Requirement for Positive Selection of γδ Receptor-Bearing T Cells

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The αβ and γδ T cell receptors for antigen (TCR) delineate distinct T cell populations. TCRαβ-bearing thymocytes must be positively selected by binding of the TCR to major histocompatibility complex (MHC) molecules on thymic epithelium. To examine the requirement for positive selection of TCR γδ T cells, mice bearing a class I MHC–specific γδ transgene (Tg) were crossed to mice with disrupted β_2 microglobulin (β_2M) genes. The $Tg^+\beta_2M^-$ (class I MHC^-) offspring had Tg^+ thymocytes that did not proliferate to antigen or Tg-specific monoclonal antibody and few peripheral Tg^+ cells. This is evidence for positive selection within the γδ T cell subset.

THE TCR MEDIATES INTRATHYMIC T cell differentiation (*1*). For example, TCRαβ recognition of self MHC and non-MHC antigens is required for phenotypic and functional maturation of $CD4^+CD8^+$ to $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocytes. This process of positive selection creates a TCRαβ repertoire that recognizes foreign antigens preferentially in association with the MHC molecules expressed on thymic epithelium during development (*2–4*). Thus, mice with no class I MHC expression because their β_2M genes were disrupted by homologous recombination ($\beta_2M^{-/-}$) have almost no $CD4^-CD8^+$ TCRαβ T cells, confirming the role of class I MHC molecules in the positive selection of this T cell subset (*3, 4*).

T cells that express the γδ receptor heterodimer are a distinct mature cell lineage (*5*). Information about the selection of the TCRγδ repertoire is limited. Studies with TCRγδ transgenic mice have shown that self tolerance (negative selection) can be established in the TCRγδ repertoire both by clonal deletion and by the induction of clonal anergy (*6, 7*).

To test directly the requirement for positive selection of TCRγδ, we crossed mice expressing a class I MHC–specific γδ transgene (Tg) (*6*) to mice with disrupted β_2M genes (*4*) to produce $Tg^+\beta_2M^-$ animals. The TCR γδ Tg was isolated from a BALB/c ($H\text{-}2^d$) *nu/nu*-derived T cell clone specific for a class I MHC alloantigen encoded in the TL region of $H\text{-}2^b$ mice (*8, 9*). We reasoned that a γδ TCR that recognizes a β_2M-associated $H\text{-}2^b$ class I MHC molecule would be positively selected during development by a homologous β_2M-associated molecule expressed in the syngeneic $H\text{-}2^d$ environment. If so, the maturation of class I MHC–specific TCRγδ T cells might be impaired in a class I MHC–deficient environment because a requisite TCR–self antigen interaction would not occur.

To examine T cell development in the γδ $Tg^+\beta_2M^-$ mice, we analyzed thymocyte populations by flow cytometry (Fig. 1). As expected, $TCR\alpha\beta^+$ $CD8^+$ T cells were present in $\beta_2M^{+/+}$ thymuses (Fig. 1A), but were absent in thymuses from $\beta_2M^{-/-}$ mice (Fig. 1B). However, substantial numbers of Tg^+ ($V_\gamma2^+$) T cells, expressing normal levels of receptor, were present in both the $\beta_2M^{-/-}$ and $\beta_2M^{+/+}$ thymuses.

TCRγδ Tg^+ $CD4^-CD8^-$ thymocytes were then purified from both the $\beta_2M^{+/+}$ and $\beta_2M^{-/-}$ mice, and their responses to alloantigen (B10 spleen cells) and receptor ($V_\gamma2$)-specific monoclonal antibody (MAb) were measured (Table 1). Tg^+ cells from β_2M^+ $H\text{-}2^d$ mice proliferated both to antigen (B10 spleen cells) and to receptor-specific MAb. In contrast, the Tg^+ thymocytes from $\beta_2M^{-/-}$ mice, including $H\text{-}2^d$ mice (experiments 4 and 5), did not proliferate in response to antigen or to MAb. The absence of proliferation is consistent with the failure of these γδ Tg^+ T cells to have received a receptor-mediated differentiative signal from a β_2M-associated self antigen.

$CD4^+CD8^+$ $TCR\alpha\beta^+$ T cells that are not positively selected do not exit from the thymus and populate peripheral lymphoid organs; we therefore examined γδ Tg expression in the periphery of $\beta_2M^{+/+}$, $\beta_2M^{+/-}$ (heterozygous for the disrupted β_2M gene), and $\beta_2M^{-/-}$ mice by flow cytometry (Fig. 2). Few $V_\gamma2$ bearing cells were present in the spleens and lymph nodes of the $\beta_2M^{-/-}$ animals, and these expressed only low amounts of receptor. The Tg^+ thymocytes from the $\beta_2M^{-/-}$ mice expressed amounts of receptor equivalent to thymocytes from the $\beta_2M^{+/-}$ and $\beta_2M^{+/+}$ control mice.

In contrast to the peripheral spleen and lymph node Tg^+ cells from Tg^+ β_2M^+ mice, which were predominantly $CD4^-CD8^-$ (Fig. 2, A and B, mice 1, 2, 4, and 5), almost no Tg-expressing lymph node or spleen cells in $\beta_2M^{-/-}$ mice (Fig. 2, A and B, mice 3 and 6) were $CD4^-CD8^-$. Although a percentage of the γδ Tg^+ thymocytes in $\beta_2M^{-/-}$ mice were $CD8^+$ (Fig. 2B, mouse 6), the peripheral spleen and lymph node dull $V_\gamma2^+$ cells in $\beta_2M^{-/-}$ mice

Table 1. Absence of proliferation to alloantigen or to Tg-specific MAb by γδ Tg^+ thymocytes that develop in $\beta_2M^{-/-}$ mice. Purified $CD4^-CD8^-$ γδ Tg^+ thymocytes (5×10^4) from $\beta_2M^{+/+}$ or $\beta_2M^{-/-}$ mice were incubated with T cell–depleted irradiated (2000 R) splenic antigen-presenting cells (APCs) (5×10^5) from allogeneic B10 ($H\text{-}2^b$) or syngeneic BALB/c ($H\text{-}2^d$) mice. Responder γδ Tg^+ thymocytes were prepared by treatment of whole thymus cell suspensions with the CD4- and CD8-specific MAbs RL172.4 and 3.155 (*21*) plus complement. Splenic APCs were depleted of T cells by treatment with a Thy 1.2–specific MAb [J1J (*21*)] and complement. For measuring MAb-induced proliferation, individual microtiter wells were coated with 30 μg/ml of purified $V_\gamma2$-specific MAb [10A6 (*9*)] and incubated for 2 hours at 37°C. After washing the MAb-coated wells three times with PBS, γδ Tg^+ thymocytes (10^5) were added. Proliferation was measured after 48 to 72 hours by labeling with [^{3}H]thymidine (1 μCi per well; specific activity 6.7 Ci/mol) for an additional 12 to 16 hours. Shown are the mean counts per minute of triplicate cultures. In general, standard errors of the mean were less than 20%. All the responder T cells from $\beta_2M^{+/+}$ mice were $H\text{-}2^d$. Tg^+ cells from $\beta_2M^{-/-}$ mice were either $H\text{-}2^{b/d}$ (experiments 1 to 3) or $H\text{-}2^d$ (experiments 4 and 5).

Stimulation	Tg^+ responder T cells									
	Expt. 1		Expt. 2		Expt. 3		Expt. 4		Expt. 5	
	β_2M^-	β_2M^+	β_2M^-	β_2M^+	β_2M^-	β_2M^+	β_2M^-	β_2M^+	β_2M^-	β_2M^+
BALB/c spleen ($H\text{-}2^d$)	696	700	867	651	–	–	226	233	323	351
B10 spleen ($H\text{-}2^b$)	849	63,965	744	28,823	–	–	1,226	31,437	376	33,183
$V_\gamma2$-specific MAb	–	–	–	–	117	23,116	–	–	182	59,728

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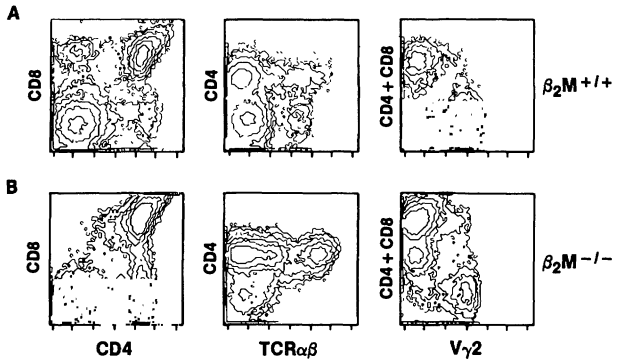

Fig. 1. Intrathymic development of γδ transgene expressing cells in $\beta_2M^{-/-}$ mice. $\beta_2M^{-/-}$ mice (*4*) were bred with TCR $\gamma\delta^+$ transgenic mice (*6*) to produce Tg^+ β_2M^- offspring. The mice were screened for the presence of the mutated β_2M gene or the γδ tg by Southern blot analysis of tail DNA as previously described (*6, 20*). The DNA constructs encoding the transgenic γ and δ chains have been described (*6*). The V_δ is a V_α11-like segment rearranged to J_δ1, and the γ chain is encoded by a V_γ2-J_γ1 rearrangement. A V_γ2-specific MAb, 10A6 (*9*), was used to assess γδ transgene expression as before (*6*). Thymocytes from these mice were examined by two-color flow cytometry to assess TCR expression and cell surface phenotype. Thymocytes were double-stained for CD4 and CD8, TCRαβ and CD4, and V_γ2 and CD4 plus CD8 (*21*). Profiles shown are from β_2M^+ H-2^d γδ Tg^+ control mice (**A**) and $Tg^+\beta_2M^{-/-}$ mice (**B**). The MHC haplotype (H-2^b or H-2^d) of the $\beta_2M^{-/-}$ mice was determined by flow cytometric analysis with appropriate class II MHC–specific MAbs (Y-3P, anti-I-A^b, and MKD6, anti–I-A^d). Identical results were obtained with H-2^d and H-2^b haplotype $\beta_2M^{-/-}$ mice. Samples for flow cytometry were prepared as single cell suspensions from thymuses of appropriate mice. Cells were stained sequentially either with FITC-labeled MAbs or unlabeled MAbs that were detected with appropriate FITC-conjugated secondary reagents, then biotinylated MAbs and streptavidin, conjugated to either allophycocyanin or phycoerythrin, with standard methodology. Control staining was performed with secondary reagents alone or with the streptavidin-conjugated reagents. Fluorescence was measured on a FACS 440 (Becton Dickenson). These results are representative of a total of eight experiments. The care of experimental animals used in this study was in full accordance with institutional guidelines.

were $CD8^-$, $CD4^+$, and $TCR\alpha\beta^+$, representing an unusual subpopulation of $TCR\alpha\beta^+$ $CD4^+$ T cells, unique to the $\beta_2M^{-/-}$ mice, that express ectopically low numbers of V_γ2 (*10*). Thus, virtually no phenotypically mature γδ Tg^+ T cells are present in peripheral lymphoid organs of Tg^+ β_2M^- mice, which is consistent with the absence of positive selection (*11*).

Differential expression of several cell surface proteins distinguishes immature from mature TCRαβ-bearing thymocytes. For example, immature αβ thymocytes express large amounts of the heat-stable antigen (HSA, J11D), whereas mature thymocytes and peripheral T cells express little or no J11D (*12*). Correspondingly, Tg^+ thymocyte populations from β_2M^+ mice consist of both $J11D^+$ and $J11D^-$ cells, whereas Tg^+ thymocytes from $\beta_2M^{-/-}$ mice are exclusively $J11D^+$ (*13*). Moreover, the Tg^+ β_2M^- thymocytes are CD5 dull, whereas the Tg^+ β_2M^+ thymocytes are CD5 bright (*13*). This pattern of CD5 expression also corresponds to immature and mature subsets of TCRαβ thymocytes, respectively (*12*).

Our data are direct functional evidence for positive selection of thymocytes expressing TCRγδ. Class I MHC–specific TCRγδ transgenic T cells that developed intrathymically in mice that do not express β_2M-associated class I MHC molecules did not proliferate in response to TCR-mediated signals and did not exit from the thymus and populate peripheral lymphoid organs. These findings parallel several fundamental parameters of TCRαβ positive selection (*2*), but it is possible that the molecular mechanisms of selection of γδ and αβ T cells may be different (*14*).

Although shown here for a γδ Tg with a single specificity, our results may apply to γδ T cell development in general. Thus, several studies have provided indirect evidence for positive selection of various γδ T cells (*15–17*). For example, strain-dependent selection has been shown for $V_\delta4^+$ receptors in murine intestinal epithelium [by class II MHC molecules (*16*)] and for the invariant $V_\delta5$-$D_\delta2$-$J_\delta1$ (BID) receptors in BALB/c mice (*17*). Our γδ Tg–bearing cells are phenotypically and functionally fully characteristic of normal γδ T cell populations. Like normal γδ T cells, the Tg^+ cells are predominantly $CD4^-CD8^-$, and the γ and δ Tgs are appropriately down-regulated in αβ T cells (*6*). We have also found (*18*) that γδ Tg^+ thymocytes develop in the presence of cyclosporin A in a fashion analogous to γδ T cells in general (*14*). Studies of TCRαβ development show that phenotypically related T cells differentiate through the same pathway (*1, 2*). Therefore, the requirement for a

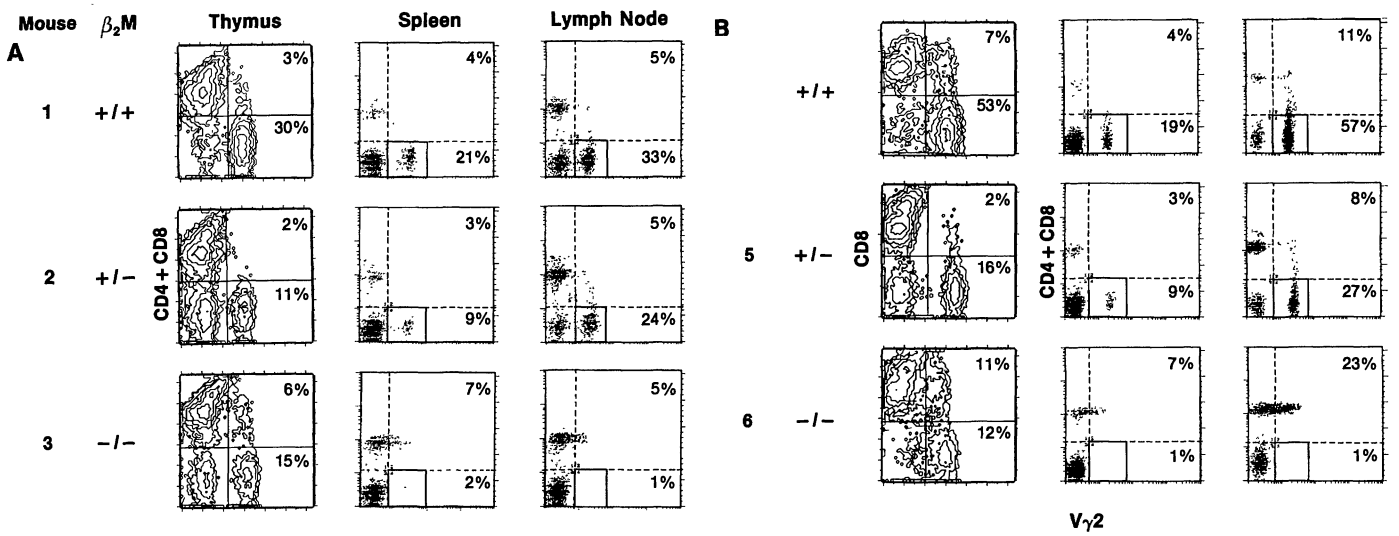

Fig. 2. Two-color cell surface phenotype analysis of thymocytes and peripheral lymphoid Tg-bearing cells from $\beta_2M^{+/+}$, $\beta_2M^{+/-}$, and $\beta_2M^{-/-}$ mice. Cell populations from the thymus, spleen, and lymph nodes of each animal were double stained for V_γ2 and CD4 and CD8 expression as described in Fig. 1. Samples were analyzed on a FACS 440 (thymuses) or FACSCAN (spleen and lymph nodes). The percentages of V_γ2-positive cells are indicated. The two representative experiments shown (**A** and **B**) were performed in identical fashion except that in (B), the thymocytes were double-stained for V_γ2 and CD8 alone.

TCR–self antigen interaction to complete differentiation may well apply generally to TCRγδ-bearing T cells, independent of their individual receptor specificities.

However, the normal maturation of γδ T cells in nontransgenic $\beta_2M^{-/-}$ mice indicates that many γδ T cells do not require class I MHC in order to differentiate (*3, 4*). Thus, if all γδ cells are subject to positive selection, non-MHC antigens might function as selecting self restriction molecules for many TCRγδ. When the cell surface molecules [for example, heat shock proteins (*19*)] that restrict the responses of non-MHC–specific γδ T cells are identified, this possibility can be tested directly. However, the fact that the self antigens that select the $V_\delta5$ BID receptor map outside the MHC is consistent with this notion (*17*). If true, then the requirement for TCR engagement might be a universal property of all T cell differentiation.

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10. F. B. Wells and L. A. Matis, unpublished data. None of the Tg^+ thymocytes from $\beta_2M^{-/-}$ mice, nor any Tg^+ thymocytes or peripheral T cells from the $\beta_2M^{+/-}$ and $\beta_2M^{+/+}$ mice coexpressed any CD4 or TCRαβ.
11. The mean percentage (±SEM) Tg^+ ($V_\gamma2^+$) cells in the thymus, spleen, and lymph nodes for the different groups of mice were determined. The mean percentage of Tg^+ thymocytes was 24 ± 4% (n = 15) for $\beta_2M^{-/-}$ mice, 28 ± 8% (n = 6) for $\beta_2M^{+/-}$ mice and 49 ± 6% (n = 15) for $\beta_2M^{+/+}$ mice. The corresponding mean absolute numbers of $V_\gamma2^+$ thymocytes were $1.5 \times 10^7 \pm 4 \times 10^6$ for $\beta_2M^{-/-}$, $2.8 \times 10^6 \pm 10^6$ for $\beta_2M^{+/-}$, and $6.2 \times 10^6 \pm 1.2 \times 10^6$ for $\beta_2M^{+/+}$. The mean percentage of $CD4^-CD8^-$ Tg^+ cells in spleen were 1 ± 0.2% (n = 5) for $\beta_2M^{-/-}$, 12 ± 3% (n = 4) for $\beta_2M^{+/-}$, and 17 ± 2.5% (n = 4) for $\beta_2M^{+/+}$. The average total number of cells per spleen (5×10^7 to 10^8) did not differ significantly among $\beta_2M^{-/-}$, $\beta_2M^{+/-}$, and $\beta_2M^{+/+}$ mice. Thus, the percentage values are directly proportional to the absolute numbers of Tg^+ splenocytes. The mean percentage of lymph node $CD4^-CD8^-$ Tg^+ cells were 1 ± 0.1% (n = 4) for $\beta_2M^{-/-}$, 32 ± 4% (n = 4) for $\beta_2M^{+/-}$ and 52 ± 6% (n = 7) for $\beta_2M^{+/+}$. Lymph nodes were quite comparable in size among different animals, indicating that the percentage values reflect the absolute numbers of Tg^+ cells present. However, the absolute numbers of Tg^+ lymph node cells were not compared because variable numbers of lymph nodes were recovered from individual animals.
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18. T cell–depleted bone marrow cells from γδ Tg^+ mice were injected into lethally irradiated (850 R) syngeneic ($H\text{-}2^d$) host mice, which were then treated with daily intraperitoneal injections of cyclosporin A (10 to 20 mg/kg) or olive oil alone as a control, exactly according to previous protocols (*14*). After 27 days, thymocyte populations were examined by flow cytometry for expression of γδ Tg, TCRαβ, CD4, and CD8. Cyclosporin A treatment abolished intrathymic expression of $TCR\alpha\beta^+$ $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells, but had no effect on development of γδ Tg^+ $CD4^-CD8^-$ thymocytes (F. Wells, S. J. Gahm, L. A. Matis, unpublished data).
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Targets for Cell Cycle Arrest by the Immunosuppressant Rapamycin in Yeast

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FK506 and rapamycin are related immunosuppressive compounds that block helper T cell activation by interfering with signal transduction. In vitro, both drugs bind and inhibit the FK506-binding protein (FKBP) proline rotamase. *Saccharomyces cerevisiae* cells treated with rapamycin irreversibly arrested in the G1 phase of the cell cycle. An FKBP-rapamycin complex is concluded to be the toxic agent because (i) strains that lack FKBP proline rotamase, encoded by *FPR1*, were viable and fully resistant to rapamycin and (ii) FK506 antagonized rapamycin toxicity in vivo. Mutations that conferred rapamycin resistance altered conserved residues in FKBP that are critical for drug binding. Two genes other than *FPR1*, named *TOR1* and *TOR2*, that participate in rapamycin toxicity were identified. Nonallelic noncomplementation between *FPR1*, *TOR1*, and *TOR2* alleles suggests that the products of these genes may interact as subunits of a protein complex. Such a complex may mediate nuclear entry of signals required for progression through the cell cycle.

IN HIGHER EUKARYOTES, THE IMMUnosuppressive compounds cyclosporin A (CsA), FK506, and rapamycin inhibit the intermediate steps in signal transduction between the cytoplasm and the nucleus. These three compounds are immunosuppressive because they block signal transduction pathways required for the activation of helper T cells (*1, 2*).

FK506 and rapamycin are related macrolides and share no chemical similarity with CsA, a cyclic undecapeptide (*2*). Although structurally unrelated, CsA and FK506 inhibit the same step in T cell activation, an antigen-stimulated, Ca^{2+}-dependent signal transduction step that acts through the nuclear factor of activated T cells (transcription factor NF-AT) (*3*). In contrast, rapamycin does not interfere with the response to an antigen but rather impairs a later step in T cell activation, the proliferative response to interleukin-2 (*4, 5*).

The effects of CsA, FK506, and rapamycin are thought to be mediated by the

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