TopBP1 deficiency impairs V(D)J recombination during lymphocyte development

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Abstract

TopBP1 was initially identified as a topoisomerase II- β -binding protein and it plays roles in DNA replication and repair. We found that TopBP1 is expressed at high levels in lymphoid tissues and is essential for early lymphocyte development. Specific abrogation of TopBP1 expression resulted in transitional blocks during early lymphocyte development. These defects were, in major part, due to aberrant V(D)J rearrangements in pro-B cells, double-negative and double-positive thymocytes. We also show that TopBP1 was located at sites of V(D)J rearrangement. In TopBP1-deficient cells, γ -H2AX foci were found to be increased. In addition, greater amount of γ -H2AX product was precipitated from the regions where TopBP1 was localized than from controls, indicating that TopBP1 deficiency results in inefficient DNA double-strand break repair. The developmental defects were rescued by introducing functional TCR $\alpha\beta$ transgenes. Our data demonstrate a novel role for TopBP1 as a crucial factor in V(D)J rearrangement during the development of B, T and iNKT cells.

Keywords lymphocyte development; TopBP1; VDJ recombination

Subject Categories Immunology

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Introduction

Adaptive immunity is based on the ability of B and T cells to recognize and ultimately eliminate a plethora of foreign invaders from the host. An individual has billions of B and T cells and each conventional B and T cell expresses a B-cell receptor (BCR) or T-cell receptor (TCR) that recognizes a single antigen. The combinatorial assembly of a limited number of BCR and TCR genes by rearrangement gives rise to the diversity of the BCR and $\alpha\beta$ TCR repertoire.

The processes of B-cell and T-cell development are very similar, except that T progenitor cells migrate to the thymus via the blood to acquire T-cell identities. Lymphocytes originate from multipotent stem cells in the fetal liver before, and in the bone marrow after birth. Common lymphoid progenitors (CLPs) differentiate to late pro-B cells after passing through pre-pro (Fr. A) and early-pro stages (Fr. B). Early-pro-B cells rearrange the immunoglobulin (Ig) heavy chain, and late-pro-B cells (Fr. C) express pre-B-cell receptor (pre-BCR) on the cell surface. Pre-BCR signaling results in a burst of proliferation and the differentiation of pro-B cells to pre-B cells.

The earliest thymic progenitor cells express neither CD4 nor CD8 co-receptors (the double-negative (DN) stage) (Godfrey *et al*, 1993). DN thymocytes are briefly arrested at late DN2 stage to rearrange the TCR β locus. After a set of somatic DNA rearrangements, a functional TCR β chain associates with pT α and CD3 complex to form the pre-TCR complex (von Boehmer & Fehling, 1997; von Boehmer *et al*, 1999). Cells that fail to produce a functional pre-TCR are eliminated by apoptosis. Therefore, pre-TCR deficient RAG^{-/-} (Mombaerts *et al*, 1992b; Shinkai *et al*, 1992), TCR β ^{-/-} (Mombaerts *et al*, 1992a), pT α ^{-/-} (Fehling *et al*, 1995) and CD3^{-/-} (Love *et al*, 1993; Malissen *et al*, 1993) mice have a developmental block at the DN3 stage. The pre-TCR signaling at the DN3 stage is critical for differentiation into the CD4⁺CD8⁺ double-positive (DP) stage. Most DP thymocytes further develop into mature CD4 or CD8 single-positive (SP) thymocytes. On the other hand, iNKT cells branch away from the conventional T-cell lineage at the DP stage and proceed through defined developmental stages (Benlagha *et al*, 2002). Although conventional T cells express a diverse repertoire of TCR β and TCR α , iNKT cells combine only the V α 14 TCR α chain with a restricted repertoire of TCR β proteins.

Diverse and functional receptors are produced by V(D)J recombination and this process is initiated by the lymphoid-specific recombinase (RAG, composed of RAG1 and RAG2). RAG proteins create double-strand breaks (DSBs) at recombination signal sequences (RSSs) that flank each variable (V), diversity (D) and joining (J)

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gene segment to release blunt signal ends and hairpin coding ends (Bassing *et al*, 2002; Schatz & Spanopoulou, 2005). These breaks are subsequently resolved via the nonhomologous end joining (NHEJ) pathway, one of the DSB repair pathways (Gellert, 2002). XRCC4, Ku70, Ku80, Ligase IV and XLF/Cernunnos are components of the NHEJ machinery. The blunt signal ends are simply joined to form precise signal joints whereas the hairpin coding ends must be opened by the Artemis/DNA-PK complex before ligation can take place (Bassing *et al*, 2002; Schatz & Spanopoulou, 2005; Dahm, 2008). The lesions introduced by RAG proteins are considered to be among the most dangerous lesions, because they present threats to genome stability. Therefore, elaborate mechanisms are crucial to ensure efficient DSB repair.

TopBP1 is topoisomerase II- β -binding protein with eight BRCT (the C-terminal domain of a breast cancer susceptibility protein) motifs that are found in proteins involved in DNA replication, DNA damage response, and cell cycle checkpoints (Bork *et al*, 1997; Callebaut & Mornon, 1997). TopBP1 is a substrate of Ataxia telangiectasia mutated (ATM) (Yamane *et al*, 2002), which coordinates DSB repair through its ability to phosphorylate its substrates including Mre11, Rad50, BRCA1, DNA-PK, 53BP1, H2AX, and p53 (Keimling *et al*, 2011). ATM-deficient mice display a reduction in number of mature CD4 and CD8 cells (Barlow *et al*, 1996). T-cell number and function were rescued by introducing functional TCR $\alpha\beta$ transgene, indicating that a defect in TCR recombination is responsible for the impaired T-cell development (Chao *et al*, 2000). TopBP1 is also known to bind to Nbs1, a component of the MRN complex which is involved in DNA DSB repair in mammals (Carney *et al*, 1998; Chen *et al*, 2000). TopBP1 colocalizes with Nbs1 following DNA damage (Yamane *et al*, 2002), and TopBP1-depleted cells display a reduced frequency of sister chromatid exchange (SCE), suggesting that TopBP1 plays a role in double-stranded DNA break-induced homologous recombination (HR) repair in association with Nbs1 (Morishima *et al*, 2007). Nbs1-deficient mice also showed defects in lymphoid development resulting in immune deficiency (Kang *et al*, 2002). Thus, it is conceivable that TopBP1 has a critical role in V(D)J recombination during lymphocyte development. Here we report that TopBP1 is a new critical factor for V(D)J recombination occurring during lymphocyte development.

Results

Expression of TopBP1 in early lymphocytes

We first determined the expression profile of TopBP1 in various tissues using Western blot analysis. TopBP1 is highly expressed in the lymphoid tissues (Supplementary Fig S1A). In B-cell subpopulations, *Topbp1* is abundant in pro- and pre-B cells when compared to immature and mature B cells (Supplementary Fig S1B). In the thymus, expression of *Topbp1* is induced at the DN2 stage, maintained at a high level until the DP stage, and down-regulated when thymocytes reach maturity (Supplementary Fig S1C). Thus, the *Topbp1* expression patterns suggest that it may be required for early lymphocyte development. The loss of TopBP1 leads to embryonic lethality at an early stage (Jeon *et al*, 2011). Thus, we used mice

conditionally deficient in TopBP1 expression and investigated the function of TopBP1 in lymphocyte development.

Deletion of TopBP1 results in profound immune deficiency in B cells

The role of TopBP1 in B-cell development was investigated using TopBP1^{f/f} mice crossed with Mb1-cre mice. Deficiency of TopBP1 in early B-cell progenitors resulted in a significant decrease in both the percentage and number of B cells, characterized by surface expression of B220 and CD19 in the bone marrow, spleen, and peripheral blood cells (Fig 1A). B-cell lymphopoiesis can be subdivided using cell-surface markers according to the classification proposed by Hardy and colleagues (Hardy *et al*, 1991). As shown Fig 1B, most B220⁺ cells present in the bone marrow of TopBP1-deficient mice were pro-B cells (Fr. A-C) and very few exhibited a pre-B-cell phenotype. These results imply that the ablation of TopBP1 impaired B-cell development during or before the pro-B cell stage. Further subfractionation of pro-B-cell populations revealed a significant increase in the percentage of CD24⁺BP1⁺ cells (Fr. A) in TopBP1-deficient mice (Fig 1C). However, pre-pro-B (Fr. A) cells were clearly quantitatively preserved in the absence of TopBP1, suggesting a critical role for TopBP1 at early-pro-B-cell stage (Fr. B).

Impaired early T-cell development in TopBP1-deficient mice

Roles of TopBP1 during thymocyte development were also investigated by analyzing thymi from TopBP1^{f/d} mice crossed with Lck-cre mice. TopBP1-deficient mice showed approximately a seven-fold reduction in the total cellularity of thymocytes compared to littermate controls (Supplementary Fig S2A and B). They did not display clear demarcations between the cortex and medullar regions (Supplementary Fig S2C). The percentage of DN cells was markedly increased, but the actual number of DN cells from TopBP1-deficient mice was not substantially different from that of littermate controls (Fig 2A). Both the percentage and the actual cell number of DP thymocytes and mature SP thymocytes were reduced, suggesting that there was a major developmental block between the DN and DP stages. Total cellularity of spleen and lymph nodes from TopBP1-deficient mice was also significantly reduced compared to that of littermate controls, reflecting the greatly reduced generation of mature SP thymocytes (Supplementary Fig S2D). However, non-T cells in peripheral organs were not affected by the T lineage cell-specific TopBP1 deficiency (Supplementary Fig S2E).

When DN thymocytes were stained for CD25 and CD44 surface markers to pinpoint more precisely the DN stage at which TopBP1 was required, we found that TopBP1 deficiency led to the accumulation of thymocytes at the DN3 stage (Fig 2B). The DN3/DN4 ratio showed a more than a three-fold increase in TopBP1-deficient mice (Supplementary Fig S3). The proportion of CD44^{int}CD25^{bright} cells significantly increased in the TopBP1-deficient DN compartment (Supplementary Fig S2F). These populations are normally present in mice bearing defects in pre-TCR signaling, such as pT α ^{-/-}, TCR β ^{-/-}, Rag1^{-/-}, and CD3 ϵ ^{-/-} mice. The presence of a large number of CD25^{bright} DN3 cells may be due to one of the following reasons; deficiency of one of the pre-TCR components, problems in pre-TCR signaling, or defects in proliferation and survival. We stained DN3

Figure 1. TopBP1 deficiency results in B cell immune deficiency.

A Flow cytometric analysis of CD19 and B220 expression on bone marrow, spleen and peripheral blood cells from control and TopBP1-deficient mice ($n = 3$). Absolute cell numbers of CD19⁺B220⁺ cells are shown (bottom).
B B220⁺IgM⁻ cells were analyzed for expression of B220 and CD43.
C Expression of BP-1 and CD24 on pro-B cells are shown, with percentages of Fr. A, B, and C.

and DN4 thymocytes for the CD27 surface marker to see how many cells properly went through β -selection (Fig 2C), which results in an upregulation of expression of CD27. These post- β -selected DN3 cells are classified as DN3b and the CD27^{low} pre- β -selected DN3 cells as DN3a (Taghon *et al*, 2006). The relatively low expression of CD27 in both DN3 and DN4 cells from TopBP1-deficient mice suggests that the developmental arrest at the DN3 stage might be due to defects during β -selection.

Next, we determined whether TopBP1 influences the formation of the pre-TCR complex with CD3 components. We found that surface CD3 expression and pT α mRNA level were not affected (Supplementary Fig S4A and B). However, we found that TopBP1-deficient mice had a decreased percentage and number of surface and intracellular TCR β ⁺ thymocytes (Fig 2D). Thus, the developmental block at the DN3 stage was mainly due to defective expression of TCR β .

Since thymus cellularity was greatly reduced and developmental blocks were seen at the DN3–DN4 and the DN–DP transitions in TopBP1-deficient mice, we also examined the cell survival of TopBP1-deficient thymocytes. Increased Annexin V⁺ thymocytes were detected in DN3 and DN3–DN4 subpopulations, suggesting

increased cell death of TopBP1-deficient cells (Supplementary Fig S5A). This was further confirmed *in vitro* using the OP9-DL1 co-culture system (Notch ligand Delta-like-1 transduced OP9 cells) (Schmitt & Zuniga-Pflucker, 2002; Schmitt *et al*, 2004; de Pooter *et al*, 2006). Purified DN3a cells from TopBP1-deficient mice and littermate controls were cultured on OP9-DL1 monolayers for 7 days. The DP population was severely reduced when TopBP1-deficient DN3a cells, rather than control cells, were co-cultured with OP9-DL1 stromal cells (Supplementary Fig S5B). Fewer DN3a cells from TopBP1-deficient mice than from controls progressed to DN3b, since the CD27 expression remained relatively lower in the TopBP1-deficient mice (Supplementary Fig S5C). This defect in the DN3a to DN3b transition in TopBP1-deficient cells appeared to be due to both decreased proliferation and increased cell death during β -selection. When we cultured DN3a cells from littermate control mice after stably labeling with carboxyfluorescein succinimidyl ester (CFSE), each cell division resulted in a sequential halving of fluorescence, which was not observed for DN3a cells from TopBP1-deficient mice (Supplementary Fig S5D). These results suggest that TopBP1-deficient DN3 thymocytes are less proliferative, which may

Figure 2. Block in early lymphocyte development in the absence of TopBP1.

A Percentages and absolute cell numbers (bottom) of DN, DP, CD4, and CD8 cells ($n = 14$).
 B Expression of CD25 and CD44 on DN thymocytes with the percentages of DN1–4 subsets.
 C Expression of CD27 in DN3 and DN4 thymocytes.
 D Analysis of surface (left) and intracellular (right) TCRβ in DN3 and DN4 thymocytes. Data shown represent four independent experiments with similar results.

contribute to the perturbation of development and maturation of thymocytes.

Impaired maturation of SP thymocytes and iNKT development in TopBP1-deficient mice

Next, we investigated the role of TopBP1 in the production of mature CD4 and CD8 SP thymocytes. We crossed mice with a floxed allele of *TopBP1* with CD4-cre mice, since very early inactivation of TopBP1 in DN thymocytes using Lck-cre mice leads to defects during the transition from DN to DP stage, thereby severely reducing thymic cellularity. The numbers of mature CD4 and CD8 SP cells were significantly reduced in TopBP1-deficient mice (Fig 3A). Since

commitment to iNKT-cell lineages as well as to CD4 and CD8 T-cell lineages is determined during the DP stage, we examined whether TopBP1 is required for iNKT-cell development. We stained thymocytes with CD1d-tet and TCRβ to evaluate iNKT-cell populations in the thymus, liver, and spleen. We observed significantly smaller numbers and frequency of iNKT cells in the thymus, spleen, and liver of TopBP1-deficient mice compared to those of controls (Fig 3B).

To determine precisely the stages of iNKT-cell development perturbed by the absence of TopBP1, we analyzed developmental subsets on the basis of expression of CD24, CD44 and NK1.1. This analysis showed a severe reduction in stage 2 and 3 cells, indicating that the developmental block occurred during stages 0 and 1. As

Figure 3. TopBP1 is required for iNKT-cell development.

A Percentages of DN, DP, CD4 and CD8 cells ($n = 7$).

B Expression of CD1d-tet and TCRβ in the thymus, liver and spleen from control and TopBP1-deficient mice ($n = 4$). Absolute cell numbers of iNKT cells are shown (bottom).

C Expression of CD44/NK1.1 (top) and CD24 (bottom) in CD1d-tet⁺TCRβ^{int} cells.

shown Fig 3C, most iNKT cells in the thymus of TopBP1-deficient mice were CD24^{high}. These results suggest that ablation of TopBP1 impairs iNKT-cell development at stage 0, indicating that they have not yet received the positive selection signal. We analyzed CD1d expression on DP thymocytes to determine whether the skewing toward stage 0 cells was due to diminished CD1d expression. Control and TopBP1-deficient thymocytes expressed similar amounts of CD1d (Supplementary Fig S6A and B). In addition, mRNA expression of Bcl-xl, the main factor controlling DP survival, did not show any significant differences between control and TopBP1-deficient DP thymocytes, suggesting that TopBP1 deficiency does not influence the survival of DP thymocytes (Supplementary Fig S6C). Therefore, decreased survival of DP cells of TopBP1-deficient mice was not the main reason for the block in iNKT-cell development.

Inefficient V(D)J recombination in TopBP1-deficient cells

From our results showing the developmental block at the time of IgH gene rearrangement during B-cell development, decrease in expression of TCRβ and impaired maturation of SP thymocytes in T-cell development, and the defects in early iNKT-cell differentiation, we deduced that TopBP1 could be involved in the V(D)J rearrangement process. Thus, we investigated whether ablation of TopBP1 affects V(D)J rearrangement. Genomic DNA from sorted

pro-B cells of control and TopBP1-deficient mice was extracted and analyzed for V_H52 and V_H558 with J rearrangements. We observed that both V_H52-J_H3 and V_H558-J_H3 rearrangements were largely absent (Fig 4A). To see whether the recombination events were normal, we sequenced V(D)J rearrangements from control and Mb1-cre; TopBP1^{fl/fl} mice pro-B cells. Although V(D)J rearrangement of Ig is significantly decreased in TopBP1-deficient pro-B cells, we observed no apparent differences in the fidelity of the coding joints between these two populations (Supplementary Table S5). It is not yet clear whether the overall normality of V(D)J rearrangement sequences is due to TopBP1-sufficient cells resulting from incomplete deletion or because TopBP1 is dispensable for these processes.

The absence of TopBP1 also affected the rearrangement of TCR genes in a similar way to Ig rearrangement. Because of low expression of TCRβ in thymocytes, we purified genomic DNA from fractionated DN3 and DN4 cells and amplified across Vβ5.1 and Vβ8.2 segments by using PCR. There was a significant decrease in the V(D)J rearrangement of TCRβ in TopBP1-deficient thymocytes compared to control cells (Fig 4B). Also, using semi-quantitative PCR to detect TCRα rearrangements, we found that pre-selected DP thymocytes from TopBP1-deficient mice showed inefficient rearrangements, including Vα14-Jα18 (Fig 4C).

In order to assess the function of TopBP1 in V(D)J recombination *in vitro*, a DNA for shRNA-targeting TopBP1 (shTopBP1) was cloned

Figure 4. Inefficient V(D)J recombination in TopBP1-deficient mice.

- A V(D)J recombination analyses of genomic DNA from purified pro-B cells (B220⁺CD43⁺IgM⁻) of control and TopBP1-deficient mice. C μ was amplified as a loading control.
- B Genomic DNA was isolated from sorted DN3 and DN4 thymocytes, from control and TopBP1-deficient mice. Serially diluted genomic DNA was assayed for V(D)J recombination in DN3 and DN4 thymocytes. Thy1 was amplified as a loading control. The experiments were repeated at least three times with consistent results.
- C V α rearrangement of purified DP cells was measured by semi-quantitative PCR. C α was amplified as a loading control.

into the retroviral vector MDH (Supplementary Fig S7A). NIH3T3 cells were infected with either MDH-shTopBP1 or empty MDH vector, and *in vitro* recombination assays were performed. We purified GFP⁺ cells from these MDH or MDH-shTopBP1-infected NIH3T3 cells. Western blotting assay confirmed that TopBP1 expression was reduced in MDH-shTopBP1-infected cells relative to MDH-infected NIH3T3 cells (Fig 5A). These cells were then transiently co-transfected with murine RAG1 and RAG2 expression vectors, as well as the extrachromosomal recombination substrate pJH289 and pJH290 (Deriano *et al*, 2009) (Supplementary Fig S7B). This system provides a specific RAG1/2-mediated V(D)J recombination model in non-lymphoid cells. Coding joints and signal joints were produced inefficiently in TopBP1-deficient cells (Fig 5B).

Impaired V(D)J recombination in TopBP1-deficient lymphocytes and NIH3T3 cells may be due to insufficient expression of components necessary for V(D)J recombination. To test this, we isolated DN3 and DN4 cells and measured mRNA levels of factors involved in the V(D)J recombination. mRNAs for *Rag1*, *Rag2*, *ku70*, *ku80*, *Xrcc4*, *Ligase4* and *Dna-pk* were expressed normally in TopBP1-deficient cells (Fig 5C).

Incomplete repair of DNA DSBs in TopBP1-deficient cells

Even though NHEJ family, *Rag1* and *Rag2* are expressed normally in TopBP1-deficient cells, V(D)J rearrangement was found to be reduced. To further verify that TopBP1 is actually involved in V(D)J recombination, we analyzed the DSB repair status around RAG-induced DSB sites by ChIP analysis using the TopBP1 antibody. We found that TopBP1 was loaded on DSBs of the TCR V β segment, at the very site of V(D)J recombination (Fig 5D). Histone H2AX is rapidly phosphorylated specifically at serine 139 (γ -H2AX) after exposure to DNA damaging agents which is a hallmark of DSBs (Rogakou *et al*, 1998; Banath & Olive, 2003; Pilch *et al*, 2003). γ -H2AX foci were accumulated in TopBP1-deficient DN3 cells compared to control cells (Fig 5E). We also examined the H2AX phosphorylation status around RAG-induced DSB sites by ChIP analysis using TopBP1-deficient and control thymocytes. We precipitated greater amount of γ -H2AX products from V regions of TCR β loci in TopBP1-deficient DN3 cells than those from controls (Fig 5F). It is possible that genomic instability exists due to increased γ -H2AX. Abnormal chromosomes are significantly

Figure 5. Aberrant V(D)J recombination and persistence of γ -H2AX around DSB sites in TopBP1-deficient cells.

- A Reduction of TopBP1 expression by shRNA-TopBP1 retrovirus. β -actin served as a loading control.
- B PCR-based *in vitro* recombination assay using recombination substrates and RAG expression vectors transfected into either control or shTopBP1 retrovirus-infected NIH3T3 cells ($n = 3$). Transfectants without RAG1 and RAG2 served as a negative control.
- C Real-time PCR analysis showing levels of *Rag1*, *Rag2*, *Ku70*, *Ku80*, *Xrcc4*, *Lig4*, and *Dna-pk* transcripts relative to β -actin control detected in cDNA prepared from purified control and TopBP1-deficient DN3 thymocytes ($n = 4$).
- D ChIP analysis using antibodies against TopBP1 and IgG. These antibodies recovered the V β region of WT (wild-type) thymocytes. PCR product of *Gapdh* intron served as a negative control. The data are representative of three experiments.
- E γ -H2AX accumulates in TopBP1-deficient DN3 cells.
- F ChIP analysis using antibody against γ -H2AX. This antibody recovered the V β region of WT and TopBP1-deficient DN3 cells. PCR product of *Gapdh* intron served as a negative control.

increased in TopBP1-deficient thymocytes when compared to control cells (Supplementary Fig S8). Because TopBP1 interacts with NBS1, we performed DuoLink assay to see whether NBS1 is recruited to the γ -H2AX foci in TopBP1-deficient cells. We found that NBS1 interacts with γ -H2AX even in the absence of TopBP1 (supplementary Fig S9).

Transgenic TCR overcomes the developmental defects caused by TopBP1 ablation

Our results suggest that reduced thymic cellularity was due to a DN3 arrest, which was caused by defective TCR β expression. To confirm this, we tested whether forced expression of a TCR transgene could overcome the DN3 arrest. TopBP1-deficient mice were crossed with OT-II TCR transgenic mice, which express a TCR specific for the chicken ovalbumin_{323–339} epitope in the context of I-A^b (Barnden *et al*, 1998). Expression of OT-II TCR transgene improved thymic

cellularity in TopBP1-deficient mice to a normal level (Fig 6A). We performed PCR with genomic DNA from total thymocytes of OT-II; Lck-TopBP1-deficient mice and found that the majority of thymocytes were TopBP1-deficient (Supplementary Fig S10). When V α 2⁺ cells were gated to study the effect of OT-II TCR introduction, TopBP1-deficient mice expressing the transgenic TCR displayed a similar thymocyte distribution compared to OT-II TCR transgenic mice. Also, there were no significant differences in the percentage of V α 2⁺ DN3 and DN4 thymocytes or DN3/4 ratio in TopBP1-deficient mice compared to control mice (Fig 6B and C), suggesting a normal DN3–DN4 transition was enabled by the presence of pre-rearranged TCR transgene in TopBP1-deficient thymocytes. These results suggest that blocked DN3 cells of TopBP1-deficient mice were truly rescued by introduction of OT-II TCR and differentiated into DN4 and DP cells. Therefore, the low expression of the TCR β chain is the primary defect in TopBP1-deficient thymocytes. These results indicate that TopBP1 is essential for V(D)J recombination.

Figure 6. Analysis of thymocytes from TCR transgenic mice; Lck-cre; TopBP1^{f/d} mice.

- A Thymic cellularity of OT-II; Lck-cre; TopBP1^{f/d} mice relative to OT-II control mice (left). Thymic cellularity of Lck-cre; TopBP1^{f/d} mice relative to control mice (right).
- B Flow cytometric analysis of DN thymocytes from control and TopBP1-deficient mice in the presence and absence of OT-II TCR. The data are representative of two experiments.
- C DN3/DN4 ratio of control and TopBP1-deficient thymocytes in the presence and absence of the TCR transgene.
- D Expression of CD1d-tet and TCRβ on total thymocytes from control and TopBP1-deficient mice in the presence and absence of Vα14 TCR.
- E Flow cytometric analysis of iNKT cells from control and TopBP1-deficient mice in the presence and absence of Vα14 TCR (top). Expression of CD44 and NK1.1 on iNKT cells from control and TopBP1-deficient mice in the presence and absence of Vα14 TCR. The data are representative of two experiments (bottom).

If the main role of TopBP1 in iNKT-cell development is also to control V(D)J recombination, provision of a rearranged Vα14 TCR transgene would also rescue the developmental block seen in TopBP1-deficient mice. To test this hypothesis, we crossed TopBP1-deficient mice with Vα14 TCR transgenic mice. Frequency as well as cell number of iNKT cells in Vα14 TCR-TopBP1-deficient mice was completely restored to the level of normal mice (Fig 6D). Moreover, TopBP1-deficient cells skewed at stage 0 differentiate normally to stage 3 in the presence of Vα14 TCR (Fig 6E). These results suggest that TopBP1 also plays a critical role during iNKT-cell development by mediating VJ recombination of the TCRα chain gene. Overall, TopBP1 is a crucial factor for V(D)J recombination because all defects observed in TopBP1-deficient mice can be rescued by a functional TCR transgene.

Discussion

In this study, we found that *Topbp1* is abundant in early stages of lymphocyte development; pro- to pre-B cells in the bone marrow and DN3 to DP cells in the thymus. These stages are crucial for the

generation of lymphocytes as they are the points at which V(D)J recombination occurs. Based on the abundant expression of *Topbp1* in the early stages of lymphocyte development and the possibility that TopBP1 may participate in DSB repair, we hypothesized that TopBP1 may be involved in V(D)J recombination, a DSB repair process which takes place during lymphocyte development. To investigate this hypothesis, we generated mice deficient in TopBP1 expression, specifically in B and T lineage cells.

TopBP1-deficient mice displayed a severe immune deficiency. B-cell generation was almost completely impaired in TopBP1-deficient mice. In bone marrow, there was a defect in pro- to pre-B-cell transition, especially in differentiation from Fr. A to B. We also found an increased percentage of DN and decreased percentage of DP cells in the thymus of TopBP1-deficient mice, reflecting that there was a defect in the DN to DP transition. Both the cell numbers and percentages of mature T cells were significantly reduced in the lymph node and spleen. In particular, TopBP1-deficient mice had a developmental block at the DN3 stage. The increase in the percentage of DN cells observed in the absence of *Topbp1* allele was not due to an enhanced γδ T-cell development (Supplementary Fig S11A and B). There was a comparable number of thymocytes expressing

$\gamma\delta$ TCRs in the thymi of TopBP1-deficient mice to those of control mice. It appears that $\gamma\delta$ T-cell development was not affected in this system probably because the Lck-cre transgene is turned on after the completion of $\gamma\delta$ T-cell specification. Defects in the generation of CD4 and CD8 SP thymocytes and iNKT cell were also observed in CD4-cre; TopBP1^{f/f} mice. All defects in the generation of B, T, and iNKT cells in TopBP1-deficient mice occurred at stages in which TopBP1 is highly expressed and V(D)J recombination occurs actively. We found that V(D)J recombination was indeed inefficient in TopBP1-deficient cells.

Since the expression of *Rag1* and *Rag2* was not affected by the deficiency of TopBP1, we conjectured that there may be defects in the DSB repair process. In fact, not only did we find that TopBP1 was loaded on the DSBs of the TCR V β segment, but we were also able to precipitate increased amounts of γ -H2AX products, a hallmark of unrepaired DSBs, from the same TCR V β segment in TopBP1-deficient thymocytes compared to those in control. These results indicate that the defects in V(D)J recombination were due to inefficient DSB repair. We also checked the expression of NHEJ components, since these are necessary for RAG-induced DSB repair. mRNAs for *ku70*, *ku80*, *Xrcc4*, *Ligase4* and *Dna-pk* were expressed as normal in TopBP1-deficient mice. It remains to be verified whether TopBP1 is involved in the control of the recruitment and/or the activation of the components participating in V(D)J recombination, or participates directly in the V(D)J recombination process. Lowered efficiency of the recombinatorial machinery is another possible explanation. Alternatively, TopBP1 may help the function of its binding factors such as 53BP1 and ATM since they have roles in V(D)J recombination (Cescutti *et al*, 2010).

RAG2-deficient mice also show immune deficiency due to defects in V(D)J recombination. However, they have a developmental block at a later stage than TopBP1-deficient mice. In order to examine reasons of the difference, we stained RAG2-deficient cells and TopBP1-deficient cells with Annexin V to see whether Fr. B cells undergo apoptosis. There were no apparent differences in Annexin V⁺ cells between Fr. A and Gr1⁺Mac1⁺ cells (Supplementary Fig S12). However, we found that increased Annexin V⁺ cells of TopBP1-deficient Fr. B cells when compared to RAG2 Fr. B cells. It appears that TopBP1-deficient Fr. B cells are more apoptotic because of impaired double-strand break repair than RAG2 deficient Fr. B cells which do not have double-strand breaks. However, this remains to be further elucidated.

Other defects observed at DN3 stage were reduced proliferation and increased cell death. There was a possibility that TopBP1 might regulate proliferation and cell survival directly. However, we found no correlation between the reduced proliferation, increased cell death, and TopBP1 deficiency when TopBP1-deficient cells from the thymus, lymph node, spleen, and NIH3T3 cell line were analyzed. Therefore, it seemed that the reduced proliferation and cell survival were due to the defective formation of the pre-TCR complex and subsequent lack of pre-TCR signaling. This was further confirmed when we analyzed TopBP1-deficient mice crossed to OT-II TCR transgenic mice. In the double transgenic mice, thymic cellularity was recovered and the transition from DN to DP stage was very similar to control mice. We also found that the defect in iNKT-cell development was completely restored by the expression of the V α 14 transgene. Therefore, it seems that the defects in the generation of

thymocytes and iNKT cells observed in TopBP1-deficient mice were mainly due to aberrant TCR gene assembly.

Unsuccessful DSB repair poses a serious threat to the integrity of the immune system. One example of a life-threatening immune deficiency is severe combined immunodeficiency (SCID). A lack of circulating B and T lymphocytes, due to a general DNA repair defect, accompanied by an increased sensitivity to agents causing DNA DSBs is a distinct feature of scid mice (Bosma *et al*, 1983). A-T patients and NBS patients also show immune deficiency and it would be interesting to test whether these immune deficiencies are due, at least in part, to malfunction of TopBP1 since it is downstream of ATM and a binding partner of Nbs1. Considering that the failure of V(D)J recombination results in immune deficiency in both ATM- and Nbs1-deficient mice and these defects are much more severe in mice lacking TopBP1, it seems a likely scenario.

In conclusion, TopBP1-deficient mice showed defects in the generation of B, T, and iNKT cells, primarily due to aberrant V(D)J rearrangements. TopBP1 was located at the site where V(D)J rearrangement occurs and TopBP1 deficiency resulted in an inefficient DSB repair. Finally, the early developmental blocks were rescued by introducing functional TCR $\alpha\beta$ transgenes. We therefore propose a new role of TopBP1 as a mediator of V(D)J recombination, thereby playing a critical role in the generation of an adaptive immune system.

Materials and Methods

Mice and cells

TopBP1^{f/f} mice were described previously (Jeon *et al*, 2011). Lck-cre and CD4-cre mice were purchased from Taconic. Mb1-cre mice were kind gifts from Dr. Michael Reth (University of Freiburg). RAG knockout mice were purchased from the Jackson Laboratory. All mice were bred and maintained in specific pathogen-free barrier facilities at Seoul National University and Institute of Molecular Biology and Genetics (IMBG) and were used according to protocols approved by the Institutional Animal Care and Use Committees (IACUC) of Seoul National University. NIH3T3 was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% bovine calf serum, and the Phoenix ecotropic packaging cell line (Phoenix-eco; ATCC) was maintained in DMEM supplemented with antibiotics and 10% fetal bovine serum.

PCR assay for V(D)J recombination

Genomic DNA was isolated from electronically sorted pro-B cells (B220⁺CD43⁺IgM⁻), DN3 (CD4⁻CD8⁻CD25⁺CD44⁻), DN4 (CD4⁻CD8⁻CD25⁻CD44⁻), and pre-selected (CD4⁺CD8⁺TCR β ^{int}CD69^{low}) DP cells. Gene rearrangement at *Ig*, *Tcra*, and *Tcrb* loci was measured by PCR assay as described (Wolfer *et al*, 2002).

Virus work

The packaging Phoenix-eco cells were transfected with either a control empty vector or MDH-shTopBP1 using a standard calcium phosphate protocol to generate retroviruses containing short-hairpin RNA targeting TopBP1. After 48 h, the retrovirus-containing

supernatant was harvested and used to infect NIH3T3 cells in the presence of 10 µg/ml polybrene by spin infection (2500 r.p.m., 1.5 h).

Antibodies and flow cytometry

Thymi were separated to single-cell suspensions using glass slides. $1\text{--}5 \times 10^5$ cells were used for each stain. mAbs against CD4, CD8, CD19, CD24, CD25, CD27, CD43, CD44, BP-1, NK1.1, B220 and TCRβ were purchased from BD Biosciences and eBiosciences. For RNA and immunoblot analysis, cellular subsets were sorted using FACSAriaII (BD Biosciences). FACSCantoII (BD Biosciences) was used for flow cytometry analysis. Data were analyzed with FACS Diva software and FlowJo. Annexin V staining was performed using the manufacturer's protocol (BD Biosciences).

Chromatin immunoprecipitation (ChIP)

Primary thymocytes were cross-linked with 1% formaldehyde and ChIP assay was carried out as described previously (Choi *et al*, 2012) using anti-TopBP1, anti-γ-H2AX (Upstate), and a control rabbit IgG antibody (Upstate). Input DNA and immunoprecipitated DNA were analyzed by PCR using primers specific to the Vβ region.

Transient V(D)J recombination assays

Retroviruses were generated by calcium phosphate transfection of MDH and MDH-shTopBP1 into Phoenix-eco. 2×10^5 of NIH3T3 murine fibroblast cells were seeded in 60-mm dishes and transduced with viral supernatant in the presence of 5 µg/ml polybrene. Murine RAG1 and RAG2 expression constructs (encoding full-length, untagged proteins) and recombination substrate (pJH289 and pJH290) were gifts from David B. Roth (New York University School of Medicine). Recombination substrates were transfected into either MDH-transfected NIH3T3 or MDH-shTopBP1-transfected NIH3T3 cells with murine RAG1 and RAG2 expression vectors by calcium phosphate transfection. Forty-eight hours after transfection, plasmid substrates were isolated from the cells and subjected to PCR analysis with specific primers for signal joints and coding joints (DR99–100) as described previously (Deriano *et al*, 2009).

CFSE dilution assays

DN3 cells were isolated and labeled with 5 µM CFSE (Invitrogen Life Technologies) at 10^6 cells/ml in PBS for 10 min at 37°C. CFSE-labeled DN3 cells were incubated for 7 days in the presence of OP9-DL1 stromal cells. After incubation, cells were stained with appropriate antibodies and analyzed for CFSE dilution by flow cytometry.

RNA isolation and RT-PCR

RT-PCR was performed using sorted fractions of thymocytes. Total RNA was extracted, purified, and DNaseI-treated (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using SuperScriptIII (Invitrogen). For these experiments, expression was normalized to β-actin or Cα expression.

Intracellular staining

Intracellular staining was performed with the Cytofix/Cytoperm kit (PharMingen). After incubation of mAbs against cell surface proteins, cells were gently resuspended in the Cytofix/Cytoperm solution for 20 min, washed with Cytoperm/Wash buffer and stained with TCRβ antibody.

OP9-DL-1 co-culture

Purified DN3 cells were seeded at 2×10^5 cells/well into 12-well tissue culture plates containing a subconfluent monolayer of OP9-DL1 cells. Co-cultures were performed in culture medium containing α-MEM supplemented with 20% FBS, 100 IU/ml streptomycin and penicillin, and in the presence of 5 ng/ml IL-7 (R&D Systems), 5 ng/ml Flt3L (R&D Systems), and 2.5 ng/ml stem cell factor. Cells were harvested every 3 days and transferred to a fresh confluent monolayer of OP9-DL1 cells.

Immunofluorescence microscopy

For immunofluorescence microscopy, freshly isolated thymocytes were plated onto coverslips, fixed in 4% paraformaldehyde for 5 min, and permeabilized in PBS-0.5% Triton X-100 (0.5% PBST) for 20 min at room temperature. Cells were then incubated in blocking solution (10% normal goat serum in 0.1% PBST) for 1 h at room temperature. Cells were incubated for 1 h with primary and 1 h with secondary antibodies in blocking buffer. After a final rinse, the cells were mounted on microscope slides in VECTASHIELD containing DAPI. Images were obtained from DeltaVision. Duolink *in situ* PLA analysis was performed using the manufacturer's protocol (Olink Biosciences). In brief, we prepared cytospin slides of control and TopBP1-deficient thymocytes. NBS1 was detected with primary rabbit anti-NBS1 antibody (NB100-143, Novus Biologicals) while γ-H2AX was detected using mouse anti-γ-H2AX antibody (JW301, Upstate). Subsequently, we used a Duolink *in situ* proximity ligation assay kit with corresponding secondary antibodies (PLUS-rabbit, MINUS-mouse). The fluorescence signal was detected using confocal microscopy.

Cytogenetics

Thymocytes of each genotype were treated with nocodazole for 4 h. After swelling with hypotonic solution, cells were fixed in fixation solution. Cells were dropped onto humidified glass slides. Slides were dried and stained with Giemsa solution. Individual metaphases were photographed.

Supplementary information for this article is available online: <http://emboj.embopress.org>

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Author contributions

JK designed and performed experiments, analyzed data, and wrote the paper; SKL and CJL performed experiments and analyzed data; YJ, JS, IK and HL contributed to the generation of mice; YK performed experiments and wrote the paper; SHJ, NK and SH analyzed data; RHS designed research and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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