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At the intersection of DNA damage and immune responses

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Abstract

DNA damage occurs on exposure to genotoxic agents and during physiological DNA transactions. DNA double-strand breaks (DSBs) are particularly dangerous lesions that activate DNA damage response (DDR) kinases, leading to initiation of a canonical DDR (cDDR). This response includes activation of cell cycle checkpoints and engagement of pathways that repair the DNA DSBs to maintain genomic integrity. In adaptive immune cells, programmed DNA DSBs are generated at precise genomic locations during the assembly and diversification of lymphocyte antigen receptor genes. In innate immune cells, the production of genotoxic agents, such as reactive nitrogen molecules, in response to pathogens can also cause genomic DNA DSBs. These DSBs in adaptive and innate immune cells activate the cDDR. However, recent studies have demonstrated that they also activate non-canonical DDRs (ncDDR) that regulate cell type-specific processes that are important for innate and adaptive immune responses. Here, we review these ncDDRs and discuss how they integrate with other signals during immune system development and function.

The development and function of innate and adaptive immune cells are regulated by diverse extracellular cues that activate a broad variety of cell surface receptors and intracellular cues emanating from cytosolic and nuclear events. Developing and mature lymphocytes generate programmed DNA double-strand breaks (DSBs) at specific locations within the genome as necessary intermediates of physiological DNA rearrangements, such as antigen receptor gene assembly by V(D)J recombination and immunoglobulin class switch recombination (CSR) (see below)^{1,2}. In addition, non-programmed DNA DSBs can be generated throughout the genome of immune cells, for example, during transcription, DNA replication and by genotoxic agents produced to eradicate pathogens^{3–5}. DNA DSBs generated in these different settings, and the responses they elicit, are emerging as important signalling events in regulating immune system development and function.

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DNA DSBs are dangerous genomic lesions that initiate a conserved canonical DNA damage response (cDDR) in all cells⁴. The cDDR promotes DNA DSB repair through either non-homologous end joining (NHEJ) or homologous recombination^{4,6,7}. NHEJ functions to rejoin broken DNA ends at all phases of the cell cycle and frequently does so imprecisely, with nucleotides gained or lost at the join⁶. By contrast, homologous recombination functions only in the S and G2 phases of the cell cycle using the sister chromatid as a template for precise repair⁷. The cDDR also activates check-points that prevent cells with DSBs from progressing through the cell cycle and ultimately kills cells with persistent DSBs that could otherwise be resolved aberrantly leading to chromosomal rearrangements and cellular transformation⁴. The G2–M check-point is regulated by serine/threonine-protein kinase CHK1, whereas the G1–S checkpoint is enforced by CHK2 and p53, which also triggers cell death if DSBs persist unrepaired⁴.

The cDDR is initiated by phosphoinositide 3-kinase-like serine threonine kinases that are activated by DSB sensor proteins, or protein complexes, once they have bound to DNA DSBs⁸. These kinases include ataxia telangiectasia mutated (ATM), DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and ataxia telangiectasia and RAD3 related (ATR)⁸. ATM and DNA-PKcs are activated by DNA DSBs at all phases of the cell cycle, whereas ATR is primarily activated by DNA ends generated during DNA replication. Upon binding to broken DNA ends, the MRE11–RAD50–NBS1 (also known as NBN) (MRN) complex activates ATM, the heterodimer of KU70 (also known as XRCC6) and KU80 (also known as XRCC5) activates DNA-PKcs, and ATRIP activates ATR^{8,9}. Once activated, the DDR kinases phosphorylate proteins that function in the different arms of the cDDR. However, these kinases can phosphorylate many other proteins with broad cellular activities and no known cDDR functions¹⁰. This suggests that, in some settings, in response to DSBs, DDR kinases may initiate cell type-specific non-canonical DNA damage responses (ncDDRs) that regulate normal cellular functions unrelated to DNA DSB repair. Indeed, as discussed in this Review, recent studies have shown that activation of DDR kinases by DNA DSBs in immune cells has been co-opted to initiate a variety of ncDDRs that regulate cell type-specific processes that are required for the normal development and function of innate and adaptive immune responses.

The ncDDR in developing lymphocytes

All developing B and T cells make and repair DNA DSBs as they assemble antigen receptor genes through the process of V(D)J recombination^{11,12}. The signals that initiate this highly ordered process leading to the generation of DSBs at antigen receptor loci are well defined and, once generated, these DSBs activate an ncDDR that regulates subsequent V(D)J recombination steps. In addition, this ncDDR activates pathways that are important for normal lymphocyte development. We focus our discussion on developing B cells, as the most is known about the function of the ncDDR to DSBs generated in these cells.

V(D)J recombination.

Lymphocyte antigen receptor genes include the immunoglobulin (Ig) heavy (H) chain genes and light (L) chain κ and λ genes that are expressed in B cells and the T cell receptor (TCR)

β , α , γ and δ chain genes that are expressed in T cells¹¹. The variable (second) exon of all these genes must be assembled during development from variable (V), joining (J) and, at some loci, diversity (D) gene segments through the process of V(D)J recombination¹¹ (FIG. 1). V(D)J recombination is initiated when two recombining gene segments are brought together in a synaptic complex with the recombination-activating gene 1 (RAG1) and RAG2 proteins, which together form the RAG endonuclease^{1,13}. RAG proteins introduce DNA DSBs at the border of the two gene segments and the flanking recombination signal sequences (RSSs) that are recognized by RAG proteins. Each RAG-mediated DSB has a hairpin-sealed coding DNA end and blunt signal DNA end. RAG DSBs are generated in developing lymphocytes only during the G1 phase of the cell cycle and must be repaired by NHEJ, which joins the two coding ends to form a coding join and the two signal ends to form a signal join¹². Signal joining is fairly precise, whereas coding join formation is imprecise, with a gain and loss of nucleotides. As a result, only one-third of the completed coding joins will be in-frame (productive) rearrangements and encode an antigen receptor chain. The generation of RAG DSBs leads to the activation of ATM and DNA-PKcs, which initiate the cDDR and are required for RAG DSB repair^{12,14}. DNA-PKcs promotes Artemis endonuclease activity in opening hairpin-sealed coding ends so they can be joined, and ATM maintains the stability of RAG DSBs in post-cleavage complexes until the DNA ends are joined^{15,16}. In response to RAG DSBs in developing lymphocytes, ATM also activates ncDDR programmes that regulate antigen receptor gene assembly and several pathways that are important for normal lymphocyte development (see below). It is conceivable that DNA-PKcs also participates in the activation of ncDDRs, but this has yet to be established.

B cell development.

B cell development proceeds through a series of discrete stages in the bone marrow, with developmental transitions orchestrated by diverse sets of signals, including ncDDRs initiated by RAG DSBs generated during lymphocyte antigen receptor gene assembly at multiple stages of B cell development¹⁷ (FIG. 2). RAG DSBs are generated in pro-B cells during *Igh* chain gene assembly, and a productive *Igh* rearrangement leads to expression of an IgH chain as a pre-B cell receptor (pre-BCR) that signals transition to the pre-B cell stage of development^{11,17,18}. In pre-B cells, RAG DSBs are generated during *Igl* chain gene (*Iglk* and *Igll*) assembly, and a productive rearrangement encoding an IgL chain that pairs with the IgH chain to form a B cell receptor (BCR) drives transition to the immature B cell stage. Antigen receptor gene assembly ceases in immature B cells unless the BCR is autoreactive, in which case RAG DSBs can be generated during rearrangements that are initiated to replace the IgL chain of the auto-reactive BCR through a process termed receptor editing (see below)^{17,19}. In response to BCR activation, mature B cells undergo CSR, leading to the generation of DNA DSBs in the constant region of the *Igh* locus (see below)²⁰ (FIG. 2). Accordingly, DNA DSBs at antigen receptor loci are generated in all pro-B cells, all pre-B cells, some immature B cells and all mature B cells once they are activated (FIG. 2). Thus, ncDDRs initiated in response to these DSBs could regulate diverse processes that are required for B cell development and function. Similarly, T cells develop through pro-T and pre-T cell stages in which the TCR chain genes are assembled and likely also activate ncDDRs that regulate developmental processes, although much less is known about these responses²¹.

Pro-B cells require IL-7 receptor (IL-7R) signalling through AKT, Janus kinases (JAKs) and signal transducer and activator of transcription 5 (STAT5) to promote proliferation, survival and transition to the large pre-B cell stage¹⁸ (FIG. 3a). *Ig* chain genes are assembled at the pre-B cell stage; however, IL-7R signals antagonize *Ig* chain gene assembly through STAT5-dependent suppression of *Ig* chain germline gene transcription, which prevents accessibility of the *Ig* locus to RAG^{11,22,23} (FIG. 3a). Furthermore, activation of AKT by IL-7R leads to phosphorylation and degradation of the transcription factor forkhead box protein O1 (FOXO1), which is required for RAG gene expression^{24,25} (FIG. 3a). STAT5 induces expression of the PIM family kinase PIM1, which along with AKT drives proliferation and promotes survival of large pre-B cells, in part, by antagonizing BAX-mediated cell death through phosphorylation of BCL-2-associated agonist of cell death (BAD)^{18,26–28} (FIG. 3a). These proliferative signals also antagonize *Ig* chain gene assembly, as V(D)J recombination is restricted to G1 phase cells¹⁴.

IL-7R signals in large pre-B cells must be attenuated to promote transition to the small pre-B cell stage of development, halt proliferation and promote induction of *RAG1* and *RAG2* expression, which depends on growth arrest and DNA damage-inducible protein GADD45α and FOXO1 (REFS^{18,24,25}) (FIG. 3b). Furthermore, FOXO1 promotes expression of B cell linker protein (BLNK), which antagonizes AKT signalling and permits pre-BCR signalling through the tyrosine protein kinase SYK^{18,25}. Pre-BCR signals induce expression of the transcription factor interferon regulatory factor 4 (IRF4), which along with PU.1 promotes germline *Ig* chain gene transcription and accessibility to RAG, allowing for *Ig* chain gene assembly^{18,29} (FIG. 3b). In small pre-B cells, the lost pro-survival signals following attenuation of IL-7R signals need to be replaced if these cells are to survive for several days as they undergo multiple rearrangements in an attempt to generate a productive *Ig* chain gene (see below)^{18,30}.

IL-7R signalling is attenuated through the repositioning of pre-B cells to regions of the bone marrow that are devoid of IL-7-producing mesenchymal stromal cells^{29,31}. Bone marrow stromal cells may produce either CXC-chemokine ligand 12 (CXCL12) or IL-7, with the expression of CXC-chemokine receptor 4 (CXCR4), the receptor for CXCL12, on large pre-B cells triggering migration away from IL-7-producing mesenchymal stromal cells and towards CXCL12-producing mesenchymal stromal cells^{29,32}. However, recent studies have demonstrated that CXCL12 and IL-7 can be made by the same stromal cells and that pre-BCR-driven reduction in focal adhesion kinase (FAK; also known as FADK1)-mediated and α4β1 integrin-mediated adhesion to vascular cell adhesion protein 1 (VCAM1) leads to detachment and diffusion of large pre-B cells away from mesenchymal stromal cells that produce both IL-7 and CXCL12 (REF.³¹).

ncDDR in pre-B cells.

Once *Ig* chain gene assembly is initiated, RAG DSBs activate ATM, leading to the cDDR and an ncDDR that includes the induction of a broadly functional genetic programme regulated, in part, by nuclear factor-κB1 (NF-κB1) and NF-κB2 (REFS^{21,33,34}) (FIG. 3c). The activation of NF-κB1 in response to RAG DSBs likely occurs through the phosphorylation of NF-κB essential modulator (NEMO) in the nucleus by ATM, as occurs

in response to genotoxic DSBs^{35,36}. This genetic programme may further reinforce the extinction of IL-7R signals. In this regard, in small pre-B cells, NF- κ B1 activation in response to RAG DSBs induces the expression of CD69, L-selectin (also known as CD62L) and switch-associated protein 70 (SWAP70)²¹. These proteins have functions in cell migration and localization that could promote the residence of small pre-B cells in regions of the bone marrow that are devoid of IL-7-producing stromal cells^{37–39}. Moreover, RAG DSB signals in pre-B cells inhibit IL-7 production by neighbouring stromal cells³¹ (FIG. 3c). This intriguing ncDDR-mediated intercellular crosstalk between pre-B cells and stromal cells would be important not only for regulating *Ig*I chain gene rearrangement but also for preserving genome stability by preventing pre-B cells with RAG DSBs from being driven into cycle by IL-7R signals.

Pre-B cell survival.

In small pre-B cells, IL-7R-mediated pro-survival signals are lost, and RAG DSBs activate a cDDR that involves p53, which can promote cell death, in part, through induction of BAX³⁴ (FIG. 3c). However, the ncDDR activated by RAG DSBs promotes survival through the NF- κ B1-dependent induction of PIM2, a kinase in the same family as PIM1 (REFS^{21,27,40}) (FIG. 3c). Like PIM1 in large pre-B cells, PIM2 promotes survival in small pre-B cells through phosphorylation of BAD^{27,41} (FIG. 3c). Indeed, *Pim2*^{-/-} mice exhibit a decrease in the fraction of *Ig*II-expressing B cells, which is consistent with a survival defect in these cells, as *Ig*II rearrangements usually occur after several failed rounds of *Ig*Ik chain gene assembly^{17,42}.

The pro-survival effects from RAG DSB-mediated induction of the ncDDR may be important for permitting multiple *Ig*I chain gene rearrangements in a single pre-B cell (see below). However, when DSBs persist unrepaired, the cDDR must promote cell death rather than allowing these DSBs the opportunity to be aberrantly resolved. Indeed, mice deficient in p53 and the NHEJ factors required for RAG DSB repair invariably develop B cell tumours with translocations formed by misrepaired RAG DSBs^{34,43,44}. Thus, a balance must be maintained between ncDDR survival signals that provide a critical time window for *Ig*I chain gene assembly and the initiation of cDDR pathways that promote the death of cells with persistent unrepaired RAG DSBs that could lead to genome instability and cellular transformation.

G1 phase arrest in pre-B cells.

Antigen receptor gene assembly occurs in developing lymphocytes at the G1 phase of the cell cycle. The transition of pre-B cells from G1 phase into S phase limits RAG activity through the degradation of RAG2 and could also promote the aberrant resolution of unrepaired RAG DSBs^{11,14,45}. In response to RAG DSBs, the cDDR activates p53 and likely also CHK2, which enforce the G1–S checkpoint^{4,34,46} (FIG. 3c). However, in pre-B cells, which can receive proliferative signals from both IL-7R and the pre-BCR, the ncDDR is required to fully enforce the G1–S checkpoint^{27,33}. In this regard, unlike PIM1, which promotes proliferation downstream of IL-7R, PIM2, which is induced by the ncDDR to RAG DSBs, promotes G1 arrest in pre-B cells by inhibiting IL-7-driven proliferation^{27,28}.

Indeed, in the presence of an intact cDDR, PIM2-deficient pre-B cells with RAG DSBs will enter S phase²⁷.

Pre-BCR signalling through SYK and BLNK can drive pre-B cell proliferation in the absence of IL-7R signals^{47,48}. The ncDDR to RAG DSBs in pre-B cells leads to the induction of NF- κ B2, which initiates a genetic programme including SPIC, a member of the PU-box binding ETS family transcription factors that is expressed primarily in B cells and macrophages^{49–51} (FIG. 3c). Unlike the PU.1 and SPIB family members that are transcriptional activators required for B cell development, SPIC has a limited ability to activate transcription and may function primarily as a transcriptional repressor by competitively inhibiting PU.1 and SPIB binding to PU-box sites^{52,53}. Indeed, in pre-B cells with RAG DSBs, SPIC binds to the promoters of both *Syk* and *Blnk*, with a concomitant decrease in PU.1 binding and transcription of these genes^{33,53,54}. In addition to inhibiting the transcription of *Syk* and *Blnk*, ncDDR-dependent post-translational regulatory pathways could be responsible for the rapid downregulation of SYK and BLNK protein in response to RAG DSBs³³ (FIG. 3c). The importance of this pathway in maintaining the G1–S checkpoint is evidenced by the ectopic expression of SYK in pre-B cells with unrepaired RAG DSBs, which drives these cells from G1 into S phase³³. Thus, the ncDDR to RAG DSBs in small pre-B cells enforces the G1–S checkpoint by blocking both IL-7R and pre-BCR signals through the induction of PIM2 and SPIC, respectively (FIG. 3c).

The molecular components of the ncDDR to RAG DSBs have been best established in pre-B cells. We expect that ncDDR pathways also function in pro-B cells and developing T cells that are assembling antigen receptor genes but that these pathways may have distinct molecular components. Indeed, SPIC is not expressed in developing thymocytes⁴⁹. In thymocytes, the ATM-dependent activation of the p38 mitogen-activated protein kinase (MAPK) in response to genotoxic DSBs and RAG DSBs leads to phosphorylation and inactivation of glycogen synthase kinase 3 β (GSK3 β), a constitutively active kinase that is known to promote cell death⁵⁵. Moreover, the induction of DSBs in thymocytes by irradiation causes an ATM-independent reduction in cyclin D3, blunting proliferation⁵⁶. Clearly, our understanding of the ncDDR pathways activated by RAG DSBs and their functions at different stages of B and T cell development remains incomplete.

Ordering antigen receptor gene assembly.

Most lymphocytes express a single heterodimeric antigen receptor, with cells generating only a single in-frame rearrangement for each antigen receptor chain. This occurs through the process termed allelic exclusion, which requires that rearrangements be initiated on one allele at a time and that newly completed antigen receptor genes have time to be tested to determine whether they are in-frame before another rearrangement is initiated on the alternative allele^{11,57}. To achieve allelic exclusion, antigen receptor gene assembly is inter-allelically ordered, which depends on the differential accessibility of the two antigen receptor alleles so that RAG cleavage is initiated on a single allele^{58–60} (FIG. 4). However, inter-allelic ordering also depends on activation of the ncDDR by the newly generated RAG DSBs, which prevents additional RAG DSBs from being generated on the alternative allele (FIG. 4). Indeed, ATM-deficient B cells, which cannot initiate an ncDDR to RAG DSBs,

exhibit a higher frequency of simultaneous RAG-mediated cleavage events at both *Igh* alleles (pro-B cells) and both *Igl* alleles (pre-B cells) than wild-type B cells⁶¹. In pre-B cells with NHEJ deficiencies, RAG DSBs are generated but not repaired, leading to persistent activation of ATM and the ncDDR. In these pre-B cells, RAG DSBs are found at approximately half of the *Iglk* alleles, which is consistent with RAG cleavage at a single allele in each cell⁶². However, loss of ATM or inhibition of ATM kinase activity in NHEJ-deficient pre-B cells leads to RAG cleavage at both *Iglk* alleles⁶². These findings are consistent with the notion that activation of ATM, and the ncDDR, by RAG DSBs functions to enforce allelic exclusion by limiting RAG cleavage to one *Iglk* allele at a time in pre-B cells. In agreement, developing B and T cells from ATM-deficient mice exhibit a partial loss of allelic exclusion^{62,63}.

Some antigen receptor loci (*Iglk* and *Tcra*) are assembled from clusters of V and J gene segments that are organized such that a VJ rearrangement can be replaced by rearrangement of an upstream V to a downstream J on the same allele^{64–66} (FIG. 4). This allele structure is advantageous, as it permits individual cells multiple sequential opportunities to successfully complete a productive antigen receptor gene on each allele. However, these sequential rearrangements must be intra-allelically ordered so that a VJ rearrangement can be completed and tested to determine whether it encodes a functional antigen receptor chain before it is deleted by another rearrangement on the same allele (FIG. 4). Promoters upstream of the J gene segment clusters in the *Iglk* and *Tcra* loci function to target initial rearrangements at these loci to the most upstream J gene segment^{67,68}. Indeed, in NHEJ-deficient pre-B cells, RAG cleavage occurs primarily at the most 5' *Jk* segment^{33,62}. However, in pre-B cells deficient in NHEJ and ATM, RAG cleavage progresses to more 3' *Jk* gene segments^{33,62}. Moreover, *Tcra* rearrangements in ATM-deficient thymocytes use more 3' *Ja* gene segments⁶⁹. Thus, once a RAG DSB is generated, the ncDDR limits subsequent RAG DSBs on the same allele.

The ncDDR can limit RAG DSBs through multiple mechanisms that impact both RAG activity and antigen receptor locus accessibility. In pro-B cells, RAG cleavage at one *Igh* allele causes an ATM-dependent repositioning of the other *Igh* allele to heterochromatic regions, presumably making it less accessible to RAG cleavage⁶¹. In pre-B cells, ATM antagonism of pre-BCR signals leads to diminished levels of IRF4 and a decrease in *Iglk* germline transcripts and *Iglk* locus accessibility³³ (FIG. 3c). Moreover, SPIC displaces PU.1 from the *Iglk* 3' enhancer, which would further reduce *Iglk* germline transcription and accessibility^{33,70,71} (FIG. 3c). The ncDDR also feeds back directly to inhibit RAG expression and activity. In pre-B cells, the ncDDR causes a reduction in GADD45α, which is required for *RAG* gene expression^{24,62} (FIG. 3c). NF-κB1, which is activated by RAG DSBs, leads to downregulation of *RAG* gene expression in response to genotoxic DSBs in developing lymphocytes^{21,72}. Moreover, genotoxic DSBs also lead to an ATM-dependent reduction in FOXO1 binding to the *RAG* enhancer, *ERAG*⁷³. ATM-dependent phosphorylation of RAG2 also leads to diminished RAG cleavage activity⁷⁴ (FIG. 3c). Thus, ncDDR signals to RAG DSBs likely feed back through multiple mechanisms that reduce RAG gene expression and RAG protein activity.

The toggle model.

How is it that a small pre-B cell undergoes iterative *Igk* chain gene rearrangements in an ordered fashion while allowing a single rearrangement to be tested to determine whether it is productive before another rearrangement is initiated? We propose that this occurs through a toggling between pre-BCR signalling and RAG DSB-mediated ncDDR signalling (FIG. 5). In small pre-B cells, the two *Igk* alleles are differentially accessible such that in response to pre-BCR signalling, RAG DSBs are efficiently generated on a single allele^{58–60}. The generation of a pair of RAG DSBs at an *Igk* allele leads to activation of ATM and the ncDDR, which antagonizes pre-BCR signalling, *Igk* locus accessibility and RAG activity, preventing initiation of additional *Igk* rearrangements. Completion of *Igk* chain gene assembly through NHEJ-mediated repair of the RAG DSBs leads to silencing of ATM activity and the ncDDR. If the completed *Igk* chain gene is productive, encoding an IgL κ chain that can pair with the IgH chain to generate a BCR, then the BCR signals promote transition to the immature B cell stage, where RAG activity and *Igk* chain gene rearrangement cease if the BCR is non-autoreactive¹⁷. If the rearrangement does not encode a functional IgL κ chain, then loss of ATM signals, and the ncDDR, would presumably lead to re-initiation of pre-BCR signalling, *Igk* locus accessibility and RAG activity, which together would trigger another *Igk* chain gene rearrangement. This rearrangement could be on the alternative *Igk* allele or the same allele, leading to deletion of the previous non-functional *Igk* rearrangement. It is tempting to speculate that reactivation of pre-BCR signals in these cells may drive them through the cell cycle, stochastically resetting the accessibility of the two *Igk* alleles during DNA replication and rendering a single *Igk* allele accessible in each of the daughter cells. In immature B cells, autoreactive BCRs can generate signals that maintain RAG expression and lead to new *Igk* chain rearrangements that replace the *Igk* chain gene responsible for the autoreactive BCR, a process termed receptor editing^{17,19}. Thus, in addition to iteratively toggling between the ncDDR and pre-BCR signals in pre-B cells, immature B cells with autoreactive BCRs may toggle between ncDDR and BCR signals as they attempt to replace this BCR. In this regard, the ncDDR may antagonize BCR signalling to regulate receptor editing in immature cells through pathways similar to those used to antagonize pre-BCR signalling in pre-B cells.

The ncDDR in mature B cells

Once activated, mature B cells generate antibodies of different isotypes through the process of CSR²⁰ (FIG. 6). Activation of a naive IgM-expressing B cell leads to transcription of the switch region (S_{μ}) upstream of the C_{μ} exon and the switch region associated with the target constant region gene (FIG. 6). This transcription leads to the formation of DNA–RNA R-loops with non-template single-strand DNA, which serves as a substrate for activation-induced deaminase (AID). AID converts cytosine to uridine, with the resulting mismatch processed primarily by the base excision repair pathway and also by the mismatch repair pathway²⁰. Uracil-DNA glycosylase (UNG) of the base excision repair pathway forms an abasic site, and apurinic/apyrimidinic endonuclease 1 (APE1) excises this abasic site to form a single-strand nick. In conjunction with the RNA exosome complex, AID also targets the template strand, although less efficiently, and it is thought that DSBs form through closely staggered single-strand nicks on the alternative strands⁷⁵. DNA ends in two switch regions

are then joined with deletion of the intervening sequence to place the new C region exons downstream of the V region exons²⁰ (FIG. 6). During CSR, the DNA ends can join by either classical or alternative NHEJ^{6,76}.

The DSBs generated during CSR activate ATM, leading to an ncDDR that, like RAG DSBs in developing B cells, regulates a broadly functional genetic programme. CSR, and the resulting DSBs, occur in rapidly proliferating germinal centre B cells. BCL-6, a key transcription factor that regulates the B cell germinal centre programme, suppresses the expression of several DDR factors, including ATR, p53 and p21, which potentially limits the cDDR to CSR DSBs^{77–79}. However, CSR DSBs activate ATM, initiating an ncDDR that includes activation of the kinase LKB1 (also known as STK11)⁸⁰. LKB1 inactivates CREB-regulated transcription co-activator 2 (CRTC2), which leads to the acquisition of a genetic programme that promotes the differentiation of activated B cells into plasma cells⁸⁰. Thus, through activation of an ncDDR, CSR DSBs provide the signals that are required for the differentiation of activated B cells into plasma cells.

Unlike RAG DSBs, which are inhibited by ATM during V(D)J recombination, during CSR, ATM augments AID-induced DSB generation at switch regions. In this regard, ATM-mediated phosphorylation of AID promotes its association with the endonuclease APE1, which is required for the generation of single-strand nicks⁸¹. Moreover, ATM activity promotes the association of two switch regions with DSBs⁸². The intriguing contrast between the activity of ATM in inhibiting RAG DSBs and its activity in promoting AID-induced DSBs could be related, in part, to the different manner in which the paired DSBs are generated by RAG and AID. RAG DSBs are generated in a synaptic complex that contains RAG and two recombining gene segments, and, as discussed above, ATM-mediated activation of the ncDDR prevents the formation of additional RAG DSBs. By contrast, AID-induced DSBs are generated independently at two switch regions that are not necessarily associated with each other^{20,83–86}. Here, ATM signalling in response to AID-induced DSBs at one switch region is used to stimulate AID-induced DSBs at another switch region and to drive association of the two switch regions with DSBs, thereby promoting CSR. Indeed, in ATM-deficient B cells, DSBs are observed at increased frequency at the most upstream switch region, S_μ, but at decreased frequency in down-stream switch regions⁸⁷. This contrast in the regulation of RAG-induced and AID-induced DSB generation by ATM provides an important example of how the ncDDR has evolved to regulate similar processes in different ways on the basis of cell type-specific and context-specific requirements.

The ncDDR in macrophages

Activated macrophages produce reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates, such as nitric oxide (NO), to eradicate pathogens⁵. These agents could also damage host cellular DNA, leading to DNA DSBs that activate the cDDR and ncDDR. Indeed, bone marrow-derived macrophages (BMDMs) activated with Toll-like receptor agonists, such as bacterial lipopolysaccharide (LPS), and IFN γ or infected with the intracellular bacterial pathogen *Listeria monocytogenes* activate both the cDDR and ncDDR⁸⁸ (FIG. 7). ROIs can activate ATM directly without the generation of DNA DSBs⁸⁹. However, the cDDR in activated BMDMs does not depend on ROIs; it depends on NO and

the generation of DNA DSBs by NO⁸⁸. Unlike ROI production, which occurs immediately upon macrophage activation, NO production is delayed owing to the requirement for inducible NO synthase (*NOS2*) gene induction⁹⁰. Thus, activation of an ncDDR will be delayed relative to the initial activation of the macrophage. In BMDMs, an optimal cDDR and ncDDR depend on both ATM and DNA-PKcs activation as well as on type I interferon signals⁸⁸. The basis for the requirement for type I interferons is not known but is not due solely to the role of type I interferons in stimulating NO production or to a requirement for type I interferon signals in promoting expression of key DDR proteins⁸⁸. The ncDDR in macrophages regulates a functional genetic programme that includes several cytokine and chemokine genes in addition to genes encoding cell surface receptors with functions in phagocytosis⁸⁸. Irradiation of macrophages also leads to ATM-dependent induction of an ncDDR that regulates the expression of many genes that are involved in innate immune responses^{91,92}. The type I interferons required for this response may come from the genotoxic DNA damage caused by irradiation⁹³. In BMDMs, ATM and DNA-PKcs also promote optimal caspase 1 activation and cleavage of pro-IL-1 β and pro-IL-18 to form IL-1 β and IL-18 during AIM2 inflammasome activation in response to *L. monocytogenes* infection^{88,94}. Indeed, ATM-deficient mice exhibit defects in innate immunity owing to impaired inflammasome activation⁹⁵. NBS1, a component of the MRN complex, is required for normal macrophage proliferation and differentiation, which could reflect MRN function in DNA replication in addition to its role in activating ATM and the ncDDR^{4,88,96}. In the setting of chronic inflammatory signals in vivo, ATR regulates pathways that promote the generation of polyploid macrophages in granuloma lesions⁹⁷. Thus, all three DDR kinases, ATM, DNA-PKcs and ATR, promote ncDDRs that regulate macrophage differentiation and function.

Heritable effects of ncDDR signalling

DNA DSBs generated during V(D)J recombination and CSR impact the cellular events that are coincident with DSB generation through initiation of ncDDRs. However, recent studies suggest that DNA DSBs generated early in immune cell development may influence the function of mature immune cells long after the DNA damage has been resolved and any damage response signalling has been extinguished. Lymphocytes and natural killer (NK) cells differentiate from common progenitors that can express RAG^{98–101}. In response to viral pathogens, NK cells derived from progenitors that expressed RAG exhibit greater fitness, proliferation and better survival than NK cells derived from progenitors that failed to express RAG^{102,103}. Although it was not established that this requires DDR kinase activity and an ncDDR, the endonuclease activity of RAG1 is required for this effect, indicative of a requirement for RAG DSB generation¹⁰². Moreover, antigen receptor gene rearrangements can be found in mature NK cells, which demonstrates that RAG-generated DNA DSBs are produced and repaired in progenitors that give rise to NK cells¹⁰¹. Thus, activation of an ncDDR by these DSBs may have long-term effects on mature NK cells, presumably through heritable alterations in their genetic programmes. In this regard, NK cells derived from progenitors that expressed RAG exhibit increased expression of several genes encoding DDR proteins¹⁰².

Determinants of ncDDRs

The unique pathways that are activated by ncDDRs in different cell types may be related, in part, to the nature of the DSB (for example, RAG DSBs versus CSR DSBs). However, in developing pre-B cells, DSBs induced by ionizing radiation trigger many of the gene expression changes induced by RAG DSBs^{21,104}. Indeed, NF- κ B1, a major determinant of this genetic programme, can be activated through the phosphorylation of NEMO by ATM in the nucleus in response to genotoxic DSBs^{35,36}. Thus, cells that undergo programmed DNA DSBs may be poised to express a genetic programme in response to DSB signals regardless of how the DSBs are generated. Activation of ATM provides the cellular cue that a DSB has been generated, initiating an ncDDR that regulates cell type-specific processes. Implied in this model is the idea that exposure of immune cells to genotoxic agents, as in the setting of cancer therapy, could alter the genetic programme of these cells, impacting immune system development and function²¹.

Conclusion

We are now just beginning to understand how ncDDRs to DNA DSBs generated in different physiological settings signal events that are important for the development and function of cells of the innate and adaptive immune system. Although most of what we know comes from the analysis of programmed DSBs generated during V(D)J recombination and CSR, DSBs made in other settings, such as during transcription and DNA replication in immune cells that have been stimulated to divide, may also initiate important ncDDRs. In addition, fragments of damaged genomic DNA that gain access to the cytosol can also regulate immune responses through activation of the cytosolic DNA sensor cyclic GMP-AMP synthase (cGAS), which leads to type I interferon production¹⁰⁵. This pathway, which evolved to respond to pathogen DNA, may also functionally intersect with the cDDR and ncDDR¹⁰⁶. The full spectrum of ncDDR pathways, how they integrate with other cellular pathways and the processes that they regulate in innate and adaptive immune cells remain to be determined. The regulation of diverse immune responses by ncDDRs raises the intriguing possibility that therapeutic agents that target DDR proteins may be used to manipulate immune responses in different disease settings.

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Programmed DNA double-strand breaks

DNA double-strand breaks made at specific genomic regions as required intermediates of physiological processes, such as V(D)J recombination or immunoglobulin class switch recombination.

Non-programmed DNA DSBs

DNA double-strand breaks (DSBs) made by genotoxic agents or nucleases throughout the genome.

Canonical DNA damage response

(cDDR). A cellular response to DNA double-strand breaks (DSBs) initiated by DNA damage response kinases in all cells that activates the pathways required for DNA DSB repair and genome stability.

Non-homologous end joining

(NHEJ). A DNA repair process that joins broken DNA ends (double-strand breaks) at all phases of the cell cycle without using homologous DNA as a template. The core components of this pathway include the proteins KU70, KU80, X-ray repair cross-complementing protein 4 (XRCC4) and DNA ligase IV.

Non-canonical DNA damage responses

(ncDDRs). Cellular responses to DNA double-strand breaks (DSBs) activated by DNA damage response kinases but that are not involved in DNA DSB repair or in maintaining genome integrity. Rather, these DSB-dependent programmes regulate cell type-specific pathways that are involved in a broad variety of cellular functions.

Pre-B cell receptor

(Pre-BCR). A receptor composed of immunoglobulin heavy chains and surrogate light chains, VpreB and $\lambda 5$, that associates with the transmembrane Ig α and Ig β proteins that have cytoplasmic domains that transduce intracellular signals through the tyrosine kinase SYK and adaptor protein B cell linker protein (BLNK).

IL-7 receptor

(IL-7R). A receptor composed of the IL-7R α chain and the cytokine receptor common γ -chain. Upon binding to IL-7, IL-7R activates the kinase AKT and the kinases Janus kinase 1 (JAK1) and JAK3, which phosphorylate and activate signal transducer and activator of transcription 5 (STAT5), to regulate cellular proliferation and survival.

Accessibility

Refers to the structure of chromatin. When chromatin is more loosely packed (or open), it is accessible for transcription, whereas tightly packed or closed chromatin is refractory to factors that need to gain access to the DNA template. Recombination-activating gene (RAG) is expressed in developing B and T cells, but it can only carry out V(D)J recombination at antigen receptor loci with an accessible chromatin structure.

ETS family transcription factors

A large family of proteins with conserved DNA binding domains that bind to GGA(A/T) (PU-box) motifs. Family members have distinct transactivation domains that dictate binding partners and regulate transcription. The PU.1 (also known as SPI1), SPIB and SPIC ETS family members all have roles in regulating transcription during B cell development.

Fig. 1: V(D)J recombination.

a The initial step of the V(D)J recombination reaction is the synapsis of two gene segments (variable (V) to joining (J) recombination is shown) and their flanking recombination signal sequences (RSSs; triangles) with recombination-activating gene 1 (RAG1) and RAG2 proteins, which recognize the RSSs. **b** DNA cleavage by RAG proteins leads to the generation of a blunt signal end and a hairpin-sealed coding end at each RAG-mediated DNA double-strand break (DSB). **c** The two coding ends are then joined to form a coding join that completes the second exon of the antigen receptor gene. This joining is imprecise,

with a gain and/or loss of nucleotides at the join leading to only one-third of completed antigen receptor genes being in-frame and productive. The signal ends are joined somewhat precisely to form a signal join. Binding of KU70, KU80 and MRE11–RAD50–NBS1 (MRN) to RAG DSBs activates the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and ataxia telangiectasia mutated (ATM) kinases, respectively. ATM and DNA-PKcs initiate a canonical DNA damage response (cDDR) to promote RAG DSB repair. ATM also activates non-canonical DNA damage response (ncDDR) signalling, which regulates B cell developmental programmes. NHEJ, non-homologous end joining.

Fig. 2:|. B cell development. B cell development proceeds in the bone marrow through several discrete steps. From a standpoint of DNA double-strand break (DSB) generation and potential non-canonical DNA damage response (ncDDR) signalling, these stages can be generally divided into the pro-B cell stage in which *IgH* gene assembly occurs in all cells, the pre-B cell stage in which *IgH* gene assembly occurs in all cells and the immature B cell stage in which receptor editing occurs in some cells. DNA DSBs are also generated in mature B cells once they are activated and initiate class switch recombination (CSR). AID, activation-induced deaminase; APE1, apurinic/apyrimidinic endonuclease 1; BCR, B cell receptor; RAG, recombination-activating gene; UNG, uracil-DNA glycosylase.

Fig. 3:|. Large pre-B cell to small pre-B cell transition.a

In large pre-B cells, IL-7 receptor (IL-7R) signalling activates AKT and the Janus kinase (JAK)–signal transducer and activator of transcription 5 (STAT5) pathway. This inhibits *Igk* gene transcription and accessibility, inhibits recombination-activating gene (RAG) expression by causing degradation of forkhead box protein O1 (FOXO1) and promotes proliferation and survival of large pre-B cells, in part, through inhibition of the apoptosis regulator BAX by PIM1-mediated and AKT-mediated phosphorylation of BCL-2-associated agonist of cell death (BAD). **b** | In small pre-B cells, loss of IL-7R signalling leads to

increased activity of tyrosine protein kinase SYK and B cell linker protein (BLNK), which augments signalling through the pre-B cell receptor (pre-BCR), leading to the induction of interferon regulatory factor 4 (IRF4) expression and induction of *Igk* gene transcription and accessibility. Loss of AKT signals results in FOXO1-driven expression of *Rag1* and *Rag2* and loss of IL-7R-mediated proliferative and survival signals. Pre-BCR signals can also drive proliferation, although not as strongly as IL-7R signals. **c** | The initiation of *Igk* gene rearrangement and the generation of RAG double-strand breaks (DSBs) leads to the activation of ataxia telangiectasia mutated (ATM), which initiates both a canonical DNA damage response (cDDR) and a non-canonical DNA damage response (ncDDR). The ncDDR is mediated, in part, by the activation of a nuclear factor- κ B1 (NF- κ B1)-dependent and NF- κ B2-dependent genetic programme. The induction of SPIC represses *Igk* gene transcription and antagonizes pre-BCR signals by inhibiting SYK and BLNK expression. This ncDDR genetic programme also promotes survival through the induction of PIM2, which phosphorylates BAD to inhibit BAX. p53 and PIM2 also enforce the G1 to S checkpoint. ATM activation leads to reduced *Rag* expression through inhibition of growth arrest and DNA damage-inducible protein GADD45 α expression and through direct phosphorylation and inhibition of RAG2. RAG DSBs also trigger an ncDDR that inhibits the production of IL-7 by local stromal cells. Collectively, these ncDDR pathways prevent the generation of additional RAG DSBs, promote survival and block proliferation in cells with existing RAG DSBs. P, phosphorylation.

Fig. 4:|. ordered assembly of antigen receptor genes.

Antigen receptor gene assembly at several loci is ordered inter-allelically, such that one allele rearranges at a time. Once completed, the rearrangement is tested to determine whether it is productive before rearrangement at the alternative allele occurs. This inter-allelic ordering is critical for enforcing allelic exclusion. Antigen receptor gene assembly is also intra-allelically ordered. After a rearrangement is initiated on one allele, the initiation of additional rearrangements on the same allele must be prevented. Cleavage events at an antigen receptor loci mediated by recombination-activating gene (RAG) proteins are regulated by accessibility of the loci. However, once RAG double-strand breaks (DSBs) are made, the non-canonical DNA damage response (ncDDR) participates in enforcing both inter-allelic and intra-allelic ordering to prevent additional RAG DSBs from being generated until the initial rearrangement has been completed and tested to determine whether it encodes a functional antigen receptor gene. ATM, ataxia telangiectasia mutated.

Fig. 5:|. The toggle model.

Small pre-B cells have two signalling states; first, pre-B cell receptor (pre-BCR) signalling that promotes *Igl* gene rearrangement and, second, signalling via ataxia telangiectasia mutated (ATM), in response to double-strand breaks (DSBs) generated by recombination-activating gene (RAG) proteins, that activates a non-canonical DNA damage response (ncDDR). The ncDDR suppresses additional RAG DSBs until the *Igl* gene assembly is complete and is tested to determine whether it encodes a functional IgL chain. Small pre-B cells may iteratively toggle between these two signalling states as they undergo multiple rounds of *Igl* chain gene rearrangements in an ordered manner as they attempt to generate a functional IgL chain.

Fig. 6:|. Immunoglobulin class switch recombination.

Immunoglobulin class switch recombination (CSR) is initiated by transcription through the switch μ (S_μ) region and a downstream switch region to which switching will occur (shown here to be S_{γ_3}). Activation-induced deaminase (AID) targets the non-template single-strand DNA (ssDNA), deaminating cytosine to form uridine. The proteins uracil-DNA glycosylase (UNG) and apurinic/apyrimidinic endonuclease 1 (APE1) of the base excision repair pathway then deglycosylate and excise the uridine to generate a single-strand nick. The same reaction occurs on the template strand, although less efficiently, and the formation of closely staggered nicks on the two DNA strands leads to a DNA double-strand break (DSB). Broken DNA ends from the two switch regions are then joined by either classical or alternative non-homologous end joining. CSR DSBs activate ataxia telangiectasia mutated (ATM), initiating a non-canonical DNA damage response (ncDDR) that regulates CSR and B cell differentiation. Phosphorylation of AID by ATM in response to CSR DSBs promotes the association of APE1 with AID, leading to more efficient nick formation. ATM activity promotes the association of the two switch regions with DSBs. ATM activates the kinase LKB1, which inhibits CREB-regulated transcription co-activator 2 (CRTC2), a transcriptional co-activator of cAMP-responsive element-binding protein 1 (CREB1), promoting a plasma cell genetic programme. cDDR, canonical DNA damage response; dsDNA, double-strand DNA; P, phosphorylation.

Fig. 7:|. Activation of the ncDDR in macrophages.

Macrophages activated by signals through Toll-like receptors (TLRs) and interferon receptors produce nitric oxide (NO), which causes genomic DNA double-strand breaks (DSBs) that activate ataxia telangiectasia mutated (ATM) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs), leading to a canonical DNA damage response (cDDR) and a non-canonical DNA damage response (ncDDR). The ncDDR promotes a broadly functional genetic programme in these cells and regulates inflammasome activation. Type I

interferon signals are required for optimal cDDR and ncDDR activation. NOS2, nitric oxide synthase.

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