Development of CD4+CD8+ Thymocytes in RAG-deficient Mice Through a T Cell Receptor β Chain-independent Pathway

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Summary

Antigen-binding diversity is generated by site-specific V(D)J recombination of the T cell receptor (TCR) and immunoglobulin loci in lymphocyte precursors. Coordinate expression of two structurally distinct recombinase activating genes, RAG-1 and RAG-2, is necessary for activation of site-specific V(D)J recombination. In mice bearing targeted disruptions of either the RAG-1 or RAG-2 genes, T and B lymphocyte development is arrested at the CD4+8− double negative (DN) thymocyte or B220+/CD43+ pro-B cell stage. Development of CD4+CD8+ double positive (DP) thymocytes is restored by expression of a functionally rearranged TCRβ transgene, suggesting that TCRβ expression is critical for this developmental transition. We have found that treatment of adult or newborn RAG-deficient mice with a single sublethal dose of γ-irradiation rescues the DN to DP transition in early thymocytes, and this is accompanied by a dramatic increase in thymus cellularity. In contrast to the observed induction of thymocyte maturation, there was no phenotypic or functional evidence of coincident B lymphocyte development in irradiated RAG-deficient mice. Interestingly, maturation of DP thymocytes occurred without expression of TCRβ protein in the cytoplasm or on the cell surface. These results suggest an in vivo pathway for DP thymocyte development which is TCRβ chain independent.

A unique facet of lymphocyte development is the requirement for a site-specific recombinase system to assemble the dispersed germline elements encoding the V, D, and J segments of clonally variable antigen receptors. During V(D)J recombination, the lymphocyte-specific recombinase activating genes (RAG)1 control introduction of double-strand DNA breaks (DSB) at recombination signal sequences (RSS) that flank V, D, and J gene segments (1–5). Joining of the free V(D)J coding ends requires a ubiquitously expressed DSB repair activity (6–9) encoded by the murine SCID gene (10–13). Mutation of the RAG-1, RAG-2, or SCID genes causes defective lymphocyte development and profound immunodeficiency (3, 4, 14). Examination of T and B lymphocyte precursors in these mutant mice has shown that development is arrested at the IL-2Rα−, double negative (DN) thymocyte or B220+/CD43+ pro-B cell stages (3, 4, 15, 16), respectively, which mark the onset of TCRβ (17–19) or Igμ (20, 21) rearrangements in wild-type mice. This developmental arrest occurs before the major proliferative expansion of T cell progenitors, and therefore thymus cellularity in mutant mice is dramatically reduced. Transgenic expression of a productively rearranged TCRβ gene restores both the development and expansion of double positive (DP) thymocytes in SCID (22, 23) or RAG-deficient mice (24, 25). These studies have given rise to the "β-selection" model of DP thymocyte maturation, which posits that expression of the TCRβ protein is required for maturation of DP thymocytes. A putative pre-TCR complex, containing TCRβ, gp33, and CD3e, γ, and δ, was recently identified on the surface of immature T cell lines and thymocytes (22, 25–29), and may transduce signals required for the DN to DP transition. However, TCRβ-deficient mice do develop DP thymocytes, though the numbers are greatly reduced relative to wild-type mice (24). Thus, TCRβ expression may be required for clonal expansion, but not for development of DP thymocytes. This notion is consistent with the finding that productive TCRβ rearrangements are highly selected for in proliferating, late stage DN thymocytes (19, 30). Recent studies also show that kk, a src-family protein tyrosine kinase, functions downstream of TCRβ in promoting thymocyte proliferation and TCRβ allelic exclusion during the DN to DP transition (31–36).

1 Abbreviations used in this paper: BM, bone marrow; DN, double negative; DP, double positive; DSB, double-strand DNA breaks; IRNB, irradiated newborn; RAG, recombinase activating gene.

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We have recently shown that treatment of newborn SCID mice with DNA damaging agents (low dose γ-radiation and bleomycin) induces maturation and expansion of DP thymocytes within 1 wk of treatment, but has no effect on B cell development (37). Remarkably, this treatment also rapidly restored TCRβ rearrangement and expression of cytoplasmic TCRβ protein in a high frequency of SCID thymocytes, but only 10-50% of the radiation-induced DP thymocytes expressed cytoplasmic TCRβ protein. Furthermore, some of these DP thymocytes had lost normal growth control, since 100% of irradiated newborn (IRNB) SCID mice eventually developed DP thymic lymphoma. These results were consistent with the proposed role of TCRβ in promoting clonal expansion of DP thymocytes, but we could not determine whether defective DSB repair also promoted proliferation, independently of TCRβ expression. In addition, the coincident development of DP thymocytes lacking TCRβ protein suggested that a TCRβ-independent DP maturation pathway may also exist. We sought to resolve these issues by examining the effect of γ-radiation on T and B cell maturation in RAG-deficient mice. We previously hypothesized that irradiation of newborn SCID mice induces one or more DNA repair activities that rescues coding joint formation after RSS-specific, RAG gene-mediated cleavage of TCRβ coding segments, leading to productive TCRβ rearrangement (37). Thus, we reasoned that in RAG-deficient animals which cannot initiate RSS-dependent DNA cleavage during V(D)J recombination, DNA damage-induced DSB repair would not restore TCRβ rearrangement and expression. As a result, RAG-deficient mice would allow us to determine the effect of radiation on DP thymocyte development in the absence of TCRβ expression. We report here that, as predicted, sublethal irradiation of newborn SCID mice induces one or more DNA repair activities that rescues coding joint formation after RSS-specific, RAG gene-mediated cleavage of TCRβ coding segments, leading to productive TCRβ rearrangement (37). Thus, we reasoned that in RAG-deficient animals which cannot initiate RSS-dependent DNA cleavage during V(D)J recombination, DNA damage-induced DSB repair would not restore TCRβ rearrangement and expression. As a result, RAG-deficient mice would allow us to determine the effect of radiation on DP thymocyte development in the absence of TCRβ expression. We report here that, as predicted, sublethal irradiation did not restore rearrangement of TCRβ or Igμ. Surprisingly, however, this treatment did restore the development and expansion of DP thymocytes in RAG-1- and RAG-2-deficient mice, but similar to our observations in SCID mice, had no effect on B cell development. These observations suggest that DP thymocytes can develop by a TCRβ-independent mechanism, and provide a new system for investigating molecular control of T cell development.

Materials and Methods

Mice. Animals carrying the RAG-1 (3) or RAG-2 (4) mutations were obtained from Dr. S. Tonegawa (Massachusetts Institute of Technology, Cambridge, MA), and GenPharm (Mountain View, CA), respectively, and bred at the Ontario Cancer Institute (RAG-1) or at the Hospital for Sick Children (RAG-2). C.B-17 SCID and wild-type B6 mice were bred and housed at the Hospital for Sick Children.

Flow Cytometry. Flow cytometric analyses of thymocytes (surface and intracellular) and bone marrow (BM) were carried out as previously described (37), using a FACScan® flow cytometer with Lysis II software (Becton Dickinson & Co., Mountain View, CA).

PCR Assay for IgH Rearrangements. Cell preparation, lysis, and PCR amplification of genomic DNA was performed essentially as described (38, 39). Briefly, 106 nucleated BM cells were aliquoted into PCR tubes, washed with PBS, and stored at -86°C until use. Cell pellets were lysed in PCR amplification buffer (10 mM Tris, pH 8.3, 2 mM MgCl2, 50 mM KCl, 0.45% NP40, 0.45% Tween 20, and 60 μg/ml Proteinase K). The samples were digested at 56°C for 1 h and then 90°C for 1 min, before PCR reagents (final concentrations: 200 μM dNTPs, 0.5 μM each oligonucleotide primer, and 2.5 U Taq polymerase) were added to a final volume of 100 μl. 30 cycles of PCR were performed: denaturation for 1 min at 94°C, annealing for 1.5 min at 60°C (DJH) or 1.5 min at 65°C (VDJH), and polymerization for 2 min at 72°C with an additional 3-s extension in each cycle. The resulting PCR reactions were resolved by agarose gel electrophoresis as four bands reflecting the VDJH1, VDJH2, VDJH3, and VDJH4, or the DJH1, DJH2, DJH3, and DJH4 products. The gels were transferred to nylon membranes, probed with a radiolabeled J4IN oligomer (39). The resulting hybridization was visualized by autoradiography and quantified using a phosphorImager and ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA).

Oligomers. All primer sequences are written in the 5' → 3' orientation. The VHα consensus primer is AGGTCA/CACA/CTGCAGAGAGAGCTGC-3'. The DHα consensus primer is GGGG/GGTTTTTGGAAGAGGGGTTCATCTA/ACGGTGACTGAGGTTCCTTG. This primer extends from the 5' end of the RS sequence of the DSP and DFL genes to the 3' end of the heptamer, and contains no DH coding sequences (40). The JH4-specific primer is AAAAGCCCGCACGAGGCCCATTCCTTACCC. It contains sequences in the J-C intron immediately 3' of the JH4 gene. The J4IN primer is GAGGAGGACCCCTGAGAGGCCATTCCTTACCC. It is specific for the JH4 coding sequence upstream of the JH4 primer site, but does not overlap the JH4 primer. The VHα/JH4 primer set amplifies >80% of the VH genes (Marshall, A., C. J. Paige, and G. E. Wu, unpublished observations).

Pre-B Cell Cultures. BM was removed from 750 cGy-irradiated, and untreated littermate adult RAG-1-/- and RAG-2-/- mice, as well as wild-type B6 controls, 3 wk after irradiation. An aliquot of

![Figure 1](image_url)
Control

B6 +/-

RAG-1 -/-

RAG-2 -/-

CD8

750 cGy

1 wk

2 wk

3 wk

Figure 2. Kinetics of DP thymocyte development in irradiated RAG-deficient mice. Thymocytes were obtained at different times after irradiation (750 cGy) of adult RAG-1 -/- or RAG-2 -/- mice and analyzed for CD4 and CD8 expression by multiparameter flow cytometry. Control, nonirradiated RAG-deficient and wild-type B6 adult animals were also analyzed and are shown for comparison. Two parameter contour plots (7% probability) show CD4 (y axis) versus CD8 (x axis) expression.

of these cells was stained and analyzed by flow cytometry as described above. Aliquots of BM cells (1 ml containing \(4 \times 10^6\) cells/well) were also cultured for 9 d in either IL-7 (250 U/ml), IL-7 + S17 stromal cells \((10^6\) irradiated [2,000 cGy] cells/well), or LPS \((15 \mu g/ml) + S17\) stromal cells following methods described previously (41). Cells were then harvested, counted, and stained to evaluate expression of B220, CD43, and Igk by flow cytometry as described above. In addition, cells expanded in IL-7 were analyzed for Ig gene rearrangement, as described above.

Results

Sublethal Irradiation Induces Development of DP Thymocytes in RAG-deficient Mice. For our initial experiments, we irradiated young adult RAG-1 -/- mice with 250, 500, or 750 cGy and analyzed thymocytes for CD4 versus CD8 expression 3 wk later. The results showed that few DP thymocytes were detected in mice given 250 cGy, but in those that received 500 or 750 cGy, 60-90% of the thymocytes were DP (Fig. 1). In addition, there was a 5-10-fold increase in thymocyte number in response to the latter two radiation doses (data not shown). To investigate the kinetics of DP rescue in this system, we irradiated young adult RAG-1 -/- and RAG-2 -/- mice with 750 cGy, and evaluated the number and phenotype of thymocytes 1, 2, 3, and 5 wk later. Within 2 wk of treatment, up to 70% of thymocytes were DP in both strains of mice (Fig. 2, Table 1). By 3 weeks post-

Table 1. Progressive Development and Expansion of DP Thymocytes after Irradiation of Adult or Newborn RAG-deficient Mice

| Age      | Weeks after irradiation | Mutant strain | Percent DP thymocytes ± SD | No. cells/thymus ± SD \((\times 10^6)\) | n*   |
|----------|------------------------|---------------|-----------------------------|--------------------------------|------|
| Adults   | 1                      | RAG-1         | <0.5%                       | 0.8 ± 0.7                      | 3    |
|          | 1                      | RAG-2         | <0.5%                       | 3 ± 0.8                        | 3    |
|          | 2                      | RAG-1         | 70, 26                      | 1, 0.1                         | 2    |
|          | 2                      | RAG-2         | 66 ± 5                      | 0.8 ± 0.2                      | 3    |
|          | 3                      | RAG-1         | 86 ± 5                      | 11 ± 6                         | 4    |
|          | 3                      | RAG-2         | 83 ± 3                      | 14 ± 6                         | 4    |
|          | 5                      | RAG-2         | 24 ± 29                     | 0.3 ± 0.2                      | 4    |
| Newborns | 3                      | RAG-2         | 64 ± 7                      | 60 ± 2                         | 3    |

RAG-deficient mice were treated with \(\gamma\)-irradiation (adults, 750 cGy; newborns, 400 cGy). At the indicated times after irradiation, thymocytes were counted and analyzed for expression of CD4 and CD8, as described in Fig. 1. The frequency of DP recovered from RAG-2 -/- mice at 5 wk after irradiation was highly variable, and ranged from 0 to 64%. Thymocyte recovery from nonirradiated control mice ranged from 0.5-3 \(\times 10^6\) cells/thymus (RAG-1 -/-) to 2-7 \(\times 10^6\) cells/thymus (RAG-2 -/-).

* Number of animals analyzed.
irradiation, thymocytes were >80% DP, and total thymus cellularity had increased 10-15-fold as compared with nontreated littermate controls (Table 1). An even more dramatic (30-60-fold) expansion in thymocyte cellularity was observed 3 wk after RAG-2-/- mice were irradiated at birth with 400 cGy, and >60% of the cells were DP (Table 1). However, by 5 wk after irradiation of young adult animals, thymus cellularity had returned to normal, but widely variable numbers of DP cells were still detected (Table 1). Mature CD4⁺ or CD8⁺ single positive (SP) thymocytes were not observed at any time point examined. These observations document a radiation dose-dependent rescue of DP development and expansion in RAG-deficient mice. However, in contrast to our observations in IRNB-SCID mice, there appeared to be a single wave of DP thymocyte development, and no thymic lymphomas have been observed to date (10 wk after irradiation).

**Development of DP Thymocytes in Irradiated RAG-deficient Mice**

Figure 3. Development of DP thymocytes in irradiated RAG-2-/- mice is TCRβ independent. (A) Thymocytes were obtained at different times after irradiation (750 cGy) of adult RAG-2-/- mice and analyzed for cell size (FSC), and expression of IL-2RIα and TCRβ by multiparameter flow cytometry. The percentage of DP thymocytes was >80% in these animals (Fig. 2). Shaded histograms in IL-2RIα and TCRβ plots represent staining profiles with isotype-matched control antibodies. Control, nonirradiated RAG-2-/- and wild-type B6 adult animals are shown for comparison. (B) Thymocytes obtained from adult irradiated RAG-2-/- mice (3 wk after irradiation) were also analyzed for expression of cytoplasmic TCRβ and CD4, as described (17). Both a young adult B6 mouse and a 2-wk-old IRNB-SCID mouse are shown for comparison.
Figure 4. Irradiation of RAG-deficient mice fails to promote B cell development in vivo or in vitro. BM was obtained from both RAG-1-/- and RAG-2-/- mice at 3 wk after irradiation (750 cGy). (A) An aliquot from each animal was analyzed immediately by multiparameter flow cytometry for surface B220 versus CD43 expression. The staining profiles of control, nonirradiated RAG-deficient and wild-type B6 mice are also shown. (B) BM cells from each group were also analyzed for expression of B220, CD43, and surface Igµ after culture for 9 d in IL-7. The top row shows staining of cultured B6 cells compared with isotype-matched control antibodies (light histograms). The middle and bottom rows show staining of each antibody on cultured cells derived from control (dark histograms) and irradiated (light histograms) RAG-1-/- and RAG-2-/- mice. For both (A) and (B), three animals were analyzed per group. Since there were no significant differences between replicates, a single animal per group is shown.
Mice Is TCRβ Independent. In wild-type mice, the DN to DP developmental transition is accompanied by downregulation of the IL-2Rα chain, surface expression of a TCRβ/CD3 (TβR) complex, and a reduction in cell size corresponding to exit from the cell cycle. The development of DP thymocytes in irradiated RAG-2-/- mice appeared normal in that it was accompanied by loss of IL-2Rα and a decrease in cell size (Fig. 3 A). However, in striking contrast to wild-type mice, progression to the DP, IL-2Rα- phenotype in irradiated RAG-deficient mice was not accompanied by detectable TβR expression. We have previously shown that irradiation of newborn SCID mice also restored development of small IL-2Rα- DP thymocytes that express cytoplasmic TCRβ (dTβR), but not TβR (37). In contrast to IRNB-SCID mice, we could not detect TβR in the CD4+ thymocytes of irradiated RAG-2-/- mice (Fig. 3 B). Correspondingly, we were unable to detect TβR rearrangement by Southern blot analysis, or to detect TCRβ transcripts by RT-PCR (data not shown). Together, these data suggest that the transition from cycling, IL-2Rα+ DN progenitors, to small, IL-2Rα- DP thymocytes in RAG-deficient mice occurred in the absence of TCRβ expression.

Irradiation Fails To Promote B Cell Development in RAG-deficient Mice. Many aspects of the DN to DP thymocyte transition are paralleled during the pro-B to pre-B transition in the BM. B cell development in RAG-deficient mice is arrested at the B220+, CD43+, large pro-B cell stage, owing to blockade of Igμ rearrangement (4). To determine whether irradiation rescued this transition, we evaluated surface expression of these markers in the BM of control and irradiated (750 cGy) RAG-1-/- and RAG-2-/- mice (Fig. 4 A). In contrast to thymocyte development, radiation did not induce phenotypic progression to the B220+, CD43- pre-B cell stage found in wild-type mice. However, BM from irradiated RAG-deficient mice might contain small numbers of developmentally competent B cell progenitors not revealed by ex vivo phenotypic analysis. Therefore, we cultured BM isolated from RAG-1-/- and RAG-2-/- animals (3 wk after irradiation) in well-defined in vitro conditions previously shown to promote the development of B cell progenitors to the stage of Ig expression (42, 43). Fig. 4 B shows the results of expansion for 9 d in the presence of IL-7. As expected, normal B6 BM contained progenitors that proliferated in response to IL-7, yielding a majority population of slgμ+ B220+, CD43+ cells, as well as a few slgμ+, B220+ cells. Both control and irradiated RAG-1-/- and RAG-2-/- mice also contained progenitors that responded to IL-7. However, consistent with the analysis of the BM cells at the initiation of culture, control and irradiated B cell progenitors were indistinguishable from each other based on the cell surface markers used. Similar results were obtained from cultures containing splenic cells, although, as expected, B cell progenitors represented relatively fewer of the total cells recovered, because myeloid progenitors also expand in these cultures (data not shown). B cells that had matured to the stage of LPS responsiveness were only found in control B6 cultures (data not shown).

To examine V(D)J recombination at the Igμ locus, a PCR assay was employed to detect rearranged DJμ and VDJμ structures in the BM of B6, control, or irradiated RAG-1-/- and RAG-2-/- mice (Fig. 5). Although products representing both DJμ (Fig. 5 A, lane 2) and VDJμ (Fig. 5 A, lane 9) rearrangements were clearly observed in B6 controls, BM DNA from RAG-1-/- (Fig. 5 A, lanes 3, 4, 10, and 11) and RAG-2-/- (Fig. 5 A, lanes 5, 6, 12, and 13) mice showed no evidence of either type of rearrangement, with or without radiation treatment. Moreover, VDJμ rearrangements were not detected in BM from irradiated RAG-deficient animals after expansion for 9 d in IL-7 (Fig. 5 B). Thus, in contrast to the observed promotion of thymocyte development in irradiated RAG-deficient mice, we could not find evidence of IgH locus recombination, or phenotypic transition from the pro-B to pre-B cell stage either in vivo, or following in vitro BM culture.

Discussion

Our experiments have revealed a novel pathway of radiation-induced development of DP thymocytes from DN precursors in RAG-deficient mice that is independent of TCRβ expression. Strikingly, the effect of radiation in RAG-deficient mice was specific to the T cell lineage, as no corresponding promotion of B cell development was observed, either by analyses of cell surface phenotype or Ig gene rearrangement. Similar analyses carried out after expansion of B cell progenitors in IL-7 confirmed this conclusion. We have previously found that radiation-induced lymphoid development in SCID mutant mice was also T-lineage specific (37). It is unlikely that the absence of radiation-induced B cell development results from a paucity of precursors in these mutant mouse strains, since both RAG-deficient and SCID BM populations contain detectable populations of cycling B220+, CD43+ pro-B cells (4, 16).

Our previous studies of IRNB-SCID mice showed that development and expansion of DP thymocytes occurred concomitantly with the restoration of normal V(D)J recombination at the TCRβ locus, but not all DP thymocytes expressed TCRβ protein (37). We report here that positive selection into the DP stage occurred in irradiated RAG-deficient mice in the complete absence of TCRβ. We envision two mechanisms by this could occur. First, radiation could alter the thymic microenvironment. This idea is consistent with reports that DP thymocyte development can be induced in SCID mice by a number of experimental maneuvers which alter the thymic microenvironment, but have no obvious effect on TCRβ expression. These maneuvers include adoptive transfer of wild-type BM cells (44, 45) and treatment with anti-asialo GM1 antibody (46). The T lineage-specific maturation reported here could also be consistent with a differential effect of radiation on the BM and thymic microenvironments. Second, radiation could activate downstream components of the TCRβ pathway, or activate an alternative, perhaps aberrant, pathway. For example, radiation could induce somatic mutation of genes that control DP thymocyte development and proliferation. We consider
this to be unlikely, given that mutations resulting from a single sublethal dose of irradiation would likely be random, not T cell specific and low frequency, in RAG-deficient mice that have wild-type DSB repair. Moreover, we have no evidence that DP proliferation is uncontrolled, since thymus cellularity had declined to normal RAG-deficient levels by 5 wk (Table 1), and we have not observed the pre-neoplastic thymocyte expansion (data not shown) always observed in IRNB-SCID mice (37). Thus, the loss of growth control in IRNB-SCID DP thymocytes is attributable to the SCID DSB repair defect, which may enhance the generation of chromosomal aberrations in proliferating thymocytes, contributing to the invariant development of thymic lymphoma.

Uncoupling of TCRβ expression and maturation of DP thymocytes has also recently been observed by experimental manipulation of intrinsic thymocyte processes. For example,
treatment of SCID, RAG-1−/−, or TCRβ-deficient thymocytes with anti-CD3ε antibody in organ culture induces development and expansion of IL-2Rα− DP thymocytes (47, 48). These data suggest that signals generated through CD3ε may substitute for TCRβ in the DN to DP transition, and potentially explain why CD3ε engagement bypasses the requirement for TCRβ expression at this stage. A second line of investigation has shown that TCRβ-independent development of DP thymocytes can also be triggered by thymocyte expression of a constitutively activated kK transgene in RAG-1−/− mice (34). Clearly, it will be important to determine whether radiation induces DP thymocyte maturation in SCID and RAG-deficient mice indirectly, by altering the thymic microenvironment, or whether radiation activates a thymocyte-intrinsic developmental program, possibly involving an kK-dependent pathway.

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