Brief Definitive Report

Sublethal γ-Radiation Induces Differentiation of CD4⁻/CD8⁻ into CD4⁺/CD8⁺ Thymocytes without T Cell Receptor β Rearrangement in Recombinase Activation Gene 2−/− Mice

By Juan Carlos Zúñiga-Pflücker, Di Jiang, Pamela L. Schwartzberg, and Michael J. Lenardo

From the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892

Summary

DNA recombination of the immunoglobulin (Ig) or T cell receptor (TCR) gene loci is an essential step in the production of lymphocytes bearing antigen-specific receptors. Mice that lack the ability to rearrange their Ig and TCR gene loci are devoid of mature B and T cells. Complete rearrangement and expression of the TCR-β chain has been suggested to allow immature thymocytes to switch from the CD4⁻/CD8⁻ to the CD4⁺/CD8⁺ stage of thymic development. Thus, thymocytes from severe combined immune deficient (SCID) mice or mice deficient in recombinase activation genes (RAG), which do not undergo proper DNA rearrangement, are arrested at the early CD4⁻/CD8⁻ stage of development. B cell precursors in SCID or RAG mice do not progress from the B220⁺/sIgM⁻/heat stable antigen (HSA)⁺/CD43⁺ to the B220⁺/sIgM⁻/HSA⁺/CD43⁻ stage. In an attempt to reconstitute RAG-2−/− mice with bone marrow- or fetal liver-derived progenitor cells, we subjected these mice to sublethal doses of γ-radiation. It is surprising that in the absence of donor cells, irradiated RAG-2−/− mice revealed a dramatic change in their lymphoid phenotype. 14 d after irradiation, the majority of thymocytes had advanced to the CD4⁺/CD8⁺ stage of T cell development and a small number of bone marrow precursors had progressed to the CD43⁻, HSA^{hi} stage of B cell development. Analysis of the resulting CD4⁺/CD8⁺ thymocytes revealed no surface expression of the TCR/CD3 complex and no V-D-J rearrangement of the TCR-β gene locus. Our findings provide evidence for a novel pathway that allows the transition of thymocytes from the CD4⁻/CD8⁻ to the CD4⁺/CD8⁺ stage and that does not appear to require TCR-β chain rearrangement.

The ability of B and T lymphocytes to rearrange their respective Ig and TCR gene loci creates the diversity required for adaptive immune responses to foreign antigens. SCID mice and RAG-deficient mice, which cannot undergo proper DNA rearrangement, display a profound absence of B and T lymphocytes, and a loss of acquired immune function (1–4). These mice show an arrest in B and T cell development corresponding to the stage before that of normal DNA rearrangement. The immature B (B220⁺/sIgM⁻/heat stable antigen [HSA]⁺/CD43⁺) and T (CD4⁻/CD8⁻/IL-2R⁺) cells in such mice cannot further differentiate because of a lack of expression of properly rearranged Ig or TCR gene products (2, 4). In fact, this requirement has been validated in the T lineage by experiments that show that thymocytes either from TCR-α−/− mice or RAG−/− mice which carry a TCR-β chain transgene can continue differentiating up to the CD4⁺/CD8⁺ stage (5, 6). These findings suggest that successful TCR-β chain rearrangement and expression trigger a pathway for the expression of CD4 and CD8, which has been termed "β-selection" (7–9). It was postulated that cells which fail to produce a β chain, i.e., "unselected" immature T cells, are eliminated. In an effort to use RAG-2−/− mice as hosts for thymic reconstitution, we were surprised to discover a striking effect of sublethal γ-irradiation on thymocyte maturation. We found that sublethal doses of γ-radiation allowed immature CD4⁻/CD8⁻/IL-2R⁺ cells to differentiate into CD4⁺/CD8⁺/IL-2R⁻ thymocytes in the absence of any detectable TCR-β gene loci rearrangement. Thus, γ-radiation appears to potently induce the maturation of CD4⁻/CD8⁻ cells into CD4⁺/CD8⁺ thymocytes, and circumvent the normal process of β-selection.

Materials and Methods

Mice. RAG-2−/− mice were obtained from Dr. Fred Alt (Howard Hughes Medical Institute, Children's Hospital, Boston, MA) (4) and bred in our animal facility. All other mice were pur-

chased from the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). Irradiation of mice was performed as previously described (10).

Flow Cytometry. Antibodies for flow cytometry were purchased from Pharmingen (San Diego, CA). Staining of cells was performed as previously described (10).

RNA Analysis. Total RNA form thymocytes was prepared with RNAzol (Tel-Test Inc., Friendswood, TX) as indicated by the manufacturer. Northern analysis was performed using standard techniques (11). Reverse transcriptase and PCR (RT-PCR) analysis of Vβ rearrangement and expression was performed as previously described (11). Briefly, RT reaction was performed with isolated RNA using random hexamers or gene-specific primers (12). The first strand cDNA product was amplified by PCR using Vβ-specific primers and a consensus Cβ primer (12). The PCR product was run on a 6% acrylamide gel, electroblotted, and probed with an internal consensus Cβ oligonucleotide (12). The PCR product corresponds to the expression of mRNA from loci bearing complete V-D-J rearrangement.

Results and Discussion

RAG-2−/− mice were subjected to sublethal γ-radiation and thymocytes were analyzed at different times after treatment by flow cytometry. The "normal" thymic phenotype of RAG-2−/− mice resembles that of immature day 15 fetal thymus, a stage in thymic development before the onset of V-D-J TCR-α/β rearrangement in which most cells are $CD3^-/CD4^-/CD8^-$ (4). As shown in Fig. 1, *a*–e, almost all RAG-2−/− thymocytes express high levels of IL-2Rα, HSA, and MHC class I, and fail to express the CD3, CD4, and CD8 surface molecules as compared to wild-type (C57BL/6) thymocytes. The RAG-2−/− thymus has 10–100-fold fewer cells than a wild-type thymus (4). 2 d after irradiation with a sublethal dose of 750 rad (7.5 Gy), RAG-2−/− mice show a further 5–10-fold decrease in thymic cellularity, however their numbers recover to preirradiation levels within 6–8 d (data not shown). Because of the phenotype of RAG-

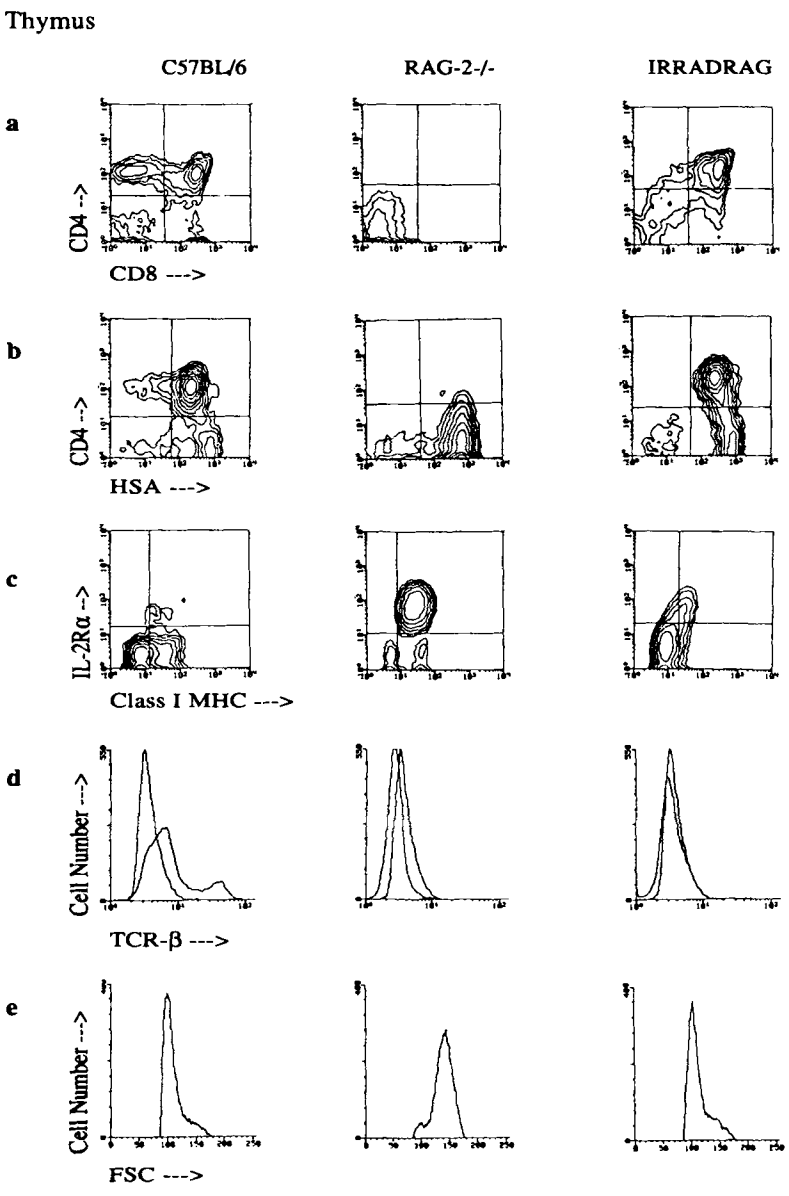

Figure 1. Flow cytometry analysis of radiation-induced differentiation in RAG-2−/− thymocytes. Thymocytes from C57BL/6 mice (*left*), RAG-2−/− mice (*center*), or day 14 irradiated RAG-2−/− mice (*right*, *IRRADRAG*) were stained for the expression of (*a*) CD4 vs. CD8, (*b*) CD4 vs. HSA, (*c*) IL-2R vs. class I-MHC (K^b), (*d*) TCR-Cβ, and (*e*) forward size scatter. Irrelevant antibody and specific anti-TCR-Cβ staining are shown (*d*).

2−/− thymocytes, the predominant cells eliminated by the radiation treatment are the immature $CD4^-/CD8^-/IL\text{-}2R^+$ thymocytes. At 14 d after irradiation, a dramatic change in the RAG-2−/− thymic phenotype becomes apparent (Fig. 1, *a–e*). Thymocytes from irradiated RAG-2−/− mice appear to have advanced to the next step in thymic development, from the $CD4^-/CD8^-$ (double negative [DN]) stage to the $CD4^+/CD8^+$ (double positive [DP]) stage.

Further analysis showed that the switch in thymic phenotype is not simply aberrant expression of CD4 and CD8 molecules induced by radiation. Rather, a shift in several aspects of their developmental status was observed (Fig. 1, *a–e*). The irradiated thymocytes have changed their size (forward scatter [FSC]), from large DN to small DP thymocytes. Also, the irradiated thymocytes showed a switch from the MHC class I^{hi} and $IL\text{-}2R^+$ stage to the MHC class I^{lo} and $IL\text{-}2R^-$ stage, which is characteristic of maturation to the DP stage. Among the irradiated thymocytes, a few cells appear to be $CD4^+/CD8^-$ and $CD4^-/CD8^+$ (single positive [SP]) thymocytes, suggesting the appearance of mature T cells. Flow cytometry analysis of these SP cells reveals that they are HSA^{hi} and MHC class I^{lo}, and do not express TCR/CD3 complex on their surface (Fig. 1 *d*, and data not shown). Therefore, these cells are not mature SP thymocytes, but may represent intermediates between the DN and DP stage that begin to preferentially express either CD4 or CD8 (7).

Recent reports (5–8) have suggested that the differentiative shift of thymocytes from the DN to the DP stage normally requires the functional rearrangement of the TCR-β chain. This requirement has been called β-selection (7–9). We were intrigued that irradiation appeared to promote an event that was proposed to require TCR-β rearrangement in mice that lack the ability to rearrange their TCR gene loci by virtue of the absence of a functional RAG-2 gene (4). Either pathways which do not involve TCR-β rearrangement permit the DN to DP shift, or RAG-2−/− mice can execute TCR-β rearrangement upon exposure to sublethal γ-radiation. For example, DNA repair genes with recombinatorial properties which become upregulated by radiation-induced DNA damage may allow for cryptic rearrangements to take place (13–15). However, flow cytometry analysis from thymocytes of irradiated RAG-2−/− mice did not show any significant expression of TCR-β or CD3 complex (Fig. 1 *d*). Moreover, Northern analysis of thymus RNA revealed only the expression of a 1.0-kb TCR-β message from normal and irradiated RAG-2−/− mice (data not shown). The 1.0-kb message has been reported to correspond to a germline-sterile transcript or to an incomplete D-J rearrangement of the TCR-β loci, which appears early in T cell development and which may precede full V-D-J rearrangement (16–18).

Furthermore, to rigorously investigate the possibility of transcripts from rearranged TCR loci, we carried out a sensitive RT-PCR assay with 5′ Vβ-specific primers and 3′ Cβ primers for 20 Vβ gene families (12). For each Vβ gene family we detected a fully rearranged V-D-J TCR-β chain within the thymus of control C57BL/6 mice but not in RAG-2−/− mice, whether or not they were irradiated (Fig. 2). The absence of fully rearranged TCR-β message in thymocytes from

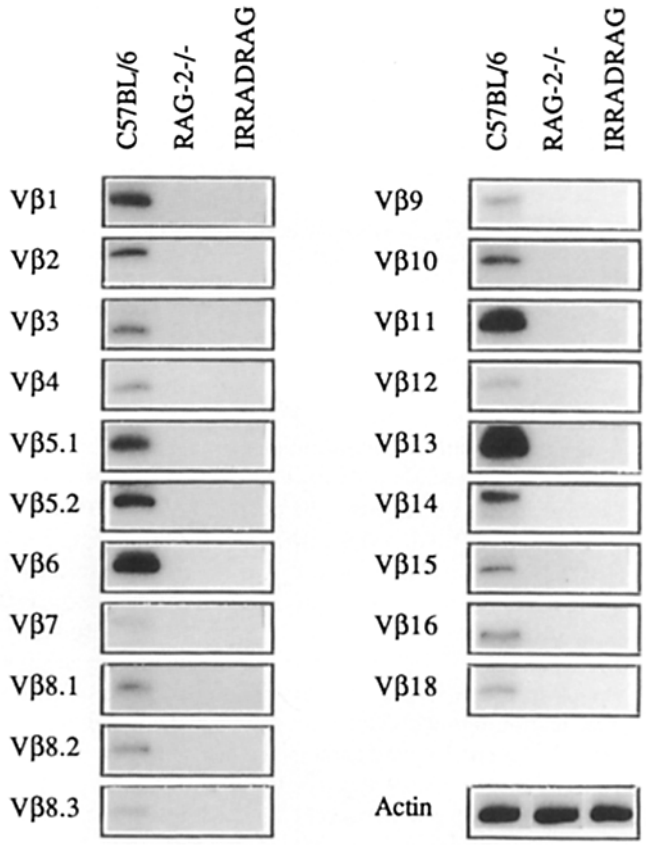

Figure 2. Irradiated RAG-2−/− thymocytes show no evidence of TCR rearrangement. RT-PCR analysis of TCR-β rearrangement of RNA from C57BL/6 mice (*left*), RAG-2−/− mice (*center*), or day 14 irradiated RAG-2−/− mice (*right, IRRADRAG*). RNA was copied into first strand cDNA using a Cβ gene–specific primer and reverse transcriptase (12). The cDNA was amplified with Vβ-specific 5′ primers and Cβ-specific 3′ primers (12). The gel was blotted and probed with an internal consensus Cβ probe (12). Each Vβ-specific PCR-amplified gene product is indicated above. The β-actin RT reaction was carried out with random hexamer primers.

irradiated RAG-2−/− mice is consistent with the requirement for RAG-2 gene expression to initiate TCR rearrangement. Nonetheless, γ-radiation appears to allow maturation of thymocytes from DN to DP in the absence of the normal process of β-selection.

In addition to the striking changes in thymic phenotype, certain features in the kinetics of appearance of DP cells merit consideration. The emergence of DP cells after a 2-wk delay suggests that the cells we are observing are derived from intrathymic radio-resistant thymocytes that can now repopulate the thymus and progress to a further stage of development (10, 19). Thus, this posed dual questions: why are irradiated cells allowed to proliferate and why do they exhibit a new differentiation potential? We speculate that these early irradiated thymocytes may have undergone low frequency somatic mutations that now enable their proliferation and differentiation. One possibility is that a mutation could be occurring in p53 or a related gene, which may participate

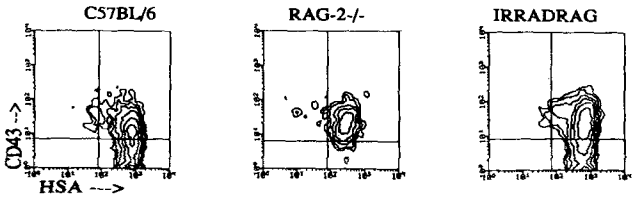

Figure 3. Flow cytometry analysis of radiation-induced differentiation in RAG-2−/− bone marrow. B220+ bone marrow cells from C57BL/6 (*left*), RAG-2−/− mice (*center*), or day 14 irradiated RAG-2−/− mice (*right, IRRADRAG*) were stained for the expression of CD43 (S7) vs. HSA. Bone marrow cells were triple stained for B220, CD43, and HSA. B220+ cells were gated and expression of CD43 vs. HSA is shown.

in checkpoints during thymic differentiation as well as during radiation-induced DNA damage (20, 21). In the absence of β-selection survival signals, the normal endpoint in the life of RAG-2−/− thymocytes is the DN/IL-2R+ stage. However, it is clear that irradiated RAG-2−/− cells are capable of progressing to the DP stage. This may be accompanied by their inability to respond to the appropriate death signals that DN cells normally receive in the absence of β-selection. Therefore, checkpoint genes may themselves become targets of irradiation-induced damage, causing a loss of developmental control. The p53 gene contains several regions that are hot spots for mutagenesis that can be mutated by γ-radiation (21). Such loss of checkpoint control has been implicated as a major mechanism in oncogenesis (20–21).

The possibility that bone marrow–derived thymic and B cell precursors were also affected was investigated. As with the thymus, the bone marrow undergoes a large decrease in cellularity a few days after irradiation (data not shown). Development of B cells in RAG-2−/− mice is arrested at a B220+/sIgM−/HSA+ stage, which is marked by the expression of CD43 (4, 22). Analysis of bone marrow of RAG-2−/− mice at day 14 after irradiation showed that a small number of B220+ pre-B cells had lost CD43 and had increased HSA expression, indicating a progression to the next step in B cell differentiation (Fig. 3). Although, this shift is small, it is consistent with the notion that irradiation may induce genetic mutations that also allow pre-B cells to survive to the next stage in development (22). The low frequency of events may reflect the differences in the target tissue that give rise to B and T cells. Analysis of peripheral lymphocytes up to 20 d after radiation did not reveal the presence of either mature B or T cells (data not shown). Although partial differentiation of thymocytes and, to a lesser degree, bone marrow cells is promoted by irradiation, the lack of mature peripheral lymphocytes is no doubt due to the inability of radiation to reconstitute the recombinatorial assembly of antigen receptor genes. Thus, it appears that DNA-repair genes, although capable of recombinatorial activity (13–15, 23), do not compensate for the lack of RAG-2 function. Moreover, the genetic alterations that we hypothesize may account for a partial lymphoid maturation and do not appear to act as second-site suppressors to deficiencies in the RAG-2 gene.

Taken together, our findings provide important evidence for a unique phenotypic effect of γ-radiation in RAG-2–deficient mice. Our data also provide evidence for alternative pathways to β-selection that can be involved in switching thymocytes from the DN to DP stage during intrathymic differentiation. The ability of the alternate pathway to permit the differentiation of DN into DP thymocytes, suggests that the function of the β-selection event may be to promote the survival of DN thymocytes that then allows further thymic differentiation to proceed unimpeded.

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Address correspondence to Dr. J. C. Zúñiga-Pflücker, Department of Immunology, University of Toronto, Medical Sciences Building, Toronto, Ontario, M5S 1A8, Canada.

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