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*J Immunol* 2019; 202:1317-1320; ;
http://www.jimmunol.org/content/202/5/1317.citation

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Restoration of T Cell Development in RAG-2-Deficient Mice by Functional TCR Transgenes

Yoichi Shinkai, Shigeo Koyasu, Kei-ichi Nakayama, Kenneth M. Murphy, Dennis Y. Loh, Ellis L. Reinherz, Frederick W. Alt*

Introduction of TCRα transgene, TCRβ transgene, or both into RAG-2−/− mice differentially rescues T cell development. RAG-2−/− mice have small numbers of TCR CD4−CD8− (double negative, DN) thymocytes that express CD3γδ and ζ proteins intracellularly. Introduction of a TCRβ transgene, but not a TCRα transgene, into the RAG-2−/− background restored normal numbers of thymocytes. These cells were CD4+CD8+ (double positive, DP) and expressed small amounts of surface TCRβ chain dimers in association with CD3γδ but not ζ. RAG-2−/− mice that expressed α and β TCR transgenes developed both DP and single positive thymocytes. Thus, the TCRβ subunit, possibly in association with a novel CD3 complex, participates in the DN to the DP transition.

Genes that encode the variable regions of immunoglobulin (Ig) and T cell receptor (TCR) molecules are assembled during the early stages of T and B cell development by a highly complex recombination process referred to as V(D)J (V, variable; D, diversity; J, joining) recombination (1). The complete antigen receptor complex comprises either Ig (on B cells) or TCR (on T cells) chains that bind antigen in association with additional subunits that have been implicated in signal transduction (2, 3). For the αβ TCR, this complex consists of a disulfide-linked αβ heterodimer that is noncovalently associated with invariant CD3 chains (γ, δ, ε, and ζ, with or without η). Expression of TCRαβ heterodimers on the cell surface requires the associated CD3 components (4).

Immature T lineage cells progress from a CD4−CD8− double negative (DN) stage to a CD4+CD8− double positive (DP) stage and, subsequently, to the mature CD4+ or CD8+ single positive (SP) stage (5). Expression of complete αβ TCR and its recognition of self major histocompatibility complex (MHC) molecules is involved in the latter stages of T cell development, in particular for mediating the transition from the DP to the SP stage through positive or negative selection in a ligand-dependent manner (6, 7). Assembly and expression of TCRβ chain gene generally occurs first and may be involved in effecting allelic exclusion at the TCRβ locus while leading to TCRα chain gene assembly or expression (or both) (8). Furthermore, introduction of a functionally rearranged TCRβ transgene into the severe combined immunodeficient (scid) background partially rescues T cell development from the DN to the DP stage (9). Thus, the β chain may be important at early stages of T cell development, independent of its recognition of ligand when it is associated with TCRα chains in the mature receptor.

Mice that lack mature B and T cells have been created by targeted disruption of either the recombination activating gene−1 (RAG-1) or RAG-2 gene (10, 11). The only known defect in RAG-deficient animals is the inability to initiate V(D)J rearrangement. To determine the effect of productively rearranged TCR genes on T cell development in RAG-2−/− mice, we introduced TCRα, TCRβ, or both TCR transgenes into the RAG-2−/− background (12). The TCRα and TCRβ transgenes encode, when expressed together, a chicken ovalbumin–specific, MHC class II(i-Aκ)–restricted TCR (13). Thymuses of RAG-2−/− mice contained, on average, 3.3 × 106 thymocytes, all of which were DN in phenotype and lacked detectable surface CD3 expression (11) (Fig. 1). In TCRα transgenic, RAG-2−/− mice (TCRα+ RAG-2−/−), the thymus was essentially indistinguishable from that of the RAG-2−/− mice (Fig. 1). These mice yielded an average of 7 × 106 thymocytes. In contrast, introduction of a productive TCRβ transgene into the RAG-2−/− background (TCRβ+ RAG-2−/−) restored the thymocyte number (4.5 × 106) to that of normal wild type or RAG-2−/− heterozygotes (3.2 × 106). More than 95% of these thymocytes were DP (Fig. 1) and weakly, but clearly, expressed TCRβ and TCRα.
CD3ε chains on their surface (as compared to RAG-2/−/− thymocytes, Fig. 2, A to C). These reactivities, as defined by hamster monoclonal antibodies (MAbs) H57 (anti-CD8) and 500A2 (anti-CD3ε), respectively, are specific because the staining was brighter than that obtained using 3A10, a hamster MAb to mouse Cβ. TCRβ and CD3ε expression were one order of magnitude less than on the DP TCRβε subset of normal mice as judged by quantitative immunofluorescence. In addition, there were no detectable SP thymocytes (Fig. 1) or peripheral DP T cells in the TCRβε,RAG-2/−/− mice.

Reconstitution of RAG-2/−/− mice with both the TCRα and β transgenes led to essentially complete rescue of all stages of T cell development; both the DP population and a significant population of CD4 SP cells were detectable in the thymus (Fig. 1). Because the used transgenic receptor is restricted to I-Aβ (13), CD4 SP but not CD8 SP mature thymocytes were positively selected in the presence of that MHC element (Fig. 1). We also detected CD4 SP T cells in spleen from the TCRβε transgenic RAG-2/−/− mice; however, no B cells were detected in any of the TCRγδ TCRβε,RAG-2/−/− mice analyzed. As expected, the RAG-2/−/− heterozygous animals had a normal thymic phenotype in terms of DP and SP subpopulations (Fig. 1).

To examine the structure of the surface TCR complex on thymocytes of RAG-2/−/− mice, TCRβ transgenic mice and TCRβε,RAG-2/−/− mice were labeled with [3H]thymidine and [35S]cysteine. Cells were lysed with digitonin to preserve any weak associations between TCR and CD3 subunits, and TCR complexes were immunoprecipitated with MAbs 3A10, H57, 2C11 (anti-CD3ε), or a mixture of 14A1 (anti-CD3γ) and 14F2 (anti-CD3δ) (designated anti-CD3γδ). Proteins were analyzed by twodimensional nondenaturing and reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3). As expected, neither anti-CD8 (Fig. 3, panel a) nor anti-CD3 (Fig. 3A, panel b) precipitated TCR heterodimers (Fig. 3A, panel b) but not CD3δ is readily detected in H57 immunoprecipitates (Fig. 3C, panel b). Anti-CD3ε immunoprecipitated the same off-diagonal protein of 40 kD as anti-CD8, as well as CD3δε (Fig. 3C, panel c), indicating that the putative TCRβε, dimers associate with CD3δε immunoprecipitated with anti-CD3ε. The absence of CD3γ in the complex is not due to the lack of expression of CD3γ because the specific anti-CD3 γ MAbs immunoprecipitated the CD3δε, dimer (Fig. 3, panel d). These results indicate that CD3γ is not detectably associated with RAG-2/−/− thymocytes.

Expression of transgenic TCRβ chains, but not TCRα chains, in RAG-2/−/− mice permits the DN thymocytes to transit to the DP stage and restores normal thymic cellularity. This finding concurs with the observation that introduction of a TCRβ transgene into SCID mice leads to the generation of some DP cells (9). However, unlike TCRβε,RAG-2/−/− mice, the number of thymocytes in TCRβ transgenic SCID mice remained low (about 2 x 105) and a substantial proportion of these thymocytes remained DN (6). The scid defect allows initiation of V(D)J recombination but impairs coding join formation and may, thereby, predispose developing lymphocytes to a high frequency of lethal double-strand breaks (14). In addition, the scid defect also affects double-strand DNA break repair in both lymphoid and nonlymphoid cells (15). Thus, the apparently inefficient maturation of TCR transgenic SCID thymocytes may result from stimulation of endogenous V(D)J recombination events leading to cell death or by unknown pleotropic effects of the scid mutation. In agreement with the conclusion that the inability to initiate V(D)J rearrangement in develop-

**Fig. 2.** Surface expression of TCRβ chain and CD3ε on TCRβε transgenic RAG-2/−/− mice. Thymocytes from RAG-2/−/− mice and TCRβ transgenic RAG-2/−/− mice (TCRβε,RAG-2/−/−) were examined for (A) TCRβ chain, (B) CD3ε, and (C) TCRβ chain expression on their cell surface. The cells were stained with biotinylated MAbs to TCRβε (H57) (24), CD3ε (500A2) (25), and TCRβε (3A10) (26), followed by peroxidase. The control in (A) represents the fluorescence intensity of the TCRβε,RAG-2/−/− thymocytes without biotinylated antibody staining. The 3A10 staining in (C) was indistinguishable from the control staining in (A).
opposing lymphocytes is the only defect in RAG-2-deficient mice, both early and later stages of T cell development can be totally rescued by the introduction of rearranged TCR transgenes into this background.

CD3 subunits are present in the thymocytes of RAG-2<sup>−/−</sup> mice. Thus, it is likely that CD3 subunits are expressed intracellularly in early thymocyte development before V(D)J recombination and are transported to the cell surface after assembly and expression of functional TCR chains. We detect a TCRβ-CD3γδε complex on the surface of TCRβ<sup>+</sup>,RAG-2<sup>−/−</sup> thymocytes.

Because we also detect CD3ζ chains in these cells, its absence from their surface TCRβ complex cannot be attributed to the relative instability of this CD3 component (16). Therefore, ability to express the surface TCRβ complex may be related to lack of association of CD3ζ. TCRβ dimers without CD3ζ are present on immature SCID thymocyte cell lines reconstituted with a rearranged TCRβ chain gene (17), but were not detected on mature T cells or T cell lines that do not have a TCRα chain. In addition, expression of surface TCRβ chains was not detected in DP thymocytes that accumulate in mice homozygous for a mutation that blocks assembly of functional TCRα chains (18). It is conceivable that endogenous TCRβ chains may have been expressed on the surface of these cells but at levels lower than those of our TCRβ transgenes and which were not readily detectable. However, these molecules need to be detected on the surface of normal thymocytes.

We do not yet know why the TCRβ complex was not expressed on the surface of thymocytes that are more mature. One possibility is that additional cellular compo-

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**Fig. 3.** Biochemical analysis of the TCR complex in TCRβ transgenic RAG-2<sup>−/−</sup> thymocytes. Thymocytes from (A) RAG-2<sup>−/−</sup> (4 × 10<sup>6</sup>), (B) TCRβ transgenic (1 × 10<sup>6</sup>), and (C) TCRβ<sup>+</sup>,RAG-2<sup>−/−</sup> (1 × 10<sup>6</sup>) mice were labeled with trans-[^35]S-label for 4 hours and then incubated for 1 hour with unlabeled amino acids (27). Cells were lysed in digitonin lysis buffer solution and postnuclear supernatants were prepared. Cell lysates were aliquoted to four tubes and immunoprecipitation was performed with (a) 3A10 (anti-CD3ε), (b) 657 (anti-CD8), (c) 2C11 (anti-CD3ζ) (28), and (d) 143A and 143B (anti-CD3ζ/η) (29). Proteins were resolved by two-dimensional (nonreducing-reducing) SDS-PAGE (12% polyacrylamide in both dimension) and visualized by fluorography. Prestained molecular weight markers were electrophoresed with the samples. Squares on the diagonal show the migration positions of molecular weight markers. Under these conditions of labeling, no CD3ζ was detected.
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