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Lymphocyte Development: Integration of DNA Damage Response Signaling

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Abstract

Lymphocytes traverse functionally discrete stages as they develop into mature B and T cells. This development is directed by cues from a variety of different cell surface receptors. To complete development, all lymphocytes must express a functional nonautoreactive heterodimeric antigen receptor. The genes that encode antigen receptor chains are assembled through the process of V(D)J recombination, a reaction that proceeds through DNA double-stranded break (DSB) intermediates. These DSBs are generated by the RAG endonuclease in G1-phase developing lymphocytes and activate ataxia-telangiectasia mutated (ATM), the kinase that orchestrates cellular DSB responses. The canonical DNA damage response includes cell cycle arrest, DNA break repair, and apoptosis of cells when DSBs are not repaired. However, recent studies have demonstrated that ATM activation in response to RAG DSBs also regulates a transcriptional program including many genes with no known function in canonical DNA damage responses. Rather, these genes have activities that would be important for lymphocyte development. Here, these findings and the broader concept that signals initiated by physiologic DNA DSBs provide cues that regulate cell type-specific processes and functions are discussed.

1. LYMPHOCYTE ANTIGEN RECEPTOR GENE ASSEMBLY

1.1. Overview

Foreign antigens are recognized by receptors on B lymphocytes (the B cell receptor or BCR) and T lymphocytes (the T cell receptor or TCR). BCRs and TCRs are heterodimers with each polypeptide chain composed of a C-terminal constant region and an N-terminal variable region that makes direct contact with antigen. The first two exons of all antigen receptor genes encode the variable region. The second exon is assembled in developing lymphocytes from component variable (*V*), joining (*J*), and, in some cases, diversity (*D*) gene segments through the process of V(D)J recombination, which requires the generation and repair of DNA double-strand breaks (DSBs) (Tonegawa, 1983). The assembly of different *V*, *D*, and *J* gene segments (combinatorial diversity) and the imprecision of the assembly process (junctional diversity) together provide the basis for the vast number of foreign antigens that the adaptive immune system is capable of recognizing. Moreover, successful assembly of the genes encoding the appropriate antigen receptor heterodimer is essential for normal B and T cell development. The BCR and TCR provide critical signals that support cell survival, proliferation, and maturation.

1.2. The V(D)J recombination reaction

The V(D)J recombination reaction can be divided into a DNA cleavage step and a DNA joining step. This reaction is initiated when RAG-1 and RAG-2 (RAG endonuclease) introduce DNA DSBs at the border of two recombining gene segments and their flanking RAG recognition sequences (recombination signals, RSs) (Fugmann, Lee, Shockett, Villey, & Schatz, 2000; Gellert, 2002; Oettinger, 1999). This DNA cleavage results in the paired formation of broken DNA signal ends and coding ends that are joined forming a signal joint and coding joint, respectively. RSs are composed of conserved heptamer and nonamer sequences separated by either 12 or 23 nonconserved base pairs. RAG cleavage occurs only in the setting of a synaptic complex that contains a pair of RSs of differing spacer lengths, referred to as the 12/23 rule. If the two gene segments are in the same transcriptional orientation, then rearrangement leads to excision of the intervening signal-end flanked DNA sequence from the chromosome (Helmink & Sleckman, 2012). During these deletional rearrangements, coding joints are formed within the chromosomal context while the joining of signal ends results in the formation of extrachromosomal circular DNA fragments. In contrast, if two gene segments are in the opposite transcriptional orientation, then rearrangement leads to inversion of the intervening sequence with both the signal and coding joints forming within the chromosome (Helmink & Sleckman, 2012). During inversional rearrangements, formation of both coding and signal joints is required for maintaining the integrity of the chromosome.

1.3. Nonhomologous end-joining pathway in V(D)J recombination

DNA cleavage by the RAG proteins is restricted to the G1-phase of the cell cycle as RAG-2 is phosphorylated and degraded upon entry into the S-phase (Desiderio, Lin, & Li, 1996). Similar to other DNA DSBs generated in G1, RAG DSBs are repaired by the nonhomologous end-joining (NHEJ) pathway of DNA DSB repair (Helmink & Sleckman, 2012; Lieber, 2010; Rooney, Chaudhuri, & Alt, 2004). The core NHEJ factors XRCC4, DNA Ligase IV, Ku70, and Ku80 are necessary for forming signal joints and coding joints (Helmink & Sleckman, 2012; Lieber, 2010; Rooney et al., 2004). The Artemis endonuclease is required to open hairpin-sealed coding ends and, therefore, is essential for coding joint formation (Lieber, 2010). Artemis activity in opening hairpin-sealed coding ends is dependent on DNA-dependent protein kinase (DNA-PKcs), a member of the phosphatidylinositol-3-kinase (PI3K)-like family of serine-threonine kinases (Goodarzi et al., 2006; Ma, Pannicke, Schwarz, & Lieber, 2002; Smith & Jackson, 1999).

RAG DSBs activate ataxia-telangiectasia mutated (ATM), which is also a member of the PI3K-like family of serine-threonine kinases (Helmink & Sleckman, 2012; Shiloh, 2003). During the repair of RAG-mediated DSBs, ATM promotes the stability of coding ends in post cleavage complexes until they can be joined (Bredemeyer et al., 2006). This function of ATM depends on its kinase activity and may be due to ATM-mediated modulation of the DNA tethering activity of the Mre11, Rad50, and Nbs1 (MRN) complex, which associates with RAG DSBs (Bredemeyer et al., 2006; Chen et al., 2000; D'Amours, & Jackson, 2002; Deriano, Stracker, Baker, Petrini, & Roth, 2009; Helmink et al., 2009; Stracker & Petrini, 2011).

Although neither ATM nor DNA-PKcs are absolutely required for signal joint formation, their combined deficiency results in a block in signal joining suggesting that these two kinases have overlapping activities in repairing signal ends (Gapud et al., 2011; Helmink & Sleckman, 2012; Zha, Jiang, et al., 2011). Additionally, combined deficiency of the XLF/Cernunnos NHEJ protein and ATM, H2AX or 53BP1 leads to significant defects in the repair of RAG DSBs that are more severe than in isolated deficiency of these proteins (Li et al., 2008; Liu et al., 2012; Oksenych et al., 2012; Zha, Guo, et al., 2011). Thus, XLF has

important redundant or synergistic activities with other factors during NHEJ-mediated repair of RAG DSBs.

1.4. B lymphocyte antigen receptor genes

The B cell receptor (BCR), or immunoglobulin (Ig), is composed of two heavy (H) chains and two light (L) chains, either *IgL κ* or *IgL λ* . The second exon of the *IgH* gene is assembled from *V*, *D*, and *J* gene segments. The mouse *IgH* locus spans 2.5 MB and has approximately 195 *V_H* gene segments in the 5' region of the locus followed by 13 *D_H* and 4 *J_H* gene segments (Max, 2008). Downstream of the *J_H* gene segments lie eight different constant region genes. The second exons of the *IgL κ* and *IgL λ* chain genes are assembled from *V* and *J* gene segments. The mouse *IgL κ* locus has approximately 140 *V κ* gene segments and four functional *J κ* gene segments followed by the *IgL κ* constant region gene (Max, 2008). Given the structure of the *V κ* and *J κ* gene segments, it is possible to replace a nonfunctional *VJ κ* rearrangement with a new *VJ κ* through the rearrangement of an upstream *V κ* to a downstream *J κ* (Gorman & Alt, 1998). The mouse *IgL λ* chain comprises three *V λ* gene segments, five *J λ* gene segments, and four constant region clusters and is structured such that each *J λ* gene segment is adjacent to a constant region (Max, 2008).

1.5. Developmental regulation of *IgH* and *IgL* chain gene assembly

IgH chain gene rearrangement is initiated at the pro-B cell stage. Productive assembly of an *IgH* chain gene results in its expression in conjunction with the surrogate light chains (VpreB and $\lambda 5$) as a pre-BCR (Herzog, Reth, & Jumaa, 2009). Signals from the pre-BCR lead to cessation of RAG expression, cellular proliferation, and transit to the pre-B cell developmental stage (Herzog et al., 2009). Additionally, pre-BCR signals prevent additional *V_H* to *DJ_H* rearrangements on the alternate allele by a process termed allelic exclusion (Bergman, 1999; Brady, Steinell, & Bassing, 2010; Herzog et al., 2009; Jung, Giallourakis, Mostoslavsky, & Alt, 2006; Sen & Oltz, 2006). Pre-B cells must generate a productive *IgL* chain gene encoding an *IgL* chain that can pair with the *IgH* chain to generate a nonautoreactive mature BCR (Nemazee et al., 2000). *IgL* gene rearrangement is regulated by pre-BCR and cytokine signals (i.e., interleukin-7 (IL-7)), which control RAG expression and *IgL* locus accessibility (Corfe & Paige, 2012; Erlandsson et al., 2005; Herzog et al., 2009). If the completed *IgL* rearrangement is productive and encodes a non-self-reactive BCR, signals from the mature receptor suppress RAG expression and support continued maturation (Nemazee et al., 2000). However, if the *IgL* chain gene rearrangement is nonproductive or forms a self-reactive BCR, additional *IgL* chain gene rearrangements can occur (Casellas et al., 2001; Halverson, Torres, & Pelanda, 2004; Nemazee, 2006). Thus, multiple RAG DSBs are generated and repaired in pre-B cells as they attempt to assemble a functional *IgL* chain gene.

2. DNA DAMAGE RESPONSES

2.1. Overview

The activation of DNA damage responses by DNA DSBs requires sensor proteins to detect DSBs and transducer kinases that phosphorylate downstream effectors (Rouse & Jackson, 2002; Zhou & Elledge, 2000). The main transducers that orchestrate DNA damage responses are the ATM kinase, the DNA-PKcs, and the ATM related kinase (ATR), which are all members of the PI3K-like family of serine-threonine kinases (Durocher & Jackson, 2001; Shiloh, 2003; Smith & Jackson, 1999). These kinases phosphorylate serines or threonines that are followed by glutamine (SQ/TQ). ATM and DNA-PKcs are activated by DNA DSBs generated at all phases of the cell cycle whereas ATR is activated primarily by DNA DSBs in the S-G2 phases of the cell cycle.

2.2. ATM

ATM is a 350-kDa kinase found in all cells as an inactive homodimer (Bakkenist & Kastan, 2003; Shiloh, 2003). Activation of ATM by DNA DSBs involves an autophosphorylation step that converts inactive dimers into catalytically active monomers (Bakkenist & Kastan, 2003). However, mice expressing a mutant form of ATM that is unable to undergo this autophosphorylation do not appear to have defects in the activation of ATM-dependent DNA damage responses, demonstrating that ATM can be activated in additional ways (Pellegrini et al., 2006). Humans deficient in ATM have ataxia-telangiectasia (A-T), which is a multisystem disease comprising cerebellar ataxia, oculocutaneous telangiectasia, genomic instability, mild immunodeficiency, and a predisposition to developing lymphoid tumors with translocations involving antigen receptor loci (Lavin & Shiloh, 1997). ATM-deficient mice share many of the lymphoid hallmarks of ataxia telangiectasia (Barlow et al., 1996; Elson et al., 1996; Xu et al., 1996).

ATM is recruited to the site of a DNA DSB by the MRN complex, which is composed of the Mre11, Rad50, and Nbs1 proteins (D'Amours & Jackson, 2002; Stracker & Petrini, 2011). Mre11 has nuclease activity and Rad50 tethers sister chromatids, both of which are important for homology-mediated DNA DSB repair. The function of Nbs1 is less well understood, but it does bind to ATM and thus may be involved in recruiting ATM to the DSB (Kobayashi, Antocchia, Tauchi, Matsuura, & Komatsu, 2004; You, Chahwan, Bailis, Hunter, & Russell, 2005). The recruitment of ATM to a DNA DSB by MRN is required for optimal activation of ATM by the DSB (Lee & Paull, 2004, 2005). Moreover, once activated ATM can phosphorylate Mre11, Rad50, and Nbs1 potentially altering their activities (Gatei, Jakob, et al., 2011; Gatei, Young, et al., 2000; Lim et al., 2000; Matsuoka et al., 2007; Wu et al., 2000). Mutations in Mre11 and Nbs1 result in A-T-like disorder and Nijmegen breakage syndrome, respectively, which are characterized by chromosomal instability and immune deficiencies similar to the abnormalities seen with ATM-deficiency (Digweed & Sperling, 2004; Taylor, Groom, & Byrd, 2004).

2.3. DNA-PKcs

DNA-PKcs is a 450-kDa kinase recruited to the site of a DNA DSB by the Ku70 and Ku80 proteins (Meek, Dang, & Lees-Miller, 2008; Smith & Jackson, 1999; Weterings & Chen, 2007). Together, these three proteins comprise the DNA-PK complex. Deficiency of DNA-PKcs in mice leads to severe combined immunodeficiency (SCID) due to the requirement for DNA-PKcs to promote coding joint formation (Helmink & Sleckman, 2012; Rooney et al., 2004). These mice also exhibit sensitivity to ionizing radiation demonstrating that, in addition to functioning in the repair of RAG-mediated DSBs, DNA-PKcs also functions in the repair of genotoxic DSBs. DNA-PKcs function is modulated by autophosphorylation and through phosphorylation by ATM (Meek et al., 2008; Weterings & Chen, 2007). Interestingly, *DNA-PKcs* null mutations have not been identified in patients with SCID, although a hypomorphic *DNA-PKcs* mutation has been described (van der Burg et al., 2009).

2.4. DNA damage responses in G1-phase cells

In response to DNA DSBs, ATM phosphorylates hundreds of targets resulting in the initiation of cell cycle arrest, the activation of DNA repair pathways and the initiation of apoptosis in cells that fail to repair DNA breaks (Matsuoka et al., 2007; Rouse & Jackson, 2002; Shiloh, 2003; Zhou & Elledge, 2000). Importantly, DNA-PKcs can also phosphorylate many of these targets and can coordinate DNA damage responses in ATM-deficient cells (Callen et al., 2009; Tomimatsu, Mukherjee, & Burma, 2009). Mice deficient in ATM and DNA-PKcs exhibit early embryonic lethality demonstrating that these proteins have important overlapping activities (Gurley & Kemp, 2001; Sekiguchi et al., 2001).

In response to DNA damage, ATM phosphorylates proteins that directly participate in canonical DNA damage responses. For example, phosphorylation of the Chk2 kinase by ATM leads to its activation and phosphorylation of Cdc25A promoting cell cycle arrest (Reinhardt & Yaffe, 2009). ATM also phosphorylates the histone protein H2AX for distances of up to several hundred kilobases in chromatin flanking DNA DSBs (Fernandez-Capetillo, Lee, Nussenzweig, & Nussenzweig, 2004; Rogakou, Pilch, Orr, Ivanova, & Bonner, 1998). H2AX is a variant of the H2A histone protein present in a fraction of nucleosomes throughout the genome (Fernandez-Capetillo et al., 2004). H2AX differs from H2A by the presence of a unique 22 amino acid C-terminal peptide that contains a serine residue that is phosphorylated by ATM or DNA-PKcs (Callen et al., 2009; Fernandez-Capetillo et al., 2004; Mannironi, Bonner, & Hatch, 1989; Stiff et al., 2004; Tomimatsu et al., 2009). Phosphorylated H2AX (γ -H2AX) recruits DNA damage response proteins to the site of the broken DNA and supports functions in several aspects of DNA damage responses (Fernandez-Capetillo et al., 2004).

ATM phosphorylates proteins that lead to the activation of specific transcriptional pathways regulating DNA damage responses. Perhaps the most notable pathways are p53 and NF- κ B. In response to DNA DSBs, ATM phosphorylates p53 on serine 15 (serine 18 in mouse), which prevents its degradation leading to increased p53 levels (Banin et al., 1998; Khanna et al., 1998; Vousden & Prives, 2009). Once stabilized, p53 regulates the transcription of numerous cell cycle and cell death genes (Vousden & Prives, 2009). For example, *p21* is upregulated and enforces G1 cell cycle arrest. Additionally, p53 activates expression of *BAX*, *PUMA*, and *NOXA*, a group of pro-apoptotic factors that trigger cell death in cells with persistent DSBs (Vousden & Prives, 2009; Youle & Strasser, 2008).

In addition to p53, genotoxic DNA DSBs activate the canonical NF- κ B pathway (Miyamoto, 2011; Wu, Shi, Tibbetts, & Miyamoto, 2006). Activation of the canonical NF- κ B pathway occurs when the IKK β (I κ B kinase β) kinase phosphorylates I κ B α causing its degradation and release of the p50/p65 (RelA) heterodimer allowing for translocation to the nucleus where it regulates gene expression (Hayden & Ghosh, 2012). The activation of the IKK β kinase is regulated by NEMO (NF- κ B essential modifier or IKK γ), which is activated in the cytoplasm by cell surface receptors through ubiquitination (Hayden & Ghosh, 2012). Interestingly, DNA damage responses utilize a unique mechanism for nuclear activation of NF- κ B transcription (Miyamoto, 2011). In conjunction with RIP1 (receptor interacting protein 1) and PIDD (p53-induced death domain), NEMO can translocate across the nuclear membrane (Hur et al., 2003; Janssens, Tinel, Lippens, & Tschopp, 2005). In responses to genotoxic DNA injury, ATM phosphorylates nuclear NEMO on serine 85 (Wu et al., 2006). Phosphorylated NEMO is then sumoylated, ubiquitinated, and relocalized to the cytoplasm where it is polyubiquitinated resulting in activation of IKK β and subsequent release of p50/p65 heterodimers (Jin et al., 2009; Mabb, Wuerzberger-Davis, & Miyamoto, 2006). NF- κ B activates expression of several genes, including pro-survival factors, in response to genotoxic DSBs (Elkon et al., 2005; Rashi-Elkeles et al., 2006; Wu et al., 2006).

3. RESPONSES TO RAG-MEDIATED DNA DSBs

3.1. Overview

Almost 20 years ago, it was demonstrated that unrepaired RAG DSBs in developing thymocytes from DNA-PKcs-deficient (SCID) mice activate p53 (Guidos et al., 1996; Nacht et al., 1996). Given that these DSBs are essential for lymphocyte development, mechanisms must exist to ensure that p53 does not generally promote the death of cells attempting to assemble antigen receptor genes. It is conceivable that RAG DSBs only activate ATM and DNA damage responses if they persist un-repaired posing a hazard to the cell. However, several studies have indicated that ATM is activated by most, if not all, RAG DSBs and is

required for the appropriate repair of these DNA breaks (Helmink & Sleckman, 2012). Moreover, in response to RAG DSBs, ATM activates transcription pathways that regulate transcriptional programs to balance pro-apoptotic and pro-survival signals (Bednarski et al., 2012; Bredemeyer et al., 2008). Remarkably, this genetic program also includes many genes that are expressed in a tissue-specific manner and function in processes important for normal lymphocyte development.

3.2. Studying the responses to RAG-mediated DNA breaks

The cellular response to chromosomal RAG DSBs has been studied using v-abl kinase transformed late pro-B/early pre-B cells (Chen, Wang, Huang, & Rosenberg, 1994; Yancopoulos et al., 1986). These immortalized B cells have *IgH* chain, but not *IgL* chain, gene rearrangements (Chen et al., 1994; Yancopoulos et al., 1986). Inactivation of the v-abl kinase results in expression of a genetic program similar to that of primary pre-B cells (Muljo & Schlissel, 2003). These cells will be referred to here as abl pre-B cells, but they are referred to elsewhere as abl pro-B cells.

Rosenberg and colleagues used a temperature sensitive v-abl kinase to generate abl pre-B cells (Mainville et al., 2001). These cells ectopically expressed Bcl2 to further promote survival, especially after inactivation of the v-abl kinase. At the permissive temperature, the v-abl kinase promotes cell division and suppresses RAG gene expression. At the nonpermissive temperature, the v-abl kinase is inactivated resulting in G1 cell cycle arrest, induction of RAG expression, and initiation of V(D)J recombination at the endogenous *IgL κ* locus, as normally occurs in pre-B cells.

More recently, an alternative approach has been developed in which abl pre-B cells with a *Bcl2* transgene are treated with the v-abl kinase inhibitor STI571 (imatinib) to inactivate the kinase (Bredemeyer et al., 2006). Treatment with STI571 triggers G1 cell cycle arrest, induction of RAG expression and rearrangement of the endogenous *IgL κ* locus or chromosomally integrated retroviral recombination substrates. Moreover, upon removal of STI571, these cells can reenter the cell cycle.

Chromosomal V(D)J recombination is also studied in primary bone marrow pre-B cells that are cultured *ex vivo* in IL-7. Culturing bone marrow cells in IL-7 leads to the proliferative expansion of pre-B cells and partial inhibition of RAG and *IgL κ* rearrangement (Corfe & Paige, 2012; Rolink, Kudo, Karasuyama, Kikuchi, & Melchers, 1991; Rolink, Streb, Melchers, 1991). Removal of IL-7 leads to cessation of proliferation, gain in RAG expression, and increase in *IgL κ* rearrangement (Corfe & Paige, 2012; Rolink, Kudo, et al., 1991; Rolink, Streb, et al., 1991). The cellular response to persistent RAG DSBs can also be studied using abl pre-B cells or IL-7 cultures generated from NHEJ-deficient mice (Helmink & Sleckman, 2012).

3.3. Activation of DNA damage responses by RAG DSBs

Cleavage by RAG results in the formation of four broken DNA ends: two blunt phosphorylated signal ends and two hairpin-sealed coding ends. Some, or all, of these broken DNA ends are capable of initiating DNA damage responses *in vivo* (Chen et al., 2000). ATM is not efficiently activated by hairpin-sealed DNA ends *in vitro* (Lee & Paull, 2005). However, experiments in Artemis-deficient abl pre-B cells demonstrate that ATM activates a gene expression program and generates γ -H2AX in chromatin flanking hairpin-sealed *J κ* coding ends (Bredemeyer et al., 2008; Savic et al., 2009). As signal joint formation proceeds normally in these cells, these findings support the notion that ATM is activated by hairpin-sealed coding ends *in vivo* (Rooney et al., 2002).

Whether signal ends directly activate DNA damage responses is less clear. In addition to differences in the structure of coding ends (hairpin-sealed) and signal ends (blunt phosphorylated), there are other important factors that could impact the ability of signal ends to activate DNA damage responses. After cleavage, the RAG proteins bind avidly to signal ends *in vitro*, which could interfere with efficient activation of ATM (Agrawal & Schatz, 1997; Hiom & Gellert, 1998; Jones & Gellert, 2001). However, ATM can be activated by streptavidin bound DNA ends (You, Bailis, Johnson, Dilworth, & Hunter, 2007). Notably, signal ends can be found in cells at the S–G2–M phases of the cell cycle (Pedraza-Alva et al., 2006). Given that these signal ends were generated by RAG cleavage in the G1-phase of the cell cycle, their presence in S-G2-M would imply that some of these DNA ends cannot activate the G1–S cell-cycle checkpoint (Desiderio et al., 1996).

Another important distinction between signal ends and coding ends is their chromosomal context. All coding ends remain within the chromosome on DNA fragments that contain either the centromere or the telomere. In contrast, most signal ends are found on extra-chromosomal DNA fragments as most rearrangements occur by deletion. There may be differences in the ability of chromosomal (coding ends) and extrachromosomal (signal ends) DNA breaks to activate DNA damage responses. In this regard, although signal joint formation on excised DNA fragments occurs with slow kinetics, the joining of chromosomal signal ends occurs with kinetics similar to coding joint formation (Bredemeyer et al., 2006; Gapud et al., 2011). Moreover, ATM and DNA-PKcs have overlapping activities required for efficient chromosomal signal joint formation (Gapud et al., 2011; Zha, Jiang, et al., 2011). Together, these findings suggest that chromosomal signal ends can activate DNA damage responses.

3.4. RAG DSBs activate a multifunctional transcriptional program

RAG DSBs generated in NHEJ-deficient pre-B cells regulate the expression of over 300 genes (Bredemeyer et al., 2008). Roughly half of these genes rely on ATM-mediated transcriptional regulation. The remaining genes could be regulated by other DNA damage response kinases, such as DNA-PKcs. DNA-PKcs regulates transcription factor activity both directly and indirectly in response to genotoxic DNA damage and stress signals (Basu, Rosenzweig, Youmell, & Price, 1998; Boehme, Kulikov, & Blattner, 2008; Callen et al., 2009; Liu et al., 1998; Schild-Poulter et al., 2007; Tomimatsu et al., 2009). For example, both Oct-1 and NF- κ B can be phosphorylated by DNA-PKcs resulting in changes in their respective transcriptional activities (Basu et al., 1998; Liu et al., 1998; Schild-Poulter et al., 2007). While DNA-PKcs has well-defined roles in the repair of broken DNA ends generated during V(D)J recombination, its participation in the regulation of gene expression in response to RAG DSBs has not been explored.

Approximately half of the ATM-dependent gene expression changes that occur in response to RAG DSBs require NF- κ B transcription factors (Bredemeyer et al., 2008). RAG DSBs activate the canonical p50/p65 NF- κ B pathway in an ATM-dependent manner (Bredemeyer et al., 2008). Similar to genotoxic DSBs, the activation of NF- κ B by RAG DSBs requires NEMO (Bredemeyer et al., 2008; Wu et al., 2006). The mechanisms of NEMO and NF- κ B activation by ATM following RAG DSBs have not been fully determined. Modification of NEMO by phosphorylation and sumoylation is required for NF- κ B activation by genotoxic DNA breaks, and similar mechanisms are likely involved in signaling downstream of RAG DSBs (Miyamoto, 2011). However, alternative pathways may participate in the regulation of NF- κ B as RAG DSBs occur in the context of extracellular signals, including the pre-BCR, which also modulate NF- κ B activity (Jimi et al., 2005; Kistler, Rolink, Marienfeld, Neumann, & Wirth, 1998; Saijo et al., 2003).

In addition to NF- κ B, ATM likely activates other transcriptional pathways in response to RAG DSBs. RAG DSBs also activate the p53 transcription factor (Bednarski et al., 2012; Guidos et al., 1996; Nacht et al., 1996). Moreover, a screen for protein targets of ATM in response to genotoxic DSBs revealed several potential transcription factors, including Stat3, HMGA1, and Foxo1, that have roles in early B cell development (Matsuoka et al., 2007). Foxo1 positively regulates RAG expression and supports IgL κ recombination in pre-B cells (Amin & Schlissel, 2008; Herzog et al., 2008; Ochiai et al., 2012). In contrast, HMGA1 negatively regulates *RAG-2* expression and cooperates with PU.1 and Ets-1 to regulate IgM expression (Fedele et al., 2006; McCarthy, McDevit, Andreucci, Reeves, & Nikolajczyk, 2003). Deficiency of Stat3 results in increased apoptosis of pro-B cells and a block in B cell development (Chou, Levy, & Lee, 2006).

3.5. RAG DSBs trigger a unique genetic program

As expected, the activation of NF- κ B by RAG DSBs upregulates the expression of antiapoptotic genes, such as *Bcl3* and the *Pim2* kinase (Bredemeyer et al., 2008; Fox et al., 2003; Kashatus, Cogswell, & Baldwin, 2006). These survival factors are important for counteracting the pro-apoptotic activities of p53 early after the generation of RAG DSBs (Bednarski et al., 2012). RAG DSBs also regulate expression of a broad group of genes that are expressed in a tissue-specific manner and have no known activities in canonical DNA damage responses (Bredemeyer et al., 2008). Rather, these genes would function in processes that are important for normal lymphocyte development. For example, RAG DSBs upregulate expression of *CD69*, *SWAP70*, and *CD62L*, which have established functions in lymphocyte migration and homing (Feng et al., 2002; Pearce et al., 2006; Rosen, 2004; Shiow et al., 2006). RAG DSBs also induce expression of the common γ -chain (*IL2r γ*), which could alter cytokine responses in maturing pre-B cells (Kovanen & Leonard, 2004; Willerford et al., 1995). These findings suggest that the physiologic (RAG) DNA DSBs generated during V(D)J recombination promote a genetic program that is important for cell type-specific processes beyond canonical DNA damage responses. The notion may appear at odds with the observation that RAG-deficient mice expressing *IgH* and *IgL* transgenes have robust numbers of mature B cells (Spanopoulou et al., 1994; Young et al., 1994). Such cells clearly traverse developmental stages without signals generated by RAG DSBs. However, we propose that the genetic program activated by RAG DSBs regulates processes important for lymphocytes that are actively assembling antigen receptor genes. In this regard, the expression of *IgH* and *IgL* chain transgenes in RAG-deficient mice bypasses these requirements just as it bypasses the requirement for RAG expression.

The regulation of gene expression by RAG DSBs is not restricted to pre-B cells as these DSBs also promote gene expression in pro-B cells and developing thymocytes (Bredemeyer et al., 2008). Moreover, recent studies have shown that DNA DSBs generated during Ig class-switch recombination also promote gene expression changes that are important for the differentiation of activated B cells into plasma cells (Sherman et al., 2010). In mature B cells, DSBs trigger ATM-mediated phosphorylation and inhibition of the transcriptional coactivator CRTC2, which is essential to stop B cell proliferation and activate plasma cell differentiation (Sherman et al., 2010). Thus, signals from physiologic DNA DSBs generated in developing and mature lymphocytes activate cell type-specific genetic programs.

3.6. Transient RAG DSBs activate DNA damage response dependent gene expression

It is clear that persistent un-repaired RAG DSBs generated in NHEJ-deficient lymphocytes can activate DNA damage responses that lead to gene expression changes. However, if these gene expression changes impact lymphocyte development, then RAG DSBs that are normally repaired, and thus exist only transiently, must be able to activate DNA damage responses and the subsequent gene expression changes.

Several lines of evidence suggest that ATM is activated by most, if not all, RAG DSBs. First, in wild-type thymocytes, DNA damage response factors, that is, Nbs1 and γ -H2AX are found associated with the *TCR α* locus (Chen et al., 2000). Second, after the induction of V(D)J recombination, γ -H2AX can be found in chromatin flanking the *IgL κ* locus in wild-type abl pre-B cells (Savic et al., 2009). Third, using an NF- κ B reporter, it was demonstrated that most wild-type abl pre-B cells that had undergone V(D)J recombination activated NF- κ B in an ATM-dependent manner (Bredemeyer et al., 2008). Finally, the surface expression of CD40 and CD69 in primary pre-B cells requires RAG DSB generation as these proteins are expressed by wild-type pre-B cells but not by RAG-deficient or ATM-deficient pre-B cells (Bredemeyer et al., 2008). Collectively, these findings demonstrate that ATM and DNA damage responses, including transcriptional programs, are activated by transient RAG DSBs generated in developing lymphocytes.

Given that RAG DSBs and associated ATM activation exist transiently, how do these signals lead to sustained effects on gene expression? Even though ATM activity is transient in response to these DSBs, the phosphorylation of downstream targets and the effects of this phosphorylation on transcription factor activity may persist after RAG DSBs have been repaired.

4. INTEGRATING RAG DSB-MEDIATED SIGNALS WITH OTHER B LYMPHOCYTE DEVELOPMENTAL SIGNALS

4.1. Overview

B cell development in the bone marrow is regulated by signals from several cell surface receptors. In pre-B cells, the IL-7 receptor (IL-7r) and the pre-BCR are the principal regulators of survival, proliferation, RAG gene expression, and *IgL* chain gene rearrangement (Corfe & Paige, 2012; Erlandsson et al., 2005; Herzog et al., 2009). Much has been learned recently about how the signals from these two receptors are integrated to direct development of early B cells (Amin & Schlissel, 2008; Herzog et al., 2008; Johnson et al., 2008; Mandal et al., 2009; Ochiai et al., 2012). Here, we will consider how signals triggered by RAG DSBs generated during antigen receptor gene assembly integrate with receptor signals to promote processes that are integral to normal B cell development.

4.2. Pre-B cell receptor

In pro-B cells, generation of a productive *IgH* chain gene leads to the cell surface expression of the pre-BCR, which associates with the signaling subunits Ig α and Ig β (Herzog et al., 2009). In a ligand-independent manner, oligomerization of the pre-BCR triggers signaling that promotes cellular proliferation, allelic exclusion at the *IgH* chain gene, and transition to the pre-B cell stage of B cell development (Bankovich et al., 2007; Bergman, 1999; Brady et al., 2010; Herzog et al., 2009).

Several lines of evidence suggest that pre-BCR signals also function in the initiation of *IgL κ* chain gene rearrangement. First, expression of an *IgH* transgene in RAG-deficient mice results in the generation of pre-B cells with an accessible *IgL κ* locus (Stanhope-Baker, Hudson, Shaffer, Constantinescu, & Schlissel, 1996). Second, expression of an active form of Ras, which signals downstream of the pre-BCR, leads to *IgL κ* rearrangement in cells that do not express a pre-BCR (Shaw, Swat, Ferrini, Davidson, & Alt, 1999). Third, loss of essential downstream pre-BCR signaling molecules, such as BLNK, Btk, and PLC- γ , results in a block in *IgL κ* gene rearrangement (Flemming, Brummer, Reth, & Jumaa, 2003; Xu, Lee, Huo, Kurosaki, & Lam, 2007). Finally, the IRF-4 and IRF-8 transcription factors function downstream of pre-BCR signals to promote *IgL κ* gene rearrangement *in vivo* (Johnson et al., 2008; Lu, Medina, Lancki, & Singh, 2003). However, *IgL κ* gene

rearrangement can occur at some level in pro-B cells that do not express a pre-BCR demonstrating that pre-BCR signals are not absolutely required for *IgL* locus rearrangement (Grawunder, Haasner, Melchers, & Rolink, 1993).

4.3. IL-7 receptor

The IL-7r is composed of a unique IL-7 α chain and the common γ -chain (IL-2 γ) (Corfe & Paige, 2012; Kovanen & Leonard, 2004). The importance of IL-7 signaling is evidenced by the uniform block in B and T cell development at early stages in mice deficient in IL-7, the IL-7 α chain, or the common γ -chain (Cao et al., 1995; DiSanto, Guy-Grand, Fisher, & Tarakhovsky, 1996; Peschon et al., 1994; von Freeden-Jeffry et al., 1995). Paradoxically, in humans, IL-7 is not critical for B cell development and mutations in the common γ -chain cause a block in T cell, but not B cell, development (Puel, Ziegler, Buckley, & Leonard, 1998).

Signals from the IL-7r are transduced primarily through the JAK-STAT pathway (Corfe & Paige, 2012; Goetz, Harmon, O'Neil, Burchill, & Farrar, 2004; Kovanen & Leonard, 2004; Malin, McManus, & Busslinger, 2010). Jak1 and Jak3 associate with the cytoplasmic domains of the IL-7r and, upon ligand binding, phosphorylate each other and the cytoplasmic domain of the IL-7 α chain (Corfe & Paige, 2012; Kovanen & Leonard, 2004). Activation of Jak1 and Jak3 leads to the phosphorylation and dimerization of STAT5, which results in its nuclear translocation and initiation of gene expression (Corfe & Paige, 2012; Goetz et al., 2004; Malin, McManus, et al., 2010). Consistent with their essential signaling role downstream of the IL-7r, mice deficient in Jak1, Jak3, or Stat5 exhibit a block in early B cell development (Hoelbl et al., 2006; Malin, McManus, Cobaleda, et al., 2010; Nosaka et al., 1995; Park et al., 1995; Rodig et al., 1998; Suzuki et al., 2000; Thomis, Gurniak, Tivol, Sharpe, & Berg, 1995; Yao et al., 2006).

In response to IL-7, Jak-Stat signals regulate pre-B cell survival and proliferation as well as *IgH* and *IgL* gene accessibility. Activation of Stat5 by IL-7 signaling induces expression of antiapoptotic genes, including *Bcl2*, *Bcl-X_L*, *Mcl-1*, and *Pim1* (Goetz et al., 2004; Malin, McManus, Cobaleda, et al., 2010). Mice deficient in *Bcl-X_L* or *Pim1* have reduced numbers of pre-B cells due to a decrease in pre-B cell survival (Domen et al., 1993; Mikkers et al., 2004; Motoyama et al., 1995). Interestingly, expression of the *Bcl2* transgene in IL-7r-deficient mice only partially rescues B cell development, suggesting that IL-7r signals may have functions in addition to promoting survival of early B cells (Akashi, Kondo, von Freeden-Jeffry, Murray, & Weissman, 1997; Corfe & Paige, 2012; Maraskovsky et al., 1997; Maraskovsky, Peschon, McKenna, Teepe, & Strasser, 1998). For instance, *Pim1* supports the IL-7-driven proliferative expansion that occurs during transit from the pro-B to pre-B cell stage (Domen et al., 1993; Mikkers et al., 2004). Additionally, Stat5 coordinates increased accessibility of the *IgH* gene locus and simultaneous inactivation of the *IgL* locus, thereby supporting pre-BCR expression but blocking light chain recombination in actively dividing cells (Bertolino et al., 2005; Malin, McManus, et al., 2010; Mandal et al., 2009).

In addition to Jak-Stat signaling, the IL-7r also activates the Akt (PKB) kinase (Corfe & Paige, 2012). In response to IL-7 signals, Akt cooperates with Jak-Stat pathways to support the survival and proliferation of pre-B cells at least in part through phosphorylation and inactivation of the pro-apoptotic protein Bad (Bednarski et al., 2012; Corfe & Paige, 2012; Li, Jiang, Khaled, Keller, & Durum, 2004). Inhibition of Akt results in augmented cell death in pre-B cells cultured *ex vivo* in IL-7 (Bednarski et al., 2012). Akt also phosphorylates and inactivates the Foxo1 and Foxo3a transcription factors, which control RAG expression and enforce G1 cell cycle arrest (Amin & Schlissel, 2008; Herzog et al., 2008; Lazorchak et al., 2010; Ochiai et al., 2012). Thus, IL-7 signaling inhibits Foxo transcription factor activity, thereby supporting cell proliferation and preventing RAG DSB generation in proliferating

cells. Attenuation of IL-7 signals leads to cessation of Akt activity and associated stabilization and activation of Foxo transcription factors, which are necessary for RAG expression and induction of *IgL* gene recombination (Amin & Schlissel, 2008; Herzog et al., 2008; Lazorchak et al., 2010; Ochiai et al., 2012). Cumulatively, IL-7 triggers multiple signaling pathways to support survival, proliferation, and clonal expansion in pre-B cells.

4.4. Integrating developmental signals in pre-B cells

Although IL-7 is needed to promote the transition of pro-B cells to pre-B cells, once the cells reach the pre-B cell stage, IL-7 signals must be attenuated to allow for RAG reexpression and *IgL κ* locus accessibility, both of which are required for *IgL κ* gene assembly (Corfe & Paige, 2012; Rolink, Kudo, et al., 1991; Rolink, Streb, et al., 1991). Moreover, proliferative signals from IL-7 must be terminated so that pre-B cells with RAG DSBs at the *IgL κ* locus are not driven into S-phase before the broken DNA ends can be repaired. Attenuation of IL-7 signals in pre-B cells may be due, in part, to a downregulation of IL-7r expression mediated by pre-BCR signals (Marshall, Fleming, Wu, & Paige, 1998; Schebesta, Pfeffer, & Busslinger, 2002). In addition, pre-B cells may be repositioned to bone marrow microenvironments devoid of IL-7-producing stromal cells and that contain cells that make CXCL12 (Tokoyoda, Egawa, Sugiyama, Choi, & Nagasawa, 2004). In this regard, pre-BCR signals lead to increased expression of CXCR4, which promotes cell migration in response to CXCL12, *in vitro* (Johnson et al., 2008; Milne, Zhang, & Paige, 2005).

IL-7 and pre-BCR signals are further interconnected in three important developmental processes. First, the attenuation of IL-7 signals leads to expression of *BLNK* (*Slp-65*) and *Syk*, two critical intracellular signaling components of the pre-BCR (Ochiai et al., 2012). Second, signals from the pre-BCR upregulate the *Aiolos* and *Ikaros* transcription factors to repress c-Myc and cyclinD3 (*CCND3*), which promote proliferation downstream of the IL-7r (Cooper et al., 2006; Fleming & Paige, 2001; Ma et al., 2010; Mandal et al., 2009). Finally, both pre-BCR signals and loss of IL-7 signals are required for efficient *IgL κ* rearrangement. Attenuation of IL-7 signals activates Foxo1 and subsequent expression of the RAG machinery (Amin & Schlissel, 2008; Herzog et al., 2008; Lazorchak et al., 2010; Ochiai et al., 2012). Pre-BCR signals induce *IRF4* expression and enhance E2A activity to initiate *IgL κ* germline transcription, which is essential for RAG accessibility to the locus and *IgL κ* recombination (Herzog et al., 2008; Johnson et al., 2008; Mandal et al., 2009).

4.5. RAG DSBs support survival of pre-B cells following IL-7 attenuation

As discussed above, pre-B cells must attenuate IL-7 signaling to rearrange *IgL* chain genes and express a mature antigen receptor. However, loss of IL-7 signaling results in a loss of survival signals and rapid induction of cell death mechanisms (Corfe & Paige, 2012; Rolink, Kudo, et al., 1991; Rolink, Streb, et al., 1991). Loss of these survival pathways would be detrimental to pre-B cells, which frequently must undergo iterative rearrangements of the *IgL* gene loci. A single rearrangement can take several hours to complete and, thus, pre-B cells may have to survive for an extended period of time as they attempt to assemble and express a functional *IgL* chain (Casellas et al., 2001). Moreover, the generation of RAG DSBs triggers the p53-associated cell death pathways, including expression of the pro-apoptotic genes *PUMA*, *NOXA*, and *BAX* (Bednarski et al., 2012; Guidos et al., 1996; Nacht et al., 1996; Vousden & Prives, 2009; Youle & Strasser, 2008).

Paradoxically, despite activation of these cell death pathways, pre-B cells have enhanced survival shortly after the generation of RAG DSBs (Bednarski et al., 2012). This early survival window is dependent on RAG DSB-induced expression of *Pim2*, a member of the family of kinases that also includes *Pim1* and *Akt* (Amaravadi & Thompson, 2005;

Bednarski et al., 2012). In contrast to the loss of Pim1 and Akt activity following attenuation of IL-7 signaling, *Pim2* expression is increased in an ATM-dependent manner in response to RAG DSBs (Bednarski et al., 2012; Bredemeyer et al., 2008; Corfe & Paige, 2012; Goetz et al., 2004). The upregulation of Pim2 maintains the phosphorylation and inactivation of Bad, which promotes increased levels of Bcl2 and associated cell survival (Bednarski et al., 2012; Youle & Strasser, 2008). Cells deficient in Pim2 do not exhibit this early survival window and Pim2-deficient mice have reduced IgL λ -expressing B cells consistent with a defect in the time available to complete *IgL* chain gene recombination (Bednarski et al., 2012; Derudder et al., 2009).

4.6. RAG DSBs suppress IL-7-driven proliferation

Intriguingly, upregulation of Pim2 by RAG DSBs results in reduced proliferation of IL-7 stimulated pre-B cells (Bednarski et al., 2012). Therefore, in striking contrast to the pro-proliferative properties of Pim1 downstream of IL-7, Pim2 enforces the G1 to S checkpoint and inhibits the proliferation of pre-B cells with RAG DSBs (Bednarski et al., 2012; Domen et al., 1993; Mikkers et al., 2004). Thus, Pim2 may antagonize proliferative signals from residual IL-7 encountered when pre-B cells have relocated to regions of the bone marrow devoid of IL-7 producing stromal cells (Johnson et al., 2008; Milne et al., 2005; Tokoyoda et al., 2004). Pim2 is not sufficient to block proliferation but, rather, cooperates with other DSB-dependent signals to maintain cell cycle arrest during V(D)J recombination (Bednarski et al., 2012). Expression of Pim2 may promote genomic stability by preventing cells with RAG DSBs from entering S-phase where these breaks would be converted into a replicated broken chromosome.

4.7. RAG DSBs support developmental programs in pre-B cells

In addition to survival and proliferation, RAG DSBs initiate signals that regulate cell type-specific developmental processes. For example, RAG DSBs induce expression of a cohort of genes with known functions in lymphocyte migration and localization, such as *SWAP-70*, *CD62L* (L-selectin), and *CD69* (Bredemeyer et al., 2008; Feng et al., 2002; Pearce et al., 2006; Rosen, 2004; Shiow et al., 2006). CD69 mediates downregulation of the S1P1 receptor, which binds to sphingosine-1-phosphate (S1P) and promotes lymphocyte migration from primary and secondary lymphoid organs into circulation (Allende et al., 2009; Pereira, Xu, & Cyster, 2010; Schwab & Cyster, 2007). L-selectin (CD62L) regulates T and B lymphocyte migration in response to chemokines to direct homing to secondary lymphoid organs (Galkina et al., 2007; Rosen, 2004; Subramanian et al., 2012). SWAP-70 regulates integrin-mediated adhesion of B cells to lymph node high endothelial venules and is essential for B cell entry into lymph nodes (Pearce et al., 2006). Taken together, the known functions of CD69, SWAP-70, and L-selectin suggest that by increasing expression of these proteins RAG DSBs may regulate the migration and localization of pre-B cells actively assembling *IgL κ* chain genes. In this regard, signals from RAG DSBs and the pre-BCR may cooperate to enforce the residence of pre-B cells in IL-7-poor regions of the bone marrow while they are attempting *IgL* chain gene assembly and/or prevent premature release of immature pre-B cells that had not yet assembled a productive *IgL* chain gene.

During B lymphocyte development, the processes of allelic exclusion ensures that each cell produces only single productive *IgH* and *IgL* chain genes (Bergman, 1999; Brady et al., 2010; Jung et al., 2006; Sen & Oltz, 2006). Recent work suggests that DNA damage responses can initiate feedback mechanisms to modulate allele accessibility and control the quantity of DSBs. In early B cells, activation of ATM by RAG cleavage at one allele causes the nuclear repositioning of the other allele to heterochromatic regions (Hewitt et al., 2009). Thus, RAG DSBs may promote a negative feedback loop that restrains the number and location of DNA breaks during V(D)J recombination.

4.8. Genotoxic DNA damage activates developmental programs

The physiologic DNA breaks generated in lymphocytes during the rearrangement and editing of the antigen receptor genes activate signaling pathways that influence cell differentiation. Interestingly, in developing B cells exogenous DNA damaging agents (γ -irradiation and etoposide) can also trigger ATM-dependent expression of many genes, including *CD40*, *CD69*, *IL-12a*, and the common γ -chain (Bredemeyer et al., 2008). Similarly, these genotoxic agents inactivate CRTC2 and modulate gene expression in germinal center B cells in a manner identical to physiologic DSBs of class-switch recombination (Sherman et al., 2010). Thus, exogenous DNA damaging agents may activate broadly functional genetic programs in mature and developing lymphocytes. This has important implications for the treatment of lymphoid malignancies where therapeutic agents that cause DNA damage alter the genetic program of tumor cells in a way that is beneficial for treatment. However, these therapies may also lead to the inappropriate regulation of genetic programs in normal lymphocytes.

5. CONCLUDING REMARKS

All developing lymphocytes generate many DNA DSBs as they attempt to assemble functional antigen receptor genes at different developmental stages. These DSBs activate canonical DNA damage responses that are required for the cellular response to these DSBs. However, recent studies have revealed that these physiologic DNA breaks also initiate a genetic program that is unique to and important for developing lymphocytes. In this regard, signals from RAG DSBs integrate with other important developmental signals, including those from the IL-7 and pre-BCR receptors, to direct lymphocyte maturation. These RAG DSB-dependent signals may directly regulate antigen receptor gene assembly by influencing allelic exclusion and the inter- and intraallelic ordering of the V(D)J recombination reaction. In addition, RAG DSBs may regulate processes that indirectly impact antigen receptor gene assembly, such as survival and proliferation of developing lymphocytes. From these studies a more general paradigm emerges: activation of DNA damage responses by DSBs generated during physiologic processes regulate cell type-specific programs. Thus, DNA breaks can provide intrinsic cellular cues akin to extrinsic cues provided by cell surface receptors. As such the generation of DNA DSBs by exposure to genotoxic agents may inappropriately activate cell type-specific genetic programs altering normal cellular functions not involved in canonical DNA damage responses.

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