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CHAPTER 1

Macrophage Proinflammatory Activation and Deactivation: A Question of Balance

Annabel F. Valledor,[*] Monica Comalada,[†] Luis F. Santamaría-Babi,[†] Jorge Lloberas,[†] and **Antonio Celada[†]**

Contents

Abstract

Macrophages play key roles in inflammation. During the onset of the inflammatory process, these phagocytic cells become activated and have destructive effects. Macrophage activation, which involves the induction of more than 400 genes, results in an increased capacity to eliminate bacteria and to regulate many other cells through the release of cytokines and chemokines.

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However, excessive activation has damaging effects, such as septic shock, which can lead to multiple organ dysfunction syndrome and death. In other situations, persistence of proinflammatory activity results in the development of chronic inflammation, such as rheumatoid arthritis, psoriasis, and inflammatory bowel disease. To prevent undesirable effects, several mechanisms have evolved to control the excess of activation, thereby leading to macrophage deactivation and the resolution of inflammation. In this review, we discuss several mechanisms that mediate macrophage deactivation.

ABBREVIATIONS

ATF2	activating transcription factor 2
BCL-3	B cell lymphoma 3
CBP	CREB-binding protein
C/EBPδ	CCAAT/enhancer-binding protein-δ
CIS	cytokine inducible SH2-containing protein
COX-2	cyclooxygenase-2
CREB1	cAMP-responsive-element-binding protein 1
DCs	dendritic cells
DUSP1	dual specificity phosphatase 1
ERK	external regulated kinase
GR	glucocorticoid receptor
HATs	histone acetyltransferases
HDACs	histone deacetylases
Hes	hairy and enhancer of split
Hey	hairy/enhancer-of-split related with YRPW motif
IFN-γ	interferon-gamma
IkB	inhibitor of nuclear factor kappaB
IRFs	IFN-regulatory factors
IL	interleukin
IL1Rl1L/ST2L	IL-1 receptor-like 1 ligand
IRAKM	IL-1 receptor-associated kinase M
JAK	janus kinase
LBP	lipoprotein binding protein
LPS	lipopolysaccharide
JNK-1	junk N-terminal kinase
LXR	liver X receptor
MAPK	mitogen-activated protein kinase
MKP-1	phosphatase 1 of MAPK
MyD88	myeloid differentiation primary response gene 88
MyD88(S)	myeloid differentiation primary response gene 88 (short isoform)

NAD	nicotinamide adenine dinucleotide
NO	nitric oxide
NOS2	nitric oxide synthase 2
PIAS	protein inhibitor of activated STAT
PKA	protein kinase A
PMNs	polymorphonuclear neutrophils
PPAR	peroxisome proliferator-activated receptor
PTEN	phosphatase and tensin homolog deleted on chromosome ten
PTP1B	protein tyrosine phosphatase 1B
RUNX1	runt-related transcription factor 1
SHIP	SH2-containing 5′-inositol phosphatase
SHP-1	SH2-containing phosphatase 1
SIRT	sirtuin
SOCS	suppressors of cytokine signaling
STAT	signal transducer and activator of transcription
sTLR4	soluble TLR4
SUMO	small ubiquitin-related modifier
TOLLIP	toll-interacting protein
TGFβ	transforming growth factor-beta
TLR-4	toll-like receptor 4
TNF-α	tumor necrosis factor-alpha.

1. INTRODUCTION

When a microorganism enters the body and remains at a local site, a reaction is initiated in order to remove or inactivate the nonself element and to repair the damage caused by this reaction. This process is called inflammation and it has been recognized by its cardinal signs (heat, redness, swelling, and pain) since the early days of medicine. Inflammation is induced by changes in the microcirculation that allow large amounts of serum proteins and leukocytes to move from the blood to the affected tissue.

The first cells that selectively distinguish between self- and nonself elements at the inflammatory loci are innate-type lymphocytes, including B1, natural killer cells, and γ/δ T lymphocytes. They recognize danger- and pathogen-associated molecular patterns and respond to them by producing cytokines. The type of cytokines released during these early stages depends on the invading microorganism and influences the nature of the subsequent immune response (Th1, Th2, or Th17 response). The first cells to move from the blood to the inflammatory site are $Ly6C^{-}$ monocytes, which secrete a number of chemokines that attract polymorphonuclear neutrophils (PMNs) (Auffray *et al.*, 2007). During the first

hours of infection, PMNs massively enter the site of inflammation, followed by Ly6C$^+$ monocytes 24 h later. The differential time-course in the recruitment of PMNs and Ly6C$^+$ monocytes has been associated with the expression of selective chemokines and leukocyte-endothelial adhesion molecules. The initial goal of these phagocytic cells is to kill and eliminate the invading microorganisms (proinflammatory activity). During this process, most of the neutrophils die by apoptosis. Once the injurious stimulus is cleared, healthy tissue structure and function is restored (anti-inflammatory activity). Depending on the amount of time required to remove the harmful agent, activation of the acquired immune system may also take place. Although the cardinal signs of inflammation have been known for a long time, the mechanisms and mediators involved in this process have been largely ignored and have only recently begun to be elucidated.

2. THE TWO FACES OF INFLAMMATION

The goal of the inflammatory process is to remove noxious agents, thus protecting the body against infection. In the early stage of inflammation, neutrophils kill microorganisms very efficiently. Soon after entering tissues, neutrophils promote the switch of arachidonic acid-derived prostaglandins and leukotrienes to lipoxins, which inhibit further neutrophil recruitment and trigger neutrophil apoptosis within the inflammatory site (Serhan and Savill, 2005). The remaining microorganisms are then eliminated by infiltrating macrophages. For this purpose, macrophages use several potent mechanisms, including the production of reactive oxygen species (ROS), nitric oxide (NO), and the release of several enzymes and cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), IL-6, etc. The duration of this proinflammatory phase depends on the balance between the capacity of the microorganisms to survive and the capacity of the macrophages to remove them. If during this period macrophages are able to control the infection and eliminate the pathogen, then a second phase is initiated in which macrophages exert anti-inflammatory activity. The release of anti-inflammatory and reparative cytokines such as transforming growth factor-beta (TGF-β) and recently identified lipid mediators such as lipoxins, resolvins, and protectins predominate during this phase. Resolvins and protectins are oxygenation products derived from omega-3 polyunsaturated fatty acids, which, together with lipoxins, act coordinately to suppress local inflammatory cell influx and enhance the clearance of apoptotic bodies (Kohli and Levy, 2009). The objective is now to repair the damage produced during the proinflammatory phase, similarly to a process of wound healing, thus

leading to the resolution of inflammation. The anti-inflammatory program ends with the departure of macrophages through the lymph.

Under normal circumstances in which macrophages kill or inactivate microorganisms through the development of a granuloma, the phases of destruction and repair are well balanced. However, under persistence of the proinflammatory phase or when macrophages trigger an altered response, acute infection may result in chronic inflammation (Kim *et al.*, 2008). An excess of activation, for example, as a response to circulating lipopolysaccharide (LPS), may lead to sepsis and subsequent septic shock. To limit inflammation-associated pathology, the cells become hyporesponsive to LPS (tolerance to LPS) during conditions of excessive inflammation. LPS-tolerant cells are refractory to the induction of inflammatory cytokines such as TNF-α and IL-6, and this is due, at least in part, to the downregulation of several inflammatory signaling molecules (Foster and Medzhitov, 2009). In an experimental model of arthritis, the signature of oxidative metabolism and the mode of macrophage activation determine the shift from acute to chronic disease (Takahashi *et al.*, 2008). Failure to resolve inflammation is also a key event in atherosclerosis (Tabas, 2010). On the other end of the spectrum, an excess of anti-inflammatory activity can result in progressive fibrosis (Wynn, 2008). Therefore, the activity of macrophages in both the proinflammatory and the resolution phases is complex and must be tightly regulated.

3. MACROPHAGE PROINFLAMMATORY ACTIVITIES

3.1. IFN-γ

In order to become activated and fully functional, macrophages at the inflammatory site must interact with a variety of Th1-type cytokines, such as interferon-gamma (IFN-γ). Upon recognition of IFN-γ, its specific receptor triggers the sequential activation of the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, which results in transcriptional upregulation of more than 400 genes (Bach *et al.*, 1997; Billiau and Matthys, 2009; Stark, 2007). Many IFN-γ-induced functions are mediated by direct activation of immune effector genes by STAT1, including genes encoding antiviral proteins, microbicidal molecules, phagocytic receptors, chemokines, cytokines, and antigen-presenting molecules. There is accumulating evidence that cytoplasmic inactive STAT1 is present predominantly as unphosphorylated homodimers in an equilibrated state between a parallel and an antiparallel configuration (Ota *et al.*, 2004). After interaction of IFN-γ with the corresponding receptor, a signaling is produced inducing JAKs to phosphorylate STAT1 on tyrosine 701. This produces a dimer configuration of STAT1 and the nuclear translocation

(Wenta *et al.*, 2008). In the nucleus, STAT1 binds to gamma-activated sequences on target gene promoters inducing a transcriptional activity. Then, STAT1 is rapidly inactivated through acetylation on lysines 410 and 413 catalyzed by histone acetyltransferases (HATs), such as CREB-binding protein (CBP)/p300. After that, STAT1 is dephosphorylated by the STAT1 phosphatase TCP45 (Ota *et al.*, 2004) and returns back to the cytoplasm. The cycle phosphorylation–acetylation ended by histone deacetylases (HDACs), such as HDAC3 and possibly Sirtuins (SIRTs), that deacetylate STAT1 (Hu and Ivashkiv, 2009). At this moment, STAT1 could be tyrosine-phosphorylated again by the IFN-γ-receptor complex and JAKs.

The transcriptional activity of STAT-1 homodimers is increased by the phosphorylation of serine 727 in the transcription activation domain, which enhances binding of this domain to the coactivator CBP/p300 and other nuclear proteins (Sun *et al.*, 2005). Several kinases, including members of the mitogen-activated protein kinase (MAPK) family, have been proposed to mediate serine phosphorylation of STAT1. Recent studies showed activation of p38 in bone marrow-derived macrophages at early time points of IFN-γ-stimulation, whereas weak activation of external regulated kinase (ERK)-1/2 and Junk N-terminal Kinase (JNK-1) was detected only at later stages (Valledor *et al.*, 2008c). MAPKs regulate IFN-γ-mediated gene expression in a selective way. p38 participates mainly in the regulation of the expression of factors required for the innate immune response, including several chemokines, cytokines, and nitric oxide synthase 2 (NOS2). In contrast, JNK-1 contributes to the expression of genes involved in antigen presentation, including class II transactivator and genes encoding molecules from the class II major histocompatibility complex. Interestingly, MAPK signaling was not found to influence the state of STAT1 serine phosphorylation in those studies. Instead, some of the changes in gene expression were based on posttranscriptional regulation of mRNA stability. Despite a major role of STAT1 in IFN-γ-induced transcriptional activation, some of the biological effects of IFN-γ occur in the absence of JAK/STAT1 signaling. Microarray analysis revealed that approximately one-third of IFN-γ-stimulated genes are still upregulated by IFN-γ in the absence of functional STAT1. The activation of other STATs (STAT3/5) and alternative signaling pathways, such as NF-κB and AP-1, may contribute to IFN-γ-mediated function (Gough *et al.*, 2008; Hu and Ivashkiv, 2009).

3.2. LPS

Another major activating agent of macrophages is LPS, a component of the cell wall of gram-negative bacteria. LPS recognition requires the binding of LPS to lipoprotein binding protein (LBP) and CD14 in the

surface of macrophages. Signal transduction to LPS is then triggered by toll-like receptor 4 (TLR-4), which results in coordinate transcriptional activation of proinflammatory gene expression. Three classes of transcription factors, as well as various transcriptional coregulators and chromatin modifying enzymes, participate in the cellular response to LPS (Medzhitov and Horng, 2009). The first category (class I) consists of transcription factors that are constitutively expressed and are activated by signal-dependent posttranslational modifications. In most cases, these transcription factors are retained in the cytoplasm in the basal state and signal-dependent activation involves their translocation to the nucleus. This class includes proteins that are known to have key roles in inflammation, such as NF-κB, IFN-regulatory factors (IRFs), and cAMP-responsive-element-binding protein 1 (CREB1). The second category of transcription factors (class II) is synthesized *de novo* during the primary response to LPS. These transcription factors regulate subsequent waves of gene expression during the secondary response to LPS, and they can do so over a prolonged period of time (Ravasi *et al.*, 2007). CCAAT/enhancer-binding protein-δ (C/EBPδ) belongs to this group. The third category of transcription factors (class III) comprises lineage-specific transcriptional regulators, the expression of which is turned on during macrophage differentiation. Members of this group include PU.1 and C/EBPβ, as well as runt-related transcription factor 1 (RUNX1) and IRF8. These transcription factors mediate cell type-specific responses to inflammatory signals by conferring a permissive chromatin state on macrophage-specific inducible genes. The transcriptional response to LPS also depends on coregulators, including coactivators and corepressors, which are transcriptional regulators that, unlike transcription factors, lack DNA-binding specificity and must be recruited to their target genes through other mechanisms. Many coregulators have histone-modifying activities that lead to chromatin remodeling at target gene promoters. Several histone modifications differentially regulate subsets of LPS-inducible genes. These modifications include phosphorylation of histone 3 at serine 10, which might facilitate NF-κB recruitment to specific inflammatory genes, deubiquitination of ubiquitinated histone 2A at lysine 119, which releases repression from a specific subset of LPS-inducible genes (Zhou *et al.*, 2008), and demethylation of trimethylated histone 3 at lysine 27, a prerequisite for the induction of certain inflammatory genes (De Santa *et al.*, 2007).

4. MACROPHAGE DEACTIVATION

The extent and duration of macrophage activation is critical to limit the detrimental effects associated with excessive inflammation. Many of the molecules generated during macrophage activation are toxic not only to

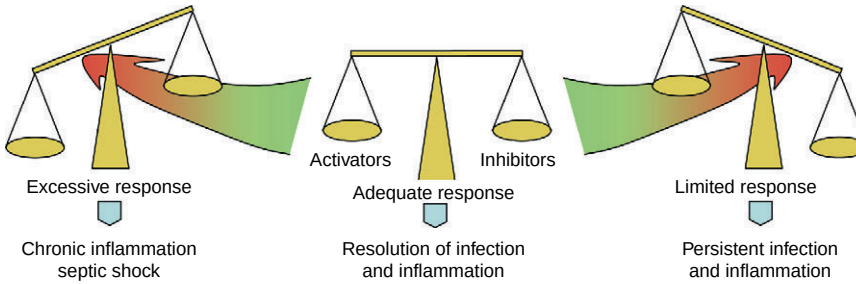

FIGURE 1.1 An unbalanced proinflammatory activation of macrophages results in inappropriate inflammation.

microorganisms but also to the macrophages themselves. For this reason, mechanisms that account for macrophage deactivation play key roles in maintaining homeostasis and keeping the immune response under control (Fig. 1.1). In general, two main categories of negative regulators of inflammation can be distinguished: signal- and gene-specific mechanisms (Medzhitov and Horng, 2009). The first group consists of regulators that inhibit signal transduction by TLRs and other inflammatory pathways. Examples of these include A20, IL-1 receptor-like 1 ligand (IL1Rl1L/ST2L), IL-1 receptor-associated kinase M (IRAKM), and suppressors of cytokine signaling (SOCSs) (Liew *et al.*, 2005) (Table 1.1). All these proteins exert global inhibitory effects on gene induction by the inflammatory signal. The second category includes transcriptional repressors or other negative regulators that selectively modulate gene expression. These can be divided in basal repressors, which are constitutively expressed and mediate basal repression of proinflammatory genes, and inducible repressors, which are transcriptionally upregulated in response to the inflammatory signal. The latter are normally part of a negative feedback loop that limits the inflammatory response.

4.1. Signal-specific mechanisms

SOCSs and protein inhibitors of activated STAT (PIAS) are key repressors of IFN-γ activity. SOCS1 directly interacts with JAKs, resulting in inhibition of its tyrosine kinase activity (Kamura *et al.*, 1998). Cytokine-induced SH2-containing protein (CIS) interacts with cytokine receptors to block the recruitment and activation of STATs. The SOCS-box motif interacts with proteins that belong to the ubiquitin-mediated proteasomal degradation pathway (Piessevaux *et al.*, 2008). For this reason, SOCS proteins seem to combine specific inhibitory actions with a general mechanism of

TABLE 1.1 Mechanisms of macrophage deactivation

Gene name/family	Expression/abundance in macrophages	Function	References
A20	Induced by LPS	Inhibits TLR signaling by deubiquitination of TRAF6	Boone *et al*. (2004)
Adenosine	Levels rise during inflammation	Blocks STAT1 phosphorylation	Barnholt *et al*. (2009), Hasko *et al*. (2008)
cAMP	Levels rise in response to adenosine receptors and other G-coupled receptors	Activates PKA, leading to phosphorylation of p105 and suppression of LPS-production of TNF-α	Wall *et al*. (2009)
CIS	Induced by LPS	Blocks recruitment of STATs, targets signaling molecules to proteasomal degradation	Baetz *et al*. (2004)
Hes1	Induced by LPS	Inhibits cytokine production	Hu *et al*. (2008)
Hey1	Induced by LPS	Inhibits cytokine production	Hu *et al*. (2008)
IL-10	Induced during infection	Attenuation of proinflammatory responses	Dagvadorj *et al*. (2009), Li and Flavell (2008), Medzhitov and Horng (2009)
IRAKM	Induced by LPS	Inhibits TLR signaling by blockage of IRAK1 phosphorylation	Liew *et al*. (2005)
MKP-1	Induced by LPS	Dephosphorylates MAPK (ERK, p38 and JNK)	Chi *et al*. (2006), Salojin *et al*. (2006), Hammer *et al*. (2006), Zhao *et al*. (2006)

(*continued*)

TABLE 1.1 (*continued*)

Gene name/family	Expression/abundance in macrophages	Function	References
MyD88(S)	Induced by LPS	Inhibits TLR4 signaling by antagonizing MyD88	Liew *et al*. (2005)
Nuclear receptors (e.g., GR, LXRs, PPARs, etc.)	Either constitutive (e.g., LXRβ) or induced by LPS (e.g., LXRα); activated by phagocytosis of apoptotic cells (e.g., LXRs, PPARδ)	Induction of MKP-1 (GR); agonist-dependent transrepression of selective NF-κB target genes	Castrillo *et al*. (2003), Cho and Kim (2009), Ghisletti *et al*. (2007)
$p21^{WAF1/CIP1}$	Constitutive	Inhibits NF-κB activity	Lloberas and Celada (2009)
PIAS1	Constitutive, activated by phosphorylation in response to inflammatory stimuli	SUMOylates and inactivates STAT1 and NF-κB; mediates PPAR-γ-anti-inflammatory responses	Liu *et al*. (2005, 2007), Pascual *et al*. (2005)
PTEN	Constitutive	Negatively regulates JAK/STAT signaling	Dalpke *et al*. (2008)
PTP1B	Constitutive	Negatively regulates JAK/STAT signaling	Heinonen *et al*. (2009)
SHIP	Induced by LPS	Negatively regulates JAK/STAT signaling	Dalpke *et al*. (2008)
SHP-1	Constitutive	Negatively regulates JAK/STAT signaling	Dalpke *et al*. (2008)
SIRT1	Constitutive	Deacetylates and blocks the transactivation potential of NF-κB p65	Yeung *et al*. (2004)

SOCS1	Induced by LPS, CpG-DNA, IL-10, and IFN-β	Suppresses IRAK and MAL during TLR signaling; inhibits JAK kinase activity during IFN-γ signaling; targets signaling molecules to proteasomal degradation	Dennis *et al*. (2006), Liew *et al*. (2005), Qing *et al*. (2005), Wormald *et al*. (2006)
ST2L	Induced by LPS	Inhibits TLR signaling by sequestering MyD88 and MAL	Brint *et al*. (2004)
TGF-β	Induced by phagocytosis of apoptotic cells	Attenuation of proinflammatory responses	Li and Flavell (2008)
TOLLIP	Constitutive	Inhibits IRAK1	Didierlaurent *et al*. (2006)

Several endogenous mechanisms participate in macrophage deactivation. The table covers some of the most representative mechanisms that negatively regulate the macrophage response to LPS or IFN-γ. Abbreviations: CIS, cytokine inducible SH2-containing protein; Hes, hairy and enhancer of split; Hey, hairy/enhancer-of-split related with YRPW motif; IL, interleukin; IRAKM, IL-1 receptor-associated kinase M; LXR, liver X receptor; MKP-1, phosphatase 1 of MAPK; MyD88(S), myeloid differentiation primary response gene 88 (short isoform); PIAS, protein inhibitor of activated STAT; PPAR, peroxisome proliferator-activated receptor; PTEN, phosphatase and tensin homolog deleted on chromosome ten; PTP1B, protein tyrosine phosphatase 1B; SHIP, SH2-containing 5′-inositol phosphatase; SHP-1, SH2-containing phosphatase 1; SIRT, sirtuin; SOCS, suppressors of cytokine signaling; TGFβ, transforming growth factor-beta; TOLLIP, toll-interacting protein.

targeting associated signaling molecules for degradation (Dalpke *et al.*, 2008). PIAS act as small ubiquitin-related modifier (SUMO)-ligases, as well as decoy receptors. PIAS SUMOylate and inactivate STAT dimers in the nucleus (Rakesh and Agrawal, 2005). Negative regulation of JAK/STAT signal transduction has also been shown to be mediated by constitutively active tyrosine phosphatases, namely SH2-containing phosphatase 1 (SHP-1) and protein tyrosine phosphatase 1B, as well as lipid phosphatases, such as phosphatase and tensin homolog deleted on chromosome ten (PTEN) and SH2-containing 5′-inositol phosphatase (SHIP) (Dalpke *et al.*, 2008).

Because LPS is a potent macrophage activator acting through a different pathway than IFN-γ, many different deactivating mechanisms have evolved in response to it. Macrophage response to LPS involves the phosphorylation of the three members of the MAPK family (ERK, p38, and JNK) (Valledor *et al.*, 2008a). MAP kinase phosphatase 1 (MKP-1), also termed dual specificity phosphatase 1 (DUSP1), is encoded by an immediate-early response gene induced in macrophages upon stimulation with LPS (Valledor *et al.*, 2008b) and is responsible for dephosphorylating tyrosine and threonine residues of MAPK, thus suppressing signaling downstream of these kinases. Genetic ablation of MKP-1 has shown that this phosphatase is a pivotal feedback control regulator macrophage activation (Chi *et al.*, 2006; Hammer *et al.*, 2006; Salojin *et al.*, 2006; Zhao *et al.*, 2006).

4.2. Gene-specific mechanisms

Members of the IκB family are examples of inducible negative regulators. For instance, IκBα inhibits the global expression of NF-κB-dependent genes, whereas IκBNS and B cell lymphoma 3 (BCL-3) limit the expression of only specific genes (Kuwata *et al.*, 2006; Wessells *et al.*, 2004). Both IκBNS and BCL-3 modulate the exchange of active NF-κB dimers for their inactive counterparts at target gene promoters. ATF3 is another transcriptional negative regulator that is induced by LPS. ATF3 recruits HDACs to target genes (Gilchrist *et al.*, 2006) and forms a regulatory circuit with NF-κB and C/EBPδ at some LPS-inducible genes (Litvak *et al.*, 2009). Loss of these inducible repressors leads to hyper-induction of a number of LPS-inducible genes and increased susceptibility to septic shock (Gilchrist *et al.*, 2006; Wessells *et al.*, 2004). Endogenous decoy molecules have also evolved to antagonize the action of TLRs or specific signaling molecules. For example, soluble TLR4 (sTLR4) antagonizes TLR4 signaling by blocking its interaction with the accessory molecule MD2, whereas the short splice variant of myeloid differentiation primary response gene 88 (MyD88(S)) antagonizes activation of the adaptor molecule MyD88 (Liew *et al.*, 2005). Other molecules that negatively regulate TLR signaling

include toll-interacting protein (TOLLIP) (Liew *et al.*, 2005), SOCS1, and PIAS1 (see Table 1.1).

Some members of the sirtuin family have been implicated in the negative control of inflammatory gene expression. These proteins are nicotinamide adenine dinucleotide (NAD)-dependent HDACs and deacetylate transcription factors and coactivators. SIRT1 p300-mediated acetylation of the transactivation domain of NF-κB p65 is counteracted by the deacetylase activity of SIRT. This leads to a blockage of p65-dependent gene expression that is independent of the binding capacity to DNA of p65 (Yeung *et al.*, 2004).

Two Notch target genes, Hes1 and Hey1, are also examples of LPS-inducible repressors, as they inhibit cytokine production, including IL-6 and IL-12 (Hu *et al.*, 2008). Notch and LPS cooperate to induce the expression of these genes through activation of the transcription factor RBP-J. Interestingly, IFN-γ signaling blocks the upregulation of Hes and Hey by LPS, which may represent a mechanism that contributes to the proinflammatory enhancing effect of IFN-γ in LPS-stimulated macrophages.

The purine nucleoside adenosine has emerged as a key endogenous regulator of macrophage activation and function. Under conditions of stress and inflammation, local extracellular concentrations of adenosine rise as a result of ATP catabolism and cell secretion (Hasko *et al.*, 2008). Most of the known immunomodulatory effects of adenosine are mediated through its interaction with specific cell surface G protein-coupled receptors. Adenosine blocks IFN-γ-induced STAT1 phosphorylation on serine 727 (Barnholt *et al.*, 2009). Interestingly, we have observed that IFN-γ upregulates the expression of the A_{2B} adenosine receptor, thus providing a mechanism for macrophage deactivation. The generation of cAMP by G-coupled receptors, including adenosine receptors, may also contribute to inhibiting inflammation. cAMP has been shown to suppress LPS production of TNF-α expression through activation of protein kinase A (PKA) and subsequent phosphorylation of p105, a member of the inhibitor of nuclear factor kappaB (IkB) family (Wall *et al.*, 2009).

$p21^{WAF1/CIP1}$ (p21) is a crucial cyclin-dependent kinase inhibitor that negatively controls progression through the cell cycle. The functional activity of p21 depends on the cellular context and is controlled through phosphorylation and protein–protein interactions. Apart from its role in regulation of the cell cycle, p21 also plays a critical role as a negative regulator of macrophage activation, in particular, by modulating NF-κB activity and therefore inhibiting the LPS-dependent induction of TNF-α and IL-1β. Consequently, $p21^{-/-}$ mice are more susceptible to septic shock (Lloberas and Celada, 2009). Negative modulation of macrophage proinflammatory activities may also underlie the molecular mechanisms by which p21 favors macrophage survival during inflammation (Lloberas and Celada, 2009).

4.3. Cytokines

The production and release of anti-inflammatory cytokines, such as IL-10 and TGF-β, are also mechanisms that mediate autocrine and paracrine inhibition of macrophage proinflammatory activities, as illustrated by inhibition of LPS-induced TNF-α production (Li and Flavell, 2008; Medzhitov and Horng, 2009). IL-10 is produced by macrophages and myeloid dendritic cells (DCs), but not by plasmacytoid DCs, in response to microbial products. The ERK1/2 pathway is involved in IL-10 production in these cells, whereas the exact signaling pathways that mediate IL-10 production in other immune cells, such as B cells, mast cells, and eosinophils, remain elusive (Saraiva and O'Garra, 2010). IL-10 requires *de novo* protein expression in order to downregulate the transcription of proinflammatory genes, as indicated by the capacity of cycloheximide treatment to block IL-10-mediated inhibition of primary response genes (Murray, 2005). Many of the proteins that function in LPS-induced negative feedback loops are indeed also upregulated by IL-10 and other pathways that inhibit inflammatory gene expression. For example, IL-10 attenuates the LPS response by inducing BCL-3, which then inhibits the LPS-induced expression of IκB-zeta, thus interfering with the production of IL-6 (Dagvadorj *et al.*, 2009). The binding or uptake of apoptotic cells by phagocytes induces the production of TGF-β and in some model systems IL-10 (Erwig and Henson, 2007). Arachidonic acid release, cyclooxygenase-2 (COX-2), and prostaglandin synthase expression were shown to be dependent on TGF-β production as well as the inhibition of thromboxane synthase, sulfidopeptide leukotrienes, NOS2, and NO. In addition to autocrine and paracrine effects mediated through cytokines and lipid mediators, direct effects of apoptotic cells have been observed on the proinflammatory transcriptional machinery of macrophages (Cvetanovic and Ucker, 2004).

4.4. Nuclear receptors

Members of the nuclear receptor superfamily, including (but not limited to) the glucocorticoid receptor (GR), liver X receptors (LXRs) (activated by oxidized forms of cholesterol or oxysterols), and peroxisome proliferator-activated receptors (PPARs) (activated by endogenous eicosanoids), also negatively regulate inflammation. Nuclear receptors are ligand-dependent transcription factors that control several aspects of metabolism and homeostasis. Nuclear receptors can both activate and inhibit gene expression. The prototypic activity of these receptors is through ligand-dependent activation of transcription by binding to specific response elements on target gene promoters. Several nuclear receptors inhibit inflammation by directly inducing gene expression programs that are anti-inflammatory.

For example, activated GR induces the expression of MKP-1, leading to decreased MAPK-dependent phosphorylation of activating transcription factor 2 (ATF2)/c-Jun complexes and repressed COX-2 gene induction by LPS (Cho and Kim, 2009). In addition, several nuclear receptors, including GR, LXRs, and PPARs, inhibit, in a ligand-dependent manner, the activity of other transcription factors, such as NF-κB, without directly binding to DNA or promoting target gene expression. This effect is referred to as transrepression. Genes affected by nuclear receptor-mediated transrepression include NOS2, COX-2, TNF-α, IL-1β and -6, matrix metalloproteinase MMP-9 and chemokines, such as monocyte chemoattractant protein-1 and -3, macrophage inflammatory protein-1β, and interferon-activated gene-10 (Castrillo *et al.*, 2003; Gough *et al.*, 2008). Recent findings highlighted the use of alternative SUMOylation-dependent mechanisms by LXRs and the isoform PPARγ to negatively regulate selective subsets of TLR-inducible proinflammatory genes in macrophages (Ghisletti *et al.*, 2007). Agonist-bound LXRs conjugate to SUMO2/3 whereas SUMO1 shows a preference for ligand-activated PPARγ. Upon conjugation to SUMO proteins, both types of nuclear receptors are targeted to the promoters of specific TLR target genes, where they prevent LPS-dependent removal of corepressor complexes and subsequent transcriptional activation. SUMO1-PPARγ and SUMO2/3-LXRs inhibit distinct corepressor clearance mechanisms, thereby allowing promoter- and TLR-specific patterns of repression.

Recent work demonstrated that the activation of the LXR pathway takes place after phagocytosis of apoptotic bodies (Gonzalez-N *et al.*, 2009), thus suggesting that ligands for LXRs are generated at intermediate stages of an infection or in other forms of tissue damage where extensive phagocytosis is required for maintenance of homeostasis. LXR agonists induce the expression of Mer, a receptor tyrosine kinase that is critical for phagocytosis. LXR-deficient macrophages exhibit defects in apoptotic cell clearance and an aberrant proinflammatory response after phagocytosis. Consequently, mice lacking LXRs manifest a breakdown in self-tolerance and developed autoantibodies and autoimmune disease. Thus, activation of LXR by apoptotic cells is part of a positive feedback loop that promotes their own clearance and couples phagocytosis to the suppression of inflammatory gene expression. The activation of LXRs also results in increased survival of macrophages during infection through the combined upregulation of AIM/Sp-α and inhibition of the expression of several caspases and other proapoptotic molecules (Dalpke *et al.*, 2008; Rakesh and Agrawal, 2005). The combination of anti-inflammatory and prosurvival effects of the LXR pathway, together with its role in mediation of apoptotic cell clearance, suggests that it may have evolved as a means to potentiate the role of the macrophage in the resolution of inflammation. PPAR-δ has been also shown to play a crucial role in phagocytosis and the maintenance of self-tolerance (Mukundan *et al.*, 2009). This isoform

is induced when macrophages engulf apoptotic cells. The lack of functional PPAR-δ results in decreased expression of opsonins, defective apoptotic cell clearance, and reduced production of anti-inflammatory cytokines. Similar to LXR-deficient mice, macrophage-specific PPAR-δ knockout mice show increased autoantibody production and are predisposed to autoimmune disease.

5. CONCLUDING REMARKS

The tuning of macrophage activation and deactivation is critical in order to limit proinflammatory responses and account for the efficient resolution of inflammation. A number of signaling pathways and transcription factors mediate the transcriptional activation of genes involved in the inflammatory response. Several mechanisms have evolved to ensure adequate macrophage deactivation including negative feedback loops induced as part of the macrophage response to inflammatory signals. Negative regulators include inhibitors of inflammatory signaling, transcriptional repressors, and anti-inflammatory cytokines. Also, members of the nuclear receptor superfamily potentiate macrophage function during the resolution of inflammation.

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CHAPTER 2

Natural Helper Cells: A New Player in the Innate Immune Response against Helminth Infection

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Abstract

The Th2-type immune response, characterized by the production of IL-4, IL-5, and IL-13, is a critical immune response against helminths invading cutaneous or mucosal sites. Th2 cytokines are induced soon after helminth infection, even before a pathogen-specific adaptive immune response is established. Although the expulsion and clearance of helminths usually requires pathogen-specific Th2-mediated immunity, early induction of Th2 cytokines during the innate immune phase is important for host protection from helminth invasion. Recent studies have shed light on such Th2 cytokine production by formerly uncharacterized innate immune cells such as a newly identified natural helper cell. We discuss here the mechanisms of innate production of Th2 cytokines in host immune responses against helminth infection.

1. INTRODUCTION: HELMINTH INFECTION AND TH2 IMMUNITY

The World Health Organization reported that more than 1 billion people suffer from neglected tropical diseases such as helminthiasis, which is a major health problem throughout developing countries and a food safety issue worldwide (Albonico *et al.*, 1999; Savioli, 2009). A major concern of helminth infection is that these metazoan parasites may also impair effective immune responses against other microbial pathogens, including *Mycobacterium tuberculosis* and human immunodeficiency virus (HIV; Hotez and Kamath, 2009; Willyard, 2009).

A wide variety of lumen/tissue-dwelling helminths infect humans including *Anisakis* spp. (*Anisakis physeteris*, *Anisakis simplex*, *Pseudoterranova decipiens*), guinea worms (*Dracunculus medinensis*), hookworms (*Ancylostoma duodenale* and *Necator americanus*), lymphatic filaria (*Brugia malayi*, *Brugia timori*, *Wuchereria bancrofti*), pinworms (*Enterobius vermicularis*), roundworms (*Ascaris lumbricoides*), subcutaneous filaria (*Mansonella streptocerca*, *Oncocerca volvulus*), threadworms (*Strongyloides stercoralis*), trichina worms (*Trichinella* spp.), and whipworms (*Trichuris trichiura*) (Albonico *et al.*, 1999). Helminth infections are also a major concern in veterinary medicine (Urban *et al.*, 2007).

Each type of helminth has a unique strategy for infecting and taking up residence in a distinct microenvironment in the host. Conversely, the host employs a variety of immune cells to expel those invading helminths. The Th2-type immune response characterized by the production of IL-4, IL-5, and IL-13 is induced against helminths invading cutaneous or mucosal sites and functions as protective immunity against those pathogens. Interestingly, Th2 cytokines are induced soon after helminth infection and before pathogen-specific Th2 cells are established. Although

the final expulsion of helminths usually requires Th2-mediated immunity, an early Th2-type innate immune response is important in the restriction of helminth invasion before the adaptive immune response initiates. Recent studies shed light on formerly uncharacterized innate immune cells that carry out such innate Th2 cytokine production.

2. HELMINTHS' INVASION AND HOST IMMUNE RESPONSES

Host immune responses to several species of helminths have been studied using animal models. Helminths have adapted to reside in the hosts during evolution and their unique life cycles have been established likely through the fierce battles raging between the invading helminths and the host's immune responses. As a result, each helminth has a unique, characteristic life cycle and the host immune system deals with each type of helminth in a distinct way (Patel *et al.*, 2009; Fig. 2.1).

2.1. Helminth life cycles

2.1.1. *Trichinella spiralis* (Fig. 2.1A)

The encysted first stage larva excyst in the host stomach a few hours after ingestion of contaminated meat and invade duodenal or jejunal epithelium. Larvae rapidly mature into adult worms and adult worms mate during the next 36 h. Adult worms reside in the intestine by forming syncytia within the epithelial cell layer. One week after infection, female worms begin to release larvae. Larvae enter intestinal lymphatics or mesenteric venules and migrate throughout host body, settling most heavily in host striated muscle. This invasion induces differentiation of muscle cells into nurse cells and encapsulation begins 17–21 days after infection. In the rodent intestine, adult worms can be expelled in less than 2 weeks while they remain in the human gut for several weeks (Capó and Despommier, 1996; Murrell, 1985).

2.1.2. *Trichuris muris* (Fig. 2.1B)

The first stage larvae hatch from environmentally resistant eggs that are orally ingested by the host. Larvae immediately invade duodenal or jejunal mucosa and reside there for a few days. The developed larvae then migrate to the cecum and proximal colon where they invade the mucosal epithelial cells at the crest of the crypt, such that the worm's head and part of its filamentous anterior portion are embedded in host mucosal epithelia. Thus, the worms survive and grow to egg-laying adult worms in an isolated tunnel-like environment (Mahida, 2003).

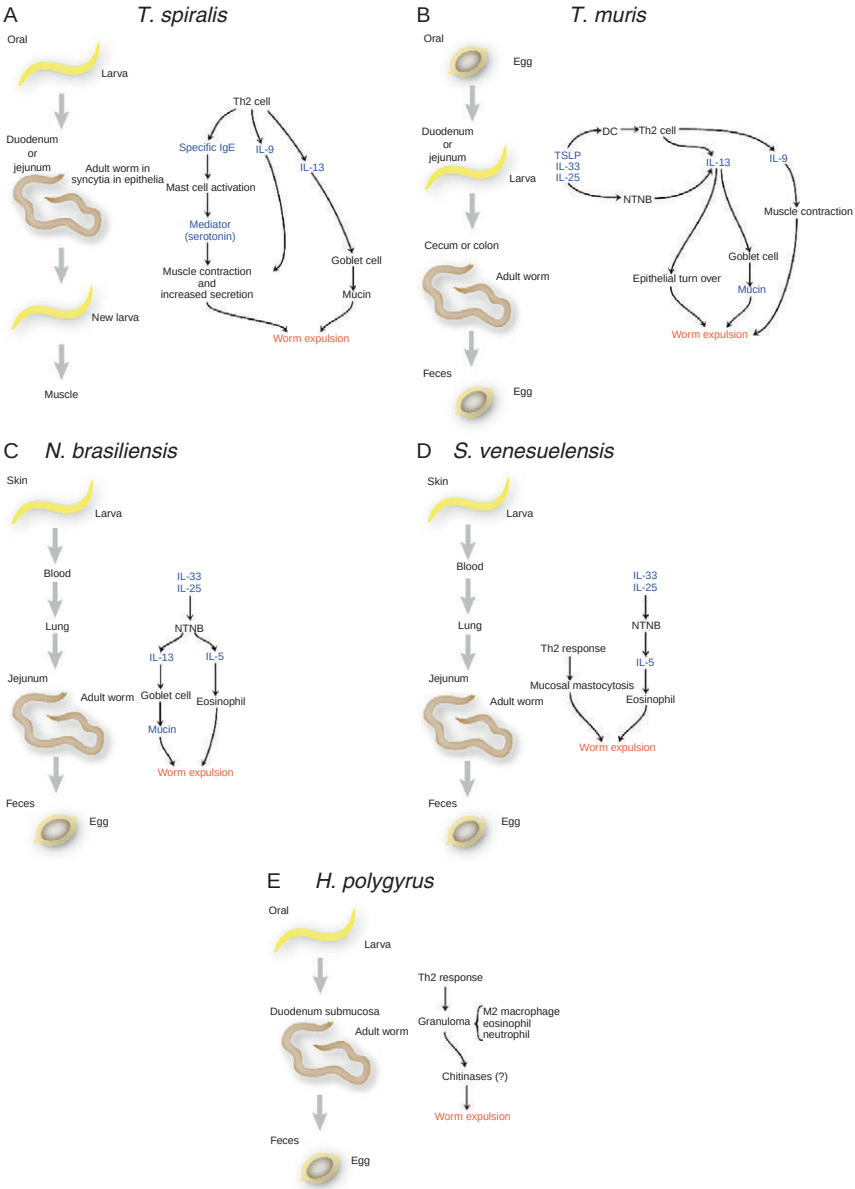

FIGURE 2.1 Schematic models of the course of helminths' infection and the path of host immunity for the expulsion of helminths. (A) *T. spiralis*, (B) *T. muris*, (C) *N. brasiliensis*, (D) *S. venezuelensis*, and (E) *H. polygyrus*.

2.1.3. *Nippostrongylus brasiliensis* and *Strongyloides venezuelensis* (Fig. 2.1C and D)

Infective third stage larvae penetrate the host skin, travel to the blood stream via subcutaneous lymphatics and eventually migrate to the lung 24–48 h later. Larvae are then coughed up and swallowed, and mature into adults in the jejunum. Adult worms begin to produce eggs 5–6 days after infection. Adult worms are usually expelled from the gut less than 2 weeks after infection (Camberis *et al.*, 2003; Negrão-Corrêa, 2001; Vadlamudi *et al.*, 2006).

2.1.4. *Heligmosomoides polygyrus* (Fig. 2.1E)

This nematode generally establishes a chronic infection and lives in the gut of mammalian hosts. Infective third stage larvae enter the wall of the anterior small intestine within 24–72 h of oral infection, reside in the duodenal mucosa and migrate to the submucosa. One week after infection, they return to the gut lumen and rapidly mature into adults. Adult worms reside in host intestinal mucosa and survive for several months (Camberis *et al.*, 2003; Negrão-Corrêa, 2001; Robinson *et al.*, 1989).

2.2. Host's immune responses

2.2.1. *Trichinella spiralis* (Fig. 2.1A)

Worm expulsion is dependent on Th2 immune responses. The Th2 cytokines, IL-4 and IL-13, are critical for worm expulsion and the inhibition of both cytokines significantly delays worm expulsion (Finkelman *et al.*, 2004). Eosinophils are able to kill larvae *in vitro* but IL-5 and eosinophilia are dispensable for *T. spiralis* expulsion (Dixon *et al.*, 2006; Gurish *et al.*, 2002).

Mast cells and *T. spiralis*-specific IgE play important roles in protective immunity (Gurish *et al.*, 2004; Knight *et al.*, 2002; McDermott *et al.*, 2003) although one report showed that *T. spiralis* can be expelled in B cell deficient mice (Finkelman *et al.*, 2004). It has been suggested that gut smooth muscle contraction induced by mast cell-derived serotonin is important for the worm expulsion (Vermillion and Collins, 1988) and that a mast cell-mediated chloride ion-dependent increase in short circuit current induces fluid secretion, which also plays a role in the expulsion of this helminth (Finkelman *et al.*, 2004; Harari *et al.*, 1987). Capture of helminth antigens by IgE bound to a high affinity Fc receptor for IgE (FcεRI) on mast cells induces mast cell degranulation, releasing various chemical mediators including histamine and serotonin. Goblet cell hyperplasia is also involved in protection from mucosal stage *T. spiralis* as mucins produced by IL-13-stimulated goblet cells block adhesion of worms on the epithelial layer (Knight *et al.*, 2008).

2.2.2. *Trichuris muris* (Fig. 2.1B)

Comparison of resistant and susceptible strains suggests that the Th2 response is also critical for the expulsion of *T. muris*. In fact, Th2 cells accumulate in the epithelium at the time of worm expulsion and macrophages accumulate in the lamina propria (Little *et al.*, 2005). Thymic stroma lymphopoietin (TSLP) derived from epithelial cells may play a role in supporting Th2 cells in the epithelium as blockade of TSLP function by deletion of TSLP receptor, suppression of TSLP production by IKKβ inhibition reduces Th2 cells and enhances Th1 and Th17 cells in the epithelia (Zaph *et al.*, 2007). IL-33 is also reported to be involved in the expulsion of *T. muris* (Humphreys *et al.*, 2008). In addition to goblet cell hyperplasia, IL-13 accelerates the migration and turnover of epithelial cells. Epithelial cell turnover dislodges *T. muris* from its niche in the lumen. Blocking of CXCL10 also enhances epithelial cell turnover and such forced epithelial cell turnover was sufficient to expel the worm in SCID mice (Cliffe *et al.*, 2005), indicating that accelerated epithelial cell turnover is an important expulsion mechanism for *T. muris*. IL-9 is also reported to be involved in the expulsion of *T. muris* and induces colonic muscle hypercontractility in a mast cell-independent manner; such muscle contraction helps the clearance of *T. muris* but not *T. spiralis* (Khan *et al.*, 2003; Richard *et al.*, 2000).

2.2.3. *Nippostrongylus brasiliensis* and *Strongyloides venezuelensis* (Fig. 2.1C and D)

Migration of these helminths to the lung induces an inflammatory response characterized by pulmonary eosinophilic infiltration. IL-5 and eosinophilia play important roles in the protective immunity against *N. brasiliensis* and *S. venezuelensis* in the lung stage as eosinophils effectively kill larvae of *N. brasiliensis* and *S. venezuelensis* (Dent *et al.*, 1999; Korenaga *et al.*, 1994). During the intestinal stages, the Th2 immune response is also critical for the expulsion of *N. brasiliensis* and *S. venezuelensis*. Mast cells are not essential in the expulsion of *N. brasiliensis* in the gut stage as *N. brasiliensis* can be expelled from W/W^v mice. Although the expulsion is slower in W/W^v mice than in wild-type mice, the slow expulsion in W/W^v mice was not corrected by wild-type bone marrow transfer, suggesting that the slow expulsion is in part due to the impaired development of the c-Kit^+ interstitial cell of Cajal, a pacemaker cell in the intestine (Ishikawa *et al.*, 1994). Goblet cell hyperplasia followed by mucin secretion is an important mechanism of *N. brasiliensis* expulsion (Fallon *et al.*, 2006; Ishikawa *et al.*, 1993; McKenzie *et al.*, 1998).

In contrast to *N. brasiliensis* infection, *S. venezuelensis* infection resolves significantly more slowly in W/W^v mice compared to *N. brasiliensis* infection in W/W^v mice and bone marrow transplantation significantly

corrected the protection against *S. venezuelensis* (Khan *et al.*, 1993). Mast cells of the mucosal type are important in this process and chondroitin sulfate secreted by mucosal mast cells is involved in the expulsion of *S. venezuelensis* (Maruyama *et al.*, 2000). Expulsion of *S. venezuelensis* was slow in mice lacking gastrointestinal mast cells but adoptive transfer of bone marrow-derived cultured mast cells (BMMCs) was sufficient to correct the protective immunity (Fukao *et al.*, 2002). It is of note that incubation of BMMCs with Th2 cytokines such as IL-4 and IL-10 was critical for the expulsion of *S. venezuelensis*, underscoring the importance of Th2 cytokines for arming mucosal mast cells (Fukao *et al.*, 2002; Ghildyal *et al.*, 1992). Mucosal mast cells also secrete β-chymase and mouse mast cell protease (mMCP)-1 for increasing the permeability of the epithelial layer by degrading tight junction proteins (McDermott *et al.*, 2003). One of the molecules secreted by goblet cells, resistin-like molecule (RELM)-β, binds to *S. stercoralis* and impairs the chemosensory mechanisms of the worm (Artis *et al.*, 2004).

2.2.4. *Heligmosomoides polygyrus* (Fig. 2.1E)

The inoculation of *H. polygyrus* results in chronic infection. At 4 days after infection, third stage larvae in the duodenum are surrounded by innate immune cells, neutrophils, and macrophages. Treatment of infected mice with antihelminth drugs clears the helminth and such mice can expel the worm upon subsequent secondary infection, indicating the establishment of adaptive immunity and immunological memory. After secondary infection, larvae in the duodenum are again surrounded by granuloma with neutrophils and M2-type or oxidative macrophages induced by Th2 cytokines (Fairweather and Cináková, 2009; Murata *et al.*, 2002). These are distinct from Th1-type granulomas consisting of M1-type or reductive macrophages induced by *M. tuberculosis*. Furthermore, dendritic cells (DCs), Th2 cells, and eosinophils are also observed around the granuloma structure (Morimoto *et al.*, 2004). Although there is no concrete evidence that such Th2-type granulomas directly function in the expulsion of worms, M2-type macrophages may be involved in the expulsion by secreting chitinase and related family members including RELM-α and -β (Anthony *et al.*, 2006).

3. INDUCTION OF TH2 IMMUNE RESPONSES

Although the final effector mechanisms that expel helminths are distinct for each helminth likely due to the different invasion strategy of each helminth, Th2 immunity is key for protective immunity to all helminths.

Naïve CD4 T cells differentiate into several different types of T helper (Th) cells, namely Th1, Th2, Th9, Th17, follicular helper T cell (Tfh), Tr1, and inducible regulatory T cell (iTreg) subsets depending upon the invading

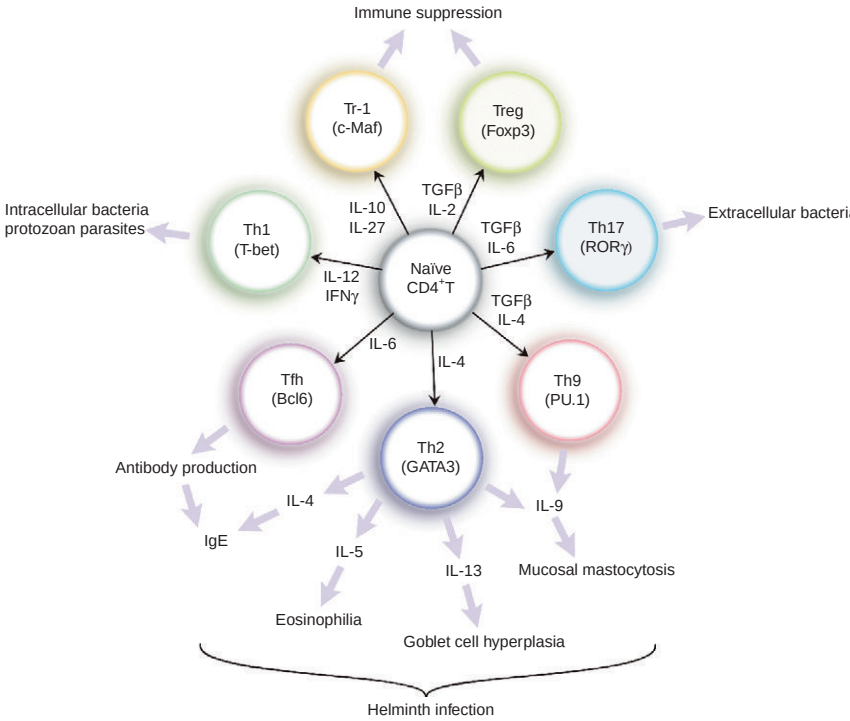

FIGURE 2.2 Differentiation and function of Th cells. Transcription factors characterizing each Th subset are shown in parentheses.

pathogen(s). Innate immune responses against invading pathogens result in the production of various cytokines and distinct cytokine milieus induce different Th cells (Zhu *et al.*, 2010; Fig. 2.2). Each cell type is induced by a specific combination of cytokines and characterized by the expression of a unique transcription factor responsible for the expression of distinct sets of cytokines upon antigen stimulation of Th cells. Among them, Th2 cells producing IL-4, IL-5, IL-9, and IL-13 are induced by IL-4, at least *in vitro*. IL-4 upregulates the expression of GATA-3, a master transcription factor characterizing Th2 cells (Zhang *et al.*, 1997; Zheng and Flavell, 1997).

Activation of adaptive immunity requires the activation of innate immunity (Novak *et al.*, 2010; Steinman and Hemmi, 2006). Invading pathogens are usually sensed by innate immune cells such as macrophages and DCs, which induce innate cytokine production. Upon bacterial and/or protozoan infection, proinflammatory cytokines such as TNFα, IFNγ and IL-1β are induced by these innate cells. DCs that engulf pathogens and are activated by Toll-like receptors (TLRs) mature and migrate to the draining lymph node and activate T cells. A subset of DCs produces IL-12 upon activation and thus induces Th1 cells (Shortman and Heath, 2010).

Because IL-4 was originally thought to be produced by Th2 cells, the initial source of this cytokine was enigmatic. It was later shown that CD1d-restricted natural killer T (NKT) cells (Arase *et al.*, 1993; Yoshimoto and Paul, 1994), mast cells (Plaut *et al.*, 1989) and basophils (Piccinni *et al.*, 1991; Seder *et al.*, 1991) are able to produce IL-4 upon stimulation. Basophils produce IL-4 and accumulate in the liver and lung after *N. brasiliensis* infection (Min *et al.*, 2004). Many allergens are associated with cysteine protease activities and incubation of basophils with one of these cysteine proteases, papain, leads to the production of IL-4 (Sokol *et al.*, 2008). Basophils also produce TSLP. TSLP was originally identified from thymic stromal cells and thought to support growth and differentiation of T and B cells but is now considered to be a Th2-inducing cytokine (Liu, 2006). Intriguingly, basophils are transiently recruited to the draining lymph node where Th2 cells are predominantly induced after *T. muris* infection and present antigens to T cells (Perrigoue *et al.*, 2009; Sokol *et al.*, 2009; Yoshimoto *et al.*, 2009). However, other studies have concluded that the Th2 induction is independent of basophils (Hammad *et al.*, 2010; Ohnmacht *et al.*, 2010; Phythian-Adams *et al.*, 2010).

4. TH2-INDUCING CYTOKINES IN INNATE IMMUNE PHASES

Recent studies also revealed the importance of epithelial cells in the production of cytokines such as TSLP, IL-25, and IL-33 in response to allergens and helminths (Eisenbarth *et al.*, 2002; Zaph *et al.*, 2007). Innate stimuli inducing these cytokines include house dust mites that stimulate lung epithelial cells through TLR4 (Hammad *et al.*, 2009).

4.1. TSLP

TSLP acts to induce DCs capable of differentiating naïve $CD4^+$ T cells to Th2 cells producing IL-4, IL-5, and IL-13 (Soumelis *et al.*, 2002; Ying *et al.*, 2005). Interestingly, DCs activated by TSLP produce IL-8 (CXCL8) attracting neutrophils, eotaxin-2 (CCL24) attracting eosinophils, and TARC (thymus and activation-regulated chemokine: CCL17) and MDC (macrophage-derived chemokine: CCL22) attracting Th2 cells. These same DCs, however, do not produce TNF, IL-1β, IL-6, IL-10, or IL-12. Induction of Th2 cells by TSLP-stimulated DCs depends on OX40L specifically induced by TSLP (Ito *et al.*, 2005). Smooth muscle also produces IL-8 and eotaxin in response to TSLP (Shan *et al.*, 2010).

4.2. IL-25

IL-25 is a member of the IL-17 family (Fort *et al.*, 2001) but, unlike other family members, induces Th2 immune responses (Hurst *et al.*, 2002). Transgenic mice expressing either mouse or human IL-25 produce

increased levels of serum IL-5 and IL-13 and induce eosinophilia (Kim *et al.*, 2002; Pan *et al.*, 2001). IL-25 was originally considered as a Th2 cytokine produced by Th2 cells (Fort *et al.*, 2001) but further studies have shown that this cytokine is also produced by epithelial cells as well such as gut epithelial cells in mice infected with *N. brasiliensis* (Angkasekwinai *et al.*, 2007) and lung epithelial cells infected with *Aspergillus fumigatus* (Hurst *et al.*, 2002). Mast cells activated through FcεRI also produce IL-25 (Ikeda *et al.*, 2003).

The importance of IL-25 in the Th2 immune response was demonstrated using mice deficient for IL-25. Such mice were unable to elicit a Th2 response upon *T. muris* infection and thus unable to control infection (Owyang *et al.*, 2006). Similarly, the expulsion of *N. brasiliensis* was significantly delayed in IL-25 deficient mice (Fallon *et al.*, 2006). It should be noted that administration of recombinant IL-25 leads to the expulsion of *N. brasiliensis* even in Rag1 deficient mice that lack both T and B cells, suggesting a pivotal role for the innate immune response in this process and involvement of IL-25 and Th2 cytokines.

4.3. IL-33

IL-33 is a member of the IL-1 family and binds to a complex formed by T1/ST2 and IL-RAP (Sanada *et al.*, 2007). IL-33 is expressed in a variety of cells including fibroblasts, epithelial cells, adipocytes and endothelial cells (Moussion *et al.*, 2008; Sanada *et al.*, 2007; Wood *et al.*, 2009). Intriguingly, IL-33 is localized in the nucleus (Carrière *et al.*, 2007). Although IL-33 can be cleaved by caspase 1 *in vitro* (Schmitz *et al.*, 2005), it is thought that caspases 3 and 7 cleave IL-33 at the IL-1-like domain and inactivate it during apoptosis (Cayrol and Girard, 2009; Lüthi *et al.*, 2009). In contrast, full-length IL-33 is released from cells upon necrotic death and functions as an alarmin to stimulate a variety of cells (Cayrol and Girard, 2009). Similar alarmins include HMGB-1 (Scaffidi *et al.*, 2002), SAP130 (Yamasaki *et al.*, 2008) and IL-1α (Cohen *et al.*, 2010) that are also present in the nucleus and released upon cellular damage. IL-33R is expressed on various types of cells including Th2 cells (Xu *et al.*, 1998), mast cells (Ali *et al.*, 2007), basophils (Smithgall *et al.*, 2008; Suzukawa *et al.*, 2008), NK cells and NKT cells (Bourgeois *et al.*, 2009; Smithgall *et al.*, 2008). Interestingly, IL-33 induces Th1 cytokine production by NK and NKT cells (Bourgeois *et al.*, 2009; Smithgall *et al.*, 2008).

Administration of TSLP, IL-25, and IL-33 induces Th2 cytokine production and associated physiological changes in mice including IgE production, eosinophilia, and goblet cell hyperplasia, suggesting that these innate cytokines are involved in the induction of Th2 immune responses.

5. CELLS PRODUCING TH2 CYTOKINES IN INNATE IMMUNE RESPONSES

Although TSLP, IL-25, and IL-33 induce Th2 cytokines *in vivo*, the identity of the cell(s) responsible for the production of Th2 cytokines has been obscure. Mast cells and basophils have been reported to respond to those cytokines as described above. A fraction of NKT cells express IL-25R and respond to IL-25 to produce IL-13 (Terashima *et al.*, 2008). In addition, non-T/non-B (NTNB) cells of unknown origin have been reported to produce Th2 cytokines in response to IL-25, IL-33, *T. muris*, and *N. brasiliensis* (Fallon *et al.*, 2006; Fort *et al.*, 2001; Humphreys *et al.*, 2008; Hurst *et al.*, 2002; Voehringer *et al.*, 2006). We have recently identified a previously unrecognized lymphocyte population that we named natural helper (NH) cells. These cells are present in the adipose tissue of the peritoneal cavity and NH cells produce a large amount of Th2 cytokines (Moro *et al.*, 2010).

5.1. Natural Helper Cell

Infection of *N. brasiliensis* in Rag2 deficient mice resulted in the production of IL-5 and IL-13 in the sera of infected mice but such cytokine production was not observed in mice lacking the cytokine receptor common γ chain (γ_c) and Rag2. These observations suggest that γ_c is required for the development or function of NTNB cells producing Th2 cytokines upon helminth infection and prompted us to examine the γ_c-dependent mucosal immune tissues.

We noted a previously unrecognized γ_c-dependent lymphoid structure located along the blood vessels in the mouse mesentery, an adipose tissue in the peritoneal cavity (Fig. 2.3A). The size of each cluster is small in young mice but increases with age and clusters are easily identified in mice over 20 weeks of age. By H&E staining, the majority of cells appear to be lymphocytes and no fibrous capsule is present around the clusters such that, unlike in lymph nodes, lymphocytes are in direct contact with ambient adipocytes (Fig. 2.3A). These clusters were structurally similar to the ''milky spot'' in the omentum, which is considered a gateway of cells between the circulation and the peritoneal cavity (Cranshaw and Leak, 1990). However, unlike the milky spot (Rangel-Moreno *et al.*, 2009), T and B cell zones or germinal center structures are not observed in lymphoid clusters in the mesentery. Similar lymphocyte clusters distinct from lymph node are found in the human mesentery. We named these lymphoid clusters ''fat associated lymphoid clusters'' or FALC. FALC are present in $ROR\gamma^{GFP/GFP}$ and *aly/aly* mice, indicating that FALC are distinct from lymph nodes and the development of FALC is independent of lymphoid tissue inducer (LTi) cells (Nishikawa *et al.*, 2003).

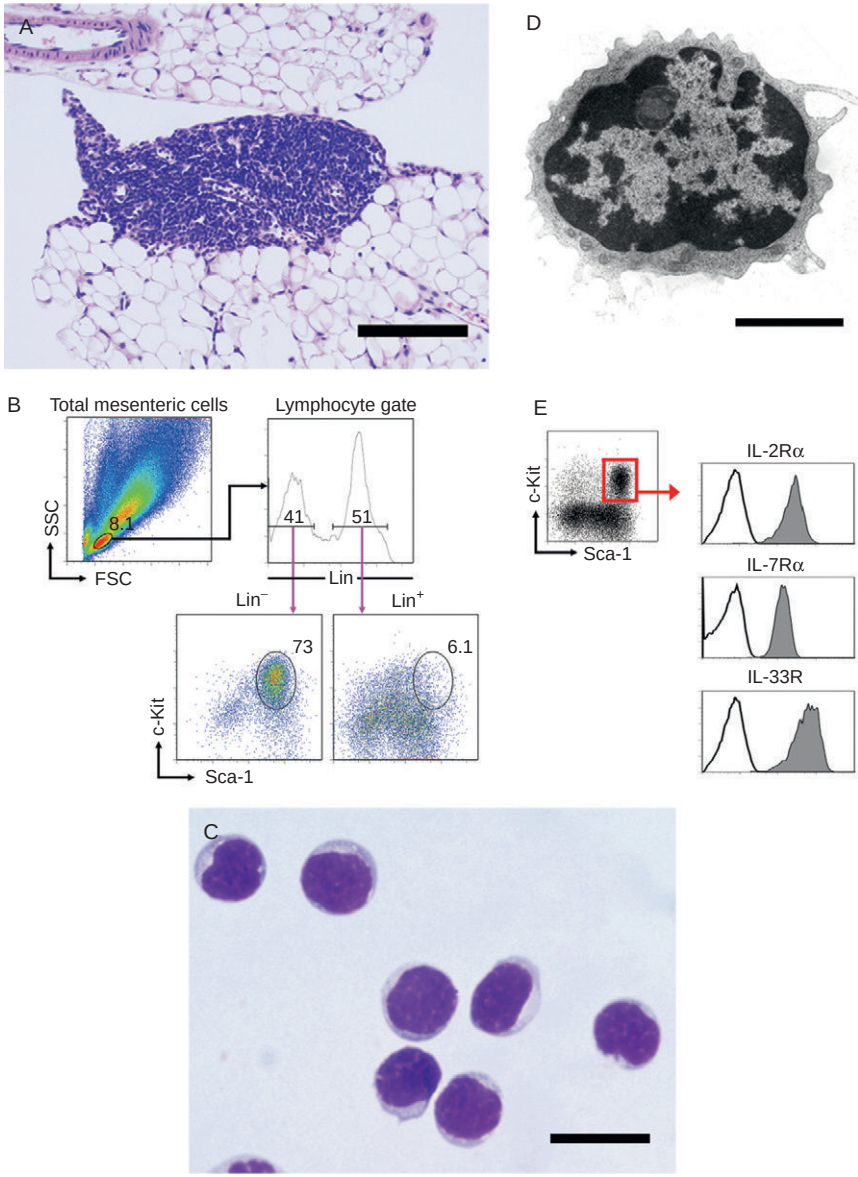

FIGURE 2.3 Fat-associated lymphoid cluster (FALC) and FALC-derived NH cells. (A) H&E stained specimen of a lymphoid cluster in the mesentery. Bar = 200 μm. (B) Flow cytometry of FALC-derived cells. FALC-derived cells were stained with monoclonal antibodies against c-Kit, Sca-1, and Lin markers (CD3, CD4, CD8α, TCRβ, TCRδ, CD5, CD19, B220, NK1.1, TER119, Gr-1, Mac-1, CD11c, FcεRIα). Staining patterns of cells in the lymphoid gate are shown. (C, D) Giemsa staining (C) and electron micrograph (D) of sorted FALC-derived NH cells. Bars = 20 μm (C) and 2 μm (D). These figures are reproduced from Moro *et al.* (2010) by the courtesy of *Nature*.

Flow cytometric analysis of FALC-derived cells shows that nearly 50% of cells in a lymphocyte gate express c-Kit, IL-2R, IL-7R, and IL-33R but lack lineage (Lin) markers (CD3, CD4, CD8α, TCRβ, TCRδ, CD5, CD19, B220, NK1.1, TER119, Gr-1, Mac-1, CD11c, FcεRIα) (Fig. 2.3B). Giemsa staining and electron microscopic analysis of sorted cells demonstrate that these cells are lymphocytes (Fig. 2.3C and D). The fact that these cells express IL-7R and are absent in $\gamma_c^{-/-}$ and IL-$7^{-/-}$ mice also supports the notion that these cells are of lymphoid origin. Among the cytokines tested including Flt3L, SCF, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-15, IL-25, IL-33, M-CSF, GM-CSF, TNFα, and TGF-β1, only SCF and IL-7 supported the survival of FALC-derived Lin^-c-Kit^+IL-$2R^+$IL-$7R^+$IL-$33R^+$ cells and IL-2 induced proliferation of these cells without changing their surface phenotype. Based on the observations that these lymphocytes proliferate in response to IL-2 and exhibit innate-type effector functions by producing Th2 cytokines (see below), we named these cells as NH cells. NH cells are also present in adipose tissues around the kidney and genitalia but very few are found in the subcutaneous fat tissue or the omentum.

NH cells constitutively produce IL-5 and IL-6. IL-5 is a critical growth factor for B1 cells, which are abundant in the peritoneal cavity and play an important role in innate-type immune responses by producing natural antibodies (Erickson *et al.*, 2001; Martin and Kearney, 2000). IL-5 and IL-6 regulate B cell antibody production (Beagley *et al.*, 1989; Sonoda *et al.*, 1989). Indeed, NH cells support the production of IgA from B cells and self-renewal of natural antibody-producing B1 cells (Moro *et al.*, 2010), which are apparently the steady-state functions of NH cells. NH cells produce large amounts of IL-5, IL-6, and IL-13 in response to IL-33 and a combination of IL-2 and IL-25 (Fig. 2.4A). Five thousand NH cells are able to produce μg amounts of IL-5 and IL-13 in response to a combination of IL-2 and IL-33 during a 5-day culture period. The amounts of IL-5 and IL-13 produced by NH cells are much higher than those from mast cells (Ali *et al.*, 2007), basophils (Smithgall *et al.*, 2008; Suzukawa *et al.*, 2008), and polarized Th2 cells (Xu *et al.*, 1998; Fig. 2.4B). It should be noted that NH cells do not respond to IL-25 without IL-2. Whereas basophils are able to produce IL-4, NH cells do not produce IL-4 in response to IL-33 or a combination of IL-2 and IL-25 although stimulation of NH cells with a combination of phorbol myristate acetate and ionomycin induced IL-4 production.

As observed in the *N. brasiliensis* infection, administration of IL-33 to $Rag2^{-/-}$ but not $\gamma_c^{-/-}Rag2^{-/-}$ mice induced the production of IL-5 and IL-13 and goblet cell hyperplasia in the intestine. Adoptive transfer of isolated NH cells into $\gamma_c^{-/-}Rag2^{-/-}$ mice restored the production of IL-5 and IL-13 and goblet cell hyperplasia in response to IL-33 administration and *N. brasiliensis* infection (Moro *et al.*, 2010). We conclude from these results that NH cells play a major role in both the innate production of IL-5 and IL-13 and in goblet cell hyperplasia upon *N. brasiliensis* infection independent of adaptive immunity (Fig. 2.5).

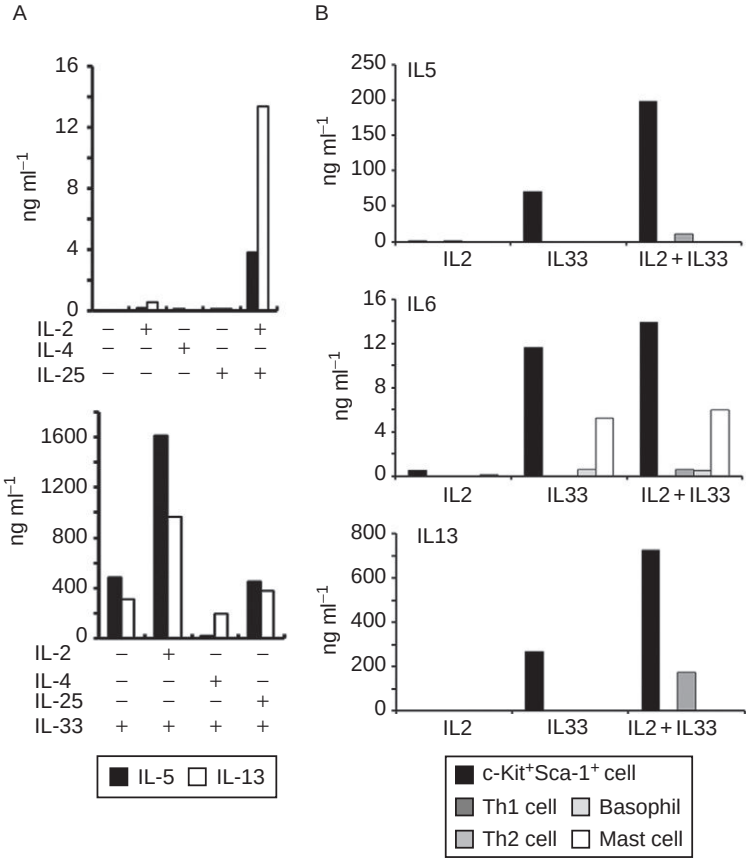

FIGURE 2.4 Th2 cytokine production from NH cells. (A) FALC-derived NH cells (5000 cells/well) were cultured with the indicated cytokines (10 ng/ml) for 5 days and culture supernatants analyzed in triplicate by ELISA. (B) Production of cytokines from various types of cells. The indicated cells (5×10^3) were stimulated with the specified cytokines for 4 days and the concentrations of IL-5, IL-6, and IL-13 in the supernatants were determined in triplicate by ELISA. Although not shown, IFNγ production was not detected in these cultures. These figures are reproduced from Moro *et al.* (2010) by the courtesy of *Nature*.

5.2. Other cells

Since our identification of NH cells, others have published papers demonstrating similar cell types. Using knock-in mice in which GFP was inserted into the IL-13 allele, Neill *et al.* reported that IL-13-producing NTNB cells (GFP^+ cells) were observed in the mesenteric lymph nodes, spleen, and small intestine of mice after administration of IL-25 or IL-33 (Neill *et al.*, 2010). They named these GFP^+ cells "nuocytes," nu being the thirteenth letter of the Greek alphabet although these cells also produce IL-5.

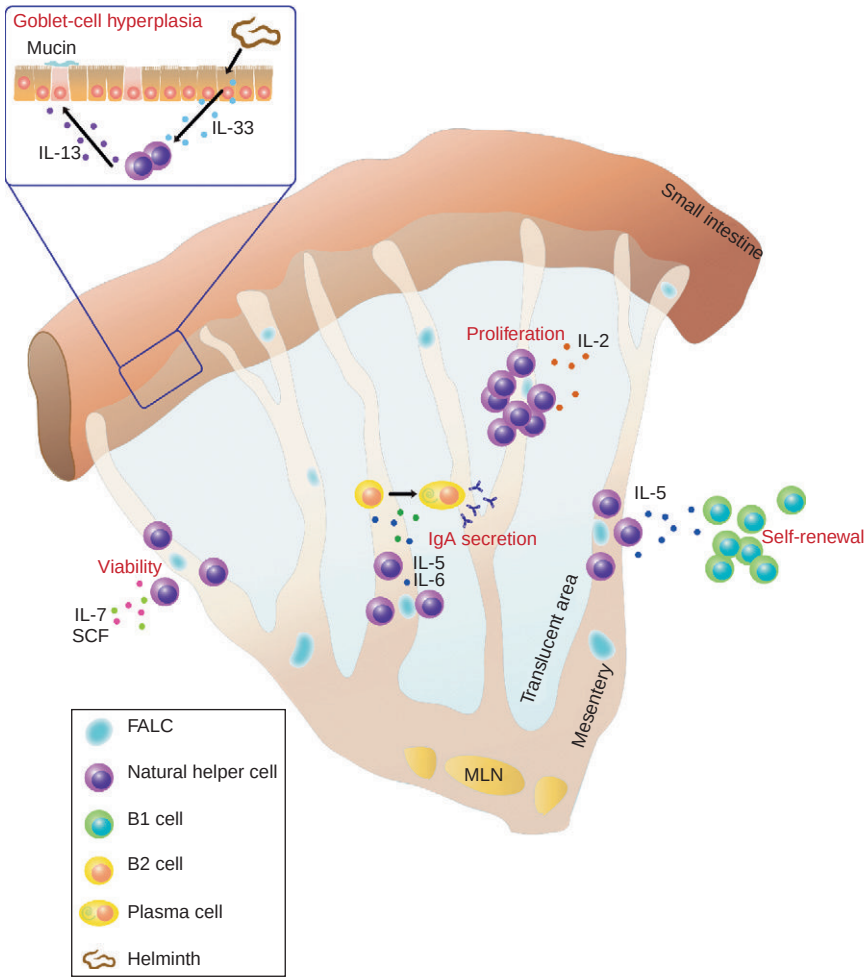

FIGURE 2.5 Schematic diagram for the role of NH cells.

As observed for NTNB cells in an earlier report (Fort *et al.*, 2001), nuocytes express MHC class II and respond to IL-25 alone, which are characteristics distinct from those of NH cells (Table 2.1). Neill *et al.* demonstrated that the adoptive transfer of wild-type nuocytes into IL-17RB deficient mice restored their ability to expel the helminth, *N. brasiliensis*. Two million per milliliter nuocytes are able to produce μg amounts of IL-5, IL-6, and IL-13 upon one-week culture with IL-7 and IL-33 (Neill *et al.*, 2010).

Using knock-in mice in which GFP is inserted into the IL-4 allele, Saenz *et al.* reported that IL-25 administration induced the expansion of IL-4-producing NTNB cells (GFP^+ cells) in gut-associated lymphoid tissues (Saenz *et al.*, 2010). These cells share cell surface markers with

TABLE 2.1 Comparison of NH cell and other innate Th2 cytokine producing cells

Markers	NH cell	Nuocyte	MPP^{type2}	Ih2 cell
c-Kit	+	+/−	+	+/−
CD45	+	+	+	+
IL-7Rα	+	lo	−/lo	?
Sca-1	+	+	+	−
Thy-1	+	+	?	+
CD34	−	−	−/lo	?
CD4	−	−	−	?
CD25	+	?[a]	?	?
CD44	+	+	?	+
CD69	+	?	−	+[b]
CD62L	−	?	−/lo	?
FcεRI	−	−	−	?
T1/ST2	+	+/−	−/lo	?
MHC class II	−	+	?[c]	?

[a] Microarray analysis showed the lack of CD25 expression on Nuocytes.
[b] Microarray analysis showed the expression of CD69 on Ih2 cells.
[c] GFP^{-} cells expressed MHC class II after cultivation with a combination of IL-3 and SCF.

hematopoietic stem cells or multipotent progenitor cells and were named "MPP^{type2}". In fact, this population seems to be heterogeneous and c-Kit^{+}GFP^{+} cells differentiate into mast cells while c-Kit^{+}GFP^{-} cells have the potential to differentiate into basophils and macrophages.

Furthermore, Price *et al.* reported using both IL-4 and IL-13 reporter mice that c-Kit^{+} NTNB cells capable of producing IL-5 and IL-13 are distributed in various tissues (Price *et al.*, 2010). Although the cells are IL-4-GFP^{+}, they do not produce IL-4 protein. The authors named these cells innate helper type 2 (Ih2) cells. It is unknown whether Ih2 cells are able to respond to IL-25 alone to produce cytokines or how much cytokines Ih2 cells produce.

At the moment, the relationship between NH cells, nuocytes, MPP^{type2}, and Ih2 cells is unclear. There are similarities and differences as summarized in Table 2.1. An important difference between NH cells and other cells mentioned above is that NH cells do not respond to IL-25 alone (Moro *et al.*, 2010; Saenz *et al.*, 2010). Future studies will uncover the relationship between these cell types.

6. PERSPECTIVES

Recent studies have shed light on the innate immune responses upon helminth infection. Invasion by helminths results in tissue destruction, leading to the secretion of alarmins including IL-25 and IL-33 from

necrotic cells. The cytokines act on innate immune cells such as NH cells to induce IL-5 and IL-13, which induce eosinophilia and goblet cell hyperplasia, respectively. Eosinophilia is important in controlling helminths in the lung stage of *N. brasiliensis* and *S. venezuelensis* and goblet cell hyperplasia in the intestine is involved in blocking the attachment of helminths during the early intestinal phase. IL-25 and IL-33 induce IL-4 from basophils, mast cells and possibly MPP^{type2} cells that support the induction of Th2 differentiation. Th2-mediated adaptive immune responses will eventually result in the expulsion and clearance of helminths.

These pictures resemble the Th1-type innate immune response before pathogen-specific Th1 cells are induced. Phagocytes, supported by NK cells, limit the growth of intracellular pathogens including protozoan parasites and viruses by providing interferon-γ, a Th1 cytokine (Korbel *et al.*, 2004). IL-22-expressing $NKp46^{+}$ NK cells deal with extracellular mucosal pathogens in the intestine as well as *M. tuberculosis* in the lung until Th1 cells are ready to work (Dhiman *et al.*, 2009; Satoh-Takayama *et al.*, 2009). From this viewpoint, it is of interest that IL-33 induces Th1 cytokines from NK cells and NKT cells (Bourgeois *et al.*, 2009; Smithgall *et al.*, 2008), implying that IL-33 induces a Th1-type innate immune response under certain circumstances.

IL-5 is a critical factor in the induction of eosinophilia and IL-13 leads to goblet cell hyperplasia, both of which are involved in the pathogenesis of allergic diseases such as asthma and allergic diarrhea. It is therefore of interest and importance to elucidate the functions of innate cells producing Th2 cytokines in various allergic diseases in future studies.

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CHAPTER 3

Mapping of Switch Recombination Junctions, a Tool for Studying DNA Repair Pathways during Immunoglobulin Class Switching

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Abstract

Class switch recombination (CSR) is induced upon B cell activation and occurs within special DNA regions, termed switch (S) regions, which consist of tandem repeats of G-rich sequences. CSR occurs by introduction of double-strand breaks (DSBs) into each S region, and recombination by nonhomologous end-joining (NHEJ). The recombination event occurs during the G1 phase of the cell cycle in cells that are rapidly dividing. By examination of patients and mouse knock-out strains lacking various DNA-damage response factors and enzymes involved in DNA repair, much has been learned about which factors are important for CSR, how DSBs are introduced into S regions, and how the donor and acceptor S regions are then recombined. One of the approaches for analyzing the steps involved in CSR is to determine the nucleotide sequence of S–S junctions. Many of the DNA repair deficiencies alter the sequence of the recombination junctions, generally increasing the use of microhomologies, interpreted as a shift from classical (C)-NHEJ to alternative end-joining (A-EJ). However, it is clear that

A-EJ, is not simply one pathway; rather, recombination is likely to occur using various subsets of end-joining factors, which will vary depending on the structure of the DSBs provided by the initial phases of CSR. Herein we review the results of analyses of S–S junctions, suggest minimal information required for these analyses, and attempt to integrate these results in order to increase our understanding of the complex process of CSR.

1. INTRODUCTION TO CLASS SWITCH RECOMBINATION

1.1. Antibody class switching occurs by a unique type of recombination

The antibody class, or isotype, is defined by the heavy chain constant (C_H) region, and this determines the antibody's effector functions. When B cells are activated during an immune response, they change isotypes, that is, they switch from expressing IgM and IgD to expressing IgG, IgE, or IgA, improving the ability of the antibody to remove the particular pathogen that induced the response. Immunoglobulin (Ig) isotype switching occurs by intrachromosomal deletional recombination between switch (S) regions located upstream of each of the C_H regions except Cδ. The human IgH locus in cells expressing IgM and/or IgD is diagrammed in Fig. 3.1A. The mouse IgH locus is diagrammed in Fig. 3.1B, along with CSR to IgA. S regions consist of tandem repeats of short G-rich sequences (20–80 bp), which differ for each isotype and with an overall length varying from ~1 to 12 kb. CSR can occur anywhere within or near the S regions (Dunnick *et al.*, 1993; Min *et al.*, 2005; Stavnezer, 1996). CSR occurs by an end-joining type of recombination, rather than by homologous recombination (Pan-Hammarstrom *et al.*, 2007; Rooney *et al.*, 2004; Stavnezer *et al.*, 2008). Enzymes and proteins involved in nonhomologous end-joining (NHEJ), and also in two other DNA repair pathways, base excision repair (BER) and mismatch repair (MMR), are involved in CSR. In this review, we will consider the effects of deletions or mutations of enzymes and proteins involved in CSR, focusing on their effects on the S–S junctions, and how this might inform us about the mechanism of CSR and the roles of these proteins.

1.2. Induction of CSR

B cells undergo antibody or Ig class switching *in vivo* after immunization or infection, or in culture upon appropriate activation. Engagement of the CD40 receptor on mouse splenic B cells and on human peripheral blood B cells by CD154 (CD40L) or with an antibody to CD40, and cytokines, for example, IL-4, IL-10, or IL-21, or, specifically for mouse B cells through the

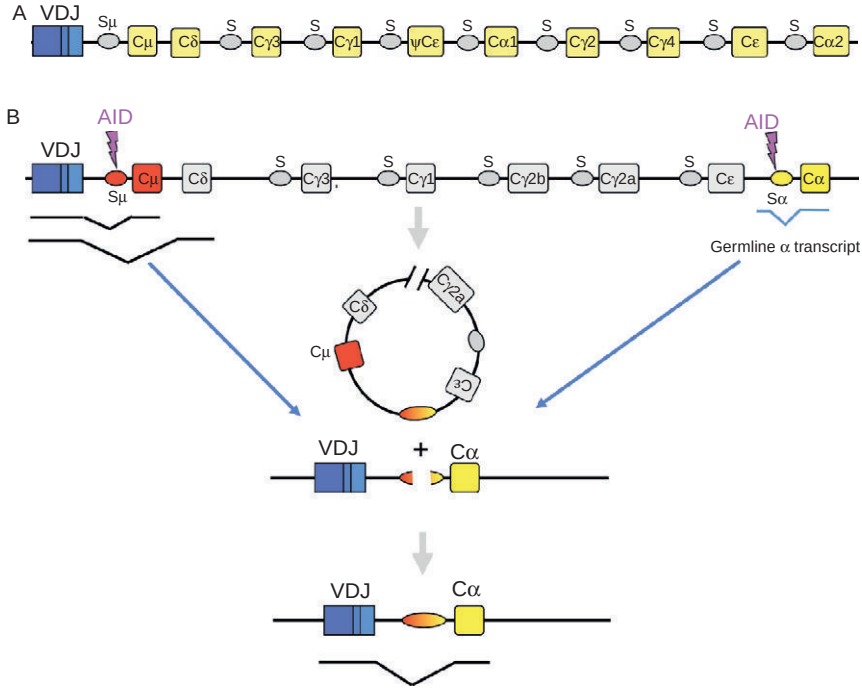


FIGURE 3.1 Diagram of Ig class switch recombination (CSR) to IgA. (A) Schematic diagram of human IgH locus: expressed VDH-Cμ and all other C_H genes in cells expressing IgM and IgD. (B) Schematic diagram of mouse IgH locus and outline of CSR. *Top*, the mouse Ig H locus in B cells expressing IgM and IgD (by alternative RNA transcription/processing). During CSR, activation-induced cytidine deaminase (AID) deaminates dC residues in the top and bottom strands of transcriptionally active S regions (Sμ and Sα in the diagram shown), initiating a process described in the text that results in DSBs in both S regions, and leading to CSR by intrachromosomal deletion (*middle*). *Bottom*, the IgH locus after CSR to IgA. Splicing diagrams for the μ, δ, and α mRNAs and for the α germline transcript are indicated below the diagrams of the locus. Similar germline transcripts are induced from unrearranged Cγ, Cε, and Cα genes, depending on the cytokine stimulation received by the B cell.

toll like receptor 4 (TLR4) by lipopolysaccharide (LPS) induces CSR (Banchereau *et al.*, 1994; Borte *et al.*, 2009; Severinson-Gronowicz *et al.*, 1979; Stavnezer *et al.*, 2008). Signals from these ligands and cytokines induce activation-induced cytidine deaminase (AID), the enzyme that initiates CSR by instigating DNA breaks in S regions (Muramatsu *et al.*, 1999, 2000; Catalan *et al.*, 2003; Schrader *et al.*, 2005). These signals induce CSR in both cultured B cells and *in vivo*. CSR requires cell proliferation, appearing to require a minimum of two complete rounds of cell division for IgG and IgA CSR in mouse B cells, and perhaps additional rounds for

IgE CSR (Deenick *et al.*, 1999; Hasbold *et al.*, 1998; Hodgkin *et al.*, 1996; Rush *et al.*, 2005). This has been hypothesized to be due to the requirement of proliferation for induction of AID expression, although the mechanism of this effect is unknown (Rush *et al.*, 2005).

Naïve B cells have the potential to switch to any isotype and cytokines secreted by T cells and antigen-presenting cells direct the isotype switch (reviewed in Cogne and Birshtein, 2004; Stavnezer, 1996). The predominant mechanism for regulating isotype specificity is by regulation of transcription through S regions, and only transcriptionally active S regions undergo CSR.

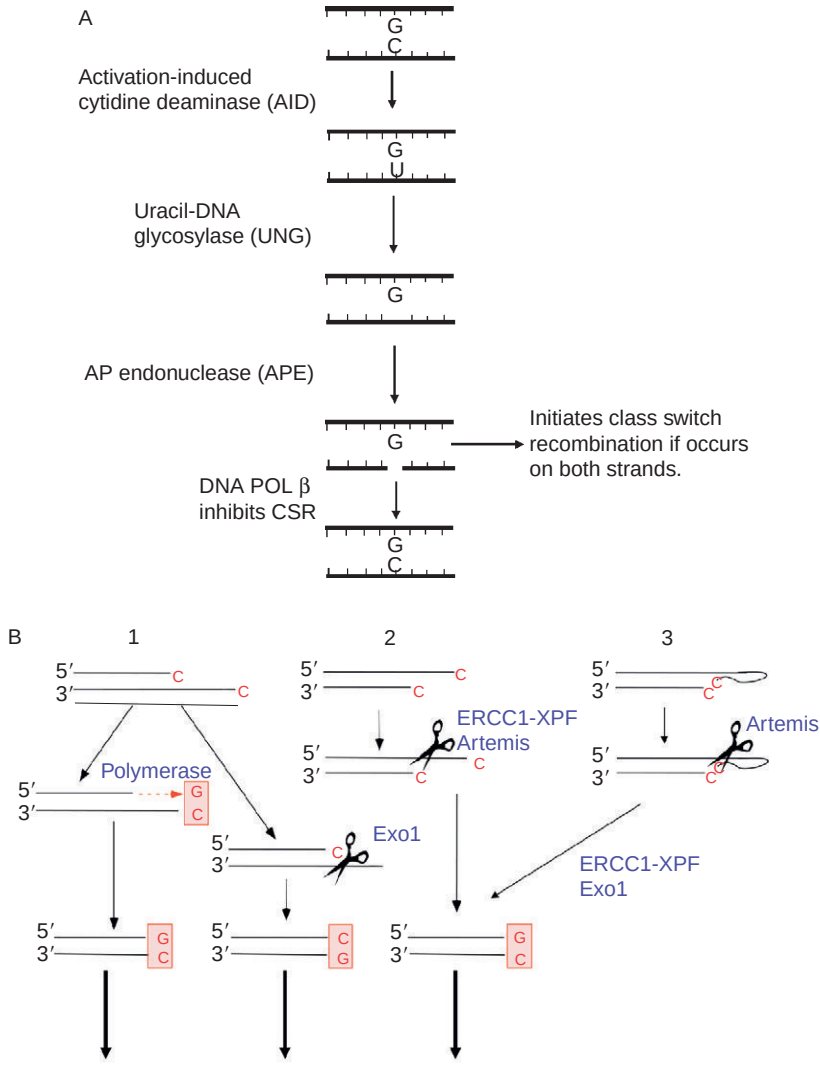
Most investigations into the roles of various proteins in CSR examine their effects in mouse splenic B cells induced to switch in culture, although studies using *ex vivo* peripheral blood B cells from humans with primary immunodeficiencies have also yielded important insights into the roles of various DNA repair proteins in CSR. Studying CSR in isolated B cells ensures that the effects of the genes are B cell intrinsic, and not due to effects on other cell types.

1.3. AID and BER enzymes initiate CSR by creating S region breaks

CSR and somatic hypermutation (SHM) are initiated by AID, which deaminates cytosines in S regions and Ig variable (V) regions, converting them to uracils (Chaudhuri *et al.*, 2003; Dickerson *et al.*, 2003; Muramatsu *et al.*, 2000; Petersen-Mahrt *et al.*, 2002; Pham *et al.*, 2003; Revy *et al.*, 2000). Mice and humans that lack AID do not undergo CSR or SHM, and do not have S region DNA breaks, as demonstrated by ligation-mediated (LM)-PCR (Catalan *et al.*, 2003; Rush *et al.*, 2004; Schrader *et al.*, 2005). The substrate for AID is single-strand DNA (ssDNA), which is generated by transcription of the Ig S regions and V regions, and dCs on both the transcribed and nontranscribed strands are deaminated by AID (Peled *et al.*, 2008).

The dU base resulting from AID activity is subsequently excised by the ubiquitous BER enzyme, uracil-DNA glycosylase (UNG), leaving an abasic site (Fig. 3.2A). B cells lacking UNG have greatly reduced CSR and S region double-strand DNA breaks (DSBs) (Imai *et al.*, 2003; Rada *et al.*, 2002; Schrader *et al.*, 2005).

The next step in the repair of these lesions is cleavage by apurinic/apyrimidic endonucleases (AP endonucleases), most commonly in mammalian cells by the enzyme APE1/Apex1, a BER enzyme which incises the phosphate backbone of DNA at abasic sites, producing single-strand DNA breaks (SSBs) (Christmann *et al.*, 2003). APE1 is essential for early embryonic development and for viability of human cell lines (Fung and Demple, 2005; Xanthoudakis *et al.*, 1996) and thus, APE1-knock-out mice



These processing pathways lead to blunt DSBs (diagrammed) but could also result in short ss tails.

cannot be generated. In mammals, there is a homologous enzyme, APE2, which is important for lymphoid cell development (Hadi *et al.*, 2002). One group tested IgG1 CSR in *ape2*$^{-/-}$ total spleen cells and found no impairment in switching (Sabouri *et al.*, 2009), whereas another group,

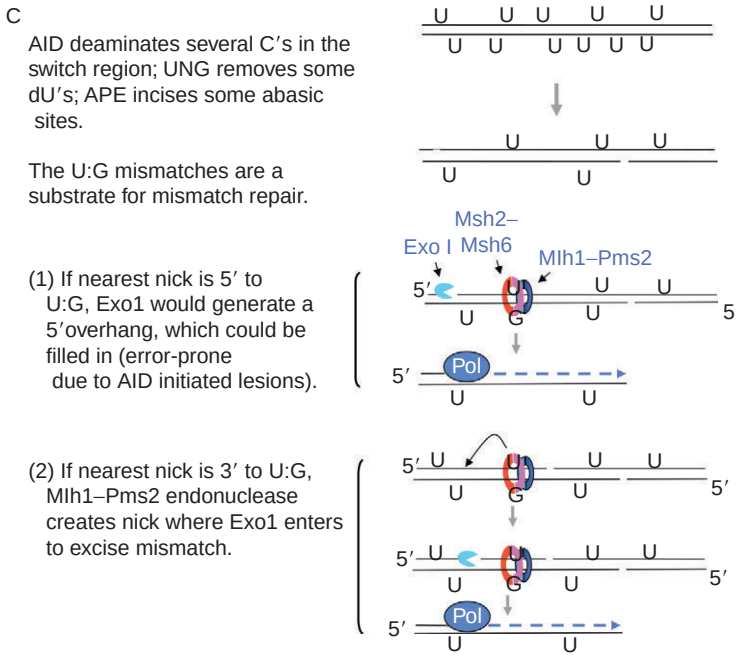


FIGURE 3.2 Diagram of models for generation of DSBs in Ig S regions. (A) AID deaminates dC, resulting in dU bases, which are excised by uracil-DNA glycosylase (UNG). Abasic sites are incised by AP-endonuclease (APE1 and perhaps also APE2), creating ss breaks (SSBs) that can spontaneously form staggered DSBs if they are near each other on opposite DNA strands (Guikema *et al.*, 2007). DNA Pol β reinserts dC at these lesions, competing with this pathway and thereby inhibiting CSR, but the large number of AID-induced lesions appear to overwhelm Pol β (Wu and Stavnezer, 2007). (B) The staggered DSBs can be converted to blunt or nearly blunt DSBs ending at G:C base pair by DNA polymerase, exonuclease 1 (Exo1), Ercc1-XPF, and/or by Artemis. These enzymes have roles in CSR, although they have not been directly demonstrated to perform the roles illustrated here. (1) 5′ overhangs can be filled in by DNA Pol, replicative or error prone. (2) 3′ overhangs can be excised by Exo1, or by the structure-specific endonucleases ERCC1-XPF and Artemis. (3) Complex ss tails, for example, hairpins due to folding back, could be excised by Artemis, perhaps followed by ERCC1-XPF or Exo1 to create a blunt DSB. (C) If the SSBs created by AID–UNG–APE are not near each other on opposite DNA strands, it is likely that they cannot form a DSB without the help of MMR. AID is hypothesized to introduce several dU residues in S regions during one cell cycle. Some of the dU residues could be excised by UNG, and some of the abasic sites nicked by APE. The U:G mismatches that remain would be substrates for Msh2–Msh6, which along with Mlh1–Pms2, recruits Exo1 (and accessory proteins) to a nearby 5′ nick, from where Exo1 begins to excise toward the mismatch, creating a DSB with a 5′ ss overhang, which can be filled in by DNA polymerase (shown in 1). Fill-in synthesis is probably performed by both replicative and translesion polymerases (possibly η and ζ), due to the presence of abasic sites which cannot be extended by replicative polymerases. Alternatively, the 5′ ss tail might be removed by Exo1. If the nearest SSB is located 3′ to the U:G mismatch (shown in 2), the nicking activity of Pms2 would create nicks surrounding the mismatch, including 5′ to the U:G. Exo1 could then excise toward the mismatch, as diagrammed.

examining splenic B cells from the same mouse knock-out line, found a 20–50% reduction in CSR to several isotypes (Guikema *et al.*, 2007). Mouse B cells haplo-insufficient for APE1 and deficient for APE2 still show only 20–50% reduced CSR, but show greatly reduced number of S region DSBs, almost to the level of DSBs in $aid^{-/-}$ cells, much more reduced than in either $ape1^{+/-}$ or $ape2^{-/-}$ cells. These results suggest that these two enzymes partially compensate for each other. These data also indicate that a few S region DSBs are sufficient to induce substantial amounts of CSR (Guikema *et al.*, 2007). Two other AP endonucleases were recently discovered, PALF/APLF/XIP-1 and ABH1, but no evidence for their involvement in CSR has been reported (Bekker-Jensen *et al.*, 2007; Iles *et al.*, 2007; Kanno *et al.*, 2007; Muller *et al.*, 2010). Taken together, it appears likely that APE1 and APE2 incise abasic sites in S regions, generating SSBs on both DNA strands, which, if sufficiently near, lead to DSB formation. Finally, during the canonical BER pathway, the single nucleotide (nt) gap generated by the action of UNG and APE is filled in by DNA polymerase (Polβ) (Fig. 3.2A) (Barnes and Lindahl, 2004; Beard and Wilson, 2006). Consistent with this, Pol β activity was shown to inhibit CSR, and Pol β was found to be associated with S regions in switching splenic B cells (Wu and Stavnezer, 2007). Splenic B cells lacking AID, UNG, or APE2, or haplo-insufficient for APE1 proliferate as well as wild-type (WT) cells, indicating that their effect on CSR is not due to reduced proliferation.

1.4. Characteristics of S region DSBs

In WT cells, LM-PCR experiments show that AID-dependent blunt and staggered DSBs in the Sμ region occur preferentially at G:C base pairs (bp) and at AID WRC target hotspots (Guikema *et al.*, 2007; Schrader *et al.*, 2005). This indicates that SSBs and DSBs occur at the dC nucleotides that are targeted by AID, as predicted by the DNA-deamination model (Fig. 3.2A and B). These results fit with the model that AID-dependent SSBs, occurring near each other on opposite DNA strands, form staggered DSBs, which are then filled in or chewed back to form blunt or nearly blunt DSBs. These DSBs form the substrate for the S–S recombination junctions required for CSR, and which are the focus of this review.

1.5. Recombination of donor (Sμ) and acceptor Sx regions occurs by an end-joining type of recombination

In all cells, DNA DSBs can be induced in response to DNA damaging agents, by ionizing radiation, and during replication. Ubiquitous proteins rapidly repair these DSBs, and many of these proteins are involved in CSR. DSBs produced during DNA replication or during the G2 phase of

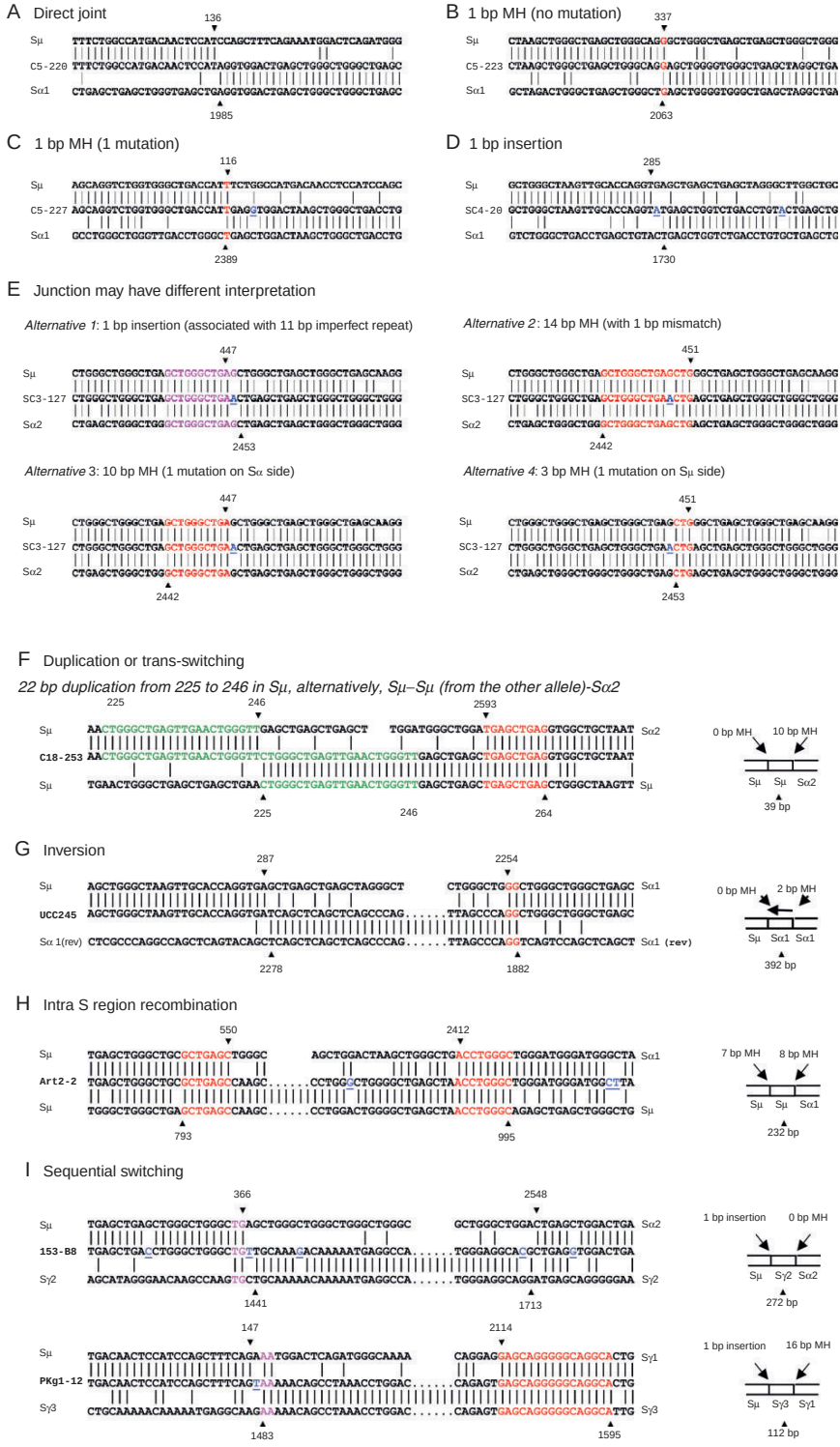
the cell cycle are generally repaired by homologous recombination, as there is a chromosomal homologue that can be copied. However, S region DSBs induced during CSR are generated and resolved during the G1 phase (Petersen *et al.*, 2001; Schrader *et al.*, 2007), and S regions lack sufficient homology to allow CSR by homologous recombination. Consistent with this, S–S recombination occurs by an end-joining type of recombination, apparently usually by NHEJ although alternative end-joining (A-EJ) pathways, involving the use of short sequence identities between donor and acceptor S regions, are also used (Fig. 3.3) (Boboila *et al.*, 2010b; Kotnis *et al.*, 2009). NHEJ can recombine a large variety of different types of DNA DSBs, blunt or with ss tails, or with hairpin ends, due to its great flexibility (Lieber, 2010b). One consequence of this flexibility is substantial diversity of junctional outcomes, even from identical starting ends. However, the junctional outcome is also influenced by the structure of the recombining DSBs.

2. CHARACTERISTICS OF S–S RECOMBINATION JUNCTIONS

2.1. Mapping the CSR junctions

Analysis of DNA sequences at or around the recombination sites has been a useful tool for studying the mechanism of CSR. A single run of PCR, or more often, a nested-PCR approach, is applied to amplify fragments of recombined S regions. The primers are often located in the 5′- or 3′-flanking regions of the repetitive donor (Sμ) or acceptor S sequences, to avoid nonspecific priming (Ehrenstein and Neuberger, 1999; Pan *et al.*, 1997, 2001). The PCR products are subsequently cloned into a vector and sequenced; alternatively, distinct bands can be gel purified and subjected to direct sequencing (Sekine *et al.*, 2007).

The Sμ–Sx breakpoints, or recombination junctions, are determined using a BLAST algorithm, and/or aligned to the corresponding germline S sequences. As S regions show extensive polymorphism (Pan-Hammarstrom *et al.*, 2007), it is important to choose the correct germline sequences for comparison. For example, the mouse Sα region from the C57BL/6J strain is much shorter than those from the 129 or BALB/c strains, although the sequences flanking the Sα regions share a very high degree of homology. In humans, the best-studied S region is Sγ4, where at least five Sγ4 alleles have been characterized that all differ in length due to deletions or insertions of varying number of 79 bp repeat units (Pan *et al.*, 1998). Using the correct germline sequences for comparison is thus not only important for mapping the actual recombination sites, but also for an appropriate identification of mutations and insertions, as described below.


A Direct joint
136
Sμ TTTCTGGCCATGACAACTCCATCAGCTTTCAGAAATGGACTCAGATGGG
C5-220 TTTCTGGCCATGACAACTCCATAGGTGGACTGAGCTGGGCTGGGCTGAGC
Sα1 CTGAGCTGAGCTGGGTGAGCTGAGGTGGACTGAGCTGGGCTGGGCTGAGC
1985
B 1 bp MH (no mutation)
337
Sμ CTAAGCTGGGCTGAGCTGGGCAGGGCTGGGCTGAGCTGAGCTGGGCTGGG
C5-223 CTAAGCTGGGCTGAGCTGGGCAGGAGCTGGGCTGAGCTAGGCTGA
Sα1 GCTAGACTGGGCTGAGCTGGGCTGAGCTGGGGTGGGCTGAGCTAGGCTGA
2063
C 1 bp MH (1 mutation)
116
Sμ AGCAGGTCTGGTGGGCTGACCATTCTGGCCATGACAACCTCCATCCAGC
C5-227 AGCAGGTCTGGTGGGCTGACCATTGAGGTGGACTAAGCTGGGCTGACCTG
Sα1 GCCTGGGCTGGGTTGACCTGGGCTGAGCTGGACTAAGCTGGGCTGACCTG
2389
D 1 bp insertion
285
Sμ GCTGGGCTAAGTTGCACCAGGTGAGCTGAGCTGAGCTAGGGCTTGGCTGC
SC4-20 GCTGGGCTAAGTTGCACCAGGTATGAGCTGGTCTGACCTGTACTGAGCTG
Sα1 GTCTGGGCTGACCTGAGCTGTACTGAGCTGGTCTGACCTGTGCTGAGCTG
1730
E Junction may have different interpretation
Alternative 1: 1 bp insertion (associated with 11 bp imperfect repeat)
447
Sμ CTGGGCTGGGCTGAGCTGGGCTGAGCTGGGCTGAGCTGGGCTGAGCAAGG
SC3-127 CTGGGCTGGGCTGAGCTGGGCTGAAACTGAGCTGAGCTGGGCTGGGCTGGG
Sα2 CTGAGCTGGGCTGGGCTGGGCTGAGCTGAGCTGAGCTGGGCTGGGCTGGG
2453
Alternative 2: 14 bp MH (with 1 bp mismatch)
451
Sμ CTGGGCTGGGCTGAGCTGGGCTGAGCTGGGCTGAGCTGGGCTGAGCAAGG
SC3-127 CTGGGCTGGGCTGAGCTGGGCTGAACTGAGCTGAGCTGGGCTGGGCTGGG
Sα2 CTGAGCTGGGCTGGGCTGGGCTGAGCTGAGCTGAGCTGGGCTGGGCTGGG
2442
Alternative 3: 10 bp MH (1 mutation on Sα side)
447
Sμ CTGGGCTGGGCTGAGCTGGGCTGAGCTGGGCTGAGCTGGGCTGAGCAAGG
SC3-127 CTGGGCTGGGCTGAGCTGGGCTGAACTGAGCTGAGCTGGGCTGGGCTGGG
Sα2 CTGAGCTGGGCTGGGCTGGGCTGAGCTGAGCTGAGCTGGGCTGGGCTGGG
2442
Alternative 4: 3 bp MH (1 mutation on Sμ side)
451
Sμ CTGGGCTGGGCTGAGCTGGGCTGAGCTGGGCTGAGCTGGGCTGAGCAAGG
SC3-127 CTGGGCTGGGCTGAGCTGGGCTGAACTGAGCTGAGCTGGGCTGGGCTGGG
Sα2 CTGAGCTGGGCTGGGCTGGGCTGAGCTGAGCTGAGCTGGGCTGGGCTGGG
2453
F Duplication or trans-switching
22 bp duplication from 225 to 246 in Sμ, alternatively, Sμ–Sμ (from the other allele)-Sα2
225
246
2593
Sμ AACTGGGCTGAGTTGAACTGGGTTGAGCTGAGCTGAGCT TGGATGGGCTGGATGAGCTGAGGTGGCTGCTAAT Sα2
C18-253 AACTGGGCTGAGTTGAACTGGGTTCTGGGCTGAGTTGAACTGGGTTGAGCTGAGCTGAGCTGAGGTGGCTGCTAAT
Sμ TGAACTGGGCTGAGCTGAGCTGAACTGGGCTGAGTTGAACTGGGTTGAGCTGAGCTGAGCTGGGCTAAGTT Sμ
225
246
264
0 bp MH
10 bp MH
Sμ Sμ Sα2
39 bp
G Inversion
287
2254
Sμ AGCTGGGCTAAGTTGCACCAGGTGAGCTGAGCTGAGCTAGGGCT CTGGGCTGGGCTGGGCTGAGC Sα1
UCC245 AGCTGGGCTAAGTTGCACCAGGTGATCAGCTCAGCTCAGCCCAG.....TTAGCCCAGGCTGGGCTGGGCTGAGC
Sα 1(rev) CTCGCCCAGGCCAGCTCAGTACAGCTCAGCTCAGCTCAGCCCAG.....TTAGCCCAGGTCAGTCCAGCTCAGCT Sα1 (rev)
2278
1882
0 bp MH
2 bp MH
Sμ Sα1 Sα1
392 bp
H Intra S region recombination
550
2412
Sμ TGAGCTGGGCTGCGCTGAGCTGGGC AGCTGGACTAAGCTGGGCTGACCTGGGCTGGGATGGGATGGGCTA Sα1
Art2-2 TGAGCTGGGCTGCGCTGAGCCAAGC.....CCTGGCCTGGGGCTGAGCTAACCTGGGCTGGGATGGATGGCTTA
Sμ TGGGCTGGGCTGAGCTGAGCCAAGC.....CCTGGACTGGGGCTGAGCTAACCTGGGCAGAGCTGAGCTGGGCTG Sμ
793
995
7 bp MH
8 bp MH
Sμ Sμ Sα1
232 bp
I Sequential switching
366
2548
Sμ TGAGCTGAGCTGGGCTGGGCTGAGCTGGGCTGGGCTGGGC GCTGGGCTGGACTGAGCTGGACTGA Sα2
153-B8 TGAGCTGACCTGGGCTGGGCTGTTGCAAAGACAAAAATGAGGCCA.....TGGGAGGCACGCTGAGGTGGACTGA
Sγ2 AGCATAGGGAACAAGCCAAGTGCTGCAAAAACAAAAATGAGGCCA.....TGGGAGGCAGGATGAGCAGGGGGAA Sγ2
1441
1713
1 bp insertion
0 bp MH
Sμ Sγ2 Sα2
272 bp
147
2114
Sμ TGACAACTCCATCCAGCTTTCAGAAATGGACTCAGATGGGCAAAA CAGGAGGAGCCAGGGGGCAGGCACTG Sγ1
PKg1-12 TGACAACTCCATCCAGCTTTCAGTAAAAACAGCCTAAACCTGGAC.....CAGAGTGAGCCAGGGGGCAGGCACTG
Sγ3 CTGCAAAAACAAAAATGAGGCAAGAAAACAGCCTAAACCTGGAC.....CAGAGTGAGCCAGGGGGCAGGCATTG Sγ3
1483
1595
1 bp insertion
16 bp MH
Sμ Sγ3 Sγ1
112 bp

2.2. S–S junctions in WT cells show limited sequence homology

Twenty to fifty percent of Sμ–Sγ, Sμ–Sε, and Sμ–Sα junctions show 0 bp of junctional homology in both normal individuals and in various WT mouse strains (Boboila *et al.*, 2010b; Du *et al.*, 2008b; Eccleston *et al.*, 2009; Li *et al.*, 2004b; Martin *et al.*, 2003; Peron *et al.*, 2008). In the remaining junctions, the precise location of the breakpoints cannot be mapped as the germline Sμ and Sx sequences are identical at the junction, typically for a few base pairs. These short sequence homologies are termed microhomologies. S–S junctions in WT cells with 1 or 2 bp of microhomology are common, but junctions with $\geq$5 bp (Sμ–Sγ) or $\geq$10 bp (Sμ–Sα) are quite rare (Ehrenstein *et al.*, 2001; Pan *et al.*, 2002; Schrader *et al.*, 2002). Sμ–Sα junctions tend to have greater microhomology than Sμ–Sγ junctions, most likely due to the greater homology between Sμ and Sα than between Sμ and Sγ regions (Pan-Hammarstrom *et al.*, 2007). As S regions are G-rich and have small regions of identity between them, 1 or 2 bp identity

FIGURE 3.3 Characterization of CSR junctions. The donor and acceptor S region germline sequences are aligned above or below the recombined switch junctional sequences. Microhomology (MH, highlighted in red) was determined by identifying the longest region at the S junction of perfect, uninterrupted donor/acceptor sequences, except as indicated. Imperfect repeats were determined by identifying the longest overlap region at the S junction by allowing one mismatch on either side of the breakpoint (highlighted in pink). Mutations or insertions are underlined (also highlighted in blue). The donor and acceptor S region breakpoints are indicated by arrowheads, and their positions in the human germline sequences (Sμ, X54713; Sα1, L19121; Sα2, AF030305; Sγ1, U39737.1; Sγ2, U39934.1; Sγ3, U39935.1) are indicated above or below the arrowhead. Sequences omitted are shown as dots. Unless specified, the S–S junction sequences shown in the figure are derived from controls. (A) Direct joint. No MH and no insertions are observed at the junction. (B) Sμ–Sα junction with 1 bp MH. (C) Sμ–Sα junction with 1 bp MH. One C > G mutation is identified on the Sα1 side. (D) Sμ–Sα junction with 1 bp insertion. (E) An Sμ–Sα junction that could be interpreted differently. Four alternatives are shown in the figure. (F) Sμ–Sα junction with a 22 bp duplicated Sμ sequence (highlighted in green). Alternatively, this could be explained by a recombination of the Sμ regions from both alleles, which can be referred to as transs-witching. (G) Inversion at the CSR junction. A stretch of 392 bp of the Sα1 sequence (in reverse orientation) is identified at the Sμ–Sα1 junction. (H) Intra-Sμ recombination shown in a switch fragment derived from an Artemis-deficient patient (Du *et al.*, 2008b). The second stretch of Sμ sequences is 232 bp long. (I) Sequential switching, illustrated by one Sμ–Sγ2–Sα2 junction derived from an Artemis-deficient patient (Q. P.-H. *et al.*, unpublished) and one Sμ–Sγ3–Sγ1 junction derived from a DNA-PKcs-deficient patient (van der Burg *et al.*, 2009). The interposed Sγ2 or Sγ3 sequence is 272 or 112 bp long, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this chapter.)

between the donor and acceptor S regions can occur by chance, as calculated by Dunnick *et al*. (1993). These data suggest that junctional homology is not favored at S–S junctions in WT cells, and therefore support data indicating that CSR occurs mostly by NHEJ (also called C-NHEJ).

Although CSR is reduced in many different mutants with DNA repair deficiencies, S–S junctions can usually still be amplified by PCR and subsequently sequenced. These junctions are found to differ in several respects from junctions amplified from WT cells. Table 3.1 reviews the characteristics of the junctions for mutants in mouse, and Table 3.2 reviews the results for human mutations. A common finding is increased lengths of microhomologies at the S–S junctions. The finding of reduced junctional microhomology relative to WT cells is rare.

In some studies, microhomology is defined as perfectly matched successive nucleotides that are shared by both the Sμ and Sx regions at the S junctions, and distinguished from imperfect repeats, in which mismatches are allowed on either side of the junction (Pan *et al*., 2002; Yan *et al*., 2007). In other studies, one or more mismatches are tolerated when defining the microhomology (Rivera-Munoz *et al*., 2009; Schrader *et al*., 2002), although the mismatch is sometimes not counted as part of the homology (Schrader *et al*., 2002). In Tables 3.1 and 3.2, we have listed the average microhomology for each study (based on the original interpretation of the authors), and studies allowing mismatches are specified in the footnotes.

2.3. Insertions and mutations frequently occur at S–S junctions

Mutations and short insertions (apparent insertion of nucleotides of unknown origin) at, or near, the S–S junctions are often observed in WT cells, and also in cells with DNA repair deficiencies (Fig. 3.3). This could reflect the error-prone characteristic of the NHEJ process, when certain types of DNA ends are involved. For instance, the repair of partially complementary DNA ends is often imprecise, and mutations and insertions are frequently introduced during the alignment and gap filling steps (Lieber, 2010b; Lieber *et al*., 2008). Nucleotide changes right at the junction are difficult to define, particularly when these are associated with sequence homology (Fig. 3.3). Some would define these as microhomology (with mismatch), while others would define these as insertions. This causes ambiguity in the interpretation if microhomology-based joining is defined as A-EJ, but junctions with insertions are counted as blunt end-joining or classical (C)-NHEJ. This will also affect the calculation of the average microhomology.

Other terms that need to be clarified are ''blunt end-joining'' or ''blunt junctions.'' In some of the earlier studies, these terms indicate any junction with 0 bp microhomology, including those with insertions (Pan *et al*., 2002;

TABLE 3.1 Switch junction analysis in mouse models

Protein	Mutation	Strain	Age (week)	References for mice	Cell source	Type of junctions (no. of junctions)	Average junctional MH (bp)	Freq. of junctional mutations	Freq. of SHM-like mutations in Sμ	Sequence available	CSR efficiency	Other features of CSR junctions	References for CSR junctions
53BP1	Truncated mutation by gene trap	n.a.	8–12	Morales *et al.* (2003)	αCD40 + IL4 stimulated splenic B cells ($CD43^+$ depletion)	Sμ–Sγ1 (26 vs. 23)	0.9 vs. 1.1[a] (n.s.)	Normal	n.s.	No	Reduced (all Ig classes)	n.a.	Manis *et al.* (2004)
53BP1	Replacing nts 3777–4048 by PGK-neo^r	C57BL/6	n.a.	Ward *et al.* (2003)	LPS + IL4 stimulated resting splenic B cells	Sμ–Sγ1 (40 vs. 40)	2.5 vs. 2.0[a] (n.s.)	Normal	Normal	Partially ($n = 2$), ≥100 bp	n.a.	Unusual insertions; increased freq. of intra-S recombination	Reina-San-Martin *et al.* (2007)
APE1 + APE2	$APE1^{+/-}$/ $APE2^{Y/-}$ (deletion of coding regions of *APE1*; disruption of ex6 of *APE2*)	C57BL/6	8–20	Ide *et al.* (2003), Meira *et al.* (2001)	T cell depleted B cells stimulated with LPS + α-δ-dextran	Sμ–Sγ3 (23 vs. 33)	1.4 vs. 2.7[a,b] (n.s.)	n.a.	Normal	No	Reduced (all Ig classes)	Reduced freq. of mutations at A:T in Sμ ($p = 0.012$)	Guikema *et al.* (2007)
APE2	Disruption of ex6	C57BL/6	11	Ide *et al.* (2003)	LPS + IL4 stimulated splenic B cells ($IgG1^+$ sorted)	Sμ–Sγ1 n.a.	n.a.[c]	Increased (in Sμ, 100 bp, $p < 0.05$)	Normal	Partially (KO, $n = 17$; WT, $n = 17$), 30 bp	Normal	Normal junctional homology	Sabouri *et al.* (2009)
Artemis	$Art^{N/N}$ HL[d] (replacing ex5–6 by neo^r)	129SvEvTac	6–8	Rooney *et al.* (2002, 2005)	αCD40 + IL4 stimulated splenic B cells	Sμ–Sγ1 (13 vs. 11)	1.2[c] (WT, n.a.)	Normal	n.a.	40 bp	Normal		Rooney *et al.* (2005)

(*continued*)

TABLE 3.1 (*continued*)

Protein	Mutation	Strain	Age (week)	References for mice	Cell source	Type of junctions (no. of junctions)	Average junctional MH (bp)	Freq. of junctional mutations	Freq. of SHM-like mutations in Sμ	Sequence available	CSR efficiency	Other features of CSR junctions	References for CSR junctions
Artemis	Conditional deletion of ex12 in B cells	C57BL/6J	n.a.	Rivera-Munoz *et al.* (2009)	LPS stimulated $IgG1^+$ and $IgG3^+$ splenic B cells	Sμ–Sγ1 (30 vs. 30)	2.0 vs. 2.0[e,f] (n.s.)	n.a.	n.a.	Partially ($n = 40$), 30 bp	Reduced (IgG3; IgA, *in vivo*)		Rivera-Munoz *et al.* (2009)
						Sμ–Sγ3 (19 vs. 18)	3.0 vs. 2.5[e,f] (n.s.)	n.a.	n.a.				
					IgA^+ B cells from Peyer patches of KLH immunized mice	Sμ–Sα (29 vs. 37)	4.0 vs. 3.0[e,f] (n.s.)	n.a.	n.a.				
ATM	Truncated at nt 5790	129/SVEv	7–12	Barlow *et al.* (1996)	CD40L+IL4 stimulated splenic B cells (CD19 enriched)	Sμ–Sγ1 (23 vs. 31)	2.6 vs. 1.2[c] ($p = 0.01$)	Normal	Normal	No	Reduced (IgA, IgG1, IgG2a, IgG3, IgE)	n.a.	Lumsden *et al.* (2004)
ATM	Truncated at nt 5790	129/SVEv (WT, C57BL/6)	8–10	Barlow *et al.* (1996)	LPS + IL4 stimulated splenic B cells ($CD43^+$ depletion)	Sμ–Sγ1 (32 vs. 40)	1.9 vs. 2.0[c] (n.s.)	Reduced ($p = 0.01$)	Normal	No	Reduced (IgG1, IgG2b, IgG3)	Normal intra-S recombination	Reina-San-Martin *et al.* (2004)
ATM + DNA-PKcs	Truncated at nt 5790 +DNA-PKcs kinase inhibitor (PKi)	n.a.	n.a.	Barlow *et al.* (1996)	LPS + IL4 stimulated splenic B cells (sorted $IgG1^+$ cells)	Sμ–Sγ1 ($ATM^{-/-}$, 50; $ATM^{-/-}$ +PKi, 56; WT, 44; WT+ PKi, 20)	Normal[a]	n.a.	n.a.	Partially ($n = 22$), 55bp + insertions	Further decrease of CSR in the presence of PKi	Increased insertions (≥2 bp)	Callen *et al.* (2009)

Cernunnos /XLF	Deletion of aa 131–196	129/Sv	n.a.	Li *et al.* (2008), Zha *et al.* (2007)	αCD40+IL4 or LPS stimulated splenic B cells ($CD43^+$ depletion)	Sμ–Sγ1 (43 vs. 31)	Increased[a]	n.a.	n.a.	No	Reduced (IgG1, IgG3)	Reduced direct joints (14 vs. 23%); increased MH usage	Li *et al.* (2008)
DNA Lig4	Deletion of aa 220–911	CH12F3 cell line	n.a.	Han and Yu (2008)	αCD40 + IL4 + TGFβ1 stimulated	Sμ–Sα (23 vs. 26)	2.5 vs. 1.5[c] (n.s.)	n.a.	n.a.	65 bp	Reduced (IgA)	Lack of direct joints (0% vs. 27%)	Han and Yu (2008)
DNA Lig4	$Lig4^{-/-}p53^{+/-}$ HL[d]	129 Sv/ev	n.a.	Yan *et al.* (2007)	αCD40+IL4 stimulated splenic B cells ($CD43^+$ depletion)	Sμ–Sγ1 (93 vs. 103) Sμ–Sε (77 vs. 69)	n.a. n.a.	n.a. n.a.	n.a. n.a.	No	Reduced (IgG1, IgE)	Almost all MH-mediated (2% direct in γ1 and 0% in ε)	Boboila *et al.* (2010b)
DNA-PKcs	$DNA\text{-}PKcs^{-/-}$ /H[d]	n.a.	6–10	Manis *et al.* (2002)	αCD40+IL4 stimulated splenic B cells	Sμ–Sγ1 (25 vs. 11)	n.a.	n.a.	n.a.	No	Impaired (all Ig classes except IgG1)	Normal appearance of junctions	Manis *et al.* (2002)
DNA-PKcs	$SW_{HEL}SCID$ (controls, SW_{HEL} $RAG1^{-/-}$)[g]	C57BL/6	n.a.	Cook *et al.* (2003)	Splenic B cells (CD19 enriched)	Sμ–Sγ1; Sμ–Sγ2b; Sμ–Sγ3; Sμ–Sε; Sγ–Sε (30 vs. 28)	3.4 vs. 2.3[c] (n.s.)	Reduced (±12 bp; $p = 1 \times 10^{-4}$)	Reduced at C ($p = 0.02$)[h]	Partially ($n = 23$), 80–100 bp	Reduced (IgG1, IgG2a, IgG2b, IgG3, IgE)	n.a.	Cook *et al.* (2003)
ERCC1	Disruption of ex7	Mixed C57BL/6 and FVBn	2–3	Niedernhofer *et al.* (2001)	LPS + α-δ-dextran stimulated splenic B cells (T cell depletion)	Sμ–Sγ3 (38 vs. 46)	n.a.	Normal (less cluster at the junctions)	Normal	Partially (KO, $n = 38$), 43 bp	Reduced (IgA, IgG1, IgG2a, IgG2b, IgG3)	Normal MH; increase in mutations at RGYW/WRYC hotspots in germline Sμ	Schrader *et al.* (2004)

(*continued*)

TABLE 3.1 (*continued*)

Protein	Mutation	Strain	Age (week)	References for mice	Cell source	Type of junctions (no. of junctions)	Average junctional MH (bp)	Freq. of junctional mutations	Freq. of SHM-like mutations in Sμ	Sequence available	CSR efficiency	Other features of CSR junctions	References for CSR junctions
ERCC1	Disruption of ex5	n.a.	1–3	McWhir *et al.* (1993)	LPS + IL4 stimulated splenic cells	Sμ–Sε (27 vs. 27)	n.a.	Normal	n.a.	No	Normal	Normal distributions of Sμ and Sε breakpoints	Winter *et al.* (2003)
Exo1	Lack of ex6	C57BL/6	8–20	Wei *et al.* (2003)	LPS stimulated splenic B cells (T cell depletion)	Sμ–Sγ3 (32 vs. 44)	Median reduced[e] (p = 0.0017)	n.a.	n.a.	45 bp	Reduced (IgG1, IgG3)	Fewer long MH and more long insertions (≥4 bp)	Bardwell *et al.* (2004)
Exo1	Lack of ex6 (disrupt the I-nuclease domain of Exo1)	C57BL/6	n.a.	Wei *et al.* (2003)	LPS + α-δ-dextran or LPS + IL4 stimulated splenic B cells (B cell enrichment)	Sμ–Sγ3 (36 vs. 38)	1.9 vs. 0.9[e,b] (p = 0.025)	n.a.	n.a.	About 50–60 bp	Reduced (IgA, IgG1, IgG2b, IgG3)	13.9% of Sμ–Sγ3 junctions have MH ≥5 nt (vs. 2.6% in control)	Eccleston *et al.* (2009)
						Sμ–Sγ1 (31 vs. 32)	2.1 vs. 0.7[e,b] (p = 0.002)	n.a.	n.a.				
H2AX	Null mutation	n.a. (WT, C57BL/6)	8–10	Petersen *et al.* (2001)	LPS + IL4 stimulated splenic B cells ($CD43^+$ depletion)	Sμ–Sγ1 (30 vs. 38)	1.8 vs. 1.7[c] (n.s.)	Reduced (n.s.)	Normal	Partially (n = 16), 50 bp	Reduced (IgG1)	Normal freq. of mutations in Sγ1; normal intra-S recombination	Reina-San-Martin *et al.* (2003)
Ku70	$Ku70^{-/-}HL$[d] (Deletion of ex4+ part of ex5)	n.a.	n.a.	Gu *et al.* (1997), Manis *et al.* (1998)	αCD40 + IL4 stimulated splenic B cells ($CD43^+$ depletion)	Sμ–Sγ1 (106 vs. 103)	n.a.	n.a.	n.a.	No	Reduced (all Ig classes)	Decreased direct joining; increased MH-mediated joining; increased internal S recombination	Boboila *et al.* (2010a,b)
						Sμ–Sε (41 vs. 69)	n.a.	n.a.	n.a.				

Ku70 + DNA Lig4	Ku70$^{-/-}$; Lig4$^{-/-}$ HL[d]	n.a.	n.a.	Boboila *et al.* (2010b), Gu *et al.* (1997)	αCD40 + IL4 stimulated splenic B cells (CD43^{+} depletion)	Sμ–Sγ1 (86 vs. 103) Sμ–Sε (47 vs. 69)	n.a. n.a.	n.a. n.a.	n.a. n.a.	No	Reduced (IgG1, IgE)	Direct joints decreased compared to WT, but increased compared to Lig4$^{-/-}$; increased internal S recombination	Boboila *et al.* (2010a,b)
MLH1	Deletion of ex4	129 and C57BL/6	n.a.	Baker *et al.* (1996)	LPS stimulated splenic B cells (T cell depletion)	Sμ–Sγ3 (23 vs. 32)	3.2 vs. 1.7[i,b]	Normal	n.a.	45 bp	Reduced	22% junctions with MH of 5 bp or more (vs. 3% in control)	Schrader *et al.* (1999, 2002)
MLH1/ MSH2	Disruption of an ex corresponding to ex11 of human *Msh2*)	Mlh1$^{-/-}$, n.a.; Msh2$^{-/-}$, C57BL/6J	n.a.	Baker *et al.* (1996), Reitmair *et al.* (1995)	LPS + α-δ-dextran stimulated splenic B cells (T cell depletion)	Sμ–Sγ3 (32 vs. 32)	2.7 vs. 1.7[i,b]	n.a.	Increased (p = 0.001) (recombined Sμ)	40 bp	Reduced (IgA, IgG1, IgG2a, IgG2b, IgG3)	Longer MH than MSH2$^{-/-}$, but similar to Mlh1$^{-/-}$; mutation freq. increased in recombined Sγ	Schrader *et al.* (2003a)
MLH1	Deletion of ex4	129 and C57BL/6	n.a.	Baker *et al.* (1996)	LPS + α-δ-dextran or LPS + IL4 stimulated splenic B cells (B cell enrichment)	Sμ–Sγ3 (63 vs. 38) Sμ–Sγ1 (54 vs. 32)	1.7 vs. 0.9[e,b] (p = 0.008) 1.4 vs. 0.7[e,b] (p = 0.025)	n.a. n.a.	n.a. n.a.	About 50–60 bp	Reduced (IgA, IgG1, IgG2a, IgG2b, IgG3)	6.3% of Sμ–Sγ3 junctions have MH ≥5 nt (vs. 2.6% in control)	Eccleston *et al.* (2009)
MLH3	Deletion of ex1–2	129 Sv/Ev	n.a.	Wu *et al.* (2006)	LPS or LPS + IL4 stimulated splenic B cells (B cell enrichment)	Sμ–Sγ1 (44 vs. 44) Sμ–Sγ3 (44 vs. 44)	2 vs. 2.4[c] 1.9 vs. 2.9[c]	n.a. n.a.	n.a. n.a.	40 bp	Normal	Increase in blunt junctions and insertions; preferential targeting of the RGYW/WRCY motif in Sγ breakpoints	Wu *et al.* (2006)

(*continued*)

TABLE 3.1 (*continued*)

Protein	Mutation	Strain	Age (week)	References for mice	Cell source	Type of junctions (no. of junctions)	Average junctional MH (bp)	Freq. of junctional mutations	Freq. of SHM-like mutations in Sμ	Sequence available	CSR efficiency	Other features of CSR junctions	References for CSR junctions
Mre11	Conditional null allele in B cells	n.a.	6–12	Buis *et al.* (2008), Dinkelmann *et al.* (2009)	αCD40 + IL4 stimulated splenic B cells ($CD5^-$ B cells)	Sμ–Sγ1 (32 vs. 20) Sμ–Sε (7 vs. 13)	1.8 vs. 1.9[i,b] (n.s.) 0.4 vs. 1.5[i,b] (n.s.)	n.a. n.a.	n.a. n.a.	43 bp	Reduced (IgG1)	Normal ratio for blunt and MH-mediated junctions	Dinkelmann *et al.* (2009)
Mre11	Nuclease deficient (H129N)	n.a.	6–12	Buis *et al.* (2008), Dinkelmann *et al.* (2009)	αCD40 + IL4 stimulated splenic B cells ($CD5^-$ B cells)	Sμ–Sγ1 (19 vs. 20) Sμ–Sε (10 vs. 13)	1.6 vs. 1.9[i,b] (n.s.) 1.7 vs. 1.5[i,b] (n.s.)	n.a. n.a.	n.a. n.a.	43 bp	Reduced (IgG1)	Normal ratio for blunt and MH-mediated junctions	Dinkelmann *et al.* (2009)
MSH2	Hygro-mycin^r gene inserted between nts 588 and 589	n.a.	8–12	DeWind *et al.* (1995)	LPS stimulated splenic cells Peyer's patch lympho-cytes	Sμ–Sγ3 (22 vs. 28) Sμ–Sα (19 vs. 27)	n.a. n.a.	n.a. n.a.	n.a. n.a.	No	Reduced (IgG3)	Increased Sμ breakpoints in GAGCT or TGGGG motifs	Ehrenstein and Neuberger (1999)
MSH2	Replacing ex7 by PGK-neo^r	129 and C57BL/6	n.a.	Smits *et al.* (2000)	LPS stimulated splenic B cells (T cell depletion)	Sμ–Sγ3 (32 vs. 32)	0.8 vs. 1.7[i,b]	Normal	Increased (p =0.046) (recombined Sμ)	40 bp	Reduced	Shorter MH[j]; increased short inserts	Schrader *et al.* (1999, 2002, 2003a)
MSH2	Msh2^{G674A} knock in (lack of ATPase activity)	C57BL/6	24	Martin *et al.* (2003)	LPS stimulated splenic B cells	Sμ–Sγ3 (58 vs. 54)	2.6 vs. 1.8[g]	n.a.	n.a.	45 bp	Reduced (IgG1, IgG3)	Increased junctions with MH ≥5 nt (24% vs. 9%); increased large insertions (≥5 nt; 15% vs. 2%)	Martin *et al.* (2003)

MSH3	PGK-neo^r insertion in ex7	C57BL/6	4–10	Edelmann *et al.* (2000)	LPS stimulated splenic B cells (T cell depletion)	Sμ–Sγ3 (50 vs. 43)	2.8 vs. 2.8[i,b] (n.s.)	n.a.	Normal	Partially (KO, $n = 48$; WT, $n = 43$), 45 bp	Normal	Increased freq. of inserts of $\geq$5 bp	Li *et al.* (2004b)
MSH4	Null mutation (disrupted ex4)	C57BL/6	6–8	Kneitz *et al.* (2000)	Splenic cells	Sμ–Sγ3 (37 vs. 33)	2.6 vs. 1.0[c,b] ($p = 0.001$)	n.a.	n.a.	70 bp	Normal	n.a.	Sekine *et al.* (2007)
						Sμ–Sα (37 vs. 29)	2.9 vs. 1.5[c,b] ($p = 0.012$)	n.a.	n.a.				
MSH5	ATPase domain replaced	FVB (H-2^q)	6–8	de Vries *et al.* (1999)	Splenic cells	Sμ–Sγ3 (33 vs. 27)	2.5 vs. 1.4[c,b] ($p = 0.039$)	n.a.	n.a.	70 bp	Normal	n.a.	Sekine *et al.* (2007)
						Sμ–Sα (30 vs. 30)	4.6 vs. 2.5[c,b] ($p = 0.021$)	n.a.	n.a.				
MSH5	H-2^e congenic MRL/lpr	MRL/lpr	6–8	Sekine *et al.* (2006)	Splenic cells	Sμ–Sγ3 (25 vs. 19)	3.3 vs. 0.6[c,b] ($p = 0.0005$)	n.a.	n.a.	70 bp	Normal *in vitro* CSR; Reduced serum IgA and IgG3	n.a.	Sekine *et al.* (2007)
						Sμ–Sα (40 vs. 27)	3.0 vs. 2.0[c,b] (n.s.)	n.a.	n.a.				
MSH5	Disrupted ex18	C57BL/6	n.a.	Edelmann *et al.* (1999)	LPS + α-δ-dextran stimulated splenic cells (T cell depletion)	Sμ–Sγ3 (55 vs. 72)	2.3 vs. 1.8[a,b] (n.s.)	n.a.	Normal	No	Normal		Guikema *et al.* (2008)
					Splenic cells	Sμ–Sγ3 (22 vs. 26)	1.8 vs. 1.9[a] (n.s.)	n.a.	Normal				
MSH6	PGK-neo^r insertion in ex4, resulting in	C57BL/6	4–10	Edelmann *et al.* (1997)	LPS stimulated splenic B	Sμ–Sγ3 (51 vs. 99)	2.6 vs. 2.2[c,b] (n.s.)	n.a.	Normal (increase in GC transitions	Partially (MSH6, $n = 47$; WT,	Reduced (IgG1, IgG3)	No increase in blunt joints; normal MH; increase in	Li *et al.* (2004b)

(*continued*)

TABLE 3.1 (*continued*)

Protein	Mutation	Strain	Age (week)	References for mice	Cell source	Type of junctions (no. of junctions)	Average junctional MH (bp)	Freq. of junctional mutations	Freq. of SHM-like mutations in Sμ	Sequence available	CSR efficiency	Other features of CSR junctions	References for CSR junctions
	a stop codon down-stream				cells (T cell depletion)				and hotspot mutations)	$n = 97$), 45 bp		targeting to the consensus repeats in Sγ3.	
MSH6	MSH6 $^{TD/TD}$ (p.T1217D)	C57BL/6	4–10	Yang *et al.* (2004)	LPS stimulated splenic B cells (T cell depletion)	Sμ–Sγ3 (148 vs. 91)	3.0 vs. 2.6[k,b] (n.s.)	n.a.	Normal (increase in GC transitions in Sμ)	45 bp	Reduced (IgG1, IgG3)	Fewer junctions have 3–4 nt MH	Li *et al.* (2006)
NBS	Conditional deletion of ex6 in B cells	C57BL/6	n.a.	Demuth *et al.* (2004), Kracker *et al.* (2005)	LPS + IL4 stimulated splenic B cells (CD43$^+$ depletion)	Sμ–Sγ1 (41 vs. 19)	2.9 vs. 2.3[c] (n.s.)	Increased (±10 bp; n.s.)	n.a.	No	Reduced (IgG1, IgG3)	Normal distribution of breakpoints	Kracker *et al.* (2005)
NBS	Conditional deletion of ex1 in B cells	n.a.	5–8	Reina-San-Martin *et al.* (2005)	LPS + IL4 stimulated splenic B cells (CD43$^+$ depletion)	Sμ–Sγ1 (29 vs. 34)	0.9 vs. 1.4[c] (n.s.)	Normal	Reduced ($p = 0.007$)	No	Reduced (IgG1)	n.a.	Reina-San-Martin *et al.* (2005)
P53	Null mutation	C57BL/6	6–12	Donehower *et al.* (1992)	LPS + α-δ-dextran stimulated splenic B cells (T cell depletion)	Sμ–Sγ3 (39 vs. 43)	Normal[a]	Increased ($p = 1 \times 10^{-5}$)	n.a.	No	Increased (IgG2a)	Normal freq. of blunt joints	Guikema *et al.* (2010)
PARP1	Disruption of ex4	C57BL/6	8–12	deMurcia *et al.* (1997)	LPS stimulated splenic B cells (CD43$^+$ depletion)	Sμ–Sγ3 (59 vs. 52)	1.0 vs. 1.8[c] (P = 0.003)	n.a.	n.a.	40 bp	Normal	Increased 0 bp MH junctions	Robert *et al.* (2009)

PARP2	Disruption of ex9	C57BL/6	8–12	Menissier de Murcia *et al.* (2003)	LPS stimulated splenic B cells ($CD43^+$ depletion)	Sμ–Sγ3 (51 vs. 52)	2.1 vs. 1.8[c] (n.s.)	n.a.	n.a.	40 bp	Normal	No increase of 0bp MH junctions	Robert *et al.* (2009)
Pol ζ	Conditional deletion of ex2 in B cells	C57BL/6	n.a.	Schenten *et al.* (2009)	LPS stimulated splenic B cells (B cell enrichment)	Sμ–Sγ3 (41 vs. 64)	n.a.	n.a.	Reduced (recombined Sμ)	No	Reduced (IgG1, IgG3)	Increased freq. of junctions with 3–7 bp MH; reduced insertions	Schenten *et al.* (2009)
PMS2	Replacing ex2 by PGK-neo^r	n.a.	n.a.	Baker *et al.* (1995)	LPS + IL4 stimulated splenic cells	Sμ–Sγ3 (55 vs. 54)	3.5 vs. 0.8[c]	n.a.	n.a.	Partially ($n = 9$), 50 bp	Reduced (IgG1, IgG3)	No increased freq. of Sμ breakpoints at GAGCT sites	Ehrenstein *et al.* (2001)
						Sμ–Sγ1 (28 vs. 26)	3.0 vs. 0.9[c]	n.a.	n.a.				
						Sμ–Sγ2b (28 vs. 26)	2.1 vs. 0.7[c]	n.a.	n.a.				
PMS2	Replacing ex2 by PGK-neo^r	129 and C57BL/6	n.a.	Baker *et al.* (1995)	LPS stimulated splenic B cells (T cell depleted)	Sμ–Sγ3 (25 vs. 32)	2.9 vs. 1.7[i,b]	Normal	n.a.	40 bp	Reduced	24% of junctions have MH of ≥5 bp (vs. 3% in control)	Schrader *et al.* (1999, 2002)
PMS2	$PMS2^{-/-}$	n.a.	n.a.	n.a.	LPS stimulated splenic cells	Sμ–Sγ3 (20 vs. 22)	5.1 vs. 2.6[a] ($p = 0.04$)	n.a.	n.a.	No	Reduced (IgG1, IgG2b, IgG3)	Increase in junctions with ≥5 bp MH; normal blunt joints and insertions	van Oers *et al.* (2010)
PMS2	Endonuclease-deficient $Pms2^{E702K}$ knock in)	n.a.	n.a.	van Oers *et al.* (2010)	LPS stimulated splenic cells	Sμ–Sγ3 (20 vs. 22)	2.5 vs. 2.6[a] (n.s.)	n.a.	n.a.	No	Reduced (IgG1, IgG2b, IgG3)	A nonsignificant tendency for preference of blunt-joining	van Oers *et al.* (2010)

(*continued*)

TABLE 3.1 (*continued*)

Protein	Mutation	Strain	Age (week)	References for mice	Cell source	Type of junctions (no. of junctions)	Average junctional MH (bp)	Freq. of junctional mutations	Freq. of SHM-like mutations in Sμ	Sequence available	CSR efficiency	Other features of CSR junctions	References for CSR junctions
PTIP	Conditional deletion of ex1 in B cells	n.a.	6–14	Kim *et al.* (2007)	LPS + IL4 stimulated splenic B cells ($CD43^+$ depletion) IgG1 sorted	Sμ–Sγ1 (17 vs. 14)	Normal[a]	n.a.	Normal	About 60 bp	Reduced (IgG1, IgG2b, IgG3)		Daniel *et al.* (2010)
RNF8	Disrupted between ex4 and ex5	C57BL/6	n.a.	Santos *et al.* (2010)	LPS + IL4 stimulated splenic B cells ($CD43^+$ depletion)	Sμ–Sγ1 (42 vs. 28)	n.a.	n.a.	Normal	No	Reduced (IgG1, IgG3)	MH mildly increased; Increased insertions (>2 nt; 24% vs. 7%)	Santos *et al.* (2010)
SμTR (tandem repeat)	Deletion of Sμ tandem repeat region	n.a.	n.a.	Luby *et al.* (2001)	$IgG1^-$ hybridomas (immunized with Ars-KLH)	Sμ–Sγ1 ($n = 10$)	n.a.	n.a.	n.a.	Partially ($n = 8$), 80 bp	Reduced (IgG1, IgG2a, IgG2b, IgG3)	Altered distribution of Sμ and Sγ breakpoints	Luby *et al.* (2001)
SμTR (tandem repeat)	Deletion of Sμ tandem repeat region	n.a.	n.a.	Luby *et al.* (2001)	LPS + α-δ-dextran or LPS + IL4 stimulated splenic B cells (T cell depletion)	Sμ–Sγ3 ($n = 16$) Sμ–Sγ1 ($n = 22$)	1.3[e,b] 1.1[e,b]	n.a. n.a.	n.a. n.a.	80 bp	Reduced (IgA, IgG1, IgG2a, IgG2b, IgG3)	Focus of Sμ–Sγ3 junctions at GAGCT or GGGGT motifs	Min *et al.* (2003)
SμTR/ MSH2	$S\mu TR^{-/-}$/ $MSH2^{-/-}$	$MSH2^{-/-}$, C57BL/	n.a.	Luby *et al.* (2001),	LPS + α-δ-dextran or LPS + IL4	Sμ–Sγ3 (21 vs. 16	2.2 vs. 1.3[e,b] (n.s.)?	n.a.	n.a.	80 bp	Greatly reduced (IgA,	About 25% of junctions have long MH, which	Min *et al.* (2003)

		6J; SμTR (n.a.)		Reitmair *et al.* (1995)	stimulated splenic B cells (T- cell depleted)	in Sμ-TR$^{-/-}$) Sμ–Sγ1 (19 vs. 22	0.9 vs. 1.1[e,b] (n.s.)	n.a.	n.a.		IgG1, IgG2a, IgG2b, IgG3)	exhibited pentamer sequences at the Sμ side	
SμTR/ Mlh1	Mlh1$^{-/-}$/ SμTR$^{-/-}$	Mlh1$^{-/-}$, n.a.; SμTR$^{-/-}$, n.a.)	n.a.	Baker *et al.* (1996), Luby *et al.* (2001)	LPS + α-δ-dextran or LPS + IL4 stimulated splenic B cells (B cell enrichment)	in Sμ-TR$^{-/-}$) Increased MH, slightly more than in Mlh1$^{-/-}$ mice	Sμ–Sγ3 (20 vs. 38) Eccleston *et al.* (2009)	2.0 vs. 0.9[e,b]	($p = 0.008$)	n.a.	n.a.	About 50–60 bp	Severe reduction (IgA, IgG1, IgG2a, IgG2b, IgG3)
Sμ–Sγ1 (27 vs. 32)	1.3 vs. 0.7[e,b] ($p = 0.004$)	n.a.	n.a.										
SμTR/ Exo1	Exo1$^{-/-}$/ SμTR$^{-/-}$	SμTR (n.a.); Exo1$^{-/-}$, C57BL/6	n.a.	Bardwell *et al.* (2004), Luby *et al.* (2001)	LPS + α-δ-dextran or LPS + IL4 stimulated splenic B cells (B cell enrichment)	Sμ–Sγ3 (20 vs. 38) Sμ–Sγ1 (18 vs. 32)	1.4 vs. 0.9[e,b] (n.s.) 1.8 vs. 0.7[e,b] ($p = 0.019$)	n.a. n.a.	n.a. n.a.	About 50–60 bp	Severe reduction (IgA, IgG1, IgG2b, IgG3)	The level of MH, similar to Exo 1$^{-/-}$	Eccleston *et al.* (2009)
XRCC4	Xrcc4$^{-/-}$p53$^{+/-}$ HL[d] (deletion of ex3 of *Xrcc4*)	129 Sv/ev	3–5	Yan *et al.* (2007)	CD40 + IL4 or LPS stimulated splenic B cells (CD43^{+} depletion)	Sμ–Sγ1 (35 vs. 16) Sμ–Sγ2b (20 vs. 17) Sμ–Sγ3 (17 vs. 10) Sμ–Sε (13 vs. 15)	2.8 vs. 1[c] ($p = 0.0005$) 2.3 vs. 0.9[c] ($p = 0.013$) 2.4 vs. 1.5[c] (n.s.) 6.7 vs. 2.9[c] ($p = 0.04$)	Normal Normal Normal Normal	n.a. n.a. n.a. n.a.	40 bp	Reduced (IgG1, IgG3)	Lack of direct joints; increased freq. of MH-mediated joints	Yan *et al.* (2007)
XRCC4	CD21-cre-Xrcc4$^{c/-}$	129 Sv/ev	6–10	Yan *et al.* (2007)	CD40 + IL4 stimulated splenic B cells (CD43^{+} depletion)	Sμ–Sγ1 (29 vs. 8)	2.5 vs. 0.9[c] ($p = 0.025$)	Normal	n.a.	40 bp	Reduced (IgG1, IgG3)	Lack of direct joints; increased freq. of MH-mediated joints	Yan *et al.* (2007)

(*continued*)

TABLE 3.1 (*continued*)

Protein	Mutation	Strain	Age (week)	References for mice	Cell source	Type of junctions (no. of junctions)	Average junctional MH (bp)	Freq. of junctional mutations	Freq. of SHM-like mutations in Sμ	Sequence available	CSR efficiency	Other features of CSR junctions	References for CSR junctions
XRCC4	Conditional deletion of *Xrcc4* in B cells	n.a.	8–12	Soulas-Sprauel *et al.* (2007)	LPS + IL4 stimulated B cells ($IgG1^+$ sorted)	Sμ–Sγ1 (50 vs. 50)	1.0 vs. 0.9[c] (n.s.)	n.a.	n.a.	50 bp	Reduced (IgG1, IgG2b, IgG3)	Slightly reduced direct joining; slightly increased 1–3 bp MH	Soulas-Sprauel *et al.* (2007)

[a] Unknown standard used when defining microhomologies.
[b] Average length of microhomology is calculated based on data shown in the original report.
[c] Microhomology (MH) is defined as perfectly matched successive nucleotides. n.a., not analyzed; n.s., not significant.
[d] HL, knock in of preassembled IgH and Igl transgenes.
[e] One mismatch is tolerated when defining the microhomologies.
[f] Median length of microhomology is calculated.
[g] SCID mice carry a recessive mutation that results in loss of last 83 aa of DNA-PKcs. SW_{HEL} mice carry rearranged IgH and Igk transgenes cloned from the HyHEL10 hybridoma specific for HEL.
[h] Mutation in "hybrid S regions" (Cook *et al.*, 2003).
[i] Two mismatches are tolerated when defining microhomologies.
[j] Reported microhomology counts junctions with inserts as 0 bp. If junctions with inserts are not included, there is no difference in microhomology between $msh2^{-/-}$ and WT cells.
[k] More than two mismatches are tolerated when defining microhomologies.

TABLE 3.2 Switch junction analysis in human disease models

Defective proteins	Mutation (no. of individuals)	Age (years)	References for patients	Cell source	Type of junctions (no. of junctions)	Junctional MH (bp)	Freq. of junctional mutations	Freq. of SHM-like mutations in Sμ	Other features of CSR junctions	Sequence available	References for CSR junctions
Artemis	Deletion of ex10–12 ($n = 1$); Deletion of ex1–3 ($n = 1$); g.1391_1395del5 ($n = 1$); g.[207_209del3] + [377G > A] ($n = 1$)	0.5–4	Evans *et al.* (2006), Noordzij *et al.* (2003), van der Burg *et al.* (2007)	PBL	Sμ–Sα (54 vs. 137) Sμ–Sγ (24 vs. 58)	8.0 vs. 3.9[a] ($p < 0.0001$) 1.4 vs. 1.4[a] (n.s.)	Reduced ($p < 0.05$) Normal	Normal (altered pattern) n.a.	Lack of "direct joining" at Sμ–Sα; increased Sμ–Sγ–Sγ sequential switching	50 bp	Du *et al.* (2008b)
Artemis	c.1328–1344del17 ($n = 2$)	n.a.	Rivera-Munoz *et al.* (2009)	PBL	Sμ–Sα (44 vs. 93)	13.5 vs. 6.0[b,c] ($p = 0.009$); 14.5 vs. 6.0[b,c] ($p < 0.0001$)	n.a.	n.a.		Partially ($n = 20$), 30 bp	Rivera-Munoz *et al.* (2009)
ATM	g.8656_8657insT ($n = 3$); g.[481C > T] + [3284G > C] ($n = 1$); g.[1567T]> C + [?] ($n = 1$); g.9170G > C ($n = 1$); g.[7768_7771del4] + [2554C > T] ($n = 2$); ND ($n = 4$)	1–31	Laake *et al.* (2000)	PBL	Sμ–Sα (44 vs. 54) Sμ–Sγ (39 vs. 44)	7.2 vs. 1.8[a] ($p < 0.001$) 2.5 vs. 1.2[a] ($p < 0.001$)	Reduced ($p < 0.01$) Reduced ($p < 0.05$)	Reduced ($p < 0.001$) n.a.	Reduced 0 bp MH (5% vs. 44%) and reduced 1 bp insertion at Sμ–Sα	30 bp[d] No	Pan *et al.* (2002), Pan-Hammarstrom *et al.* (2006)
ATR	g.2101A > G ($n = 2$)	13, 25	O'Driscoll *et al.* (2003)	PBL	Sμ–Sα (39 vs. 154) Sμ–Sγ (47 vs. 59)	3.0 vs. 1.8[a] ($p < 0.05$) 1.8 vs. 1.2[a] ($p < 0.05$)	Reduced ($p < 0.05$) Reduced (n.s.)	Reduced ($p < 0.001$) n.a.	Normal "blunt end-joining"; increased 4–6 bp MH at Sμ–Sα	50 bp No	Pan-Hammarstrom *et al.* (2006)

(*continued*)

TABLE 3.2 (*continued*)

Defective proteins	Mutation (no. of individuals)	Age (years)	References for patients	Cell source	Type of junctions (no. of junctions)	Junctional MH (bp)	Freq. of junctional mutations	Freq. of SHM-like mutations in Sμ	Other features of CSR junctions	Sequence available	References for CSR junctions
DNA Lig4	g.[8C > T + 26C > T + 833G > A] ($n = 1$), g.[1738C>T] + [2440C > T] ($n = 1$)	9, 48	O'Driscoll *et al.* (2001)	PBL	Sμ–Sα (30 vs. 154)	9.8 vs. 1.8[a] ($p < 0.00001$)	Reduced ($p < 0.001$)	Reduced ($p < 0.001$)	Lack of "direct joining" at Sμ–Sα; increased 1 bp insertion at Sμ–Sγ	50 bp No	Pan-Hammarstrom *et al.* (2005)
					Sμ–Sγ (34 vs. 59)	1.3 vs. 1.2[a] (n.s.)	Normal	n.a.			
HIGM IV	n.d. ($n = 4$)	4–8	Peron *et al.* (2007)	PBL	Sμ–Sα (43 vs. 154)	7.2 vs. 1.8[a] ($p = 1.2 \times 10^{-9}$)	Normal	n.a.	Reduced "direct joining"; reduced insertions	Partially ($n = 8$), 50 bp	Peron *et al.* (2007)
Mre11	g.1897T > C ($n = 2$), g.[350G > A] + [1714T > C] ($n = 2$)	23–36	Stewart *et al.* (1999)	PBL	Sμ–Sα (47 vs. 154)	2.6 vs. 1.8[a] (n.s.)	Normal	Normal	Reduced C to T mutation and increased insertions at Sμ–Sα	50 bp[d] No	Lahdesmaki *et al.* (2004) Unpublished
					Sμ–Sγ (15 vs. 44)	1.8 vs. 1.2[a] (n.s.)	Normal	n.a.			
MSH5	p.[L85F;P786S] + [?] ($n = 10$)	Adults	Sekine *et al.* (2007)	PBL	Sμ–Sα (55 vs. 54)	9.3 vs. 3.8[a] ($p = 9.3 \times 10^{-7}$)	Reduced ($p = 2.4 \times 10^{-4}$)	Reduced ($p = 2.0 \times 10^{-12}$)	More breakpoints targeted to pentamers at Sμ	No	Sekine *et al.* (2007)
					Sμ–Sγ3 (18 vs. 24)	1.4 vs. 1.9[a] (n.s.)	Reduced (n.s.)	Normal			
NBS	g.657_661del5 ($n = 9$)	2–16	Varon *et al.* (1998)	PBL	Sμ–Sα (27 vs. 154)	3.6 vs. 1.8[a] ($p < 0.05$)	Normal	Normal	Significantly more Sμ–Sα flanked by long imperfect repeats	30 bp[d] No	Lahdesmaki *et al.* (2004), Pan *et al.* (2002)
					Sμ–Sγ (28 vs. 44)	2.3 vs. 1.2[a] ($p = 0.05$)	Normal	Normal			

PMS2	p.N412DfsX6 ($n = 1$), p.R802X ($n = 1$), p.S436KfsX22 ($n = 1$)	9–22	Peron *et al.* (2008)	PBL	Sμ–Sα (60 vs. 38)	6.9 vs. 1.7[b,e] ($p = 3.4 \times 10^{-7}$)	n.a.	n.a.	Reduced "blunt" joining at Sμ–Sα; reduced insertions	50 bp	Peron *et al.* (2008)
					Sμ–Sγ1 (24 vs. 20)	0.7 vs. 2[b,e] ($p = 0.025$)	n.a.	n.a.			
RNF168	c.[397dupG] + [1323_1326del4] ($n = 1$)	n.a.	Stewart *et al.* (2009)	PBL	Sμ–Sα (16 vs. 17)	10.0 vs. 1.4[e] ($p = 0.0002$)	Reduced ($p < 0.001$)	n.a.	93% vs. 17% of junctions with $\geq$4 bp MH	No	Stewart *et al.* (2009)

[a] Microhomology (MH) is defined as perfectly matched successive nucleotides. n.a., not analyzed; n.s., not significant.
[b] One mismatch is tolerated when defining the microhomologies.
[c] Median length of microhomology is calculated.
[d] The original pdf files illustrating sequences of Sμ–Sα junctions from A-T, NBS, and ATLD patients have been moved to authors' current website: http:/public.me.com/qiangpan.
[e] Average length of microhomology is calculated based on data shown in the original report.

Schrader *et al.*, 2002). Lately, the term ''direct joining'' is also used, where junctions with insertions are excluded from the 0 bp microhomology group (Yan *et al.*, 2007). Direct joining is totally absent in XRCC4 or Ligase IV (Lig4)-deficient cells, two proteins required for C-NHEJ, whereas insertions are still present or even increased in certain types of junctions from the mutants (Pan-Hammarstrom *et al.*, 2007; Yan *et al.*, 2007). This suggests that insertions can be generated by XRCC4–Lig4-independent EJ, a form of A-EJ. Thus, as the source of insertions is unclear, and they could theoretically be generated by either C-NHEJ or by A-EJ, it seems better to calculate this group of junctions separately. We will use the term direct joining to characterize junctions with 0 bp homology and no inserts.

In addition to short insertions, there are occasionally longer insertions (ranging from three to a few hundred base pairs), and these can be found in both WT and DNA repair-deficient B cells (Li *et al.*, 2004a; Reina-San-Martin *et al.*, 2007; Schrader *et al.*, 2002, 2004; Wu and Stavnezer, 2007). It is likely that AID attacks several S region sites, based on the finding of internal Sμ deletions in both WT and mutant B cells that have undergone CSR. In many cases, the inserted fragments can be clearly mapped to one of the S regions (occasionally in a reversed direction), and are thus presented as Sμ–Sμ–Sx junctions or Sμ–Sx–Sx junctions (Fig. 3.3), and referred to as intra-S region recombination, instead of insertions (Pan-Hammarstrom *et al.*, 2006). However, not all inserted sequences are derived from S regions (Reina-San-Martin *et al.*, 2007), suggesting that the inserted fragments can come from other genomic sites, consistent with suggestions that AID attacks other sites in the genome besides Ig genes (Liu *et al.*, 2008; Ramiro *et al.*, 2006).

2.4. Mutations occur in the region surrounding S–S junctions

Mutations have also been observed upstream or downstream of the S junctions and the frequency decreases with increasing distance from the junctions (Pan-Hammarstrom *et al.*, 2003; Schrader *et al.*, 2003b, 2004). It is notable that the mutation pattern in the Sμ region (upstream of the breakpoints) is clearly different from those at, or close to, the S breakpoints, suggesting that a different mechanism is involved (Pan-Hammarstrom *et al.*, 2003; Schrader *et al.*, 2003b). It is possible that mutations at, or near to, the S breakpoints are mainly generated during the formation of DSBs and during the recombination process itself, whereas mutations away from the breakpoints, like those observed in unrecombined (germline) Sμ regions, are interpreted as evidence that AID has attacked the Sμ region, but that CSR was not completed (Nagaoka *et al.*, 2002; Petersen *et al.*, 2001). The mutations away from the junctions or in the germline Sμ sequences are sometimes referred to as ''SHM-like'' mutations, as they show a spectrum similar to those associated with the V(D)J regions and

often occur in the predicted SHM hotspots (Pan-Hammarstrom *et al.*, 2003; Petersen *et al.*, 2001; Schrader *et al.*, 2003b).

2.5. Sequential switching results in Sμ–Sx–Sy junctions

In addition to switch recombination between Sμ and a downstream S region, switching may also occur between downstream S regions, in a 5′ to 3′ direction. Several studies have shown that switching to IgE in both humans and mice may occur via a sequential switch through the γ isotype, most often γ1, although the other three Sγ regions also participate (Baskin *et al.*, 1997; Mills *et al.*, 1995; Yoshida *et al.*, 1990; Zhang *et al.*, 1994). Sequential switching in human B cells from IgM via IgG to IgA, or from IgM via IgA1 to IgA2 has also been characterized at the DNA level (Pan *et al.*, 2001; Zan *et al.*, 1998). Sequential switching involving two different Sγ regions is occasionally identified in WT cells (Sμ–Sγ1–Sγ4) (Pan *et al.*, 1998) or in patients infected with *Schistosomiasis mansoni* (Sμ–Sγ1–Sγ2–Sε) (Baskin *et al.*, 1997), but is more frequently observed in patients with Artemis deficiency (Du *et al.*, 2008b). Therefore, when large insertions are observed at the S junctions, sequential switching should be considered.

3. EFFECT OF DNA REPAIR DEFICIENCIES ON S–S JUNCTIONS

3.1. NHEJ proteins are involved in CSR

Several proteins are required for NHEJ: Ku70, Ku80, and the ligase complex XRCC4–Ligase IV(Lig4)-XLF/Cernunnos. The involvement of these five proteins in a recombination reaction defines the reaction as occurring by NHEJ, also sometimes referred to as C-NHEJ. In addition, Artemis–DNA-PKcs and DNA Pol λ and μ are involved in recombination by NHEJ at a subset of DSBs, depending on the initial structure of the DSBs, that is, whether the DSBs require end-processing by these enzymes in order to undergo recombination (Lieber, 2010b). All these proteins, except Pol λ and μ, have been shown to contribute to varying extents to CSR (Boboila *et al.*, 2010b; Casellas *et al.*, 1998; Han and Yu, 2008; Ma *et al.*, 2005; Manis *et al.*, 1998; Pan-Hammarstrom *et al.*, 2005; Reina-San-Martin *et al.*, 2003; Rooney *et al.*, 2004; Sonoda *et al.*, 2006; Soulas-Sprauel *et al.*, 2007; Yan *et al.*, 2007).

The Ku70–Ku80 heterodimer is most likely the first protein to bind to DSBs due to its abundance and high affinity for DSBs (Lieber, 2010b). This heterodimer binds to DNA ends, forms a ring which encircles the DNA, and mediates synapsis of the two DNA ends, positioning the ends to allow end-processing and direct end-to-end-joining. Ku70–Ku80 also serves as a tool belt for the end-joining reaction by recruiting enzymes

that perform the recombination (Lieber *et al.*, 2003; Meek *et al.*, 2004). The Ku heterodimer binds to the nuclear matrix, which might localize the DSBs and telomeres to the matrix (Soutoglou *et al.*, 2007). After binding, Ku slides away from the ends, allowing the catalytic subunit, the kinase DNA-PKcs, to bind to each end (Spagnolo *et al.*, 2006). The heterotrimer DNA-PK acts both as an activator and scaffold during the actual ligation event (Lieber, 2010b; Meek *et al.*, 2004, 2007). Ku70–Ku80 improves the binding of XRCC4–Lig4 to DNA ends (Chen *et al.*, 2000; Costantini *et al.*, 2007; Nick McElhinny *et al.*, 2000). In the absence of Ku70–Ku80 or XRCC4–Lig4, another ligase (Ligase I or III) is involved in the end-joining process (Boboila *et al.*, 2010b; Lieber, 2010a). XRCC4–Lig4 in the presence of Ku70–Ku80 has been shown to ligate incompatible ends, consistent with the lack of microhomology observed at S–S junctions in WT cells (Gu *et al.*, 2007a).

3.1.1. XRCC4–Lig4 deficiencies reduce CSR

Complete deficiency of either XRCC4 or Lig4 alone is embryonically lethal due to impaired brain development (Frank *et al.*, 1998), although patients with hypomorphic mutations have been described (Buck *et al.*, 2006b; O'Driscoll *et al.*, 2001; van der Burg *et al.*, 2006). However, mice entirely lacking XRCC4 have been produced by mating $xrcc4^{+/-}$ mice with p53-deficient mice, allowing creation and survival of $xrcc4^{-/-}p53^{+/-}$ mice and $xrcc4^{-/-}p53^{-/-}$ mice. These mice were bred with Ig heavy (H) and light (L) chain knock-in (HL) mice to circumvent the fact that XRCC4 is required for generation of mature B cells owing to its involvement in V(D)J recombination. The resulting XRCC4-deficient cells were shown to have ~25% of normal levels of CSR, indicating that XRCC4 is important, but not essential, for CSR (Yan *et al.*, 2007). Conditional deletion of XRCC4 in $p53^{+/+}$ mature B cells also reduced, but did not abolish CSR (Soulas-Sprauel *et al.*, 2007; Yan *et al.*, 2007). Also, humans with mutations in *Lig4* have fewer peripheral blood B cells that have undergone CSR than normal controls (Pan-Hammarstrom *et al.*, 2005). Deletion of the *Lig4* gene reduces IgA CSR in the CH12F3 B lymphoma cell line (Han and Yu, 2008). At the earliest timepoint assayed (24 h), IgA CSR in the knock-out (KO) cells was only 13% of WT cells, but at 72 h it was 50% of WT levels. From these kinetic studies and from cell proliferation data, Han and Yu (2008) suggested that CSR is highly dependent upon C-NHEJ, and that cells that cannot recombine DSBs die, and thus cells that have undergone CSR outgrow in the cultures, increasing the apparent proportion of IgA switched cells with time. Taken together, the above studies suggest that C-NHEJ is the primary mechanism for CSR, but in its absence A-EJ pathways can perform S–S recombination, although at slower rates.

3.1.2. S–S junctions in XRCC4–Lig4-deficient B cells

S–S junctions in patients with hypomorphic *Lig4* mutations, in *xrcc4*$^{-/-}$ *p53*$^{+/-}$ or *xrcc4*$^{-/-}$*p53*$^{-/-}$ mouse B cells, and in Lig4-deficient CH12F3 cells show greatly increased lengths of microhomology (Han and Yu, 2008; Pan-Hammarstrom *et al*., 2005; Yan *et al*., 2007). In the *xrcc4*$^{-/-}$ mice and human Lig4 hypomorphs, many junctions (Sμ–Sε or Sμ–Sα) have up to 10 bp or more of identity. Most striking is the fact that although in WT cells in these experiments, 30–50% of junctions are direct, that is, have 0 bp of microhomology and no insertions, there are almost no (0–2%) direct junctions in cells lacking XRCC4 or Lig4 (Boboila *et al*., 2010b; Han and Yu, 2008; Pan-Hammarstrom *et al*., 2005; Yan *et al*., 2007). Perhaps in the absence of the XRCC4–Lig4 complex, end-joining is slower and DSBs are exposed and subject to end-processing, resulting in ss tails which use microhomology to recombine with the other S region DSBs.

Short insertions (mostly 1 bp) are significantly reduced at Sμ–Sα junctions in Lig4-deficient patients, but increased at the Sμ–Sγ junctions from the same individuals. In fact, a significantly increased frequency of insertions is the only altered feature of Sμ–Sγ junctions in these patients (Pan-Hammarstrom *et al*., 2005). Insertions are also increased at the Sμ–Sγ junctions in *xrcc4*$^{-/-}$ mice (Yan *et al*., 2007). Thus, as already discussed above, short insertions could theoretically be generated by C-NHEJ, but are also generated during A-EJ. This also suggests that in the absence of XRCC4–Lig4, and as found for many other DNA repair factors (in Tables 3.1 and 3.2), the Sμ–Sα and Sμ–Sγ junctions might be resolved differently. Perhaps the lower amount of homology between Sμ and Sγ results in generation of more mutations/insertions during the recombination process.

Thus, increased length of microhomology and lack of direct joining seem to be hallmarks for A-EJ, or more specifically, XRCC4–Lig4-independent end-joining. However, an increased or reduced frequency of insertions, depending on the S regions involved, might also be typical features.

3.1.3. Ku70–Ku80 deficiencies reduce CSR

As these NHEJ proteins are very important for cell viability and are essential for development of B cells, studies to determine whether they are crucial for CSR are challenging. Transgenic Ig H and L genes need to be supplied in order to obtain B cells. In early studies, two groups found very little CSR in Ku70- or Ku80-deficient B cells, but in both of these studies, cells proliferated very poorly (Casellas *et al*., 1998; Manis *et al*., 1998). However, under robust induction conditions, Ku70- and Ku80-deficient cells will undergo CSR, although the levels are 20–50% of WT (Boboila *et al*., 2010b).

3.1.4. S–S junctions in Ku-deficient cells

Similar to cells deficient in XRCC4–Lig4, the amount of microhomology found at S–S junctions in Ku70 cells is increased, and fewer direct junctions are observed, about one-third of that in WT cells (Boboila *et al.*, 2010b). Therefore, although Ku70–Ku80 focus recombination to the DNA ends and are important components of NHEJ machinery, direct junctions are still detected in its absence, unlike in XRCC4 or Lig4 deficiencies. Surprisingly, junctions in cells lacking both Ku70 and Lig4 have more direct joins than in cells lacking only Lig4, suggesting that Ku still functions at DSBs in Lig4-deficient cells, but that a different ligase is used (Boboila *et al.*, 2010b). Ku thus seems to promote microhomology-mediated A-EJ at S–S junctions in the absence of Lig4. To explain this unexpected result, Boboila *et al.* (2010a,b) suggested that Ku influences whether Ligase I or Ligase III is used; Ligase III has been shown to permit direct end-joining unlike Ligase I (Chen *et al.*, 2000; Cotner-Gohara *et al.*, 2008). Another possibility suggested by the authors is differential recruitment of DNA Pols μ or λ by Ku, although whether these Pols participate in CSR has not been demonstrated.

3.1.5. DNA-PKcs contributes to CSR

Several studies have examined the contribution of DNA-PKcs to CSR, and they all found some degree of impairment of CSR, although the extent varied. Similar to Ku or XRCC4–Lig4 KO mice, DNA-PKcs KO mice require transgenic IgH and IgL chains in order for B cells to develop. Some studies used the natural mouse mutant of DNA-PKcs, severe combined immunodeficiency (SCID). These mice have very low levels of protein that lacks kinase activity (Bosma *et al.*, 2002; Cook *et al.*, 2003). Also examined were mice with a targeted deletion of DNA-PKcs (Manis *et al.*, 2002), or mice lacking the C terminus of DNA-PKcs (Kiefer *et al.*, 2007). CSR efficiency was either nearly normal (Kiefer *et al.*, 2007), or reduced about two- to threefold (Bosma *et al.*, 2002; Cook *et al.*, 2003), or completely absent, except for IgG1 CSR (Manis *et al.*, 2002). The difference between the DNA-PKcs-null and SCID mice suggests that the kinase activity of DNA-PKcs might be replaceable, perhaps by ATM during CSR (Callen *et al.*, 2009; Shrivastav *et al.*, 2009), and that DNA-PKcs might have a noncatalytic role in CSR, for instance, by mediating synapse formation between DSBs in two different S regions (DeFazio *et al.*, 2002). It is also possible that the variances might be due to differences in CSR induction conditions, the mouse strains used, and the controls used between labs (Cook *et al.*, 2003; Kaminski and Stavnezer, 2007; Kiefer *et al.*, 2007).

3.1.6. S–S junctions in DNA-PKcs-deficient mice

In DNA-PKcs-null cells, the Sμ–Sγ1 junctions appear to be indistinguishable from controls, perhaps consistent with the finding that this isotype showed little or no reduction in CSR frequency in this study (Manis *et al.*, 2002). S–S

junctions were analyzed in one of the studies on SCID mice, and a small increase in microhomology usage was observed (3.4 vs. 2.3 bp) (Cook *et al.*, 2003). However, this analysis was performed on a mixture of junctions (Sμ–Sγ, Sγ–Sε, and Sμ–Sε), which may skew the results because Sμ–Sε and Sμ–Sα junctions tend to show longer microhomologies than Sμ–Sγ junctions when NHEJ is impaired (Pan-Hammarstrom *et al.*, 2005; Yan *et al.*, 2007). A separate analysis of Sμ–Sα junctions from SCID mice indeed showed a significant increase of microhomology (Q. Pan-Hammarström., unpublished). These results suggest that the kinase activity of DNA-PKcs might have a role in the end-joining process during CSR, probably through its ability to activate the endonuclease activity of Artemis, which is important for preparing a subset of DSBs for recombination (Ma *et al.*, 2002), and/or by its ability to regulate the unraveling of DNA ends at DSBs for end-processing by other enzymes (Meek *et al.*, 2007). Both of these hypotheses are supported by recent studies showing that DNA-PKcs and Artemis are required for preventing accumulation of chromosome breaks at the IgH locus in B cells induced to switch in culture (Franco *et al.*, 2008), and that the pattern of S–S junctions is altered in Artemis-deficient patient B cells (see below) (Du *et al.*, 2008b). However, the CSR defect is more severe and the frequency of AID-dependent IgH locus breaks is higher in DNA-PKcs-null as compared to the Artemis null-mice. This suggests that there is an Artemis-independent role for DNA-PKcs, consistent with findings for DSB repair in other systems (Lieber, 2010b; Meek *et al.*, 2007).

3.1.7. Cernunnos/XLF stimulates XRCC4–Lig4

Cernunnos/XLF is the latest addition to the NHEJ machinery (Ahnesorg *et al.*, 2006; Buck *et al.*, 2006a). In humans, mutations in the *Cernunnos/xlf* gene result in a rare, autosomal recessive disorder characterized by microcephaly and immunodeficiency. The profound T and B cell lymphopenia of these patients can be explained by defective V(D)J recombination (Buck *et al.*, 2006a). Although V(D)J recombination in a transiently transfected plasmid assay is severely impaired in *cernunnos/xlf*$^{-/-}$ mouse ES cells (Zha *et al.*, 2007), chromosomal V(D)J recombination is similar to WT in pro B cells from the KO mice, and only a small decrease in mature lymphocytes was observed. The results suggest there might be a lymphocyte-specific compensatory mechanism in mice (Li *et al.*, 2008). *In vitro* biochemical studies indicate that Cernunnos/XLF stimulates incompatible DNA end ligation by XRCC4–Lig4 (Gu *et al.*, 2007b; Lu *et al.*, 2007; Tsai *et al.*, 2007).

CSR in cultured mouse splenic *cernunnos/xlf*$^{-/-}$ B cells is reduced by 50% (Li *et al.*, 2008). Similar to Ku70-deficient cells, the frequency of Sμ–Sγ1 direct joints is reduced, along with a small increase in the average length of junctional microhomology (Li *et al.*, 2008). As some direct joints are still observed, this indicates that a subset of DNA ends can be directly joined in the absence of Cernunnos/XLF. It is unclear though whether this type of

Cernunnos/XLF-independent joining mechanism is similar to the Lig4–Ku70-independent A-EJ.

3.1.8. Artemis appears to be important for recombination of a subset of AID-induced DSBs during CSR

Artemis is an ssDNA 5′ to 3′ exonuclease, but when phosphorylated by DNA-PKcs, it acquires endonuclease activity on hairpins and 3′ overhangs (Ma *et al.*, 2002). The DNA-damage response factors ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad3-related) kinases have also been shown to phosphorylate Artemis (Zhang *et al.*, 2004). Artemis is essential for V(D)J recombination due to its ability to open hairpins, and it appears to be involved at the end-processing step in NHEJ repair of a subset of lesions caused by γ irradiation (Kurosawa and Adachi, 2010; Riballo *et al.*, 2004). Artemis KO mice need to be supplied with preassembled IgH and L chains for B cell development to proceed normally (Rooney *et al.*, 2005). In humans, Artemis deficiency causes RS-SCID (radiosensitive severe combined immune deficiency) or combined immunodeficiency, diseases characterized by an increased sensitivity to γ irradiation and defective V(D)J recombination (Evans *et al.*, 2006; Noordzij *et al.*, 2003; van Zelm *et al.*, 2008).

The role for Artemis in class switching was first thought to be insignificant since both immunoglobulin levels and Sμ–Sγ1 junctions in Artemis-deficient mice were normal (Rooney *et al.*, 2005). However, in subsequent studies, Artemis was shown to be involved in class switching. The number of switch junctions in peripheral blood from Artemis-deficient patients recovered by PCR is lower than in healthy individuals (Du *et al.*, 2008b). Furthermore, the proportion of IgA-expressing Peyers patch cells is decreased by ∼40% in immunized conditional Artemis KO mice (Rivera-Munoz *et al.*, 2009). In mouse B cell cultures, using conditional Artemis KOs, CSR to IgG3 is reduced by ∼25%, although switching to other IgG isotypes is similar to WT (Rivera-Munoz *et al.*, 2009). Most important, Artemis-deficient mouse B cells exhibit more AID-dependent IgH locus chromosome breaks than WT cells, suggesting that Artemis is involved in recombination of S region DSBs during CSR (Franco *et al.*, 2008).

3.1.9. Sμ–Sα junctions in Artemis-deficient mouse and human B cells show increased microhomology

The Sμ–Sα junctions in Artemis-deficient patients show a complete lack of direct junctions, and increased usage of long microhomologies (Du *et al.*, 2008b; Rivera-Munoz *et al.*, 2009). However, the Sμ–Sγ junctions are normal in mice (Rooney *et al.*, 2005). No Sμ–Sγ junctions could be amplified from Artemis-null patients, but a few junctions could be detected in a patient carrying hypomorphic mutations. Although the Sμ–Sγ junctions from this latter patient are normal, a high frequency of sequential Sγx to Sγy CSR (Sμ–Sγ1–Sγ2, Sμ–Sγ3–Sγ1, and Sμ–Sγ3–Sγ2) was observed, and the resulting

Sγx–Sγy junctions showed increased junctional microhomology (Du *et al.*, 2008b). Perhaps the increased microhomology observed at Sγx–Sγy junctions is due to the high amount of homology between the different Sγ regions, unlike between Sμ and Sγ. Note that this type of sequential CSR is rarely observed in WT cells, indicating that a direct switch from Sμ to one of the downstream of Sγ is strongly preferred in normal IgG switching. These results, together with the finding of increased CSR-associated chromosome IgH breaks in Artemis-deficient cells, are consistent with the hypothesis that Artemis is involved in processing a subset of DSBs. Although the structure and chemistry of the DNA ends involved in this subset of DSBs are unknown, it has been proposed that these ends might be ''damaged'' or more ''complex'' and thus require more time for repair and an involvement of the nuclease activity of Artemis (Riballo *et al.*, 2004). The key function of Artemis in V(D)J recombination is to open the hairpin structures at the coding ends. However, generation of DSBs by AID–UNG–APE does not predict such a structure. It is possible that the palindromic sequences present in the S regions could generate stem-loop structures, or hairpin-like structures (see Fig. 3.2B). Alternatively, Artemis can cleave other types of ss tails. Both ATM and DNA-PKcs can regulate the nuclease activity of Artemis; it remains unclear, however, which kinase or whether both are required for this activity during CSR.

3.2. ATM is important for repair of DSBs during CSR

3.2.1. Function of ATM during CSR

ATM is a ser/thr protein kinase, a member of the phosphoinositol 3-kinase-like kinase (PIKK) family, which includes DNA-PKcs and ATR, all of which function in DNA break responses. Upon activation by binding of the Mre11–Rad50–Nbs1 (MRN) complex to DSBs, ATM accumulates at repair foci and orchestrates binding and activation of DSB repair proteins and subsequent repair of the DSBs. ATM also initiates a cell-cycle checkpoint until repair is complete (Bakkenist and Kastan, 2003; Downs *et al.*, 2007). In $atm^{-/-}$ mice, V(D)J recombination is impaired, resulting in DSBs in Ig loci that are maintained over several cell generations (Bredemeyer *et al.*, 2006; Callen *et al.*, 2007; Vacchio *et al.*, 2007). Both repair and the cell-cycle checkpoints are compromised, as $atm^{-/-}$ lymphocytes continue to replicate despite chromosomal breaks (Franco *et al.*, 2006; Ramiro *et al.*, 2006).

CSR is reduced about threefold at each cell division cycle in cultured $atm^{-/-}$ splenic B cells relative to WT B cells (Lumsden *et al.*, 2004; Reina-San-Martin *et al.*, 2004). In B cells induced to switch in culture, the Sμ region undergoes translocations with the *c-myc* gene eight times more frequently in $atm^{-/-}$ cells than in WT cells (Ramiro *et al.*, 2006). Thus, it seems likely that when $atm^{-/-}$ B cells are activated to switch, DSBs are generated as usual, are maintained longer than usual, and do not undergo normal Sμ–Sx recombination. This can result in aberrant recombinations with other chromosomes.

Ataxia telangiectasia (A-T) patients often have IgA and IgG subclass deficiencies, and have peripheral blood lymphocytes with fewer Sμ–Sα junctions than normal individuals, similar to patients with mutated Nbs1 or Mre11, and consistent with reduced CSR (Lahdesmaki *et al.*, 2004; Pan *et al.*, 2002). Taken together, it appears possible that during CSR, ATM organizes the repair complex and might contribute to the correct juxtaposition of DSBs during the long-range interaction required for accurate S–S recombination. However, ATM does not appear to affect the cell cycle and cell proliferation during CSR, consistent with data suggesting that the G1 checkpoint is suppressed during CSR (Guikema *et al.*, 2010; Lumsden *et al.*, 2004; Phan and Dalla-Favera, 2004).

3.2.2. S–S junctions have increased microhomology in *atm*$^{-/-}$ cells

Sμ–Sα recombination junctions in A-T patients' B cells are characterized by a strong dependence on microhomologies and are devoid of normally occurring mutations and insertions around the junctions (Pan *et al.*, 2002). More than 60% of the junctions exhibit a microhomology of $\geq$4 bp with the longest being 21 bp and direct joints are markedly reduced (2% vs. 18% in WT) (Du *et al.*, 2008b).

Although the pattern of Sμ–Sα recombination junctions in A-T or DNA Lig4-deficient patients is almost identical, the pattern of Sμ–Sγ junctions is, however, different between the two groups. Significantly increased microhomology at the Sμ–Sγ junctions is only observed in A-T patients (Pan-Hammarstrom *et al.*, 2005). Furthermore, the frequency of mutations near the S junctions is reduced in A-T but normal in Lig4 deficient patients. Thus, in the absence of ATM or Lig4, depending on the S regions, the alternative, or backup repair mechanism appears to be different.

Only one of the two ATM KO mouse studies (Lumsden *et al.*, 2004) found a significant, but small, increase in microhomology length at Sμ–Sγ1 junctions, similar to the Sμ–Sγ junctions from A-T patients (Pan *et al.*, 2002) (Tables 3.1 and 3.2). If the microhomology-based pathway is a more attractive alternative for Sμ–Sα recombination due to the higher degree of homology between Sμ and Sα, as compared to Sμ and Sγ, one should observe a more pronounced increase in microhomologies in ATM-deficient mouse B cells when the Sμ–Sα junctions are analyzed. Indeed, increased microhomology usage is observed at Sμ–Sα junctions (5.6 vs. 2.7 bp in WT cells) (Q. Pan-Hammarström. *et al.*, unpublished data). Thus, the differences between human and mouse studies appear to be due to the different types of S–S junctions analyzed, and as true for other DNA repair mutants, the effect of ATM deficiency on increasing junctional microhomology is much more pronounced at Sμ–Sα junctions than at Sμ–Sγ junctions.

The exact function of ATM in the predominant C-NHEJ pathway in CSR remains unclear. One role of ATM is to recruit and/or activate other

DNA-damage response factors, such as γH2AX, 53BP1, MDC1, the MRN complex, and to activate the ubiquitin signaling cascade, thus configuring the DNA termini for subsequent repair steps (Lieber, 1999, 2003). Although the S–S junctions from cells deficient in either γH2AX, 53BP1, Mre11, or NBS differ from those in ATM-deficient cells, all these proteins are affected simultaneously in ATM-deficient cells. This might result in unprotected DSBs that recombine very slowly, and therefore become subject to end-processing, which results in exposed ss ends that recombine using microhomology. Another possibility is that ATM may have a more direct role in the end-processing step through regulation of nucleases that participate in C-NHEJ or A-EJ, for example, Mre11, CtIP, and Artemis. Mre11 and CtIP process DSBs to produce ss tails (Buis *et al.*, 2008; Mimitou and Symington, 2009). ATM has been shown to inhibit Mre11-dependent end-degradation activity, thereby inhibiting microhomology-mediated end-joining (Rahal *et al.*, 2010). It seems likely that whether microhomology is observed at S–S junctions is determined by the types of substrates available for recombination. Artemis is activated by the ATM signaling pathway and is required for repair of a subset of radiation-induced DSBs (Riballo *et al.*, 2004). Although the Sμ–Sα junctions from Artemis-deficient patients share many features of those from A-T patients (Du *et al.*, 2008b), this cannot explain the major effect of ATM, as Artemis has only a small effect on CSR efficiency. Also, the MMR proteins MSH2, MSH6, MLH1, PMS2, Exo1, and RPA1 have been identified as ATM/ATR substrates (Matsuoka *et al.*, 2007), and mice deficient in these proteins have reduced CSR (discussed below). Similar to A-T patients, increased microhomology at Sμ–Sα or Sμ–Sγ junctions is found in B cells from PMS2-deficient patients, in Mlh1- or Pms2-deficient mice, and in Mlh1–Msh2 doubly deficient mice (Ehrenstein *et al.*, 2001; Peron *et al.*, 2008; Schrader *et al.*, 2002, 2003a). Finally, ATM and DNA-PKcs have overlapping functions during CSR, as ablation of the kinase activity of both proteins results in a greater defect in CSR than expected by addition of the effects of the individual mutants. Interestingly, double ablation results in an increased frequency of large insertions at Sμ–Sγ1 junctions relative to single deficiencies (Callen *et al.*, 2009). Perhaps ATM/DNA-PKcs are important for correct synapsis and rapid recombination of Sμ–Sx region DSBs, thereby preventing incorporation of exogenous DNA segments. These possible roles of ATM during CSR are not mutually exclusive and might all contribute to its effect on CSR.

3.3. Mre11–Rad50–Nbs1 (MRN) might have multiple roles during CSR

3.3.1. Cell proliferation and CSR are reduced in MRN-deficient cells

The MRN complex continuously scans the DNA duplex for DNA breaks, and binds DSBs very rapidly after their formation (Berkovich *et al.*, 2007; Lee and Paull, 2005; Moreno-Herrero *et al.*, 2005). Mre11 is a globular

protein that binds to DNA, whereas Rad50 forms a long coiled-coil that at its middle abruptly reverses direction, forming a loop with a zinc hook at its apex. The hooks from two Rad50 molecules associate homotypically, and this is thought to be important for holding two DNA duplexes together at the DSB (de Jager *et al.*, 2001; Hopfner *et al.*, 2000). The finding of increased translocations of Ig genes with *c-myc* in MRN-deficient B cells suggests that the MRN complex is involved in organizing efficient and accurate S–S recombination (Reina-San-Martin *et al.*, 2005).

Once MRN binds a DSB, ATM binds the complex via Nbs1, becomes activated, and phosphorylates several substrates, including Nbs1, 53BP1, p53, Chk2, and H2AX. This causes a further accumulation of MRN and other repair proteins, and also activates cell-cycle checkpoints (Cerosaletti *et al.*, 2006; Difilippantonio *et al.*, 2005; Falck *et al.*, 2005). Thus, MRN is upstream of a cascade of events that function to sense the DSB, resulting in repair by end-joining or by homologous recombination. When a DSB is encountered, the conformation of Mre11 and Rad50 changes, resulting in unwinding of the DNA ends at the break. Mre11 promotes microhomology-mediated end-joining (Rass *et al.*, 2009; Xie *et al.*, 2009). Mre11 is an endonuclease and an exonuclease which can create ss tails (Paull and Gellert, 2000), and MRN recruits the 5′ ss exonuclease CtIP, which creates 3′ ss tails that can participate in homologous recombination or perhaps in A-EJ during CSR (Bunting *et al.*, 2010; Mimitou and Symington, 2009; Sartori *et al.*, 2007). Consistent with this latter hypothesis, knockdown of CtIP in the CH12F3 cell line results in reduced microhomology at Sμ–Sα junctions (Lee-Theilen *et al.*, in press). Null mutations in any component of MRN are lethal, and hypomorphic mutations result in aberrant chromosomes and translocations (Reina-San-Martin *et al.*, 2005). Nbs1 mutations cause the Nijmegen breakage syndrome (NBS) and Mre11 mutations cause ataxia-telangiectasia-like disorder (ATLD) in patients, both characterized by immunodeficiency and increased sensitivity to ionizing radiation and other DSB-inducing agents (Carney *et al.*, 1998; Varon *et al.*, 1998). Very recently, Rad50 mutations have been reported in two patients, one with NBS-like disorder (Waltes *et al.*, 2009), and the other with common variable immunodeficiency (CVID) (Offer *et al.*, 2010).

CSR is reduced two- to threefold in cultured splenic B cells from mice in which the Nbs1 gene is inactivated by a conditional mutation (Kracker *et al.*, 2005; Reina-San-Martin *et al.*, 2005). Mice with a conditional KO of the Mre11 gene or expressing an exonuclease-deficient mutant of the Mre11 gene, knocked into the normal genomic locus, have approximately four- and twofold reduced CSR, respectively (Dinkelmann *et al.*, 2009). In order to control for the effect of Nbs1 or Mre11 mutations on cell proliferation, CSR was assayed in cells stained with CFSE, measuring switched cells at each cell division. However, it is still likely that the CSR defect observed in MRN-deficient cells is partially due to impaired cell proliferation, as discussed

above for Ku70- and Ku80-deficient B cells. Patients with hypomorphic mutations in Nbs1 or Mre11 also have a lower percentage of peripheral blood lymphocytes that have undergone CSR, as assayed by detection of Sμ–Sα junctions in these cells (Lahdesmaki *et al.*, 2004; Pan *et al.*, 2002).

The relationship between MRN and Ku70–Ku80, both of which bind to DSBs, is unclear. Although Ku70–Ku80, due to its great abundance, is likely to bind DSBs even faster than MRN, AID-induced DSBs have been shown to colocalize with Nbs1 foci by immunofluorescence *in situ* hybridization (*in situ*-FISH) in mouse splenic B cells induced to undergo CSR (Petersen *et al.*, 2001). It is possible that Ku70–Ku80 binding might interfere with the unwinding of DNA ends by MRN.

3.3.2. Junctions in MRN-deficient cells

Sμ–Sα junctions in B cells from patients with NBS and ATLD show a trend toward the use of longer microhomologies, although not as striking as in those derived from A-T patients (A-T > NBS > ATLD > control). The increase is significant in NBS but not in ATLD patients (Lahdesmaki *et al.*, 2004; Pan *et al.*, 2002). Unlike A-T patients, the proportion of Sμ–Sα junctions with direct joints is not significantly reduced in NBS or ATLD patients. Furthermore, unlike A-T patients (in whom insertions are lacking), insertions are seen at a normal frequency in NBS patients (30%), and they are significantly increased in ATLD patients (40% in ATLD vs. 25% in controls). The frequency of mutations surrounding the Sμ–Sα junctions is also normal in NBS and ATLD patients. However, C to T transitions, the substitutions occurring most often in controls, are never observed at, or near the Sμ–Sα junctions in ATLD patients (Lahdesmaki *et al.*, 2004). The increased frequency of insertions and altered mutation pattern at CSR junctions may suggest that the end-processing activity of Mre11 is involved in resection at DSBs during CSR. This activity is, however, specific for CSR, as the SHM pattern in the V regions from these patients is normal (Du *et al.*, 2008a). These results support the hypothesis that DSBs are not involved during SHM in V regions.

The Sμ–Sγ junctions show a smaller increase in microhomology in Nbs1 patients, with borderline significance, but are normal in ATLD-deficient patients. Sμ–Sγ junctions derived from Nbs1-hypomorphic mice and from Mre11-deficient or Mre11-exonuclease mutant mice do not differ significantly from those from WT mice (Dinkelmann *et al.*, 2009; Kracker *et al.*, 2005; Reina-San-Martin *et al.*, 2005).

To explain these results, it is likely that the MRN complex has multiple functions in CSR. The trend toward increased microhomology at Sμ–Sα junctions in both NBS1 and ATLD patients might be explained by the fact that MRN deficiency reduces ATM activation. In cells with impaired MRN function, DSBs are repaired more slowly, due to reduced activation of ATM and perhaps reduced synapsis of the DSBs. The delayed recombination

might allow other nucleases to end-process the DSBs, and also provide time for homology searching. The increase in microhomology at the CSR junction, compared to ATM-deficient cells is, however, rather moderate and the mutation pattern at CSR junction is altered in ATLD patients, suggesting that MRN is not functioning simply to recruit and activate ATM during CSR. One possibility is that the nuclease activities of Mre11 and its ability to recruit CtIP will promote the use of A-EJ during CSR. Thus, in the absence of the MRN complex, both the ATM-dependent end-joining and the microhomology-based A-EJ will be impaired, resulting in reduced CSR efficiency, but moderate changes in the resulting CSR junctions.

3.4. 53BP1 performs multiple functions during CSR

3.4.1. CSR is greatly reduced in 53BP1-deficient cells

53BP1 accumulates at DSBs within 2 min after exposure to ionizing radiation (Pryde *et al.*, 2005), and mice deficient in this protein are highly sensitive to ionizing radiation (Morales *et al.*, 2006), but very little is known about the function of 53BP1. Its initial recruitment to DSBs is not dependent on any other known protein (Schultz *et al.*, 2000). However, subsequent accumulation of 53BP1 is dependent on ATM-phosphorylated γH2AX and MDC1 (Adams and Carpenter, 2006; Ward *et al.*, 2003). Phosphorylated MDC1 recruits the E3 ubiquitin ligase RNF8 which ubiquitinates RNF168, which in turn catalyzes polyubiquitin chains at DNA-damage sites, recruiting 53BP1 to DSBs (Doil *et al.*, 2009). 53BP1 also induces phosphorylation of ATM and ATR (Mochan *et al.*, 2004), and thus, it might also increase MRN activity and its accumulation at DSBs. 53BP1 is also a substrate of ATM and DNA-PKcs (Bothmer *et al.*, 2010). Both RNF8 and RNF168 have been shown to contribute to DSB repair and to CSR, as expected from their ability to recruit 53BP1 (Li *et al.*, 2010; Ramachandran *et al.*, 2010; Santos *et al.*, 2010; Stewart *et al.*, 2003, 2007, 2009). However, 53BP1-deficient B cells show a much greater reduction in CSR (<10% of WT B cells) than the RNF8-deficient B cells (~30–50% of WT) (Manis *et al.*, 2004; Ramachandran *et al.*, 2010; Santos *et al.*, 2010). Thus, 53BP1 has functions in CSR that do not depend upon RNF8. Of all the DNA-damage response factors involved in class switching, the absence of 53BP1 has the most severe impact.

53BP1-deficient cells do not have a dramatic increase in general chromosome instability, unlike $atm^{-/-}$ and $h2ax^{-/-}$ cells, but a much higher proportion of the chromosomal aberrancies in $53bp1^{-/-}$ cells involve the IgH locus, suggesting that 53BP1 has a special role at this locus (Adams and Carpenter, 2006; Franco *et al.*, 2006). It is possible that 53BP1 enhances the interaction between donor and acceptor S regions (Adams and Carpenter, 2006; Manis *et al.*, 2004). This is supported by the observation that mice lacking 53BP1 have decreased V(D)J recombination involving distal V genes (Difilippantonio *et al.*, 2008). In addition, intra-S region

recombination is enhanced in these mice compared to Sμ–Sx recombination (Reina-San-Martin *et al.*, 2007). The yeast homolog of 53BP1, Rad9, has been shown to oligomerize in association with DNA lesions, perhaps resulting in binding of the DNA to a nuclear scaffold (Usui *et al.*, 2009). If also true for 53BP1, this might promote interactions between two different S region breaks. Interestingly, intra-S region deletional recombination is also greatly increased in B cells lacking the C-NHEJ proteins, Ku70 and even more in cells lacking both Lig4 and Ku70 (Boboila *et al.*, 2010a). In these cells, both general chromosome and chromosome 12 breaks are greatly increased. The increased internal S region deletions might fit with the hypothesis that Ku70–Ku80 is involved in holding DSBs to the nuclear scaffold and synapsis of DSBs during end-joining. However, it is also possible that DSBs are exposed and inefficiently recombined in both 53BP1- and in Ku70- and Lig4-deficient cells, resulting in an increased end resection and generation of DNA ends with terminal microhomology. Due to the repetitive nature of S regions, there might thus be more chances to generate DNA ends with the same terminal microhomology within a single S region than two different S regions, resulting in a preference for internal S region recombination in these cells. These possibilities are not mutually exclusive.

3.4.2. S–S junctions in *53bp1*$^{-/-}$ cells show increased insertions

The data on S junctions are somewhat inconclusive. Sμ–Sγ1 switch junctions in *53bp1*$^{-/-}$ mice are normal with regard to microhomology usage and mutation frequency (Manis *et al.*, 2004; Reina-San-Martin *et al.*, 2007). However, one study found unusually long insertions at Sμ–Sγ1 junctions (Reina-San-Martin *et al.*, 2007). This appears consistent with improper synapsis of donor and acceptor S regions, allowing incorporation of other DNA segments.

In a more recent study, paired DSBs were introduced using the I-Sce1 meganuclease into the IgH locus (outside S regions; located upstream of Sμ and downstream of Sγ1) in *aid*$^{-/-}$ cells. Deficiency of 53BP1 in this experimental system resulted in increased DNA end resection at the I-Sce1-induced DSBs, reduced numbers of precise joints, and the resected DNA was preferentially repaired by microhomology-mediated A-EJ (Bothmer *et al.*, 2010). The authors thus suggested that, in addition to its role in synapsis of distal DSBs, 53BP1 might interfere with end resection and A-EJ, and therefore promote C-NHEJ. 53BP1 has also been shown to inhibit end resection during homologous recombination in a different system (Bunting *et al.*, 2010). A surprising finding in the Bothmer *et al.* (2010) study was that inhibition of ATM kinase decreases DNA end resection at I-Sce1-induced DSBs in *aid*$^{-/-}$ and *aid*$^{-/-}$*53bp1*$^{-/-}$ cells. As described above, ATM deficiency causes significant increases in microhomology at CSR junctions. Perhaps processing of the I-Sce1-induced

DSBs is different from AID-induced S region DSBs. In these experiments the amount of recombination at the I-Sce1-induced DSBs was very low, occurring in less than 1% of cells, unlike normal S–S recombination. Bothmer *et al.* (2010) also found that inhibition of ATM partially ameliorates the severe defect in chromosomal CSR observed in the absence of 53BP1. Perhaps this is due to the fact that ATM recruits MRN and CtIP to DSBs, and that in the absence of 53BP1 this results in rampant resection that interferes with CSR. To address this possibility, it would be useful to examine the S–S junctions in $atm^{-/-}53bp1^{-/-}$ cells.

3.4.3. S–S junctions in RNF8-deficient mice show increased microhomology and insertions

Sμ–Sγ1 junctions in RNF8-deficient B cells have increased microhomology: 34% of the junctions have 0 or 1 bp identity in $rnf8^{-/-}$ cells, compared to 61% of WT cells. Also, 24% of junctions in the KO cells have insertions that are $\geq$2 bp, compared to 7% of the WT junctions (Santos *et al.*, 2010). This differs from $53bp1^{-/-}$ cells, suggesting that RNF8 has a role apart from its ability to recruit 53BP1.

3.4.4. S–S junctions in RNF168-deficient patients (RIDDLE syndrome) show increased microhomology

There is thus far no human disease that has been linked to mutations in the gene encoding 53BP1. A recent study has, however, described a patient who suffered from the RIDDLE syndrome (radiosensitivity, immunodeficiency, dysmorphic features, and learning difficulties) (Stewart *et al.*, 2007). Cells from this patient lack the ability to recruit 53BP1 to the site of DSBs. Mutations in the gene encoding an ubiquitin ligase RNF168 were later identified in this patient (Stewart *et al.*, 2009). Significantly increased microhomology is observed at Sμ–Sα junctions amplified from this patient, with 94% of junctions exhibiting a microhomology of 4 bp or more (Stewart *et al.*, 2007). Also, the frequency of insertions at and mutations around Sμ–Sα junctions are reduced. The altered pattern of Sμ–Sα junctions in this patient and the increased microhomology at the Sμ–Sγ1 junction in the RNF8-deficient cells (Santos *et al.*, 2010) suggest that there could be a shift toward the use of A-EJ when RNF8/RNF168 are defective.

3.5. H2AX, activated in response to DSBs, contributes to CSR

3.5.1. H2AX-deficient B cells have reduced CSR

H2AX is a variant of histone H2A, representing about 15% of the cellular pool of H2A. It is randomly incorporated into nucleosomes (Rogakou *et al.*, 1998). Within seconds after formation of a DSB induced by ionizing radiation or by a restriction enzyme, the extended C terminal tail of H2AX

is phosphorylated by a PIKK, most frequently ATM (Burma *et al.*, 2001; Lou *et al.*, 2006). This phosphorylation spreads over a region estimated to span up to a megabase surrounding the break (Berkovich *et al.*, 2007; Downs *et al.*, 2007). ATM also phosphorylates 53BP1, Nbs1, and Mdc1, which then all bind to the phosphorylated tail of γH2AX, which serves as a docking site for these proteins. This results in a rapid recruitment of these factors, plus Mre11, Rad50, RNF8, and Brca1. γH2AX is required for the accumulation of these proteins into foci near DSBs (Celeste *et al.*, 2002; Fernandez-Capetillo *et al.*, 2003). However, mice lacking H2AX can still repair DSBs, although with lower efficiency, and they can still induce cell-cycle checkpoints (Celeste *et al.*, 2002), probably because the initial assembly of repair proteins, including MRN, ATM, and 53BP1, does not depend on γH2AX (Celeste *et al.*, 2003).

CSR to IgG3 and IgG1 in *h2ax*$^{-/-}$ B cell cultures is ~25–30% of WT, and this is not due to defective cell proliferation. The antigen specific IgG1 response to immunization is reduced to about 30% of WT mice (Celeste *et al.*, 2002; Franco *et al.*, 2006; Petersen *et al.*, 2001; Reina-San-Martin *et al.*, 2003). RNF8 and H2AX double KO mice show impairment of CSR at levels similar to those found in H2AX-deficient mice, suggesting that these two molecules function epistatically in the same pathway during CSR (Santos *et al.*, 2010).

Similar to cells deficient in either ATM, 53BP1, Mdc1, or having Mre11 or Nbs1 hypomorphic mutations, *h2ax*$^{-/-}$ B cells show numerous chromosome breaks and aberrant recombination events (Bassing *et al.*, 2003; Celeste *et al.*, 2002; Franco *et al.*, 2006; Ramiro *et al.*, 2006). In *h2ax*$^{-/-}$ B cells induced to switch in culture, there is a >10-fold increase in AID-dependent chromosome breaks within the IgH locus relative to WT cells, resulting in separation of the V genes and the 3′ end of the C_H genes and also translocations in metaphase chromosome spreads (Franco *et al.*, 2006). The breaks occur on both chromatids, indicating that they occur prior to the S phase, consistent with evidence that S region DSBs are present in the G1 phase (Petersen *et al.*, 2001; Schrader *et al.*, 2005).

3.5.2. S–S junctions are similar in WT and *h2ax*$^{-/-}$ cells

Although CSR is reduced, there is no decrease in the frequency of internal Sμ deletions in mouse H2AX-deficient cells induced to switch in culture, suggesting that H2AX is important for association between Sμ and acceptor S regions but not for short distance recombination. However, unlike several other DSB repair proteins, the amount of microhomology at Sμ–Sγ1 junctions is similar between h2ax$^{-/-}$ and WT mice, suggesting that H2AX does not regulate end-processing at DSBs. Since the structure of the DSBs is unaffected, H2AX does not determine whether recombination will occur by the C-NHEJ or A-EJ pathways. However, it is important to examine Sμ–Sα junctions to confirm this conclusion.

3.6. Altered S–S junctions in patients with AID or UNG deficiency

Very recently, Sμ–Sα junctions in humans with deficiencies in AID or UNG have been characterized. Sμ–Sα junctions from patients with a deletion of the C terminus of AID (preventing CSR) or lacking UNG show greatly increased microhomology (S. Kracker and A. Durandy, personal communication). This is also true for patients with haploid amounts of AID. In all these patients, there is a four- to ninefold increase in junctions with $\geq$10 bp microhomology. The most striking result is that 43% of the junctions show $\geq$10 bp microhomology in $ung^{-/-}$ patients. The increased microhomology may be because S–S recombination is very inefficient in these patients, allowing time for end-processing and preferential use of microhomology. $Ung^{-/-}$ cells have very few S region DSBs, and thus recombination might be delayed for this reason. Also, as the rare SSBs formed in UNG-deficient cells will not usually be near another SSB on the opposite chromosome, extensive processing from the SSB will be required in order to form a DSB. This could result in relatively long ss tails at DSBs. However, Sμ DSBs are at normal levels in mouse B cells expressing the C terminal AID deletion (Doi *et al.*, 2009), and only about twofold reduced in cells expressing haploid amounts of AID (J. E. J. Guikema and J. Stavnezer, unpublished data). Thus, the increased lengths of junctional microhomology found in these patients suggest that AID is providing a function that promotes efficient S–S recombination, thereby decreasing end-processing, or that AID actually recruits NHEJ proteins, thereby directing CSR toward the C-NHEJ pathway.

3.7. Mismatch repair-deficient cells have reduced CSR

3.7.1. Mismatch repair converts SSBs to DSBs during CSR

The MMR pathway contributes to induction of DNA breaks in S regions. In B cells deficient in various MMR proteins, CSR is reduced to 15–50% of WT levels (Bardwell *et al.*, 2004; Ehrenstein and Neuberger, 1999; Ehrenstein *et al.*, 2001; Li *et al.*, 2004b; Martin *et al.*, 2003; Martomo *et al.*, 2004; Schrader *et al.*, 1999). The major role of MMR in all cells is to correct misincorporated nts during DNA synthesis (Kunkel and Erie, 2005). This process involves recognition of the mismatch by a heterodimer of Msh2–Msh6 (for nucleotide substitutions and small loops) or by Msh2–Msh3 (for larger loops), followed by recruitment of the Mlh1–Pms2 heterodimer (Kunkel and Erie, 2005). The combined heterotetramer recruits replication factor C (RFC), the processivity factor PCNA, and exonuclease1 (Exo1) to a nearby nick, and together they excise the ss segment containing the mutated nucleotide (Genschel and Modrich, 2003; Genschel *et al.*, 2002). The excised ss patch can be hundreds of nucleotides in length *in vitro*, but the length *in vivo* is

unknown. MMR specifically repairs the newly synthesized DNA strand, thought to be due to its predilection to excise and resynthesize the nicked DNA strand (Kadyrov *et al.*, 2006).

The most attractive model for the role of MMR during CSR is to convert SSBs that are not near each other on opposite DNA strands to DSBs (Fig. 3.2C) (Schrader *et al.*, 2007; Stavnezer and Schrader, 2006). If the SSBs that are introduced by AID–UNG–APE are near each other on opposite DNA strands, they can spontaneously form a DSB, but if not, the SSBs do not form a DSB and are simply repaired. As S regions are large and the S–S junctions and DSBs appear to occur anywhere within S regions (Dunnick *et al.*, 1993; Min *et al.*, 2005; Schrader *et al.*, 2005), it seems unlikely that the SSBs would be sufficiently proximal to form a DSB in most instances. MMR could convert these distal SSBs to the DSBs that are required for CSR. Msh2–Msh6 can recognize and bind G:U mismatches created by AID activity (Wilson *et al.*, 2005). Mlh1–Pms2 and Exo1 would then be recruited, and Exo1 would excise from the nearest 5′ SSB created by AID–UNG–APE activity, toward the mismatched dU:dG. Exo1 is hypothesized to continue past the mismatch until it reaches an SSB on the other strand, thus creating a DSB. Although Exo1 can only excise in the 5′ to 3′ direction, Pms2 has nicking activity on dsDNA, creating nicks on the same strand as the initial SSB. This activity results in creation of additional SSBs located 5′ to the U:G mismatch, which serve as entrance sites for Exo1 (Kadyrov *et al.*, 2006).

Several experimental results support this model. First, B cells in which the tandem repeats of Sμ have been deleted ($S\mu TR^{-/-}$), which thus have very few AID hotspot targets, have only an approximately twofold reduction in CSR (Luby *et al.*, 2001). However, in these B cells, CSR is nearly ablated in the absence of Msh2, Mlh1, or Exo1 (Eccleston *et al.*, 2009; Min *et al.*, 2003). Second, the vast majority of S–S junctions in $msh2^{-/-}$ B cells occur within the Sμ tandem repeat region, whereas in WT cells they can also occur upstream of Sμ, where the AID target hotspots are infrequent (Ehrenstein and Neuberger, 1999; Min *et al.*, 2005). Third, and most importantly, LM-PCR experiments show that MMR-deficient B cells have fewer S region DSBs than WT B cells, and in Msh2- or Mlh1-deficient $S\mu TR^{-/-}$ cells, the DSBs are reduced nearly to levels found in $aid^{-/-}$ cells (Schrader *et al.*, 2007). These results are all consistent with the hypothesis that MMR is involved in end-processing from the SSBs sites, resulting in DSBs. This will create DSBs from SSBs that are too distal to spontaneously form a DSB.

3.7.2. MMR mutants have altered S–S junctions

3.7.2.1. Mlh1–Pms2 Many studies have examined S–S junctions from different MMR-deficient B cells in mice and humans, and found that the junctions differ between WT and MMR-deficient B cells as to the lengths of microhomology and the presence of insertions (Bardwell *et al.*, 2004;

Eccleston *et al.*, 2009; Ehrenstein *et al.*, 2001; Li *et al.*, 2004b; Martin *et al.*, 2003; Peron *et al.*, 2008; Schrader *et al.*, 2002). The clearest finding is that there is generally a large and highly significant increase in junctional microhomology in B cells lacking Mlh1 or Pms2. The effect appears greatest in Pms2-deficient cells, with average microhomology increasing by three- to fourfold relative to WT cells (Ehrenstein *et al.*, 2001). Also, in Pms2-deficient mice and humans, 16% of Sμ–Sγ3 (mouse) and ~50% of Sμ–Sα (human) junctions have ≥10 bp microhomology (Peron *et al.*, 2008; Schrader *et al.*, 2002). Although in Mlh1-deficient mouse B cells the increased microhomology is not as great as in Pms2-deficient cells, it is still significant (Eccleston *et al.*, 2009; Schrader *et al.*, 2002). The increase in junctional microhomology observed in Mlh1- or Pms2-deficient B cells might be due to the reduced numbers of DSBs, resulting in inefficient CSR and therefore additional end-processing at DSBs. It has also been proposed that Mlh1–Pms2 recruits NHEJ proteins, and thus in its absence A-EJ is used. A third possibility arises from the finding that reconstitution of MMR *in vitro* has demonstrated that Msh2–Msh6 can recruit Exo1 and repair nucleotide mismatches in the absence of Mlh1–Pms2 when there is a DNA nick 5′ of the mismatch. The Mlh1–Pms2 heterodimer limits the processing ability of the Exo1 protein, thereby decreasing the length of excision tracts in WT cells (Zhang *et al.*, 2005). Thus, it is possible that in the absence of Mlh1–Pms2, Exo1 might create longer ss tails that can be used for a microhomology search. These three possibilities are not mutually exclusive. Note, however, that Sμ–Sγ1 junctions in peripheral blood B cells from Pms2-mutant patients have decreased microhomology, although Sμ–Sα junctions from the same patients have increased microhomology (Table 3.2) (Peron *et al.*, 2008). As the patients do not have null mutations, perhaps some function of the heterodimer is retained and this might explain the different results from those in *pms2*$^{-/-}$ mice. However, these results suggest that loss of Pms2 function might have more than one effect on the structure of the DSBs, and that differential preference for use of homologies alters which effect dominates.

3.7.2.2. Msh2–Msh6 The results for the Msh2–Msh6 heterodimer and for Exo1 are not as clear. Only one study has reported the structure of S–S junctions in *msh2*$^{-/-}$ mouse B cells, and the results appear inconsistent with other studies using Msh2-deficient humans, mutant Msh2 (ATPase-deficient), or Msh6-deficient mice. Mouse *msh2*$^{-/-}$ B cells were reported to have a highly significant reduction in Sμ–Sγ3 microhomology lengths, with 81% having 0 or 1 bp, whereas 53% of WT cells had 0 or 1 bp of microhomology (Schrader *et al.*, 2002). Note, however, that these results included junctions with inserts in the 0 bp category, and 20% of the junctions in *msh2*$^{-/-}$ cells had inserts, whereas WT cells did not. If we exclude the junctions with inserts, then 59% of the junctions in *msh2*$^{-/-}$ cells have 0 or 1 bp microhomology, which is similar to WT. Also, patients with a mutation that

results in a complete lack of Msh2 show increased microhomology at Sμ–Sα junctions (Q. Pan-Hammarström *et al.*, unpublished data). Deletions of Msh2 eliminate MMR function and result in fewer S region DSBs (Schrader *et al.*, 2007). All the S region DSBs in these cells must arise from SSBs that are sufficiently near on opposite DNA strands to spontaneously form a DSB, or else end-processed by other enzymes to form a DSB.

Msh2 deficiency has been bred onto mice with the SμTR deletion. As described above, this nearly ablates CSR, yet junctions can be analyzed. The junctions show increased junctional microhomology (Eccleston *et al.*, 2009). In both Msh2-deficient cells and in $S\mu TR^{-/-}$ B cells, Sμ DSBs are reduced by about 50%, but in the $msh2^{-/-}S\mu TR^{-/-}$ cells, Sμ DSBs are greatly reduced, almost to the level observed in $aid^{-/-}$ cells (Schrader *et al.*, 2007). In these cells, S–S recombination is likely to be very inefficient, as the donor and acceptor S regions must have DSBs simultaneously. As hypothesized for other mutants above, it is possible that this delay in recombination of the DSBs would allow time for end-processing at the DSBs, resulting in ss DNA that could preferentially recombine by use of microhomologies. We hypothesize that differences in frequency of SSBs, and therefore the likelihood of spontaneously forming a DSB among different S regions, differences in activation conditions, and different preference for microhomology might explain the variations in results among different studies. Activation of human B cells in culture has been found to alter the pattern of S–S junctions (Q. Pan-Hammarström, unpublished).

Msh2 has ATPase activity, which is necessary for recruitment of Mlh1–Pms2, but an ATPase mutant Msh2–Msh6 heterodimer still binds mismatches (Lin *et al.*, 2004). B cells from mice with a mutation in the ATPase domain (Msh2–G674A) have 50% of WT CSR, not as low as in KO B cells (20% of WT in these experiments) (Martin *et al.*, 2003). The Sμ–Sγ3 junctions showed a trend toward increased junctional microhomology, although the increase was not significant. Similar to Sμ–Sγ3 junctions in $msh2^{-/-}$ cells, there was an increase in large junctional insertions in these cells (15% vs. 2% in WT cells).

Further evidence for a role for Msh2–Msh6 heterodimer in the absence of Mlh1–Pms2 comes from recent results in which the endonuclease activity of Pms2 was ablated (Pms2–E705K) in a knock-in mutant (van Oers *et al.*, 2010). CSR was reduced as much as in $pms2^{-/-}$ mice, but the S–S junctions did not show increased Sμ–Sγ3 microhomology. This finding is consistent with the hypothesis that the Mlh1–Pms2 proteins inhibit Exo1 processivity, and that the mutant Pms2 retains this function, thus preventing long ss tails at DSBs.

3.7.2.3. Exo1 Exo1-deficiency should eliminate all MMR function, as there is no other exonuclease known to substitute for Exo1. CSR to IgG and IgA is reduced to 20–30% of WT in $exo1^{-/-}$ mouse B cells (Bardwell *et al.*, 2004;

Eccleston *et al.*, 2009). Similar to the analysis of *msh2*$^{-/-}$ junctions when excluding insertions, Sμ–Sγ3 junctions did not show decreased microhomology. Bardwell *et al.* (2004) found no difference from WT, whereas Eccleston *et al.* (2009) found a modest increase in junctional microhomology (2.2 vs. 1.3 bp); both labs used the identical KO strain. However, as pointed out by the latter group, the difference between the two labs was the microhomology in WT cells, averaging 2.2 bp in the Bardwell study and 1.3 bp in the Eccleston study. In conclusion, the lack of all MMR function might result in a few DSBs with nearly blunt ends, due to nearby SSBS on opposite DNA strands, but also the reduced numbers of DSBs and ensuing delayed recombination efficiency might also allow increased end-processing of the SSBS and DSBs. These two effects might be differentially dominant, depending on induction conditions. Note, however, that neither is the cell cycle delayed nor is there increased apoptosis in MMR-deficient B cells relative to WT cells induced to undergo CSR.

3.7.2.4. Msh3 and Msh6 The Msh2–Msh6 heterodimer is specialized for recognition of single base pair mismatches, and has been shown to bind U:G mismatches (Wilson *et al.*, 2005). Msh6-deficiency does not significantly alter the Sμ–Sγ3 junctions in mouse splenic B cells, although there is slight increase in the use of microhomology (Li *et al.*, 2004b). Msh2 can heterodimerize with Msh3, in addition to Msh6. Although Msh3 deficiency does not reduce CSR, cells lacking Msh3 have increased insertions at S–S junctions (Li *et al.*, 2004b). As Msh2–Msh3 recognizes loop structures and can help to excise ss tails at DSBs, it is possible that it contributes to CSR, perhaps after DSB formation, helping to create DSBs that can recombine by direct joining, but not detectably affecting CSR frequency. However, it is difficult to make strong conclusions about the role of Msh3 in preventing long inserts, because in this same study WT littermates of *msh6*$^{-/-}$ mice also had long inserts at their S junctions.

3.7.2.5. Msh4–Msh5 This heterodimer binds Holliday junctions during meiosis and is essential for crossing-over during meiosis in spermatogenesis and oogenesis, and therefore deficiency of either of these proteins causes sterility (Edelmann *et al.*, 1999). By an examination of RNA transcripts by RT-PCR, it has been shown that no full-length normal Msh5 mRNA is expressed in splenic B cells in two mouse strains (C57BL/6 and BALB/c), nor was any Msh5 protein detected (Guikema *et al.*, 2008). The mRNA mutations identified were such that protein would not be expressed in the mouse B cells (Guikema *et al.*, 2008; Santucci-Darmanin *et al.*, 1999). Consistent with this, *msh5*$^{-/-}$ splenic B cells activated in culture switch at normal levels when the C57BL/6 strain was examined (Guikema *et al.*, 2008). Furthermore, the Sμ–Sγ3 junctions in *msh5*$^{-/-}$ and WT B cells induced to switch in culture are similar (Guikema *et al.*, 2008). However, when other *msh5*$^{-/-}$ mutants were analyzed on an MRL/Ipr or

FVB background, although no CSR defect was observed *in vitro*, both Sμ–Sγ3 and Sμ–Sα junctions amplified from *ex vivo* splenic B cells showed significantly increased microhomology relative to WT littermates (Sekine *et al.*, 2007). Significant increased usage of microhomology was also shown in junctions amplified from *ex vivo* splenic B cells in *msh4*$^{-/-}$ deficient cells (C57BL/6), although CSR in cells induced to switch in culture was identical to WT (Sekine *et al.*, 2007). To reconcile these differences, we note that mice lacking testis or ovaries, as true for Msh4- and Msh5-deficient mice, have low amounts of estrogen and progesterone (Carreau *et al.*, 2007; Oettel and Mukhopadhyay, 2004). Estrogen has been shown to stimulate antibody production and to stimulate AID gene transcription and protein levels (Erlandsson *et al.*, 2003; Pauklin *et al.*, 2009). However, progesterone has been shown to inhibit AID transcription and protein levels (Pauklin and Petersen-Mahrt, 2009). Therefore, one cannot predict whether lower levels of these hormones might be responsible for the *in vivo* effects on switch junctions. If AID levels were indeed reduced in B cells *in vivo*, this could result in increased junctional microhomology, as haploid levels of AID have been shown to result in increased lengths of microhomology in B cells from human peripheral blood, as described above (S. Kracker and A. Durandy, personal communication). Although not demonstrated to be full-length mRNA, Msh5 RNA is expressed at highly variable levels in B cells from different mouse strains, and it is possible that some strains do indeed express Msh5 protein; thus, the difference in results between different groups might be related to the different genetic background of the mice studied (Sekine *et al.*, 2009).

There is no null *MSH5* mutation described in patients. Genetic variations in the *MSH5* gene are, however, associated with a subset of patients with IgA deficiency (IgAD) or CVID (Sekine *et al.*, 2007). One of the disease-associated alleles identified contains two nonsynonymous polymorphisms, L85F/P786S, and the variant protein encoded by this allele shows impaired binding to MSH4. Furthermore, the Sμ–Sα junctions from CVID and IgAD patients carrying this allele show increased donor/acceptor microhomology as compared to controls, suggesting a role of MSH5 in CSR. However, in light of the controversial results from the mouse studies (Guikema *et al.*, 2009; Sekine *et al.*, 2009), it will be very important to determine whether human B cells express full-length Msh4 and Msh5 transcripts and proteins.

3.8. ERCC1-XPF has a modest effect on CSR, no effect on junctional microhomology, and suppresses mutations near junctions

After DSB formation, 5′ or 3′ ss overhangs can remain, as shown in Fig. 3.2B. These tails must either be excised or filled-in to create blunt DSBs appropriate for an end-joining recombination with the other

S region. The structure-specific endonuclease ERCC1-XPF excises 3′ ss tails at the junction with ds DNA, and this might be its role in CSR (Schrader *et al.*, 2004). *Ercc1*$^{-/-}$ B cells switch 50–80% as well as WT cells, depending on the isotype. Junctional microhomology and insertions are similar to WT. However, mutations near the junctions are decreased on both the Sμ and Sγ3 side, but are increased away from the junctions on the Sμ side (Schrader *et al.*, 2004). Also Artemis, which might be involved at a similar step during CSR, but perhaps at more complex DSB structures, may be associated with the generation of mutations near Sμ–Sα junctions, as in its absence mutations are reduced (Du *et al.*, 2008b).

3.9. DNA Polymerase ζ contributes to formation of S–S junctions

DNA Pol ζ is an error-prone DNA polymerase that is able to extend from mismatched primer-template termini (Johnson *et al.*, 2000). Deletion of this enzyme causes embryonic lethality and genomic instability, but recently, a Pol ζ conditional KO mouse was created, using CD21-Cre to delete the gene in mature B cells (Schenten *et al.*, 2009). Due to the fact that it is an error-prone polymerase, it was first hypothesized to contribute to SHM, but found not to directly do so (Schenten *et al.*, 2009). However, due to the requirement of Pol ζ for cell proliferation, Pol ζ-deficient cells proliferate poorly and therefore accumulate fewer V region mutations.

Pol ζ does, however, participate directly in CSR. CSR occurs at ∼30% the frequency of WT cells that have proliferated equivalently, and Sμ–Sγ3 junctions show modest changes (Schenten *et al.*, 2009). Sμ–Sγ3 junctions from the Pol ζ-deficient B cells have slightly increased microhomology and reduced frequency of insertions (10% in KO and 25% in WT). The most striking finding is that Pol ζ-deficient cells induced to switch in culture showed ∼5- to 12-fold increased frequency of chromosome breaks, as assayed by FISH, and these breaks occurred about four times more frequently within the IgH locus than elsewhere in the genome in Pol ζ-deficient cells relative to WT cells. These data suggest that Pol ζ is involved in filling in the staggered DSBs created by AID–UNG–APE-MMR activity. Without this activity, DSBs remain unrepaired, causing chromosome breaks and translocations. Thus, Pol ζ might have a role similar to the roles of DNA Pols μ and λ, which perform fill-in DNA synthesis during C-NHEJ in V(D)J recombination (Fan and Wu, 2004; Mahajan *et al.*, 2002). This role is consistent with the lack of a role for Pol ζ in SHM, as SHM does not appear to involve DSBs.

4. DISCUSSION

4.1. Suggested minimal information required for S–S junction analysis

Over the past few years, more than 6700 CSR junctions have been characterized in WT cells as well as in cells deficient in various DNA repair proteins (Tables 3.1 and 3.2). As we have already discussed in various parts of this review, differences in experimental design and in data analysis might lead to different, sometimes opposite conclusions. To facilitate comparison of data generated from different models and to facilitate a correct interpretation of the results, sufficient details need to be provided in CSR junction analysis. In this section, we will discuss the experimental conditions that might influence the results and will provide our thoughts on the minimal information required for CSR junction analysis.

4.1.1. Genetic background/mouse strains

It has previously been shown that CSR differs among Swiss James Lambert (SJL), C57BL/6 and 129 mice (Kaminski and Stavnezer, 2007). Switching to IgG3 in cultured splenic B cells from SJL and 129/Sv mice is two- to sixfold less efficient compared with C57BL/6 mice, whereas switching to IgA is higher in 129/Sv than in C57BL/6 mice when minimal stimulation is used (BLyS/LPS/TGFβ). Although there is no study that focuses on a comparison of S–S junctions derived from different mouse strains, the S region sequence for a given class or subclass differs among different mouse strains. For example, the Sμ and Sα regions from 129 are both longer than the respective S regions from C57BL/6, judging from the length of repetitive sequences (Q. P.-H., unpublished). The length of the S region may affect the efficiency of CSR (Pan *et al.*, 1998; Zarrin *et al.*, 2005), which might be one of the underlying causes for a higher level of IgA switching observed in 129 mice. Furthermore, Sμ and Sα sequences share more homology in 129 as compared to the C57BL/6 strain, which might influence the balance of different repair pathways used, and thus the resulting pattern of CSR junctions. The average length of microhomology at Sμ–Sα junctions is indeed significantly longer in the WT cells from 129 than from C57BL/6 (Q. Pan-Hammarström, unpublished). Thus, genetically matched controls should be used whenever possible, and the corresponding germline S sequences should be used in the analysis.

4.1.2. Age

Although the proportion of Sμ–Sα junctions with 1-bp insertion or direct joints is similar between adult and pediatric controls (Du *et al.*, 2008b), the average length of microhomology is significantly longer in the latter (Du *et al.*, 2008b; Rivera-Munoz *et al.*, 2009). Also, there was an increase in the length of microhomology and insertions in 3-week-old as compared to 3-month-old WT mice

(Schrader *et al.*, 2004). Although it is unclear what might cause the age-related differences, these studies demonstrate the importance of using appropriate age-matched controls or WT littermates when analyzing S junctions.

4.1.3. Cell source and activation conditions

Most of the studies on CSR junctions in human are performed on *ex vivo* peripheral blood lymphocytes, whereas the majority of the mouse studies use cultured splenic B cells (resting B cells or T cell depleted-B cells) activated to switch in culture. Theoretically, using cells from peripheral blood or spleen should not make a difference, as at least in humans, the pattern of Sμ–Sα junctions is very similar when using either source of cells (Q. Pan-Hammarström, unpublished). However, the culture conditions might affect the pattern of the junction: IL10 + CD40L stimulated human peripheral blood cells show a significantly increased average length of microhomology at Sμ–Sα junctions relative to untreated cells, whereas IL21 + IL4 + anti-CD40 antibody stimulated cells have a dramatically reduced level of microhomology usage at the Sμ–Sε junctions relative to unstimulated peripheral B cells (Q. Pan-Hammarström, unpublished data). Although no data are available on mouse B cells, activation condition might be a factor that needs to be controlled.

4.1.4. Type of junctions

In WT human cells, the Sμ–Sα junctions tend to have longer microhomology than Sμ–Sγ junctions (Du *et al.*, 2008b; Peron *et al.*, 2008; Sekine *et al.*, 2007), a finding which might be explained by the greater homology between Sμ and Sα than Sμ and Sγ. The proportion of junctions with direct joints is, however, very similar between these two types of junctions (Du *et al.*, 2008b). In the absence of certain DNA repair factors, the shift in microhomology usage is usually much more pronounced at the Sμ–Sα as compared to the Sμ–Sγ junctions, and the latter are characterized by a slightly increased length of microhomology (ATM-, ATR-deficiency), an increased frequency of 1-bp insertion (Lig4-deficiency), an absolutely normal pattern (Artemis-, NBS-, or Mre11-deficiency), or even a reduced length of microhomology (Pms2-deficient) relative to WT Sμ–Sγ junctions (Table 3.2). It is thus evident that in human B cells, Sμ–Sα and Sμ–Sγ junctions are resolved differently in WT cells as well as in cells with deficiencies in various DNA repair factors.

Most of the studies on KO mice have focused on Sμ–Sγ junctions (Table 3.1). In WT mouse cells, the Sμ–Sα or Sμ–Sε junctions also tend to show longer microhomology than the Sμ–Sγ junctions (Rivera-Munoz *et al.*, 2009; Sekine *et al.*, 2007; Yan *et al.*, 2007). Therefore, studying a single type of CSR junction appears to be insufficient, in particular when the observation is a ''normal pattern.'' Analysis of more than one type of CSR junctions is therefore recommended.

4.1.5. Nomenclature, interpretation, and sequence availability

As already described in earlier sections, microhomology, imperfect repeats, insertions, mutations, and blunt end-joining are sometimes defined differently in various studies. Therefore, we think it is important to include a clear definition of these terms in each study. For example, for microhomology, if a mismatch is allowed, the number of mismatches and when they are allowed should be indicated. As the mutation pattern might be different in the donor and acceptor S regions, in germline or in recombined S regions, near to or away from the S junctions, the region where the mutation pattern is analyzed also needs to be clarified. For certain complicated junctions, even with the same definition, the interpretations from different investigators might be different. Thus, presenting the actual junctional sequences in a supplementary format is important. In general, we suggest that at least 30 bp of junctional sequences should be available to the scientific community, to allow a meaningful comparison of the data and, perhaps, an independent interpretation.

4.2. C-NHEJ and A-EJ

CSR junctional analyses have provided evidence that NHEJ is required for CSR. Two major pathways have been proposed, the predominant C-NHEJ and the A-EJ pathways (Fig. 3.4A). The former requires not only components of the C-NHEJ machinery, that is, Ku70–Ku80, XRCC4–Lig4–Cernonnus/XLF, but also additional factors such as the nuclease complex Artemis–DNA-PKcs, a number of DNA-damage sensors or adaptors, including ATM, ATR, 53BP1, γH2AX, MDC1, RNF8, RNF168, and the MRN complex (Fig. 3.4A). A-EJ in CSR, like in other NHEJ reactions, is, however, still not well characterized. Three factors have thus far been implicated in the A-EJ pathway during CSR: the MRN complex (Dinkelmann *et al.*, 2009), Poly (ADP-ribose) polymerase 1 (PARP1) (Robert *et al.*, 2009), and CtIP (Lee-Theilen *et al.*, in press) (Fig. 3.4A). In addition, Ligase I and III are thought to be involved in A-EJ (Lieber, 2010a,b).

Microhomology, insertions, and deletions (end resections) are normally associated with A-EJ. However, we do not know how long a microhomology needs to be in order to recombine by A-EJ (more than 1, 2, or 4 bp or even longer). A-EJ does not appear to be a unique pathway, as depending on the specific component that is missing, the S–S junctions differ. Also, the identification of factors in the A-EJ pathway is complicated by the likelihood that a short microhomology (few base pairs) could be the result of either the predominant C-NHEJ or an A-EJ pathway, and the lack of microhomology may not necessarily exclude the involvement of A-EJ (as observed in Lig4–Ku70 double KO cells). Furthermore, most factors regulating the C-NHEJ pathway may also be involved in A-EJ. The kinetics

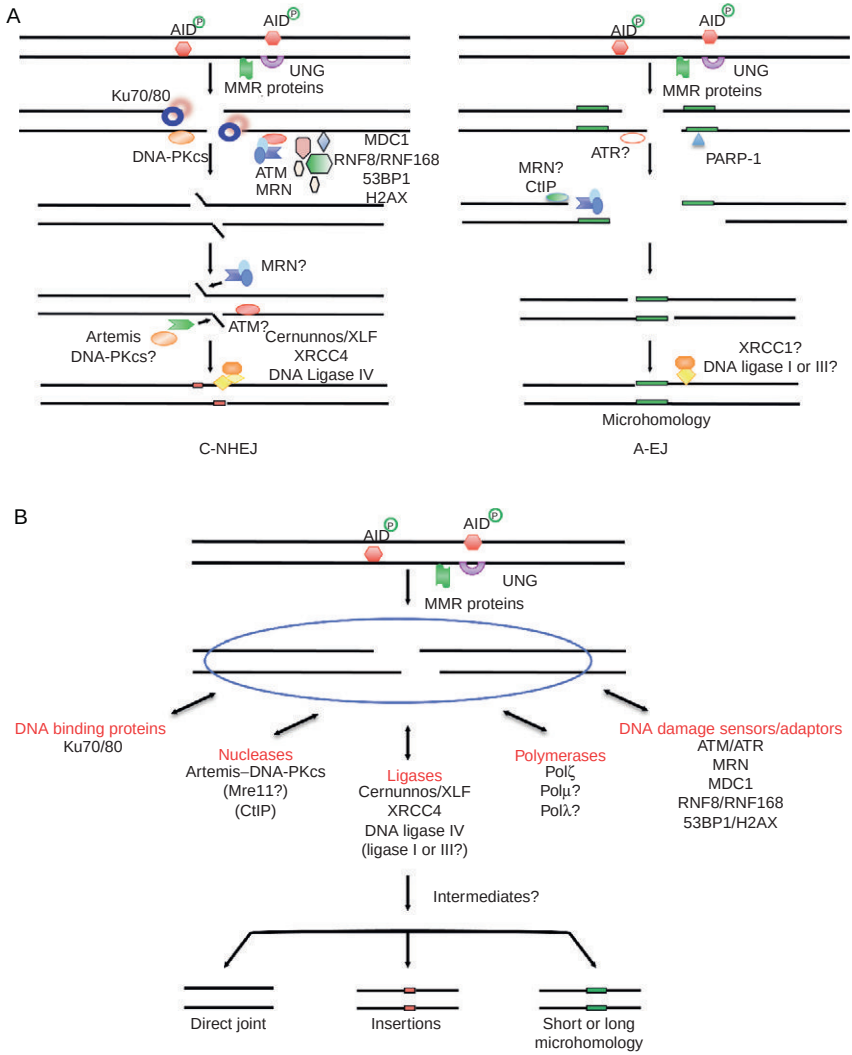


FIGURE 3.4 Hypothetical models for the end-joining mechanism during CSR. AID initiates CSR through deamination of dC residues in the S regions. The activity of AID is regulated at a posttranslational level, including phosphorylation. The dG:dU mismatches can then be processed by either the MMR pathway, or by UNG-dependent BER, leading to production of DSBs in the S regions. (A) Models for CSR by C-NHEJ and A-EJ. In the predominant C-NHEJ pathway, Ku70–Ku80 binds to DNA ends and recruits and activates DNA-PKcs. This is probably important for the synapsis process. A number of DNA-damage sensors or adaptors are activated and recruited to the DSBs, including ATM, MDC1, RNF8, RNF168, 53BP1, and γH2AX. Together they configure the DNA termini for subsequent repair steps and they also regulate the cell-cycle response, although the cell-cycle response might not be important during CSR. ATM and DNA-PKcs may also have a direct

are likely to be slower than for C-NHEJ, due to the finding that CSR appears to be delayed in Lig4-deficient CH12F3 cells (Han and Yu, 2008). This would favor the use of C-NHEJ, when the components are available.

When components of NHEJ are defective, the flexible nature of the NHEJ machinery permits substitutions by other enzymes (Lieber, 2010a,b; Lieber *et al.*, 2008). Instead of designating such substitutions as separate pathways, such as alternative NHEJ, backup NHEJ or microhomology-mediated NHEJ (MMEJ), Lieber has proposed to include them as part of NHEJ, but refer to these as Lig4-independent, Ku70–Lig4-independent or X factor-independent end-joining (Lieber, 2010b). At the current stage, when little is known about the A-EJ pathway(s) in CSR, Lieber's model is an appealing proposal (Fig. 3.4B).

5. CONCLUDING REMARKS

It appears highly likely that the choice between the use of C-NHEJ or A-EJ depends both on the structure of the DSBs available to the recombination machinery and on the availability of the C-NHEJ components. In turn, the DSB structure might be determined by the frequency of SSBs, as in cases where fewer SSBs lead to fewer DSBs, the efficiency of the recombination is likely to be slower, allowing resection of DSBs and therefore the use of

role in the end-processing step by phosphorylation of Artemis, a nuclease that might convert nonligatable DSBs into DSBs that can recombine by C-NHEJ. The Mre11–Nbs1–Rad50 (MRN) complex may contribute to CSR by activating ATM, by recruiting the CtIP exonuclease, and also by the nuclease activity of Mre11. The ligase complex XRCC4–DNA Lig4–Cernunnos/XLF performs the ligation. When the C-NHEJ pathway is impaired, A-EJ pathways are used, and microhomology is often, but not always, associated with these pathways. PARP1, CtIP, MRN, and DNA Ligase I and III are probably involved in A-EJ. Both Mre11 and CtIP nucleases can resect DSBs and promote microhomology-mediated end-joining. (B) NHEJ is mechanistically a flexible type of recombination. The factors involved in sensing and repair of the DSBs in the S regions can be organized into several groups, DNA binding proteins, DNA-damage sensors/adaptors, nuclease(s), ligase(s), and polymerase(s). These factors act iteratively, may act in different orders and can function independently at the two DNA ends being joined (Lieber, 2010b). The flexibility of the NHEJ process allows repair and joining of a wide range of DSB end configurations, generating various intermediates, and finally leading to recombination of the paired DNA ends. This can result in different-appearing junctions, from identical starting DSB ends. Also, different types of DSBs can be recombined by NHEJ, and this can also lead to different types of junction appearance. When some of the key enzymes are defective, for instance DNA Lig4, other enzymes, such as DNA Ligase III, can substitute and can interact with other components of the NHEJ machinery. This substitution might result in a less efficient recombination, and generate alternative intermediates, and thus, the resulting CSR junctions might show different phenotypes (for instance a longer microhomology).

microhomology, which is then termed A-EJ. A possible example of this is the finding that UNG-deficient and AID heterozygous patients have increased lengths of microhomology (S. Kracker and A. Durandy, personal communication). This might be due to inefficient recombination, although the authors also proposed that these proteins recruit C-NHEJ factors. MMR enzymes are important for converting SSBs to DSBs, and in the absence of some of them, microhomologies are increased. This might be simply due to inefficient recombination because of infrequent DSBs. Another possibility is that the activities of Msh2–Msh6 and Exo1 result in long ss tails in the absence of Mlh1–Pms2, due to increased Exo1 processivity (Zhang *et al.*, 2005).

Several factors are likely to determine the structure of the DSBs, once they have formed. They could be protected from end-processing by Ku70–Ku80, or they might be resected by CtIP and Mre11, creating ss tails. On the other hand, ss tails or complex ss structures might be excised by ERCC1-XPF and Artemis–DNA-PKcs, respectively. In addition, several DNA-damage response factors, for example, ATM, 53BP1, RNF8, RNF168, and MRN, directly or indirectly regulate the resecting enzymes. Also, DNA polymerases fill in the staggered DSBs creating blunt or nearly blunt substrates for C-NHEJ. Thus, when Pol ζ is deleted, junctional microhomologies are increased.

However, there are many examples of factors with multiple functions, and the effect of these factors on the junctions is often difficult to explain; examples are ATM, 53BP1, and MRN. 53BP1 does not have a marked effect on S–S junctional microhomology, although it suppresses insertions. 53BP1 inhibits end resection, and also appears to be important for synapsis of distal S regions, which might increase the efficiency of recombination. This predicts that in its absence, there should be more end-processing and slower recombination, and yet the junctions do not reflect this. Thus, there is much more to learn about the role of 53BP1 during CSR. One of the most complicated examples of this type of factor is ATM, which is activated by MRN, and also inhibits end-processing by Mre11, yet in its absence microhomologies are increased. Thus, although a tremendous amount of work in this field has been performed during the past few years, and many advances have been made, many pieces of the puzzle are still missing and further work will be required to ultimately delineate the proteins and mechanism involved in A-EJ.

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CHAPTER 4

How Tolerogenic Dendritic Cells Induce Regulatory T Cells

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Contents

Abstract

Since their discovery by Steinman and Cohn in 1973, dendritic cells (DCs) have become increasingly recognized for their crucial role as regulators of innate and adaptive immunity. DCs are exquisitely adept at acquiring, processing, and presenting antigens to T cells. They also adjust the context (and hence the outcome) of antigen presentation in response to a plethora of environmental inputs that signal the occurrence of pathogens or tissue damage. Such signals generally boost DC maturation, which promotes their migration from peripheral tissues into and within secondary lymphoid organs

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and their capacity to induce and regulate effector T cell responses. Conversely, more recent observations indicate that DCs are also crucial to ensure immunological peace. Indeed, DCs constantly present innocuous self- and nonself-antigens in a fashion that promotes tolerance, at least in part, through the control of regulatory T cells (Tregs). Tregs are specialized T cells that exert their immunosuppressive function through a variety of mechanisms affecting both DCs and effector cells. Here, we review recent advances in our understanding of the relationship between tolerogenic DCs and Tregs.

1. INTRODUCTION

Dendritic cells (DCs) are a family of leukocytes that have mostly been studied as potent stimulators of adaptive immunity, but there is mounting evidence that DCs also establish and maintain immunological tolerance (Steinman *et al.*, 2003). Indeed, DCs can prevent, inhibit, or modulate T cell-mediated effector responses through a variety of mechanisms, ranging from the production of pleiotropic anti-inflammatory factors that exert broadly attenuating effects to the induction of antigen-specific T cell responses resulting in anergy, deletion, or instruction of regulatory T cells (Tregs; Fig. 4.1). Here, we will focus on the mechanisms by which DCs induce and control tolerance, particularly the function and differentiation of Tregs, which are crucial to contain autoimmunity and chronic inflammation. Failure of Treg function has been implicated in the development of many autoimmune processes, whereas cellular therapy by adoptive transfer of Tregs has shown efficacy in these disorders (Roncarolo and Battaglia, 2007). However, Treg-mediated suppressive activity can also contribute to the immune escape of pathogens or tumors. Indeed, elimination of Tregs in mice carrying malignancies can improve antitumor immune responses and survival (Zou, 2006). Therefore, understanding the role of DCs in Treg activation and differentiation is critical for the development of therapeutic strategies in many disease settings.

At steady-state, tissue-resident DCs are immature (henceforth called iDCs); these cells are poised to acquire antigenic material from their environment, but they are poorly immunogenic because they express only modest levels of MHC molecules and little or no costimulatory molecules and proinflammatory cytokines. iDCs sense the presence of infectious microbes using specific receptors that detect pathogen-associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) that are released within tissues as a consequence of cellular distress. These ''danger'' signals trigger signaling cascades in iDCs that result in their maturation, a profound phenotypic and functional metamorphosis driven by changes in gene expression (McIlroy

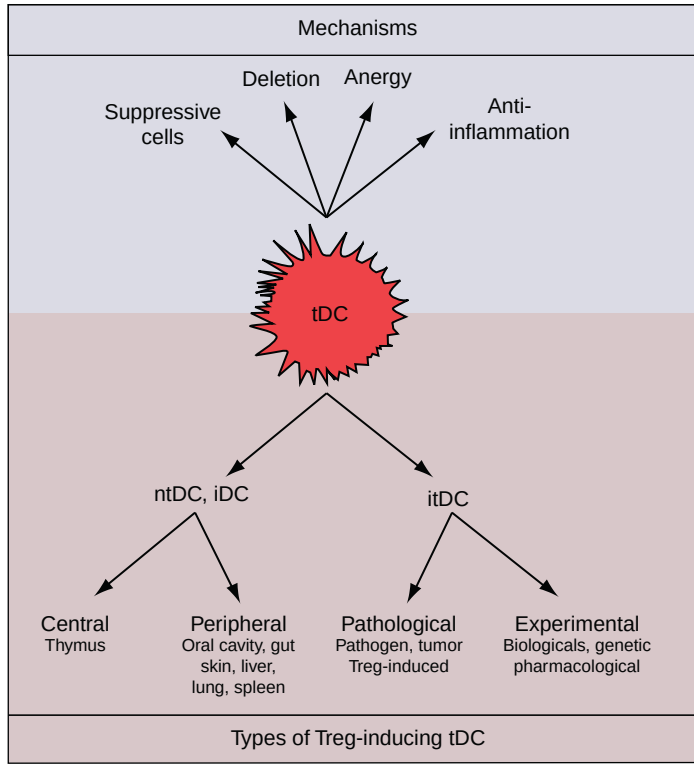

FIGURE 4.1 Types of tolerogenic DCs and their mechanisms of action. Tolerogenic DCs (tDCs) participate to the establishment of T cell tolerance by a variety of mechanisms, including the induction of anergy, deletion of antigen-reactive T cells, stimulation of suppressive regulatory T cells (Tregs) either by activation of existing Tregs or *de novo* differentiation of Tregs from Tns and production of anti-inflammatory cytokines and other factors. Depending on the differentiation state of the DC and the site of tolerogenic instruction, tDCs can be separated in natural tolerogenic DCs (ntDCs) and induced tolerogenic DCs (itDCs). The steady state environment instructs ntDCs (and includes iDCs) while itDCs arise during pathologies or after manipulation.

et al., 2005; Türeci *et al.*, 2003). During the maturation process, DCs lose their capacity to acquire soluble antigen but gain T cell stimulatory capacity due to increased antigen processing and upregulation of MHC, costimulatory molecules and cytokines (Banchereau *et al.*, 2000). Maturation signals also trigger in iDCs a profound change in their repertoire of traffic molecules, such as the upregulation of CCR7, a chemokine receptor that enables DCs in peripheral tissues to access local lymph vessels and migrate to the draining lymph nodes (Alvarez *et al.*, 2008). Here, the now fully mature DCs (mDCs) report the inflammatory and antigenic status of their source tissue to recirculating lymphocytes (Banchereau *et al.*, 2000).

Whereas newly generated mDCs are generally believed to possess primarily immunogenic functions, the role of iDCs is less well defined as they are not in a final differentiation state and can give rise to both immunogenic, proinflammatory mDCs as well as semimature DCs that share some phenotypic features of mDCs, such as CCR7 expression, but possess the capacity to establish and maintain tolerance.

Clues that iDCs themselves can either convert conventional naïve T cells (Tns) to assume a Treg phenotype and/or promote the function of existing Tregs have been gleaned from experiments in which antigen was administered to mice without a concomitant maturation signal (Apostolou and von Boehmer, 2004; de Heer *et al.*, 2004; Kretschmer *et al.*, 2005; Lambrecht and Hammad, 2009; Ostroukhova *et al.*, 2004; Tsuji and Kosaka, 2008; Vermaelen *et al.*, 2001). Under these conditions, antigen accumulated on DCs in secondary lymphoid organs (SLOs) and triggered the differentiation and/or proliferation of Tregs resulting in antigen-specific tolerance that could prevent or reverse autoimmune processes (Table 4.1). Animals that lack functional iDCs develop severe autoimmunity, possibly due, at least in part, to reduced numbers of circulating Tregs (Bar-On and Jung, 2010; Birnberg *et al.*, 2008; Darrasse-Jeze *et al.*, 2009; Ohnmacht *et al.*, 2009). Similarly, a DC-restricted genetic deficiency in $\alpha_v\beta_8$ integrin, which activates TGFβ, a key cytokine for the induction and maintenance of Tregs (Travis *et al.*, 2007), or disruption of DC-expressed TGFβ receptor (TGFβR) impairs the tolerogenic function of DCs and fosters autoimmunity (Laouar *et al.*, 2008). However, increased DC numbers are accompanied by a concomitant increase in Tregs, whereas elimination of Tregs elevates the number of DCs (Darrasse-Jeze *et al.*, 2009; Liu *et al.*, 2009; Lund *et al.*, 2008) suggesting that DCs and Tregs regulate each other's homeostasis.

It must be noted that neither iDCs nor mDCs are homogenous cell populations. Several distinct subsets that express discrete surface markers have been identified nearly two decades ago (Vremec *et al.*, 1992). The phenotypic diversity of the DC family is reflected in distinct functional properties that are rooted, in part, in the expression of different PAMP and DAMP receptors, divergent antigen presentation and crosspresentation capacities, as well as differential propensities to induce tolerance and Treg differentiation.

It is thus apparent that DCs encompass a heterogeneous mix of antigen presenting cells that differ not only with regard to phenotype, differentiation, and maturation status but also with regard to tolerance-inducing capacity. For the purpose of this chapter, we will functionally (rather than phenotypically) define two subsets of DCs based on their net effect on T cells: one subset is represented by immunogenic DCs that induce effector responses, while the other subset induces or enhances tolerance (Fig. 4.2). We will refer to the former as stimulatory DCs (sDCs) and the latter as tolerogenic DCs (tDCs). tDCs not only comprise most iDCs but also include

TABLE 4.1 Natural tolerogenic DC

Mechanism of t-DC induction	Treg phenotype	Origin of DC	DC phenotype	Mechanism of Treg induction	Disease model	Reference
Central suppressive tolerance						
TSLP	CD4+CD25+Foxp3+	Thymus	mDC			Watanabe *et al.* (2005)
	CD4+CD25+Foxp3+	Thymus	pDC			Proietto *et al.* (2008, 2009)
Peripheral suppressive tolerance						
Dermal tolerance						
Retinoic acid	CD4+Foxp3+	Skin DC	CD103− iDC		IBD	Guilliams *et al.* (2010)
	CD4+CTLA4+Foxp3+ IL-10+TGFβ+	Skin LN	DEC-205+ iDC		T1D	Bruder *et al.* (2005)
	CD4+CD25+CTLA4+	Skin LN	DEC-205+ iDC			Mahnke *et al.* (2003)
Oral tolerance						
	CD4+CD25+*	Peyer's patches	CD11c+ CD11b+		CIA	Min *et al.* (2006)
	CD25+*	Peyer's patches	pDC-like-CD8α+			Bilsborough *et al.* (2003)
	CD25+IL-10+INFγ+*	Oral cavity	CD11c+			Mascarell *et al.* (2008)
	CD25+Foxp3+*	Peyer's patches	CD11c+ IDO+		CIA	Park *et al.* (2008)
	CD25+CD103+Foxp3+	LP		(RA, TGFβ)		Sun *et al.* (2007)

(*continued*)

TABLE 4.1 (*continued*)

Mechanism of t-DC induction	Treg phenotype	Origin of DC	DC phenotype	Mechanism of Treg induction	Disease model	Reference
	CD4+Foxp3+	MLN and LP	CD103+	(RA, TGFβ)		Coombes *et al.* (2007)
	CD4+Foxp3+	MLN	CD103+	IDO	IBD	Matteoli *et al.* (2010)
IEC secreting TGFβ, RA	CD4+CD25+Foxp3+*	BMDC or SpDC	CD103+		IBD	Iliev *et al.* (2009b)
IEC secreting TGFβ, RA	CD4+CD25+Foxp3+*	MLN	CD103+			Iliev *et al.* (2009a)
Systemic tolerance						
	CD4+IL-10+	Spleen	CD11clow CD45RB+			Wakkach *et al.* (2003)
	CD4+*	Spleen	pDCs			Martín *et al.* (2002)
	CD4+Foxp3+*	Spleen	CD8α+		EAE	Smith *et al.* (2010)
	CD4+CD25+Foxp3+*	Spleen	DEC-205+			Kretschmer *et al.* (2005)
	CD4+CD25−*	hu-PBMC-pDC	BDCA4+Lin-CD123+	IDO		Chen *et al.* (2008)
	CD4+CD25+Fopx3+ IL-10+TGFβ+	hu-PBMC-pDC	BDCA4+Lin-CD123+			Moseman *et al.* (2004)
	IL-10+*	hu-PBMC-pDC	BDCA4+Lin-CD123+	CD275		Ito *et al.* (2007)

	CCR4+CD25+Foxp3+*	Allograft draining LN	pDCs		HA	Ochando *et al.* (2006)
	CD4+CD25+Foxp3+	Spleen and LN	pDC CCR9+		aGVHD	Hadeiba *et al.* (2008)
Inhaled tolerance						
	CD4+IL-10+*	Lung LN		IL-10		Akbari *et al.* (2001)
	CD4+IL-10+*	Lung LN		IL-10 CD275	EA	Akbari *et al.* (2002)
***In vitro* immature**						
	CD4+CTLA-4+IL-10+*	huMoDC	CD83−			Jonuleit *et al.* (2000)
	CD4+IL-10+	huMoDC	CD83−			Dhodapkar *et al.* (2001)
	CD8+IL-10+*	huMoDC				Dhodapkar and Steinman (2002)
	CD4+IL-10+*	huMoDC	CD1a+CD83-ILT3+ILT4+	IL-10		Levings *et al.* (2005)
	CD4+IL-10+	huMoDC	iDC	CD275		Tuettenberg *et al.* (2009)
	CD4+CD25+Foxp3+IL-10+TGFβ+	huMoDC				Cools *et al.* (2008)
	CD4+CD25+Foxp3+	BMDC			PA	Stepkowski *et al.* (2006)

aGVHD: acute Graft Versus Host Disease, CIA: Collagen-Induced Arthritis, EA: Experimental asthma, EAE: Experimental Autoimmune Encephalomyelitis, HA: Heart Allograft, IBD: Intestinal Bowel Disease, T1D: Type 1 Diabetes, PA: Pancreatic Allograft, * with suppressive activity.

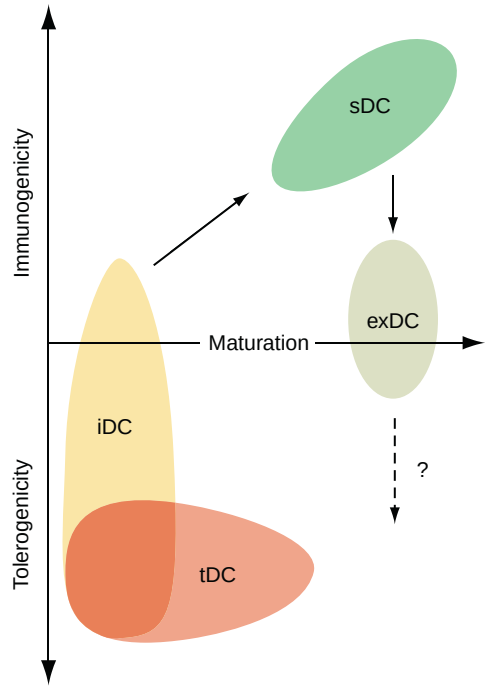

FIGURE 4.2 Relationship of maturation status, tolerogenicity, and immunogenicity among DC subsets. Immature DCs (iDCs) receive activation signals from microbial byproducts or tissue distress to acquire a mature phenotype, including the ability to migrate to lymph nodes and enhanced antigen presentation and costimulatory capacities. These mature DCs are highly stimulatory (sDC) and induce effector responses. Tolerogenic DCs (tDCs) include most iDCs but also comprise some cells with advanced maturation status. Only iDCs can give rise to mDCs. mDCs may lose their immunostimulatory capacity to become exhausted (exDC); however, their role in the induction of Tregs remains uncertain.

other DCs covering a spectrum of different maturation states. This review will summarize current knowledge of the origins and phenotypes of tDCs, the factors maintaining or inducing their tolerogenicity, and how these cells promote the expansion, function, or differentiation of Tregs.

2. WHAT IS THE ORIGIN OF TREG-INDUCING TDCS?

2.1. Tregs induction sites

Mammals, including humans, that lack functional Tregs succumb to fatal autoimmune disorders (Paust and Cantor, 2005), highlighting the importance of Tregs in controlling immune responses. In general, we

discriminate between two major types of Tregs based on their origin (Bluestone and Abbas, 2003). Natural Tregs (nTregs) originate during thymic development and first appear in the fetal circulation (Lio and Hsieh, 2008; Min *et al.*, 2007; Mold *et al.*, 2008). The phenotype and suppressive program of CD4+ nTregs is controlled by the transcription factor Foxp3, which is upregulated in developing T cells upon recognition of self-antigens in the thymus (Bensinger *et al.*, 2001; Kim and Rudensky, 2006; Ribot *et al.*, 2006). Innocuous self- and nonself-antigens that appear postnatally (like hormones, food, and commensal flora) can drive the differentiation of additional Tregs (Vigouroux *et al.*, 2004). Some of these antigens may be transported into the thymus by migratory iDCs (Bonasio *et al.*, 2006) that may then induce new nTregs. In addition, conventional Tns can be converted to so-called adaptive Tregs (aTregs) in extrathymic sites such as SLOs. aTregs are phenotypically heterogeneous and include both CD4+ and CD8+ T cells, most (but not all) of which also express Foxp3 (Table 4.1). A common trait of all Tregs is the expression of one or more anti-inflammatory molecules, such as IL-10, TGFβ, or IL-35 and/or inhibitory receptors, such as cytotoxic T-lymphocyte antigen 4 (CTLA4), lymphocyte-activation gene-3 (LAG-3), glucocorticoid-induced tumor necrosis factor receptor (GITR), CD39, or CD73, among others (Tang and Bluestone, 2008; Vignali *et al.*, 2008).

2.2. The phenotype of tDCs

The mechanisms by which tDCs exert their activity are varied and incompletely understood. As mentioned above, iDCs are typically tolerogenic (Steinman *et al.*, 2003), so the maturation status, or rather, the absence of maturation provides a hint for the tolerogenic capacity of DCs. However, iDCs comprise several different subsets that possess distinct abilities to present antigen, secrete cytokines, and induce tolerance (Ueno *et al.*, 2007). Thus, the various subsets of iDCs and mDCs do not fill a well-defined functional niche, but cover a spectrum of immunological properties, wherein iDCs primarily maintain tolerance, whereas mDCs initiate and control predominantly (but not exclusively) effector responses (Fig. 4.2).

2.2.1. Maturation phenotype

DCs receive maturation signals by a variety of inputs, including PAMP and DAMP receptors that sense certain microbial and tissue damage signatures. Such sensors include toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and others (Barton and Medzhitov, 2003; Franchi *et al.*, 2010; Pétrilli *et al.*, 2007; Re and Strominger, 2004). Additionally, inflammatory cytokines (e.g., TNFα and IL-1β) or the ligation of surface-expressed activating receptors such as CD40 can trigger DC maturation (Aggarwal, 2003; Elgueta *et al.*, 2009;

Sims and Smith, 2010). One key consequence of DC recognition of ''danger'' signals is the activation of members of the nuclear factor kappa B (NFκB) and interferon responsive factor (IRF) families (Meylan and Tschopp, 2006; Re and Strominger, 2004; Salter and Watkins, 2009). Upon maturation, DCs upregulate a plethora of gene products involved in antigen presentation and costimulation including MHC-II, CD40, CD80, CD86, OX40L, and inducible T cell costimulator ligand (ICOSL or CD275), as well as cytokines that promote and modulate inflammation and effector cell functions, including IL-1β, IL-2, IL-6, IL-8, IL-12, and IL-18 (Banchereau *et al.*, 2000). These changes are necessary for DCs to initiate T cell responses because Tns require three concomitant inputs to differentiate into full-fledged effector cells (Teffs): signal 1 is the antigenic stimulus provided by MHC molecules displaying a cognate peptide; signal 2 is provided by costimulatory molecules; and signal 3 is provided by cytokines produced by DCs or other microenvironmental sources (Cronin and Penninger, 2007). Since many tDCs have an immature phenotype (Tables 4.1 and 4.2; Fig. 4.2), it has been suggested that a major mechanism of their tolerogenicity is a consequence of their presentation of an antigen (signal 1) to T cells without concomitant costimulation or cytokines (signals 2 and 3). However, when iDCs are subjected to certain *in vitro* manipulations, such as exposure to TNFα or IFNγ or inhibition of E-cadherin, they assume phenotypic features of mDCs, including high levels of MHC and costimulatory molecules (Reis and Sousa, 2006; Tisch, 2010, and our unpublished results). Nevertheless, Tns that are exposed to such treated DCs preferentially differentiate into aTregs (Table 4.3). Moreover, although CCR7 is usually considered an indicator of DC maturation, some iDCs in peripheral tissues can also upregulate CCR7, which allows them to migrate to lymph nodes without assuming a fully mature phenotype. These migratory DCs favor the induction of aTregs rather than effector cells (Hintzen *et al.*, 2006; Jang *et al.*, 2006; Ohl *et al.*, 2004; Worbs *et al.*, 2006). CCR7 deficiency impairs lymphatic migration of iDCs and compromises the induction of inhaled and oral tolerance (Förster *et al.*, 2008; Martin-Fontecha *et al.*, 2003).

Thus, while immaturity appears to be a good indicator of DC tolerogenicity, phenotypically mDCs do not always induce immunity but, depending upon prior exposure to certain differentiation signals, may retain their tolerogenic function. This suggests that tolerance is not always a mere consequence of T cells perceiving insufficient signal 2 or 3, but additional DC-derived tolerance-promoting factors are likely to play a role. A case in point are so-called exhausted DCs (exDCs), which were observed to arise *in vitro* following an extended interval after exposure to maturation signals, such as bacterial lipopolysaccharide (LPS). The term ''exhaustion'' was proposed because exDCs, unlike freshly activated mDCs, have lost their initial capacity to induce Tn differentiation into T helper (Th)-1 cells. Instead, exDCs secrete immunosuppressive IL-10 and

TABLE 4.2 Disease-induced tolerogenic DC

Mechanism of t-DC induction	Treg phenotype	Origin of DC	DC phenotype	Mechanism of Treg induction	Disease model	Reference
Pathogen-induced tolerogenic DC						
F. hepatica products	CD4+CD25+Foxp3+	BMDC	iDC			Falcón *et al.* (2010)
S. japonicum SJMHE1 peptide	CD4+CD25+*	BMDC	iDC		DTH	Wang *et al.* (2009)
C. albicans	CD4+Foxp3+IL-10+	BMDC			IBD	Bonifazi *et al.* (2009)
Monophosphoryl lipid A	CD4+Foxp3+ IL-10+TGFβ+	Oral cavity	Oral-m-LC			Allam *et al.* (2008)
LPS	CD4+CD25+Foxp3+	BMDC			EAU	Lau *et al.* (2008)
Cryptococcus neoformans glucuronoxylomannan	CD4+Foxp3+	BMDC				Liu *et al.* (2008)
Curcuma longa L. products (Curcumin)	CD4+CD25+ Foxp3+IL10+*	BMDC			IBD	Cong *et al.* (2009)
Yersinia virulence factor	CD4+IL-10+	BMDC				Depaolo *et al.* (2008)
Tumor-induced tolerogenic DC						
Pancreatic tumor-derived mucins	*	huMoDC				Monti *et al.* (2004)
B16 Melanoma	CD4+CD25+Foxp3-*	Spleen	iDC	TGFβ	TI	Ghiringhelli *et al.* (2005b)
P815 Mastocytoma	CD4+IL-10+	Tumor-infiltrating	CD4-CD8−		TI	Liu *et al.* (2005)
MO4 Carcinoma	CD4+IL-10+*	Spleen	CD4-CD8−		TI	Zhang *et al.* (2005)
Necrotic myeloma cells	CD4+IL-10+	huMoDC				Fiore *et al.* (2005)
Ovarian carcinoma			pDC		CP	Wei *et al.* (2005)

(*continued*)

TABLE 4.2 (*continued*)

Mechanism of t-DC induction	Treg phenotype	Origin of DC	DC phenotype	Mechanism of Treg induction	Disease model	Reference
	CD8+CCR7+ CD45RO+IL-10+*	Ovary ascite				
Lung carcinoma cells	CD4+CD25+Foxp3+*	huMoDC	iDC			Dumitriu *et al.* (2009)
Retrocontrol-induced tolerogenic DC						
CD8+CD28− suppressor	CD4+	huMoDC	ILT3+ILT4+			Chang *et al.* (2002)
CD8+CD28− suppressor	CD4+CD45RO+ CD25+	huMoDC	ILT3+ILT4+			Manavalan *et al.* (2003)
CD4+ Tregs	CD4+*	BMDC				Martin *et al.* (2003)

CP: Cancer-bearing patients, DTH: delayed-type hypersensitivity, EAU: Experimental Autoimmune Uveoretinitis, IBD: Intestinal Bowel Disease, TI: Tumor implantation,
* with suppressive activity.

TABLE 4.3 Experimentally induced tolerogenic DC

Mechanism of t-DC induction	Treg phenotype	Origin of DC	DC phenotype	Mechanism of Treg induction	Disease model	Reference
Biologically induced tolerogenic DC						
Galectin 1	CD4+IL-10+	BMDC		IL-27	EAE	Ilarregui *et al.* (2009)
CD40L+IL-3	CD4+CD25+Foxp3+ IL-10+TGFβ+	Thymus	pDC			Martín-Gayo *et al.* (2010)
IL-10+IFNα	CD8+CD28−	huMoDC				Qin *et al.* (2008)
Blocking CD200R	CD4+CD25+[a]	BMDC			SA	Gorczynski *et al.* (2004)
Thymosin α1+TLR9	CD4+CD25+[a]IL-10+[a]	BMDC				Romani *et al.* (2006)
In vivo induced GM-CSF DC	CD4+Foxp3+IL-10+[a]	Spleen	iCD8α−		EAT	Ganesh *et al.* (2009)
Vitamin D3	CD4+IL-10+[a] CD4+IL-10+	huMoDC huMoDC	smDC CCR7+	PD-L1		Unger *et al.* (2009)
Vitamin D3+ dexamethasone + LPS						Anderson *et al.* (2009)
Vitamin D3	CD4+Foxp3+[a]	huMoDC				Penna *et al.* (2005b)
Vitamin D3	CD4+CD25+ Foxp3+CD62L+	BMDC	iDC			Ureta *et al.* (2007)
P-selectin	CD4+CD25+CD25+ Foxp3+[a]	huMoDC				Urzainqui *et al.* (2007)
CTLA4–Ig fusion protein	CD4+CD25+Foxp3+[a]	Spleen		TGFβ	CIA	Ko *et al.* (2010)
Estrogen	CD28-[a]	Spleen			EAE	Pettersson *et al.* (2004)
VIP	CD4+TGFβ+IL-10+[a]	BMDC			IBD	Gonzalez-Rey and Delgado (2006)

(*continued*)

TABLE 4.3 (*continued*)

Mechanism of t-DC induction	Treg phenotype	Origin of DC	DC phenotype	Mechanism of Treg induction	Disease model	Reference
VIP	CD4+TGFβ+IL-10+[a] CD8+CD28−[a]	huMoDC	iDC			Gonzalez-Rey *et al.* (2006)
VIP	CD4+IL-10+	BMDC	iDC IL-10+		DTH	Delgado *et al.* (2005)
VIP	CD4+TGFβ+IL-10+[a]	BMDC	iDC IL-10+		EAE RA	Chorny *et al.* (2005)
VIP	CD4+IL-10+[a]	BMDC	iDC		GVHD	Chorny *et al.* (2006)
BiP	CD4+CD25+CD27+[a]	huMoDC		IDO, IL-10		Corrigall *et al.* (2009)
HGF	CD4+CD25+Foxp3+ IL-10+	Spleen			EAE	Benkhoucha *et al.* (2010)
HGF	CD4+CD25+ Foxp3+IL-10+[a]	huMoDC		ILT3, IL-10		Rutella *et al.* (2006)
TSLP	CD25+Foxp3+[a]	BMDC	iDC		T1D	Besin *et al.* (2008)
HLA-G	CD25+CTLA-4+[a]	huMoDC				Ristich *et al.* (2005)
ILT3	CD8+CD28−[a]	BMDC				Vlad *et al.* (2010)
IL-10	CD4+CTLA-4+[a] CD8+[a]	huMoDC				Steinbrink *et al.* (2002)
IL-10	CD25+Foxp3+ LAG3+CTLA4+[a]	huMoDC	iDC ILT2+ IL-10+			Li *et al.* (2010)
IL-10	IL-10+Vα24+iNKT[a]	huMoDC	smDC			Yamaura *et al.* (2008)

IL-10	CD4+CD25+IL-10+[a]	huMoDC	iDC		xGVHD	Sato *et al.* (2003a)
IL-10	CD4+IL-10+	PBMC	DC-10	ILT4		Gregori *et al.* (2010)
IL-10	CD4+[a]	huMoDC				Pacciani *et al.* (2010)
IL-10	CD4+[a]	huMoDC	iDC IL-10+			Torres-Aguilar *et al.* (2010)
IL-10	CD4+IL-10+	BMDC	CD11clow CD45RB+			Wakkach *et al.* (2003)
IL-10	CD4+[a]	huMoDC				Kubsch *et al.* (2003)
IL-10+TGFβ	CD4+CD25+Foxp3+	BMDC	CD200R3+ CD49+		cGVHD	Sato *et al.* (2009)
IL-10+TGFβ	CD4+CD25+Foxp3+[a]	BMDC	iDC			Fujita *et al.* (2007)
IL-10+TGFβ	CD4+[a]	huMoDC	iDC IL-10+			Torres-Aguilar *et al.* (2010)
TGFβ	CD4+CD25+CTLA-4+[a]	BMDC	iDC		aGVHD	Sato *et al.* (2003b)
TNFα	CD4+CD25+[a]	BMDC	smDC		SA	Fu *et al.* (2010)
TNFα	CD4+CD25+[a]	BMDC	smDC IL-10+		SA	Fu *et al.* (2009)
TNFα	CD4+Foxp3+[a]	BMDC	smDC		EAE	Zozulya *et al.* (2009)
TNFα	CD4+CD25+ IL-10+CTLA4+ GITR+Foxp3+*	BMDC	smDC		EAT	Verginis *et al.* (2005)
TNFα	CD4+IL-10+	BMDC	smDC		EAE	Menges *et al.* (2002)
IFNγ	CD4+Foxp3+[a]	huMoDC	smDC			Eljaafari *et al.* (2009)

(*continued*)

TABLE 4.3 (*continued*)

Mechanism of t-DC induction	Treg phenotype	Origin of DC	DC phenotype	Mechanism of Treg induction	Disease model	Reference
Anti-CD45RB+LF150195	CD25+[a]	Spleen	iDC		HA	Min *et al.* (2003)
E-cadherin	CD4+IL-10+	BMDC	mDC		EAE	Jiang *et al.* (2007)
Pharmacologically induced tolerogenic DC						
Aspirin	CD25+Foxp3+[a]	huMoDC	iDC			Buckland *et al.* (2006b)
Dexamethasone	CD4+IL-10+[a]	huMoDC	smDC			Unger *et al.* (2009)
Dexamethasone	CD4+IL-10+[a]	huMoDC	smDC			Anderson *et al.* (2008)
Resveratrol	CD4+IL-10+	huMoDC	iDC			Svajger *et al.* (2010)
Rosiglitazone (NFkB inhibitor)	Foxp3+	BMDC	iDC		EAE	Iruretagoyena *et al.* (2006)
LF 15-0195 (IKK inhibitor)	CD4+CD25+ CTLA4+Foxp3+[a]	BMDC	iDC		HA	Zhang *et al.* (2008)
Curcumin	CD4+CD25+ Foxp3+IL10+[a]	BMDC		IL-10, TGFβ, RA	IBD	Cong *et al.* (2009)
Prednisolone	[a]	huMoDC	iDC		MG	Luther *et al.* (2009)
Genetically induced tolerogenic DC						
SOCS3KO	CD25+Foxp3+[a]	BMDC	iDC	TGFβ	EAE	Matsumura *et al.* (2007)
Dominant negative IKK2 transduction	[a]	BMDC	iDC			Tomasoni *et al.* (2005)

Foxp3 transduction	CD25+[a]	huMoDC		TGFβ		Lipscomb *et al.* (2010)
IL-10 transduced	CD4+CD25+ Foxp3+IL-10+[a]	BMDC	smDC	IL-10	EA	Henry *et al.* (2008)
CD40/80/86 KD	[a]	BMDC			CIA	Zheng *et al.* (2010)
RelB KD	Foxp3+	BMDC			EAMG	Yang *et al.* (2010)
RelBKO	CD4+IL-10+[a]	BMDC	iDC			Martin *et al.* (2003)
RelB KD	CD4+Foxp3+		iDC			Zhang *et al.* (2009a)
CD40 KD	IL-10+[a]	BMDC		IL-10	EAMG	Martin *et al.* (2003)

aGVHD: acute Graft Versus Host Disease, CIA: Collagen-Induced Arthritis, cGVHD: chronic Graft Versus Host Disease, DTH: delayed-type hypersensitivity, EA: Experimental asthma, EAE: Experimental Autoimmune Encephalomyelitis, EAMG: Experimental Autoimmune Myasthenia Gravis, EAT: Experimental Autoimmune Thyroiditis,GVHD: Graft Versus Host Disease, HA: Heart Allograft, IBD: Intestinal Bowel Disease, MG: Myasthenia Gravis, RA: Rheumatoid Arthritis, SA: Skin Allograft, T1D: Type 1 Diabetes,xGVHD: xenogeneic graft-versus-host disease,*with suppressive activity.

elicit nonpolarized memory cells and/or Th2 responses (Langenkamp *et al.*, 2000, 2002). Whether exDCs can also induce Tregs *in vivo* remains to be determined.

2.2.2. tDC subsets

In mice, at least seven different DC subpopulations can be identified, which are distinguishable by both surface and intracellular markers that govern their function (Coquerelle and Moser, 2010; Liu and Nussenzweig, 2010; Milling *et al.*, 2010; Pulendran *et al.*, 2008; Shortman and Heath, 2010; Siddiqui and Powrie, 2008; Steinman and Idoyaga, 2010; Swiecki and Colonna, 2010; Ueno *et al.*, 2007). Murine lymphoid tissue-resident DC subsets include CD8α+, CD4+, CD8α−,CD4− (DN), and plasmacytoid DCs (pDCs). Migratory DCs that carry antigen from peripheral organs to SLOs include CD103+ DCs that have been identified in the lung, the gastrointestinal tract, and the skin, CD11b+ ''myeloid DCs'' and epidermal Langerhans cells (LCs). *In vitro* assays suggest that there may be a hierarchy of tolerogenic potential that is highest for pDCs followed by CD103+ DCs and CD8α+ DCs with CD11b+ DCs having low activity in most assays.

It should be cautioned, however, that the tolerogenicity of DC subsets is context dependent. For instance, CD8α+ DCs preferentially promote aTreg differentiation in the presence of TGFβ (Shortman and Heath, 2010; Yamazaki *et al.*, 2008), although it should be noted that addition of TGFβ to activated Tns induces aTreg differentiation even in absence of DCs (Chen *et al.*, 2003). pDCs are key participants in the establishment of oral and transplant tolerance (Goubier *et al.*, 2008; Ochando *et al.*, 2006; Swiecki and Colonna, 2010), presumably owing to their expression of indoleamine 2,3-dioxygenase (IDO), an enzyme that inhibits effector T cell proliferation (Puccetti and Grohmann, 2007). Intestinal CD103+ DCs also express IDO and secrete all-trans retinoic acid (RA), which promotes Tn differentiation into aTreg (Matteoli *et al.*, 2010; Siddiqui and Powrie, 2008). Some skin-derived CD103− DCs and other DCs can also produce RA (Guilliams *et al.*, 2010), while IDO expression is inducible in DCs by a variety of signals, including TGFβ, interferons (Belladonna *et al.*, 2008; Guilliams *et al.*, 2010; Matteoli *et al.*, 2010; Puccetti and Grohmann, 2007), and engagement of GITR (Grohmann *et al.*, 2007), among others. Therefore, although DCs subpopulations have different tolerogenic capacities *a priori*, they can adapt their function according to environmental inputs.

3. INSTRUCTIVE SIGNALS FOR TREG-INDUCING TDCS

In addition to the fact that immature tDCs present little or no signals 2 and 3 (see above), they can receive tolerance-promoting molecular ''reminders'' that counteract sDC differentiation in response to maturation stimuli (Fig. 4.3). These signals can be mimicked *in vitro* to induce tDCs

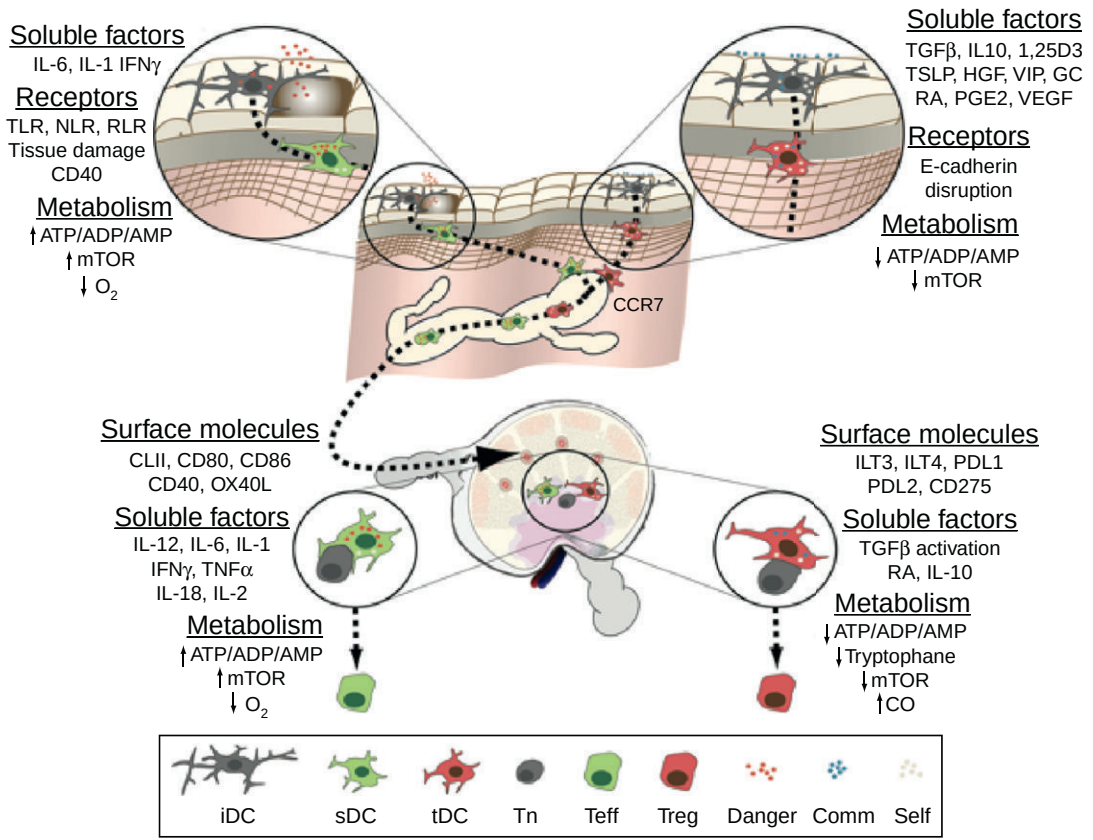

FIGURE 4.3 Education of immunogenic or tolerogenic DCs by environmental signals. Immature DCs (iDCs) perceive a myriad of inputs leading to their differentiation into sDCs or tDCs. Upon engagement of danger signal receptors by microbes or cellular distress, the presence of activating cytokines or changes in the abundance of certain metabolites, these cells mature and become sDCs that migrate to the draining

under tissue culture conditions. Thus, we can differentiate between tDCs that arise naturally from hematopoietic precursors, and tDCs that have received instructive signals that may cement or modulate their tolerogenic phenotype. To facilitate discussion, we will refer to natural versus induced tDCs as ntDCs and itDCs, respectively (Fig. 4.1). While ntDCs maintain tolerance constitutively within a steady-state environment, itDCs have received inputs from their environment, such as experimental or pharmacological interventions, infectious agents or other pathophysiological conditions. It should be emphasized that this terminology is merely meant to offer a conceptual frame of reference and does not imply that ntDCs and itDCs are strictly separate populations. Both subsets overlap and likely coexist and cooperate within tissues, making a real-life distinction between them often difficult.

3.1. Natural tolerogenic DCs

As discussed above, nTreg and aTreg originate from different anatomic compartments and in response to distinct immunological processes. The rules governing the function of tDCs in the thymus, where central tolerance is established by selection of Tns and generation of nTregs, and in peripheral tissues, where tDCs convert Tns into aTregs, are only beginning to be understood.

3.1.1. Central suppressive tolerance

Although thymic epithelial cells contribute to self-antigen-reactive nTreg commitment (Aschenbrenner *et al.*, 2007; Bensinger *et al.*, 2001; Liston *et al.*, 2008), thymic DCs and, in particular, thymic pDCs also promote the induction of Foxp3+ nTreg (Table 4.1; Atibalentja *et al.*, 2009; Martín-Gayo *et al.*, 2010; Proietto *et al.*, 2008, 2009). The mechanism(s) by which

secondary lymphoid organs (SLOs) using CCR7. Through presentation of cognate antigen and costimulatory surface receptors as well as production of cytokines and the regulation of metabolites, sDCs coerce naïve T cells (Tns) to become effector cells (Teffs). However, at steady state, commensals and structural cells produce anti-inflammatory cytokines that in combination with regular levels of metabolites and minute quantities of danger signals imprint tDCs to migrate to SLOs using CCR7. Upon contact with antigen-specific cells, tDCs induce the differentiation of regulatory T cells (Tregs) through a variety of mechanisms. Toll-like receptors (TLR), NOD-like receptors (NLR), RIG-I-like receptors (RLR), mammalian target of rapamycin (mTOR), 1,25-dihydroxyvitamin D3 (1,25D3), thymic stromal lymphoietin (TSLP), hepatocyte growth factor (HGF), vasoactive intestinal peptide (VIP), glucocorticoid (GC), all-trans retinoic acid (RA), prostaglandin E2 (PGE2), vascular endothelial growth factor (VEGF), programmed death-1 ligand (PDL), carbon monoxide (CO), and commensal (Comm).

the thymic environment promotes this capacity on DCs involves IL-7-related thymic stromal lymphoietin (TSLP) produced by Hassall's corpuscles in the thymic medulla (Besin *et al.*, 2008; Liu *et al.*, 2007; Mazzucchelli *et al.*, 2008; Wang and Xing, 2008; Watanabe *et al.*, 2005). By contrast, in extrathymic sites, such as the lung and skin (Ziegler and Artis, 2010), TSLP biases DCs and Tns toward a Th2 response, suggesting that other, as yet unknown, factors may contribute to tDC instruction or function in the thymus.

3.1.2. Peripheral suppressive tolerance

Oral intake of antigenic material, such as food and commensal microorganisms, efficiently generates antigen-specific systemic tolerance (Tsuji and Kosaka, 2008). Recent reviews have summarized the current knowledge of intestinal tract-associated Tregs and DCs and their role in oral tolerance (Belkaid and Oldenhove, 2008; Coombes and Powrie, 2008; Milling *et al.*, 2010; Siddiqui and Powrie, 2008). DCs within the intestinal mucosa directly sample the lumen of the intestinal tract (Chieppa *et al.*, 2006) and transport antigen to mesenteric lymph nodes (MLNs) in a CCR7-dependent manner. Here, the antigen-laden DCs promote the differentiation of Tns into Foxp3+ aTregs (Coombes *et al.*, 2007; Hultkrantz *et al.*, 2005; Miyamoto *et al.*, 2005; Zhang *et al.*, 2001). DCs from the lamina propria (LP) are also thought to induce Foxp3+ aTregs (Sun *et al.*, 2007). This tolerogenic ability of intestinal DCs is presumably controlled by the mucosal environment, which is rich in anti-inflammatory factors such as TGFβ, RA, IL-10, vasoactive intestinal peptide (VIP), TSLP, and hepatocyte growth factor (HGF). When these agents are added to iDCs *in vitro*, they promote the differentiation of itDC, which elicit more efficient Tn-to-aTreg conversion than iDCs (Table 4.3; Göke *et al.*, 1998; Grider and Rivier, 1990; Iwata, 2009; Nilsen *et al.*, 1998; Taylor *et al.*, 2009a). Intestinal tDCs with the most potent aTreg inductive capacity express CD103 (alpha-E), an integrin chain, whose expression is regulated by TGFβ signaling (Robinson *et al.*, 2001). In addition, TGFβ and RA also act directly on activated Tns and promote aTreg differentiation, even in the absence of DCs (Chen *et al.*, 2003; Mucida *et al.*, 2009; Nolting *et al.*, 2009).

Intestinal epithelial cells (IECs) are central for the local milieu that fosters tolerogenic responses by both DCs and activated T cells. Not only are IECs a rich source of TSLP, TGFβ, and RA (Dignass and Podolsky, 1993; Iliev *et al.*, 2009a,b; Rimoldi *et al.*, 2005; Shale and Ghosh, 2009) but also IEC-derived RA induces in DCs the expression of retinal dehydrogenases (RALDH). This presumably enables intestinal DCs to metabolize food-derived vitamin A to produce RA by themselves. However, RA- and/or TGFβ-conditioned splenic DCs fail to promote significant Foxp3+ aTreg differentiation *in vitro* (our unpublished results),

suggesting that other instructive elements are necessary for full-fledged tDC induction in the intestine.

Like intestinal DCs, lung DCs, which capture antigens from the airways, are tasked with balancing immune responses to pathogens with those to the regular microbial flora and harmless inhaled antigens (Lambrecht and Hammad, 2009). Pulmonary DCs traffic continuously from the lungs to the draining mediastinal and peribronchial LNs, but to do so they are thought to require subtle maturation signals presumably from the local flora (Jakubzick *et al.*, 2008). Thus, DCs surveilling the airways acquire a semimature phenotype, whereby they upregulate CCR7, which enables their migration to lymph nodes (Hintzen *et al.*, 2006) and induction of aTregs that control pulmonary tolerance and homeostasis (Bakocević *et al.*, 2010; Curotto de Lafaille *et al.*, 2008; Lloyd and Hawrylowicz, 2009; Ostroukhova *et al.*, 2004). Similar to IECs, resting pulmonary stromal cells promote TGFβ-dependent differentiation of tDCs that promote the differentiation of Tregs *in vitro* (Li *et al.*, 2008). However, upon exposure to TLR ligands, lung stroma cells are critical initiators of inflammatory responses to infections by generating cytokines that instruct immunogenic sDCs (Hammad *et al.*, 2009).

In the skin, DCs function is influenced by vitamin D3, which is activated by ultraviolet radiation and then enzymatically converted to 1,25-dihydroxyvitamin D3 (1,25D3). *Ex vivo* treatment of DCs with vitamin D receptor agonists elicits Treg-inducing tDC (Adorini and Penna, 2009; Anderson *et al.*, 2008, 2009; Farquhar *et al.*, 2010; Mora *et al.*, 2008; Penna *et al.*, 2005a, 2007; Unger *et al.*, 2009; Ureta *et al.*, 2007). Of note, vitamin D signaling appears to engage an autonomous transcriptional program in DCs that is distinct and independent from the transcriptional pathways that underlie DC maturation (Griffin *et al.*, 2001; Széles *et al.*, 2009). Some DCs in skin-draining lymph nodes induce Foxp3+ aTregs through the production of RA (Guilliams *et al.*, 2010), but dermal lymph nodes contain much fewer RA-producing DCs (which are CD103−) than the intestinal tract (Iwata *et al.*, 2004).

The liver arguably provides the quintessential tolerogenic environment for T cells and DCs (Tiegs and Lohse, 2010). Thus, liver allografts typically require much less immunosuppression for long-term survival (Crispe *et al.*, 2006), and targeted expression of antigens in the liver can establish tolerance by inducing antigen-specific Foxp3+ Tregs (Cao *et al.*, 2007; Lüth *et al.*, 2008; Martino *et al.*, 2009). Although the liver is a major reservoir for RA, vitamin D3, and TSLP (Friedman *et al.*, 1992), the role of these factors in hepatic tDC function is unclear. Liver sinusoidal endothelial cells elicit tolerogenic functions in cocultured DCs *in vitro* (Schildberg *et al.*, 2008), and they are also implicated in the conversion of adoptively transferred DC precursors into hepatic tDCs *in vivo* (Xia *et al.*, 2008). Hepatic DCs can induce both T cell anergy and deletional tolerance (Goubier *et al.*, 2008).

They also regulate inflammatory processes during liver fibrosis and hepatic ischemia by producing cytokines, such as TNFα or IL-10 (Bamboat *et al.*, 2009, 2010; Connolly *et al.*, 2009; Goddard *et al.*, 2004).

In summary, while the factors implicated in DC instruction to promote Treg differentiation seem to possess organ-specific flavors, TGFβ, RA, and vitamin D3 appear to play a major role. Moreover, the balance of tDCs and sDCs in peripheral organs is the result of continuous intimate cross-talk between iDCs and their local surroundings. Stromal, epithelial, and endothelial cells are particularly well positioned to perceive homeostatic changes at body surfaces, the extracellular environment, and the blood stream. Therefore, it makes sense that these cells communicate with DCs through cytokines and direct contact and apparently contribute to the regulation of DC function and tolerance.

3.2. Induced tolerogenic DCs

A variety of inputs have been implicated in the induction of tDCs, including pathological conditions and specific molecular manipulations of iDCs or DC precursors. For example, many pathogens and tumors can mimic or produce tolerogenic factors and instruct tDCs as an immune escape mechanism. Preexisting Tregs can also educate iDCs to become tolerogenic and induce more Tregs, a phenomenon termed ''infectious tolerance.'' The tolerogenic potential of DCs has also been harnessed by modifying their biology using compounds and introducing genetic alterations.

3.2.1. Disease-induced tolerogenic DC

3.2.1.1. Pathogen-induced tolerogenic DC Certain pathogens have evolved immune escape mechanisms that exploit Tregs (Belkaid, 2007; Grainger *et al.*, 2010; Mills and McGuirk, 2004). In most cases, the contribution of tDCs to these infectious settings is still unclear, although different modalities have been described by which pathogens can modify DCs. For example, products from *Fasciola hepatica*, *Candida albicans*, *Schistosoma japonicum*, *Schistosoma mansoni*, *Bordetella pertussis*, and *Vibrio cholerae* all promote DC tolerogenicity and induce Treg differentiation (Table 4.2), but the molecular basis for their recognition and signaling remains largely unknown. One mechanism involves microbial and parasite byproducts or toxins that prompt DCs to produce anti-inflammatory cytokines, like IL-10 and TGFβ. Examples for these compounds include cyclosporin, FK506 (Tacrolimus), FK520, ISA247 (voclosporin), and rapamycin (Sirolimus), which have been harnessed as immunosuppressive drugs to treat immune disorders and transplant rejection (Cooper and Wiseman, 2010; Korom *et al.*, 2009). Cholera toxin (CTx), an exotoxin secreted by *V. cholerae*, is a multimeric complex of six protein subunits recognized and internalized

by membrane-bound gangliosides. Within the cell it increases cytosolic cyclic AMP levels (Fishman and Orlandi, 2003). DC treatment with CTx B subunit (CTB) inhibits their maturation and production of IL-12 while increasing IL-10 secretion and aTreg differentiation (D'Ambrosio *et al.*, 2008; Lavelle *et al.*, 2003). Other pathogens, such as helminths, also release factors that mimic immunosuppressive molecules like TGFβ and promote itDCs, thereby staging a permissive microenvironment. Helminth infection *in vivo* is associated with increased numbers of Tregs whose depletion enhances parasite clearance (Gomez-Escobar *et al.*, 2000; Grainger *et al.*, 2010b). However, whether and how helminth-derived products act on DCs to induce Tregs has not been determined. Similarly, some viruses encode analogs of IL-10 that are produced by infected cells (Fleming *et al.*, 2000; Hsu *et al.*, 1990; Kotenko *et al.*, 2000) and attenuate DC's immunogenicity (Chang *et al.*, 2004, 2009); however, a direct effect on Treg differentiation remains to be demonstrated.

3.2.1.2. Tumor-induced tolerogenic DC Cancer cells as well as the associated tumor stroma can confer tolerogenic properties on DCs resulting in differentiation and accumulation of aTregs within the tumor mass and in the draining lymph nodes (Table 4.2; Dumitriu *et al.*, 2009; Fiore *et al.*, 2005; Gabrilovich, 2004; Ghiringhelli *et al.*, 2005; Liu *et al.*, 2005; Wei *et al.*, 2005; Zhang *et al.*, 2005). Remarkably, the presence of DCs is crucial for the vascularization of some tumors, and DC depletion can enhance the elimination of malignant cells in animal models (Fainaru *et al.*, 2008, 2010). The mechanisms by which tumors instruct DCs to become itDCs involve the production of IL-10, vascular endothelial growth factor (VEGF), prostaglandin E2, TGFβ, and other tolerogenic factors by cancerous cells (Bernabeu *et al.*, 2009; Bierie and Moses, 2010; Gabrilovich, 2004; Ikushima and Miyazono, 2010; Kelly and Morris, 2010; Yigit *et al.*, 2010).

3.2.1.3. Treg-induced tolerogenic DC Even immune challenges that induce a potent effector response can trigger concomitant differentiation of aTregs (Bilenki *et al.*, 2010; Curotto de Lafaille *et al.*, 2008; Lanteri *et al.*, 2009; Lund *et al.*, 2008). The role of these inflammation-induced aTregs remains unclear but might limit immunopathology, suppress autoaggressive responses, and/or promote restitution of tissue homeostasis (via TGFβ) or T and B cell memory generation (via IL-10). Antigen-specific Tregs, either activated nTregs that expand when exposed to cognate antigen (Fisson *et al.*, 2003) or newly converted aTregs, can spread their tolerance-promoting message to local DCs and Tns through a mechanism termed ''infectious tolerance.'' This has been elegantly demonstrated by Waldmann and colleagues who transferred CD4+ T cells from tolerized animals to new recipients which, in turn, developed tolerance. Tregs contributed directly to Tn differentiation into aTreg by producing IL-10

and TGFβ and retained this capacity during multiple transfers to successive hosts (Andersson *et al.*, 2008; Belladonna *et al.*, 2009; Jonuleit *et al.*, 2002; Mekala *et al.*, 2005; Waldmann *et al.*, 2006). Similarly, McGuirk *et al.* (2002) showed that conditioning of DCs by Tregs confers them the ability to induce Tregs in an IL-10-dependent manner, suggesting that tDCs may be key players during Treg-induced ''infectious tolerance.''

3.2.2. Experimentally induced tolerogenic DC

Given their potent activity, researchers have attempted to emulate the conditions leading to tDC differentiation and function in order to understand the underlying biology and to utilize tDCs for immune therapy (Hackstein and Thomson, 2004; Morelli and Thomson, 2003, 2007; Steinman *et al.*, 2003). Indeed, tDCs can be induced *in vitro* by (1) anti-inflammatory biologicals, (2) pharmacologic agents, and (3) genetic modification (Table 4.3). Reports on this subject are dominated by work with murine or human DCs that were differentiated *in vitro* from blood or bone marrow progenitors (Inaba *et al.*, 1992) or blood monocytes (Sallusto and Lanzavecchia, 1994), respectively.

3.2.2.1. Induction of tolerogenic DCs using biologics A number of biomolecules that are physiologically encountered in tolerogenic situations can induce tDC differentiation *in vitro* (Fig. 4.4). For example, incubation of murine splenic or bone marrow-derived DCs (BMDCs), or of human monocyte-derived DCs (huMoDC) or rat BMDC with IL-10 alone or in combination with other cytokines confers a certain capacity to induce suppressive lymphocytes, including CD4+CD25+, CD8+, and Valpha24+ invariant natural killer T (iNKT). The suppressive capacity of these cells has been extensively tested in models of allograft rejection, allergies, and xenogeneic, acute, and chronic allogeneic graft-versus-host disease (Table 4.3). Signaling through the IL-10 receptor (IL10R) maintains iDCs in their immature state even in the presence of maturation signals (Lang *et al.*, 2002; Moore *et al.*, 2001). IL10R ligation triggers janus kinases (JAK)-mediated phosphorylation of signal transducer and activator of transcription 3 (Stat3; Murray, 2006). Activated phospho-Stat3 is translocated to the nucleus, where it represses genes associated with DC maturation and immunogenicity (Moore *et al.*, 2001; Murray, 2005). A few genes are specifically induced by IL-10, including suppressor of cytokine signaling 3 (SOCS3) and signaling lymphocytic activation molecule (SLAM; Perrier *et al.*, 2004). SOCS3 negatively regulates Stat-dependent signaling of inflammatory cytokines (Croker *et al.*, 2003), particularly IL-6, which can inhibit Tregs-mediated suppression (Pasare and Medzhitov, 2003). SLAM signaling activates src homology 2 domain-containing protein tyrosine phosphatase-1 (SHP-1), which inactivates costimulatory receptors by dephosphorylating their cytoplasmic tail (Akdis and

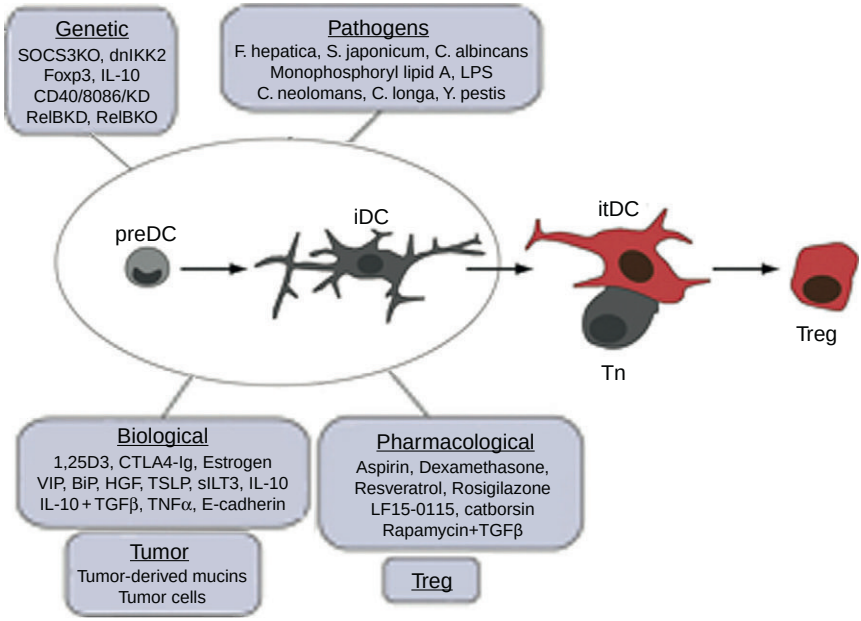

FIGURE 4.4 Induced-tolerogenic DCs. DCs progenitors (preDCs) and immature DCs (iDCs) from multiple sources are susceptible to tolerogenic instruction by multiple strategies. These cells can be used as therapeutic tools for the induction of antigen-specific tolerance.

Blaser, 2001; Veillette and Latour, 2003). More studies will be necessary to elucidate the effects of IL-10 on DCs *in vivo*.

TGFβ, a cytokine produced by Tregs and other sources in many tissues, has also profound effects on DCs *in vitro*. Using animals that express a dominant negative form of the TGFβR complex (dnTGFβR) specifically on DCs, the Flavell group has shown that the action of TGFβ allows DCs to attenuate the neuropathology associated with experimental autoimmune encephalomyelitis (EAE; Laouar *et al.*, 2008). Functional TGFβR (and TGFβ-producing Tregs; Feuerer *et al.*, 2009) is also required on NK cells to restrain their proinflammatory activity (Laouar *et al.*, 2005). Thus, the TGFβ pathway is a major mechanism by which Tregs control both NK cells and DCs. Ligation of TGFβR leads to heterodimerization of Smad2 and Smad4, which regulate gene expression in the nucleus (Miyazono, 2000; Rubtsov and Rudensky, 2007). The downstream consequences appear similar to those of IL-10 and include inhibition of DC the maturation through blockade of NFκB signaling. However, in contrast to IL-10, TGFβ signaling induces a much larger set of genes in DCs (Karlsson *et al.*, 2005). The TGFβ-induced transcriptional program in

tDCs includes TGFβ production itself as well as TGFβR, CXCL14, IL-18, the transcription factors peroxisome proliferator-activated receptor γ (PPARγ) and plasminogen activator inhibitor 1 (Fainaru *et al.*, 2007; Sargent *et al.*, 2010). The specific role of each of these factors in tDC function remains to be analyzed.

Other bioderivatives instructing itDCs are HGF and the vitamin D3 metabolite, 1,25D3. When treated *in vitro* with these compounds, DCs initiate the expression of gene products that have been implicated immune tolerance, including IDO, C5R1, CCL2, IL-10, TGFβ, TRAIL, inhibin, and the inhibitory receptors CD300LF and CYP24A1 (Rutella *et al.*, 2006; Széles *et al.*, 2009). Several other factors, such as estrogen, VIP, binding immunoglobulin protein (BiP), TSLP, GM-CSF, G-CSF, IFNα/β/γ, IL-6, PGE2, and TNFα, may also promote Treg-inducing capacities on tDCs.

Antibodies and synthetic soluble ligands of specific surface receptors have also been used to produce itDCs. For example, human MoDC treated with HLA-G, a nonclassical histocompatibility molecule associated with tolerance, induced suppressive autologous T cells that expressed CD25 and CTLA4, two markers commonly found on Tregs (Liang *et al.*, 2008; Ristich *et al.*, 2005). Similarly, the antibody-mediated activation of the suppressive receptor CD200R boosts the tolerogenicity of mouse BMDCs by activating Tregs *in vivo* (Gorczynski, 2006; Gorczynski *et al.*, 2004, 2005, 2008).

3.2.2.2. Pharmacologically induced tolerogenic DCs The use of immunosuppressive drugs has been crucial for the treatment of many diseases. Not surprisingly, immunosuppressants frequently affect DC immunogenicity often by intervening with their maturation, although the specific contribution of such drug effects on DCs relative to their influence over other target cells is not known. Nevertheless, immunosuppressive compounds have been successfully employed to manipulate DC function in many disease models (Hackstein and Thomson, 2004).

Glucocorticoids (GCs) were the first immunosuppressants to be used in a clinical setting (Leung and Bloom, 2003). Treatment of human MoDC or mouse BMDC with prednisolone or dexamethasone conditions these cells for tolerogenic instruction of aTregs (Table 4.3). GC binding to the glucocorticoid receptor (GR) regulates DC activation through nuclear glucocorticoid response elements (GRE) that negatively regulate promoters for members of the canonical NFκB pathway, inflammatory cytokines, chemokines, their receptors, and antigen presentation molecules (Leung and Bloom, 2003). In addition to repressing DC maturation, dexamethasone also induces a discrete set of anti-inflammatory gene products and chemoattractants, including IL-10, GITRL, IDO, CCL2 (MCP-1), CCL8 (MCP-2), CCR2, CCL9 (MIP-1c), and CCLl2 (MIP-2) (Grohmann *et al.*, 2007; Roca *et al.*, 2007). This impairs the DCs' ability to migrate and

provokes them to assume a tolerogenic phenotype capable of instructing Tns to express CD25, Foxp3, and IL-10.

Many maturation signals for DCs induce phosphorylation and proteolysis of the inhibitor of NFκBα (IκBα) by the inhibitor kinase-β (IKKβ), thereby releasing Rel-A (or p65; a subunit of NFκB) for nuclear translocation. In contrast, the noncanonical pathway operational during tolerogenic instruction activates NFκB-inducing kinase (NIK) and IKKα resulting in the formation of Rel-B dimers (Bonizzi and Karin, 2004; Puccetti and Grohmann, 2007). The inhibitory effect of GCs on the canonical NFκB pathway likely plays a key role in the conversion of DCs to itDCs. Accordingly, inhibition of NFκB or IKKβ by small molecule antagonists produces itDCs with the capacity to stimulate Foxp3+CD25+ aTregs that alleviate disease symptoms in EAE, heart allograft rejection, and intestinal bowel disease (IBD; Buckland and Lombardi, 2009; Buckland *et al.*, 2006a,b; Cong *et al.*, 2009; Iruretagoyena *et al.*, 2006; Zhang *et al.*, 2008).

Recent observations suggest that cellular metabolism also plays a role in DC immunogenicity. For example, treatment of human MoDCs with resveratrol induces tDCs that stimulate IL-10-secreting aTregs (Kim *et al.*, 2004; Svajger *et al.*, 2010). Resveratrol activates sirtuin 1 (SIRT-1) and PPARγ coactivator (PGC)-1α, which are involved in energy metabolism (Pervaiz and Holme, 2009). Another pathway affecting metabolism and DC immunogenicity is represented by the serine/threonine kinase mammalian target of rapamycin (mTOR). This kinase forms signaling complexes that sense oxygen supply, free amino acids, ATP levels, growth factors, cytokines, and cellular stress (Hay, 2004). Inhibition of mTOR by rapamycin, a macrolide from *Streptomyces hygroscopicus*, exerts immunosuppressive effects in humans and animals (Augustine *et al.*, 2007) and has shown efficacy in both clinical and preclinical settings of autoimmunity and inflammatory disease (Battaglia *et al.*, 2006; Esposito *et al.*, 2010; Fu *et al.*, 2010; Ge *et al.*, 2009; Massey *et al.*, 2008; Monti *et al.*, 2008; Raimondi *et al.*, 2010; Valle *et al.*, 2009; Zang *et al.*, 2008). Treatment of DCs with rapamycin stimulates Treg expansion *in vivo* and *in vitro* (Battaglia *et al.*, 2005; Horibe *et al.*, 2008; Ohtani *et al.*, 2008; Thomson *et al.*, 2009; Turnquist *et al.*, 2007). We will further discuss this subject in Section 4.3 below.

3.2.2.3. Genetically induced tolerogenic DCs Various genetic manipulations have been used, including gene knock-out, knock-down, and transgenic overexpression of active or dominant negative mutants of molecules involved in DC maturation to enhance or inhibit DC tolerogenicity (Morelli and Thomson, 2007). Genetically induced tDCs can induce hyporesponsiveness and prolong allograft survival when transferred to transplant recipients, but a mechanistic role for tDC-induced Treg differentiation has only been established in a few cases. For instance, RelB deficient DCs induce CD40+ Tregs that suppressed delayed-type

hypersensitivity (DTH) and experimental autoimmune myasthenia gravis (EAMG; Martin *et al.*, 2003; Yang *et al.*, 2010; Zhang *et al.*, 2009a). This provides yet another example for the importance of NFκB (and presumably CD40) activation in a DC's decision on whether to exert immunogenic or Treg-inducing effects. Similarly, BMDCs that overexpressed dominant negative IKKβ were refractory to maturation and prone to induce Tregs that enhanced kidney allograft survival (Tomasoni *et al.*, 2005). Another approach to target NFκB-dependent effects in maturing DCs is to eliminate the expression of downstream target genes. Silencing of IL-12, CD80, CD86, and/or CD40 results in DCs that stimulate Treg differentiation and alleviates disease symptoms in collagen-induced arthritis (CIA) and EAMG (Martin *et al.*, 2003; Zheng *et al.*, 2010).

An alternative approach to silencing immunogenic molecules is the forced expression of tolerogenic factors. For example, treatment with IL-10-transduced DCs prevents the development of experimental asthma (EA) by boosting CD4+CD25+Foxp3+, IL-10 secreting Tregs that effectively transfer tolerance to naïve animals. IL-10 produced by recipient cells is required to establish this infectious tolerance demonstrating that Tregs require other supporting cell populations to suppress immune responses (Henry *et al.*, 2008). Remarkably, transduction of DCs with ectopic Foxp3 also results in itDCs that stimulate CD4+Foxp3+ aTregs (Lipscomb *et al.*, 2010). The mechanism by which Foxp3 controls the tolerogenic potential of DCs remains unknown but likely involves pathways similar to those that induce Tregs (Kim and Rudensky, 2006).

4. HOW ARE TDCS INDUCING TREGS?

tDCs can induce Tregs by several different pathways that may act either alone or in combination. As discussed above (Section 2.2), a relatively simple Treg-promoting condition involves presentation of modest levels of a cognate antigen in the absence of signals 2 and 3, which is thought to be employed by iDCs but probably applies also to tDCs (Fig. 4.2). In addition, tDCs can produce anti-inflammatory molecules that may be secreted, membrane bound, or both. Such signals may act directly on T cells and/or modify environmental conditions, such as the metabolic state of a tissue to fine-tune T cell differentiation.

4.1. Influence of the maturation status of DC in the induction of Tregs

Studies by several laboratories have shown that presentation of very low levels of antigen in the absence of other stimuli promotes Treg differentiation *in vitro* and *in vivo* (Apostolou and von Boehmer, 2004; Hermann-

Kleiter and Baier, 2010; Kretschmer *et al.*, 2005, 2006; Picca *et al.*, 2006). Another key factor for efficient differentiation of aTregs and function of nTregs is a milieu containing little or no inflammatory cytokines, such as IL-6 and IL-12, or costimulatory membrane receptors (CD80/86/40), which counteract the tolerogenic effect of iDCs and enhance effector differentiation of Tns (King and Segal, 2005; Pandiyan *et al.*, 2007; Pasare and Medzhitov, 2003). TCR signals in conjunction with costimulation precipitate a signaling cascade resulting in intracellular calcium (Ca^2+) flux and the activation of the transcription factors nuclear factor of activated T cells (NFAT), activator protein 1 (AP-1), and NFκB that coordinate gene expression in nascent Teffs (Hogan *et al.*, 2010). While activated T cells that acquire effector functions express IL-2, IL-4, IL-17, T-bet, Edg3, and CD69 among others (Fontenot *et al.*, 2005), differentiating Tregs present a different transcriptional signature (Feuerer *et al.*, 2010; Fontenot *et al.*, 2005; Hill *et al.*, 2007; Sadlon *et al.*, 2010) driven by NFAT, Foxp3, and runt-related transcription factor 1 (Runx-1 or myeloid leukemia factor, AML1; Hermann-Kleiter and Baier, 2010, Hu *et al.*, 2007; Sakaguchi *et al.*, 2008). Indeed, the Treg transcriptome is enriched with gene products implicated in their suppressive function like IL-10, CD103, Killer cell lectin-like receptor subfamily G member 1 (Klrg1), neuropilin 1 (Nrp1), GITR, ICOS (CD278), fibrinogen-like protein 2 (Fgl2), probable G-protein coupled receptor 83 (Gpr83), and CTLA-4. However, it is still unclear, how exactly iDCs or tDCs skew the TCR signaling cascade in Tns to accomplish the subsequent selection of Treg-associated transcription factors. Furthermore, as discussed above, some mature and semimature DC expressing high levels of costimulatory molecules can also induce suppressive function on T cells (Reis and Sousa, 2006). Thus, the magnitude of antigen presentation/costimulation or activating cytokines alone cannot fully explain the function of all tDCs subsets.

4.2. Tolerogenic factors produced by tDC

The presence of IL-10 has been identified in numerous settings of tolerance (Tables 4.1–4.3). Indeed, secretion of IL-10 by tDCs is necessary for tolerance in a variety of models of Treg differentiation (Akbari *et al.*, 2001; McGuirk *et al.*, 2002; Wakkach *et al.*, 2003). IL-10 can initiate a powerful anti-inflammatory positive feedback loop because it can both modify and be produced by leukocytes and structural cells within tissues (e.g., IECs, AECs, and LSECs). Thus, when tDCs are induced by IL-10 in peripheral tissues, they acquire the ability to secrete IL-10 themselves and migrate to lymphoid organs, where tDC-derived IL-10 then contributes to Treg differentiation and proliferation. Having been instructed by tDCs, the activated Tregs enter the blood stream and home to the peripheral organ, where antigen recognition triggers their production of even more

IL-10 (Scott-Browne *et al.*, 2007; Shafiani *et al.*, 2010; Sharma *et al.*, 2009; Zhang *et al.*, 2009b). In the presence of this cytokine proliferation, cytokine production and migratory capacities of effector T cells are impaired (Moore *et al.*, 2001). Mechanistically, the Akdis and Blaser groups have shown that ligation of IL10R overrides costimulatory signaling via activation of SHP-1, which dephosphorylates the cytoplasmic tails of CD28, ICOS, and CD2, thus inhibiting the recruitment of phosphatidylinositol-3-kinase (PI3K; Akdis and Blaser, 2001; Akdis *et al.*, 2000, 2001; Taylor *et al.*, 2007, 2009b). Additionally, IL-10 signaling is also required for the stabilization of the suppressive phenotype of Tregs in the face of strong inflammatory signals (Murai *et al.*, 2009).

TGFβ is unique among cytokines in that it can induce Foxp3 expression and aTreg differentiation in the absence of DCs (Chen *et al.*, 2003). However, it is not clear whether and to what extent the tolerogenic capacity of tDCs relies on TGFβ production. Exploring this question is complicated by the fact that TGFβ effects are highly pleiotropic, and genetic mutants present complex phenotypes with multiple immune disorders and poor survival (Rubtsov and Rudensky, 2007). A strong argument for the importance of TGFβ production by tDCs has come from animals with a DC-restricted deletion of the TGFβ-activating integrin, $\alpha_v\beta_8$. These mutant mice develop autoimmunity similar to animals in which DCs are chronically depleted or TGFβR signaling is dysfunctional in T cells, suggesting that DCs are important to ensure the bioavailability of active TGFβ (Birnberg *et al.*, 2008; Gorelik and Flavell, 2000; Kim *et al.*, 2006; Ohnmacht *et al.*, 2009; Travis *et al.*, 2007). Antigen presentation by DCs in the presence of TGFβ results in the differentiation of Foxp3+ aTregs (Yamazaki *et al.*, 2008), which present a transcriptional signature that is similar to, but distinct from that of nTregs (Chen and Konkel, 2010; Feuerer *et al.*, 2010; Rubtsov and Rudensky, 2007). A recent study has shown that activation of Foxo3a and Foxo1 by TGFβ signaling precedes Foxp3 expression in aTregs (Harada *et al.*, 2010). However, we are only beginning to understand how Treg differentiation is controlled upstream of Foxp3.

Some DCs can synthesize RA, a metabolite of vitamin A that is generated by RALDH. Most intestinal DCs express at least one of the three isoforms of this enzyme, while most DCs in other lymphoid tissues express little or no RALDH (Iwata *et al.*, 2004). When T or B cells are activated in the presence of DC-derived RA, they are ''imprinted'' to express gut homing receptors (Iwata *et al.*, 2004; Mora *et al.*, 2006). In addition, exposure of activated CD4 T cells to RA promotes their differentiation into Foxp3+ aTregs (Belkaid and Oldenhove, 2008; Benson *et al.*, 2007; Hill *et al.*, 2008; Mora *et al.*, 2008; Mucida *et al.*, 2009; Nolting *et al.*, 2009; Siddiqui and Powrie, 2008; von Boehmer, 2007). RA binds the nuclear RA receptor α (RARα) and regulates the expression of Foxp3

and Smad3 in T cells (Nolting *et al.*, 2009; Takaki *et al.*, 2008), but whether RARα is necessary for differentiation of Tregs *in vivo* is unclear. It has been suggested that RA is particularly relevant in aTreg differentiation in mucosal environments because the continuous exposure to commensal antigens requires a fine balance between tolerance and immunity (Manicassamy and Pulendran, 2009). Recent observations suggest that some DCs in the skin also express RALDH and may produce RA for dermal Treg differentiation (Guilliams *et al.*, 2010). More experimentation will be necessary to evaluate the exact role of RA-producing DCs for tolerance versus immunity *in vivo*.

tDCs also express several membrane receptors that may instruct antigen-specific Tns during their activation. Among these are the immunoglobulin-like transcript (ILT) receptors, which are found on tDCs that stimulate Treg differentiation (Gregori *et al.*, 2009, 2010; Vlad *et al.*, 2010). The proximal signaling cascade for ILTs is not known and the impact of ILT recognition by T cells is also not well established. However, multiple groups have shown an important role for these molecules in cancer, transplantation, and autoimmunity by using animals deficient for the expression of ILTs, blocking antibodies, and recombinant ILT3 (Vlad *et al.*, 2009, 2010; Wu and Horuzsko, 2009). DCs also express programmed death-1 ligands (PD-Ls), PD-L1 and PD-L-2, which control T cell activation through engagement of PD-1 and CD80 (in case of PD-L1) (Keir *et al.*, 2007). PD-1 is a critical determinant of ''exhausted'' T cells that arise during chronic viral infections, and it also contributes to Treg differentiation (Francisco *et al.*, 2009; Keir *et al.*, 2007; Riley, 2009; Wang *et al.*, 2008). The effects of PD-1 signaling resemble those of the IL10R by limiting PI3K activation and shutting down costimulatory signaling through SHP-1. However, PD-1 is not thought to be expressed by Tns, but is only upregulated during activation, so its role (if any) in the initial phase of Treg education is uncertain.

4.3. DCs and metabolism

Immune responses precipitate dramatic changes in the metabolic state of many cells. Changes in intra- and extracellular metabolites are becoming increasingly recognized as integral part of the ''information content'' of tissues in which immune responses are induced. For example, differentiation of inflammatory cells and the induction of T cell memory *in vivo* can be modified by the dietary abundance of amino acid and fatty acid metabolism (Pearce, 2010; Pearce *et al.*, 2009; Sundrud *et al.*, 2009). DCs also modulate T cell differentiation by modifying metabolic parameters surrounding T cells. DCs can release IDO and heme oxygenase-1 (HO-1) to control the abundance of environmental tryptophan and carbon monoxide (CO), respectively. In the presence of extracellular IDO, T cells

proliferation is compromised and aTregs differentiation is enhanced, although the precise molecular basis for this effect is unclear (Belladonna *et al.*, 2009; Curti *et al.*, 2009; Katz *et al.*, 2008; Löb and Königsrainer, 2009; Mellor and Munn, 2004). IDO expression by DCs is induced by IFNγ and TGFβ suggesting that this enzyme may represent a feedback mechanism by which DCs modulate their own immunogenicity during inflammation (Jurgens *et al.*, 2009; Orabona *et al.*, 2006). HO-1 degrades heme, thereby producing CO, which inhibits DC immunogenicity (Rémy *et al.*, 2009). Indeed, HO-1 has a potent anti-inflammatory effect that may be mediated through Treg activity (Chora *et al.*, 2007; Yamashita *et al.*, 2006), but the mechanisms are still incompletely understood.

The serine/threonine kinase mTOR plays a pivotal role in DC immunogenicity and the control Treg differentiation. Activation of TLR signaling stimulates mTOR and promotes sDC function (Cao *et al.*, 2008; Schmitz *et al.*, 2008), whereas blockade of mTOR activity by hypoxia, amino acid starvation, or rapamycin enhances Tregs (Ben-Shoshan *et al.*, 2008; Cobbold *et al.*, 2009; Haxhinasto *et al.*, 2008; Sauer *et al.*, 2008; Thomson *et al.*, 2009). mTOR is involved in the regulation of numerous essential cellular processes, such as cell cycle progression, protein synthesis, lipid metabolism, and mitochondrial biogenesis (Delgoffe and Powell, 2009; Laplante and Sabatini, 2009; Thomson *et al.*, 2009). Treatment of DCs with the mTOR inhibitor rapamycin interferes with antigen processing and presentation, partly by regulating autophagy and production of MHC complexes, and also alters the response to cytokines, chemokines, growth factors, and TLRs agonists (Thomson *et al.*, 2009). It has been reported that rapamycin-treated DCs do not directly induce aTreg differentiation (Turnquist *et al.*, 2007); however, DC exposure to a combination of rapamycin and TGFβ effectively potentiates the capacity of DCs to induce aTreg differentiation (our unpublished results). It will be important to assess whether and how maturation and differentiation signal alter the metabolic state (e.g., oxidative vs. glycolytic) of iDCs that give rise to either sDCs or tDCs, and how such metabolic changes may be linked to the phenotypic and functional characteristics of these versatile cells.

5. CONCLUDING REMARKS

It is becoming increasingly clear that both mature and immature DC subsets can support immunological tolerance through Tregs and other mechanisms. A variety of environmental cues that may arise naturally or by pharmacological or experimental intervention can coerce iDCs to acquire a stable tolerogenic disposition that is preserved, even in the face of concomitant maturation signals. These tDCs can induce or enhance the suppressive function of existing Tregs and convert activated Tns into

aTregs. At present, we have only rudimentary knowledge of the rules that govern tolerogenic versus immunogenic functions of DCs, and the signals that tDCs use to transmit their suppressive message to T cells are also still incompletely understood. A better understanding of these issues may offer new opportunities for the treatment of autoimmunity, allograft rejection, allergy, asthma, and various forms of hypersensitivity. Therapeutic applications of tDCs, either by cellular therapy or by targeting of endogenous DCs with novel drugs, could accomplish effects that elude traditional strategies for immune suppression. Specifically, while systemic immunosuppressants exert broadly paralyzing effects on immune cells, tDCs can induce tolerance to the specific antigens that elicit pathologic immune responses in a patient without compromising the immune defense against pathogens or tumors. While the prospect of clinical translation is exciting and seems almost within reach, substantial gaps in our knowledge remain to be filled before we will be able to exploit the full potential of tDC-based therapy.

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