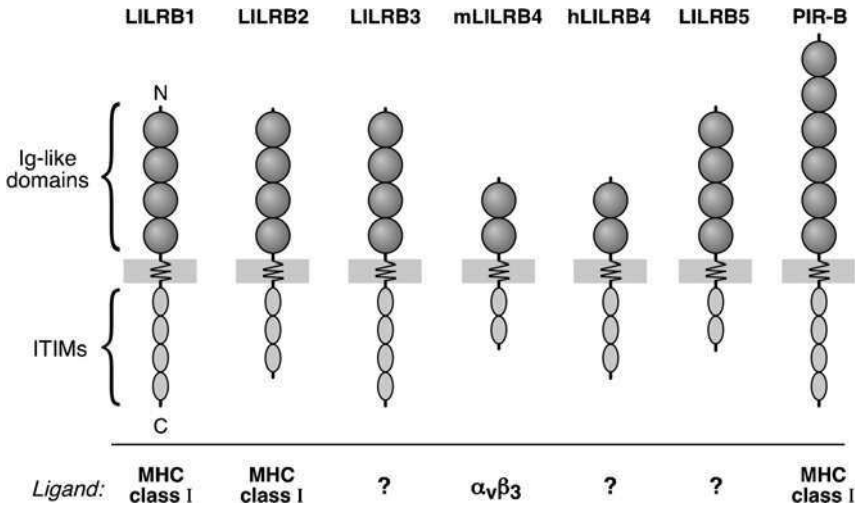

Figure 1 Structures and ligands of LILRB family members.

Besides being expressed on the NK cell line noted earlier, LILRB1 is expressed on primary NK cells as well as on B cells and various populations of $CD4^+$ and $CD8^+$ T cells (Colonna *et al.*, 1997; Cosman *et al.*, 1997; Saverino *et al.*, 2000; Young *et al.*, 2001). Indeed, LILRB1 is the only human LILRB-expressed on resting T cells. The molecule is also expressed on monocytes/macrophages, eosinophils and basophils (from some individuals), primary plasmacytoid dendritic cells (DCs), and on DCs derived in culture from monocytes or $CD34^+$ cells obtained from cord blood (Colonna *et al.*, 1997; Cosman *et al.*, 1997; Ju *et al.*, 2004; Saverino *et al.*, 2000; Sloane *et al.*, 2004; Tedla *et al.*, 2003).

The cytoplasmic region of LILRB1 has four ITIM or ITIM-like sequences that have a consensus sequence of N/S/V x Y x x L/V, where x is any amino acid (Table 2) (Colonna *et al.*, 1997; Cosman *et al.*, 1997). As described later, phosphorylation of ITIM tyrosines of LILRB1 leads to the binding of the SH2 domains of the cytosolic tyrosine phosphatase SHP-1 (Colonna *et al.*, 1997), which by analogy to studies in mouse LILRB4 (Lu-Kuo *et al.*, 1999) is likely to be the receptor-proximal inhibitory step.

The respective contributions of the four ITIMs to SHP-1 recruitment and inhibitory functions of LILRB1 have been defined in rat basophilic leukemia (RBL) cells, a mast cell line (Seldin *et al.*, 1985), transfected to express LILRB1 (Bellon *et al.*, 2002). Cross-linking LILRB1 with an anti-LILRB1 mAb and anti-Ig induces tyrosine phosphorylation of LILRB1, as does the binding of MHC class I to the transfected LILRB1. Furthermore, the binding of LILRB1 to MHC class I inhibits cell activation induced by IgE and Ag, as does copresentation of anti-LILRB1 with solid phase IgE. The tyrosines in $ITIM_{612–617}$ (*VTYAQL*) and $ITIM_{642–647}$ (*SIYATL*) (Table 2) are required for maximal binding of SHP-1 and inhibitory function in the solid phase activation assay. Accordingly, analogous pairs of ITIMs, with exactly 24 amino acids between them, are present in all five human LILRBs and mouse paired Ig-like receptor (PIR) B, a mouse LILRB analogue (see later) (Table 2). In contrast, the tyrosine in $ITIM_{560–565}$ (*VTYAEV*) of LILRB1 does not contribute to SHP-1 binding or inhibition of activation in this RBL cell transfection system. Nevertheless, this phosphorylated ITIM, when presented as a phosphopeptide bound to a solid phase, binds SHP-1 in cell extracts (Dietrich *et al.*, 2001). Certain phosphorylated ITIM peptides, when presented at sufficiently high densities on a solid phase, can bind SHP-1 in detergent extracts of cells even though that ITIM in the context of the native receptor does not do so when inhibiting cell activation (Lesourne *et al.*, 2001). Taken together, the data suggest that Y562 in the context of full-length LILRB1 may not recruit SHP-1 under physiologic conditions.

The membrane-proximal ITIM of LILRB1, namely, $ITIM_{531–536}$, does not contribute to the binding of SHP-1 (Bellon *et al.*, 2002). Nevertheless, mutation

Table 2 ITIMs of LIRBs and PIR-B

	ITIM	# AAs[a]	ITIM	# AAs	ITIM	# AAs	ITIM
LILRB1	531-NLYAAV-536	23	560-VTYAEV-565	46	612-VTYAQL-617	24	642-SIYATL-647
LILRB2	531-NLYAAV-536	23	N/A	N/A	560-VTYAQL-565	24	590-SIYATL-595
LILRB3	512-NLYAAV-517	23	541-VTYAPV-546	46	593-VTYAQL-598	24	623-SIYATL-628
LILRB4 (MOUSE)	N/A	N/A	298-IVYAQV-303	16	320-VTYAQL-325	N/A	N/A
LILRB4 (HUMAN)	N/A	N/A	358-VTYAKV-363	46	410-VTYARL-415	24	440-SVYATL-445
LILRB5	N/A	N/A	N/A	N/A	552-VTYAQL-557	24	582-SIYAPL-587
PIR-B	711-SLYASV-716	23	740-ETYAQV-745	46	792-VTYAQL-797	24	822-SVYATL-827

[a]Number of amino acids between ITIMs.

of Y533 impairs all tyrosine phosphorylation of LILRB1, possibly by decreasing the ability of *Src* family kinases to phosphorylate the cytoplasmic domain of LILRB1. Accordingly, mutation of Y533 impairs the ability of LILRB1 to inhibit cell activation. This ITIM, which has an asparagine at the −2 position relative to the tyrosine, is also present in LILRB2 and LILRB3 (Table 2). The presence of this ITIM may serve to decrease the threshold for activation-induced phosphorylation of $ITIM_{612–617}$ and $ITIM_{642–647}$ of LILRB1 that bind SHP-1 and are required for the counterregulatory functions of LILRB1 (and likely, for LILRB2 and LILRB3). In contrast, LILRB4 and LILRB5 might become maximally inhibitory only in response to greater amounts of cell activation and concomitantly elevated activation of *Src* family kinases.

Counterregulatory roles for LILRB1 have also been defined in a number of T cell activation systems. mAb-mediated coligation of LILRB1 with the TCR suppresses signaling by inhibiting tyrosine phosphorylation of TCRζ (Dietrich *et al*., 2001), which is likely a direct effect of SHP-1 in proximity to the ITAMs of the TCR. Inhibition of the ligand-proximal activation step would be expected to suppress essentially all downstream signals from the TCR. Increases in calcium flux and T cell proliferation induced with anti-CD3 are inhibited when anti-LILRB1 is coligated with anti-CD3 (Saverino *et al*., 2000). The binding of LILRB1 to MHC class I inhibits superantigen-induced T cell cytotoxicity, as determined by the ability of anti-LILRB1 to reverse the suppression, presumably by blocking the interaction between LILRB1 and MHC class I (Colonna *et al*., 1997). The addition of monomeric anti-LILRB1 augments Ag-induced T cell proliferation and the production of IL-2, IFN-γ, and IL-13 (Saverino *et al.*, 2002), as well as enhancing T cell-mediated cytolysis (Saverino *et al*., 2000). In another approach, cross-linking LILRB1 with anti-LILRB1 and anti-Ig inhibits Ag-induced proliferation and reduces IL-2 and IFN-γ production by T cells or T cell clones, while increasing IL-10 and TGF-β production (Merlo *et al.*, 2001; Saverino *et al*., 2002). Antibody (Ab)-mediated cross-linking of LILRB1 also inhibits T cell cytotoxicity, as well as T cell proliferation in response to recall Ags (Saverino *et al*., 2000). The ability of particular mAbs to effect inhibition of cell activation by cross-linking LILRB1 to itself as opposed to a requirement for the coligation of LILRB1 with anti-TCR likely reflects differences in the redistribution of LILRB1 relative to the TCR in the cell membrane in response to the particular epitopes recognized, and has implications for which epitopes of LILRB1 may serve as useful therapeutic targets. Overall, it is clear from a variety of experimental approaches that LILRB1 potently inhibits many aspects of T cell activation that include the promotion of Ab production, cell cytotoxicity, and the generation of immunoregulatory and proinflammatory mediators. Hence, LILRB1 likely contributes to the counterregulation of T cell-initiated inflammation *in vivo*.

In addition to T cells, inhibitory roles for LILRB1 have been demonstrated in other cell types that express the receptor. LILRB1 contributes to inhibition of NK cell-mediated cytotoxicity and adhesion to target cells via recognition of HLA-G (Forte *et al.*, 2001; Navarro *et al.*, 1999). In particular, decidual NK cells and macrophages express LILRB1 that through recognition of HLA-G on trophoblasts may suppress cytolysis and inflammation directed toward fetal cells and tissue (Gonen-Gross *et al.*, 2003; Petroff *et al.*, 2002; Ponte *et al.*, 1999). Tyrosine phosphorylation of LILRB1 in monocytes leads to recruitment of SHP-1, and coligation of LILRB1 with cross-linked FcγRI on human monocytes inhibits tyrosine phosphorylation of the FcRγ chain and downstream activation signaling (Colonna *et al.*, 1997; Fanger *et al.*, 1998). Cross-linking of LILRB1 with anti-LILRB1 and anti-Ig inhibits B cell activation induced by Ag, CD40L + IL-4, and LPS + IL-4, as measured by suppression of Ig production, including IgE (Merlo *et al.*, 2005). Human DCs derived from monocytes cultured in GM-CSF + IL-4 express osteoclast-associated receptor (OSCAR) that associates with FcRγ chain and induces production of IL-8 and IL-12 as well as enhancing T cell activation (Tenca *et al.*, 2005). Simultaneous ligation of OSCAR and LILRB1 by means of solid phase antibodies inhibits the cytokine production, and coligation of OSCAR and LILRB1 with fluid phase anti-LILRB1 and anti-Ig inhibits the anti-OSCAR–mediated boost in T cell activation provided by DCs (Tenca *et al.*, 2005).

Increases in the expression of LILRB1 have been associated with certain pathologic conditions. The appearance of a ligand for LILRB1, namely HLA-G, in psoriatic skin as well as on infiltrating $CD4^+$ T cells expressing LILRB1 raises the possibility that the molecule may provide *in situ* counterregulation of activated T cells in this form of dermal inflammation (Aractingi *et al.*, 2001), and presumably, mitigate deleterious effects of the chronic inflammation. Indeed, expression of LILRB1 is upregulated by HLA-G (LeMaoult *et al.*, 2005). Expression of HLA-G is normally restricted primarily to fetal cytotrophoblast cells (Ellis *et al.*, 1990) suggesting a role for the upregulated LILRBs in maternal tolerance to fetal tissue. However, expression of HLA-G becomes more widespread during inflammation (Aractingi *et al.*, 2001; Khosrotehrani *et al.*, 2001; Pangault *et al.*, 2002), viral infection (Lozano *et al.*, 2002), and graft acceptance (Lila *et al.*, 2002), which may lead to potentially beneficial contributions of the upregulated LILRBs, depending on the particular context. On the other hand, LILRB1 expression is greater on peripheral blood lymphocytes of lung transplant recipients that subsequently developed human CMV infection, suggesting that UL18 expression driven by the viral infection may upregulate LILRB1 (Berg *et al.*, 2003), leading to a LILRB1-mediated reduction in the lysis of infected cells. The expression of LILRB1 is also upregulated on $CD8^+$ T cells specific for Ag from CMV, EBV, and HIV (Ince *et al.*, 2004), and anti-LILRB1

increases IFN-γ production, but not cytolytic activity by those cells. The early absence of HLA-G in inflammation and viral infection might provide a window of time for effective innate defense before the subsequent increase in expression that, via LILRB1, may reduce collateral destruction of host tissues, and may similarly provide extended downregulation of cytotoxic alloimmune responses after tissue transplantation. Of particular note, LILRB1 binds HLA-B27 (Allen *et al*., 2001), the expression of which is associated with spondyloarthropathies such as ankylosing spondylitis (Allen *et al.*, 1999). This interaction presents the possibility that LILRB1 may play a counterregulatory role in the initiation and/or perpetuation of these arthritides. In mice transgenic for HLA-B27 heavy chains that develop symptoms akin to human spondyloarthritis, an Ab that ameliorates the inflammation *in vivo* also blocks the interaction between HLA-B27 and PIR-B, suggesting that an LILRB may contribute to amelioration of HLA-B27-associated inflammation in humans (Kollnberger *et al.*, 2004).

3. LILRB2

LILRB2 was initially defined by two groups of investigators using similar methods. In one approach, LILRB2 was cloned from a human monocyte cDNA library screened with cDNA probes encoding members of the LILR family, including LILRB1 (Borges *et al.*, 1997). In the other approach, cDNA encoding LILRB2 was amplified by reverse transcriptase PCR from myelomonocytic cells using primers for LILRB1 (Colonna *et al*., 1997). The clonings revealed that LILRB2, like LILRB1, has four Ig-like domains (Fig. 1), and they are ~82% identical at the amino acid level. LILRB2 binds UL18, but with a three-log lower affinity than LILRB1 (Borges *et al*., 1997; Willcox *et al.*, 2002). LILRB2 resembles LILRB1 in binding many of the same MHC class I molecules as LILRB1, including HLA-B27 (Allen *et al*., 2001), albeit with two- to threefold lower affinities (Shiroishi *et al*., 2003), suggesting that the thresholds of MHC class I expression for LILRB1 and LILRB2 inhibitory functions may be different. In addition, certain MHC class I alleles are recognized uniquely by LILRB1 or LILRB2 (Allen *et al*., 2001; Chapman *et al*., 1999; Fanger *et al.*, 1998). Like LILRB1, LILRB2 is upregulated by HLA-G (LeMaoult *et al*., 2005) and competes with CD8 for binding to MHC class I (Shiroishi *et al*., 2003).

In contrast with LILRB1, expression of LILRB2 is restricted to cells of the myelomonocytic lineage, such as monocytes/macrophages and DCs, and LILRB2 is also expressed on the basophils of some individuals (Allen *et al*., 2001; Chang *et al.*, 2002; Sloane *et al*., 2004; Vlad *et al.*, 2003). LILRB2 is expressed on synovial macrophages and neutrophils in the early stages of rheumatoid arthritis, whereas expression is low in synovial tissue from patients

with osteoarthritis or chronic rheumatoid arthritis (Tedla *et al.*, 2002). These data suggest that endogenous inhibitory influences of LILRB2 may be lost or not amplified in certain chronic inflammatory settings. Expression of LILRB2 is also induced on endothelial cells cocultured with tolerogenic $CD8^+$ T cells, and is expressed on endothelial cells in endomyocardial biopsies obtained from heart transplant recipients that have not undergone allograft rejection (Manavalan *et al.*, 2004). In that situation, the upregulated LILRB2 may be contributing to long-term graft acceptance and represents a rare example of LILRB expression on cells outside the immune system.

LILRB2 has three ITIMs, each of which is identical to analogous sequences in LILRB1 (Table 2) (Borges *et al.*, 1997). As noted earlier, an ITIM corresponding to $ITIM_{560–565}$ of LILRB1, which does not bind SHP-1 under inhibiting conditions, is not present in LILRB2. As with LILRB1, Ab-mediated coligation of LILRB2 with FcγRI in monocytes leads to recruitment of SHP-1 and inhibition of cell activation (Fanger *et al.*, 1998). However, LILRB1 and LILRB2 may have distinct functions on DCs. DCs cultured from monocytes in GM-CSF and IL-4 become tolerogenic in response to coculture with $CD8^+$ $CD28^-$ T suppressor cells or by addition of IL-10 and IFN-α, and LILRB2 is highly expressed on tolerogenic DCs, upregulated by IL-10 and IFN-α, and contributes appreciably to the tolerization process (as does LILRB4) (Beinhauer *et al.*, 2004; Chang *et al.*, 2002; Manavalan *et al.*, 2003; Vlad *et al.*, 2003). The tolerogenic antigen presenting cells (APCs) foster the production of regulatory T cells, which not only inhibit activation of other T cells but also perpetuate the tolerogenic state of the APCs. It is not clear which activating receptor and downstream biochemical events in the APCs are altered by LILRB2 and LILRB4 so as to create the tolerogenic state, but it does not appear to be due to a concomitant decrease in the expression of costimulatory molecules (Beinhauer *et al.*, 2004).

IL-10 appears to be particularly potent in increasing the expression of the LILRBs on DCs. When DCs are generated by culture of peripheral blood monocytes in GM-CSF and IL-4 followed by a cocktail of maturation-inducing cytokines and mediators, the addition of IL-10 generates greater amounts of mRNA encoding not only LILRB2 but also LILRB1, LILRB3, and LILRB4 (Velten *et al.*, 2004). The upregulation of LILRB2 induced by IL-10 on DCs generated in GM-CSF and IL-4 alone is further increased by LPS (Beinhauer *et al.*, 2004; Ju *et al.*, 2004). Hence, at least some of the suppressive immunoregulatory functions of IL-10 may be realized through upregulated expression and inhibitory functions of LILRBs on DCs. In addition, stimuli of the innate immune system that are traditionally associated with maturation of DCs may, in certain contexts, also provide counterregulatory functions.

4. LILRB3

A cDNA encoding LILRB3 was initially defined by reverse transcriptase PCR from myelomonocytic cells using primers for LILRB1 (Colonna *et al*., 1997). In contrast with LILRB1 and LILRB2, LILRB3 does not bind MHC class I (Allan *et al.*, 1999; Colonna *et al*., 1998), and structural predictions do not favor MHC class I serving as a ligand for LILRB3 (Willcox *et al*., 2003). Hence, the immunobiology of LILRB3 may be more distinct from the roles of LILRB1 and LILRB2 than the latter two receptors are from each other.

LILRB3 is expressed on human basophils, and when coligated by means of Ab with either FcεRI or LILRA2, an activating LILR (Borges *et al*., 1997), LILRB3 inhibits degranulation (histamine release) and the generation of cysteinyl leukotrienes (e.g., LTC_4) and IL-4 (Sloane *et al*., 2004). Hence, LILRB3 is an endogenous inhibitor of allergic inflammation that may reduce allergic disease susceptibility and severity in humans.

5. LILRB4

5.1. Mouse LILRB4

Mouse LILRB4 was previously termed gp49B1 but was recently renamed LILRB4 by the Mouse Genome Informatics Group at The Jackson Laboratory. The molecule was initially defined in 1983 by a mAb that recognizes an epitope on macrophages and mast cells (Katz *et al.*, 1983; LeBlanc *et al.*, 1984). The molecule was subsequently defined in mast cells as a 49-kDa glycoprotein (Katz *et al.*, 1989), which was immunoaffinity purified to determine its amino-terminal sequence and clone its cDNA (Arm *et al.*, 1991; Castells *et al.*, 1994). The gene encoding mouse (m) LILRB4 was cloned with a cDNA probe (Castells *et al*., 1994) and subsequently localized to mouse chromosome 10 (Kuroiwa *et al.*, 1998). The cDNA and gene sequences revealed two Ig-like domains. mLILRB4 does not appear to bind MHC class I (Wang *et al.*, 2000), which is analogous to predictions for human (h) LILRB4 (Willcox *et al*., 2003). Rather, mLILRB4 binds integrin αvβ3 (Castells *et al*., 2001), which is expressed on a wide variety of cell populations, including those of the vasculature (Eliceiri and Cheresh, 1999). In addition to macrophages and mast cells, mLILRB4 is also expressed constitutively on neutrophils (Zhou *et al.*, 2003). Although mLILRB4 is not expressed on naïve NK cells or T cells, viral infection *in vivo* elicits strong upregulation of mLILRB4 expression on these cell populations (Gu *et al.*, 2003; Wang *et al*., 2000), reminiscent of the virus-induced upregulation of expression of LILRB1 on $CD8^+$ T cells (Ince *et al.*, 2004). Depending on the timing of these increases, they may reflect either a host defense response to

prevent collateral damage to host tissue from activated cytotoxic cells, or a viral mechanism that has evolved to evade protective host responses.

The cytoplasmic region of mLILRB4 has two ITIMs, the first of which is most analogous to the ITIM that precedes the two C-terminal-most ITIMs in LILRB1, LILRB3, hLILRB4, and PIR-B (Table 2). The second ITIM of mLILRB4 is identical to an ITIM that is essentially constant in all LILRBs. The spacing between the two ITIMs in mLILRB4 is shorter than in other LILRBs. Nevertheless, it seems likely the two SH2 domains of SHP-1 can bind the two ITIMs of mLILRB4 so as to activate the enzyme (Wang *et al.*, 1999).

In mast cells, Ab-mediated coligation of mLILRB4 with cross-linked FcεRI induces the recruitment of SHP-1 to mLILRB4 and inhibits calcium mobilization, degranulation (release of preformed vasoactive mediators, such as histamine, from secretory granules), *de novo* generation of inflammatory and immunomodulatory cysteinyl leukotrienes such as LTC_4 (Katz *et al.*, 1996; Lu-Kuo *et al.*, 1999), and secretion of several cytokines and chemokines (Katz *et al.*, unpublished). Both ITIM tyrosines must be present for mLILRB4 to provide maximal inhibition of mast cell activation (Lu-Kuo *et al.*, 1999), consistent with the binding of both SH2 domains of SHP-1 to LIRB4. In addition, a sufficient amount of enzymatically active SHP-1 must be present in mast cells for mLILRB4 to inhibit activation maximally (Lu-Kuo *et al.*, 1999).

Mice homozygous for a disruption in the *mLilrb4* gene ($mLilrb4^{-/-}$) exhibit a significantly greater increase in vascular permeability in the mast cell-dependent, IgE-passive cutaneous anaphylaxis reaction than $mLilrb4^{+/+}$ mice, demonstrating that mLILRB4 inhibits IgE-initiation mast cell activation *in vivo* (Daheshia *et al.*, 2001). In addition, when $mLilrb4^{-/-}$ mice are actively immunized and challenged locally with Ag, they exhibit more tissue swelling at the site of challenge than $mLilrb4^{+/+}$ mice, and when challenged systemically, undergo significantly more anaphylactic shock (Daheshia *et al.*, 2001). Hence, mLILRB4 is an endogenous inhibitor of IgE-mediated mast cell activation and attendant immediate inflammatory responses.

Although *in vitro* studies of LILRBs suggest that the inhibitory spectrum of these receptors might be primarily focused on activating receptors bearing ITAMs, *in vivo* studies with $mLilrb4^{-/-}$ mice revealed that Stem Cell Factor, which activates mast cells through the receptor tyrosine kinase Kit leading to immediate inflammatory reactions, is also counterregulated by mLILRB4 (Feldweg *et al.*, 2003). Furthermore, a single intradermal injection of LPS in $mLilrb4^{-/-}$ mice induces a thrombohemorrhagic reaction that does not occur in $mLilrb4^{+/+}$ mice and is dependent on neutrophils, β2 integrins, and the

blood coagulation system (Zhou *et al*., 2003). In wild-type mice, a second, systemic injection of LPS is required for this response, termed the local Shwartzman reaction (Stetson and Good, 1951). The expression of mLILRB4 on neutrophils is upregulated by LPS *in vivo* (Zhou *et al*., 2003). Hence, the pathologic response of *mLilrb4*$^{-/-}$ mice to a single LPS exposure is attributed to the lack of basal and LPS-upregulated expression of mLILRB4 on neutrophils, which leads to greater adhesion molecule avidity and attendant formation of venous thrombi consisting of neutrophils, platelets, and fibrin (Zhou *et al*., 2003). mLILRB4 also attenuates inflammatory arthritis induced by the injection of a mixture of antitype II collagen mAbs followed three days later by the injection of LPS (Zhou *et al*., 2005). Neutrophilia and neutrophil-dependent joint swelling, synovial thickening, and cartilage matrix depletion are all significantly greater in *mLilrb4*$^{-/-}$ mice compared with *mLilrb4*$^{+/+}$ mice. In addition, the amounts of IL-1β, MIP-1α, and MIP-2, each of which contributes to the articular inflammation and pathology, are greater in the inflamed joints of *mLilrb4*$^{-/-}$ mice. Hence, mLILRB4 counterregulates cytokine and chemokine induction and attendant neutrophilia that are each essential for synovitis and cartilage matrix depletion in this model of inflammatory arthritis. This effect is not limited to joint inflammation induced with LPS, because *mLilrb4*$^{-/-}$ mice also have exacerbated joint swelling in response to the injection of larger amounts of anticollagen mAbs alone (Zhou *et al*., 2005). Thus, inflammation induced by effectors of both the innate and adaptive immune systems is subject to counterregulation by mLILRB4.

5.2. Human LILRB4

cDNA encoding hLILRB4 was initially cloned from EBV-transformed B cell lines (Cella *et al*., 1997). Shortly thereafter, a cDNA was also cloned from monocytes based on its homology with mouse gp49A, a close homologue of mLILRB4 that does not have cytoplasmic ITIMs (Arm *et al*., 1991, 1997). A third cDNA encoding hLILRB4 was cloned from monocyte and DCs based on its homology with LIRB1 (Borges *et al*., 1997). Like mLILRB4, the human analogue has two Ig-like domains (Fig. 1). The D1 and D2 domains of human and mouse LIRB4 are 62% and 38% identical, respectively. Similar to LILRB3, hLILRB4 has not been found to bind MHC class I (Allan *et al*., 1999; Cella *et al*., 1997), and its structure does not support the possibility that MHC class I is a ligand (Willcox *et al*., 2003). Hence, neither mouse nor human LILRB4 bind MHC class I and the ligand for hLILRB4 remains undefined. hLILRB4 is expressed on monocytes as well as on plasmacytoid and myeloid DCs (Cella *et al*., 1997, 1999).

As noted earlier, hLILRB4 does not have an ITIM equivalent to $ITIM_{531–536}$ of LILRB1 that fosters *Src* kinase-mediated phosphorylation of all the ITIMs (Table 2) (Bellon *et al*., 2002). However, the two relatively invariant ITIMs that in LILRB1 bind SHP-1 and are required for inhibitory function, are present in hLILRB4, as is a third ITIM ($ITIM_{358–363}$) that is similar to $ITIM_{560–565}$ of LILRB1 whose function is unknown. Coligation of anti-hLILRB4 with anti-FcγRIII on macrophages or with an anti-MHC class II mAb on monocytes inhibits the calcium flux initiated by the two activating mAbs alone (Cella *et al*., 1997). Like LILRB2, hLILRB4 on DCs cultured from monocytes in GM-CSF and IL-4 and on HUVECs contributes appreciably to their conversion to tolerogenic DCs by $CD8^+$ $CD28^-$ T suppressor cells or by IL-10 and IFN-α, which also upregulate the expression of hLILRB4 on the DCs, although IL-10 may be less effective for increasing hLILRB4 presentation than it is for LILRB2 (Beinhauer *et al*., 2004; Chang *et al*., 2002; Manavalan *et al*., 2003, 2004; Vlad *et al*., 2003). It is unknown whether LILRB2 and hLILRB4 have distinct inhibitory functions in tolerogenic DCs, but it seems likely from an immunobiologic standpoint because they appear to have different ligands.

6. LILRB5

LILRB5 was initially defined by cDNA cloning from a human DC library using LILR family probes (Borges *et al*., 1997). NK cells express mRNA encoding LILRB5, but little is known about the expression of LILRB5 at the protein level. LIRB5 is unique among the human LILRBs in having only the two ITIMs analogous to those in LILRB1 that bind SHP-1, and in that regard, the cytoplasmic domain of LILRB5 resembles that of mLILRB4. However, LILRB5 has four Ig-like domains like LILRB1, LILRB2, and LILRB3. Surprisingly, little is known about the immunobiology of LILRB5, despite the amount of time that has passed since its discovery.

7. PIR-B

Although mouse PIR-B has not been given an LILRB designation, it has a number of striking similarities with the human LILRBs, which make it likely that it is a reasonable homologue, if not orthologue, of one or more human LILRB. PIR-B was originally discovered during attempts to clone the mouse orthologue of the human myeloid IgA receptor (a member of the human LRC on chromosome 19) by cross-hybridization screening of mouse genomic DNA with a cDNA probe encoding the human receptor (Hayami *et al*., 1997;

Kubagawa *et al.*, 1997). In contrast with the LILRBs described earlier, PIR-B has six Ig-like domains (Fig. 1).

Initial data about potential ligands for PIR-B came from studies suggesting that the amount of constitutive tyrosine phosphorylation of PIR-B in primary cells was lower in β2μ-deficient mice, suggesting that an MHC class I molecule(s) was a ligand for PIR-B (Ho *et al.*, 1999). Subsequent studies revealed that human HLA-G or HLA-B27 tetrameric molecules bound to and induced inhibitory signaling by mouse PIR-B, consistent with the possibility that PIR-B might also bind murine MHC class I (Kollnberger *et al.*, 2004; Liang *et al.*, 2002). Ultimately, the binding to PIR-B of mouse MHC class I tetramers representative of several class I loci and alleles and their ability to induce signaling was shown directly (Nakamura *et al.*, 2004). Hence, PIR-B resembles LILRB1 and LILRB2 in binding a variety of MHC class I molecules.

PIR-B is broadly expressed on cells of the hematopoietic system, including B cells, macrophages, DCs, mast cells, neutrophils, and certain NK cell populations (Hayami *et al.*, 1997; Kubagawa *et al.*, 1997, 1999). The expression of PIR-B increases modestly on B cell and myeloid lineage cells as they mature in bone marrow (Kubagawa *et al.*, 1999). The factors that regulate expression levels of PIR-B are largely unknown.

PIR-B has four ITIMs with sequences very similar to the four ITIMs in LILRB1 and LILRB3 (Table 2). PIR-B is constitutively tyrosine phosphorylated in primary B cells, macrophages, and mast cells, and furthermore, is constitutively associated with SHP-1 (Berg *et al.*, 1998; Ho *et al.*, 1999; Timms *et al.*, 1998; Uehara *et al.*, 2001). Basal levels of PIR-B phosphorylation are lower in continuous cell lines, but phosphorylation in both primary cells and continuous cell lines increases when anti-PIR-B is coligated to ITAM-bearing receptors such as FcεRI (Blery *et al.*, 1998; Uehara *et al.*, 2001). A modest increase is also seen when B cells interact with MHC class I tetramers (Nakamura *et al.*, 2004). In concordance with the other LILRBs, the two C-terminal-most, "invariant" ITIM tyrosines provide the bulk of inhibitory capacity, as defined by suppression of FcεRI-induced degranulation of PIR-B RBL cell transfectants. Although this points to the involvement of SHP-1 in those cells, it has also been reported that PIR-B fully inhibits IgE-induced activation in SHP-1-deficient bone marrow culture–derived mouse mast cells (Uehara *et al.*, 2001). However, the results with only a single concentration of IgE were reported in that study. The loss of mLILRB4-mediated inhibition of IgE-mediated activation in SHP-1 deficient mast cells is only apparent with amounts of IgE that are not supersaturating (Lu-Kuo *et al.*, 1999). Hence, it remains possible that SHP-1 contributes to PIR-B-mediated inhibition of mast cell activation under certain conditions, as it does in B cells, where a contribution of SHP-2 was also detected

(Maeda *et al.*, 1998). $ITIM_{740–745}$ of PIR-B, which corresponds to the ITIM with the greatest sequence variability among the receptors (Table 2), also contributes to the inhibition through an undefined mechanism that apparently does not involve SHP-1 (Uehara *et al.*, 2001). This contribution may relate to the unique presence of a negatively charged amino acid at position −2 relative to the tyrosine. $ITIM_{711–716}$ of PIR-B is analogous in position, and somewhat in sequence, to $ITIM_{531–536}$ of LILRB1 that is a master regulator of the phosphorylation of the other ITIMs of LILRB1 (Bellon *et al.*, 2002). However $ITIM_{711–716}$ of PIR-B does not contribute to its ability to inhibit mast cell activation (Uehara *et al.*, 2001), perhaps reflecting the absence of an asparagine at position −2 relative to the tyrosine.

Contributions of PIR-B to downregulating the *in vitro* activation of cells in the myelomonocytic lineages were surmised by a series of observations concerning negative regulation of cell activation by certain *Src* family kinases. Phosphorylation of PIR-B and association of SHP-1 are greater in bone marrow culture–derived macrophages expressing hyperactive Lyn and are decreased in $Lyn^{-/-}$ macrophages and bone marrow culture–derived DCs (Beavitt *et al.*, 2005; Harder *et al.*, 2001). Furthermore, phosphorylation of PIR-B induced by adhesion of macrophages is lower in $Lyn^{-/-}$ cells concomitant with increases in cell adhesion and spreading (Pereira and Lowell, 2003). Chemokine-induced phosphorylation of PIR-B in neutrophils and DCs is attenuated, and functional responses are greater, in cells deficient in the two *Src* family kinases *Fgr* and *Hck* (Zhang *et al.*, 2005). Accordingly, $Pirb^{-/-}$ neutrophils, macrophages, and DCs demonstrate phenotypes similar to those observed in cells deficient in the respective *Src* family kinases that phosphorylate PIR-B (Pereira *et al.*, 2004; Zhang *et al.*, 2005). An intriguing aspect of the chemokine studies is that they showed a reduction, rather than an increase, in the amount of induced phosphorylation of PIR-B and its association with SHP-1 in wild-type cells, at least at the time point examined (Zhang *et al.*, 2005). This finding suggests that certain activation responses can overcome the apparent tonic inhibition by PIR-B, despite the fact that the activating agents stimulate the *Src* kinases that phosphorylate PIR-B, which is an event that would be predicted to maintain or even increase the amount of inhibition. Presumably, the MHC class I molecules on adjacent cells provide the requisite ligand for the constitutive PIR-B phosphorylation, association of SHP-1, and attendant inhibition in $Pirb^{+/+}$ cells. The observation that continuous cell lines exhibit less constitutive phosphorylation of PIR-B (Ho *et al.*, 1999) may reflect lower amounts of MHC class I on the transformed cells. A significant aspect of the studies that link inhibitory functions of *Src* family kinases with PIR-B is that they demonstrate that the inhibitory capacity of this ITIM receptor *in vitro* extends beyond ITAM-bearing receptors and includes activation

induced by integrin-mediated adhesion alone in macrophages or in combination with TNF-α in neutrophils, as well as by chemokines in neutrophils and DCs (Pereira and Lowell, 2003; Pereira *et al*., 2004; Zhang *et al*., 2005). Hence, SHP-1 and/or SHP-2 recruited by PIR-B may dephosphorylate substrates induced by a variety of activating receptor pathways, and/or PIR-B may use as yet unknown proximal inhibitory effector molecules depending on the nature of the activating receptor.

The ability of PIR-B to inhibit cell activation and attendant immune and inflammatory responses *in vivo* has been established with $Pirb^{-/-}$ mice (Ujike *et al*., 2002). In accordance with the *in vitro* studies, B cells from $Pirb^{-/-}$ mice exhibit greater basal and BCR-mediated activation than cells from $Pirb^{+/+}$ mice (Ujike *et al*., 2002). In addition, the immune responses of $Pirb^{-/-}$ mice show greater Th2 polarization in terms of both the profile of cytokines produced by activated T cells *in vitro* and the increased generation of IgG1 and IgE *in vivo*. The Th2 skewing is accompanied by a reduction in DC maturation, with a parallel decrease in IL-12 production that could account for the increase in Th2 polarization. Hence, PIR-B downregulates conditions that favor allergic inflammation *in vivo* at the afferent level of DCs and B cells. $Pirb^{-/-}$ mice also exhibit exacerbated allogeneic graft-versus-host disease, leading to excess mortality (Nakamura *et al*., 2004). This is attributed to a greater activation of donor $CD4^+$ and $CD8^+$ T cells by $Pirb^{-/-}$ host DCs, possibly as a result of upregulated expression of the activating receptor PIR-A on the latter cells (Nakamura *et al*., 2004). It remains to be determined whether PIR-B inhibits allergic responses *in vivo* at the level of effector mast cells.

8. Conclusions

The accumulated *in vitro* and *in vivo* data leave little doubt that the constitutively expressed LILRBs play significant roles in preventing pathologic inflammation that could otherwise ensue from unchecked activation of cells of the innate and adaptive immune systems. Cells must overcome an appreciable level of inhibitory signals to initiate and sustain inflammation *in vivo* because of the presence of multiple LILRBs on relevant cell populations, the constitutive or readily inducible endogenous ligands for many of the LILRBs, and the ability of the earliest signaling steps of certain activation processes to stimulate the LILRBs through incremental phosphorylation of ITIMs.

Significant issues about the immunobiology and immunopathology of the LILRBs remain to be addressed. For example, the ligands for LILRB3, hLILRB4, and LILRB5 need to be identified and located *in vivo* to define the requirements for, and sites of action of, inhibition. Associations between

inflammatory disease states and LILRB expression levels *in vivo* need to be defined for the receptors expressed in humans. Similarly, single nucleotide polymorphisms and other allotypic differences in *LILRB* genes that are associated with pathologic inflammation in affected individuals need to be defined to reveal potential alterations in receptor expression or function that may have predictive and/or therapeutic value. Strategies for stimulating or reducing the inhibitory capacity of individual LILRBs or groups of LILRBs *in vivo* need to be developed. Addressing these important challenges will take the LILRB field to the next level of understanding and provide interventions that harness the remarkable anti-inflammatory capacity of these endogenous regulatory receptors.

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