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A Novel T Cell Subset with Trans-Rearranged Vγ-Cβ TCRs Shows Vβ Expression Is Dispensable for Lineage Choice and MHC Restriction

Steven Bowen,* Peter Sun,† Ferenc Livak,‡ Susan Sharrow,* and Richard J. Hodes*

αβ T cells, which express the α-β TCR heterodimer, express CD4 or CD8 coreceptors on cells that are MHC class I or MHC class II dependent. In contrast, γδ T cells do not express CD4 or CD8 and develop independently of MHC interaction. The factors that determine αβ and γδ lineage choice are not fully understood, and the determinants of MHC restriction of TCR specificity have been controversial. In this study we have identified a naturally occurring population of T cells expressing Vγ-Cβ receptor chains on the cell surface, the products of genomic trans-rearrangement between the Vγ2 gene and a variety of Dβ or Jβ genes, in place of an intact TCRβ-chain and in association with TCRα. Identification of this population allowed an analysis of the role of TCR variable regions in determining T cell lineage choice and MHC restriction. We found that Vγ2-Cβ+ cells are positive for either CD4 or CD8 and are selected in an MHC class II– or MHC class I-dependent manner, respectively, thus following the differentiation pathway of αβ and not γδ cells and demonstrating that Vβ V region sequences are not required for selection of an MHC-restricted repertoire. The Journal of Immunology, 2014, 192: 169–177.

The development of T lymphocytes in the thymus is dependent on the successful expression of an Ag-specific TCR. TCRs are heterodimers of either α- and β-chains (αβ T cells) or γ- and δ-chains (γδ T cells). Each chain consists of two major Ig superfamily domains: the membrane-distal V domain, which is encoded by somatically recombined V, D, and J gene segments, and the membrane-proximal C domain. Ag recognition by a TCR complex is mediated in large part by three CDRs on each variable domain. CDRs 1 and 2 are encoded within the TCRβ gene and are created by the imprecise V(D)J junction, making it highly diverse and accounting for the tremendous variability of the TCR repertoire. Despite sharing similar mechanisms of receptor rearrangement and expression, αβ and γδ T cell lineages have distinct requirements for development and selection in the thymus (1). αβ TCRs generally recognize peptide Ags presented in the context of MHC molecules, and during the CD4+CD8+ double-positive (DP) stage of development they are selected by peptides associated with MHC class I (MHC I) and MHC class II (MHC II) on thymic stromal cells. CD8 or CD4 coreceptor engagement in concert with TCR interaction with MHC I or MHC II, respectively, determines lineage selection to the CD8 or CD4 single-positive (SP) phenotype of mature αβ T cells (2). It has been proposed that CDR1 and CDR2 of the TCRβ variable domain play a critical role in mediating TCR interaction with MHC molecules through residues that are highly conserved between Vβ gene segments and throughout evolution (3–6), but this remains controversial (7). In contrast, γδ T cells do not express CD4 or CD8 coreceptors, remaining CD4/CD8 double-negative (DN) throughout development and are selected independently of the classical MHC I and MHC II molecules (8).

V(D)J recombination at particular TCR loci is tightly restricted to specific developmental stages. The TCRβ, TCRγ, and TCRδ loci rearrange simultaneously early in T cell development during the DN stage (9). TCRα locus recombination occurs subsequently during the DP stage (10). Typical V(D)J rearrangement occurs in cis between gene segments within a single TCR locus. However, simultaneously rearranging β, γ, and δ loci in DN thymocytes may also recombine in trans, creating hybrid genes with elements from multiple TCR loci. Previously it has been shown that interchromosomal trans-rearrangements involving TCRβ, TCRγ, and TCRδ occur in T cells of mice and humans and are dramatically increased in the absence of the DNA damage response mediator ataxia telangiectasia mutated (ATM) (9, 11–13). Sequence analysis of these trans-rearrangement junctions reveals features similar to those of normal cis-rearrangements (small coding flank deletions, non–template N nucleotide additions) (9, 11–13). The existence of in-frame Vγ-(Dβ)-Jβ trans-rearrangements in peripheral T cells of both wild-type (WT) and ATM−/− mice raises the question of whether a trans-rearranged locus can be transcribed and expressed on the cell surface. Furthermore, if such cells develop and survive in the periphery, it would be of interest to determine the factors required for their development and selection, as well as their functionality.

In this study, we have identified and characterized a naturally occurring T cell population expressing a cell surface TCR chain encoded by trans-rearrangement between the Vγ2 gene and various (Dβ)Jβ genes. We found that these Vγ-Cβ hybrid chains are capable of mediating TCRβ allelic exclusion, thus being expressed in
the absence of intact Vβ-chains, and they are developmentally dependent on TCRβ expression. The identification of these cells provided an opportunity to determine the requirements for TCR V gene products in determining T cell lineage fate and MHC restriction. We found that Vγ2Vβ+ cells in the periphery are CD4 or CD8 SP and are dependent on selection by MHC I or MHC II. These results provide evidence that the conserved CDR1 and CDR2 residues of the Vβ domain are dispensable for MHC restriction and lineage choice, as Vγ domains typically expressed in non-MHC-restricted γδ cells are capable of mediating MHC-restricted selection by CD4 and CD8 coreceptor-positive T cells.

Materials and Methods

Mice

ATM−/− mice were generated through a mutation of the ATM gene at nucleotide 5790 resulting in a truncation of the protein (14). ATM−/− breeders were used to generate ATM−/− progeny as well as ATM+/+ littermates that were used as WT controls. All analysis was done at 6–12 wk of age. MHC II−/− (H2Ab1-Eq1) (The Jackson Laboratory, Bar Harbor, ME), β2M−/− (The Jackson Laboratory), TCRα−/− (15), Foxp3-GFP (16), and ThP0K-YFP (17) mice were maintained by homozygous breeding. Animals were maintained at Bioqual (Rockville, MD) and at SAIC-Frederick. All procedures were approved by the National Cancer Institute Animal Care and Use Committee.

Flow cytometry

Flow cytometry for analysis only was done on an LSR Fortessa (BD Biosciences). Cell sorting was done on a FACSAria (BD Biosciences). For cell sorting a pool of 5–10 mice was used for each sort. Total splenocytes were pre-enriched on a CD19 negative selection column (Miltenyi Biotec, Auburn, CA) and subsequently stained with Abs for Vγ2 (UC3-10A6; BioLegend), B220 (RA3-6B2), TCR Cβ (GL3), Vβ2, 3, 5–8, 11, and 12, and Cβ (H57) (BD Biosciences). Additional Abs used in this study include CD4 (RM4-5), CD8 (53-6.7), CD44 (Ly24), and CD62L (ME-L14) (BD Biosciences). Flow cytometry data were analyzed with FlowJo software.

Real-time PCR analysis of Vγ-Cβ transcripts

RNA was isolated from sorted cells using an RNeasy Mini Kit (Qiagen). The reverse transcription into cDNA was done using a SuperScript III first-strand synthesis system (Invitrogen). Real-time PCR was performed using RT SYBR Green PCR mastermix (Invitrogen). The Vγ-Cβ reaction was normalized to a control amplification on the same DNA sample of the ubiquitously expressed β-actin gene using the following formula: 1.9−ctVγ2+Cβ/1.9−ctβ-actin, where ct is the cycle threshold of amplification. The Vγ2 forward primer is 5′-AAATGGGACGAGGTAATAATCC-3′; the Cβ reverse primer is 5′-AGGCGATCCACAGTCTGCTCG-3′.

Thymine-adenine cloning of PCR products and sequencing

DNA was purified from PCR reactions using a QIAquick PCR purification kit (Qiagen) and ligated into the PCR 2.1 vector using a TOPO TA cloning kit (Invitrogen). Ligation was transformed into chemically competent bacteria by heat shocking at 42°C for 30 s. Transformed bacteria were screened using a blue-white selection (18) and DNA was purified from PCR reactions using a QIAquick PCR purification kit (Qiagen). Ligations were transformed into chemically competent bacteria by heat shocking at 42°C for 30 s. Transformed bacteria were selected by X-Gal staining after incubation for 2 days at 37°C. Blue-white colonies were picked and sequenced by Sanger sequencing using the M13 forward and reverse primer is 5′-CTGCTGCTCG-3′.

In vitro proliferation of Vγ2Vβ cells

Total splenocytes from four to five WT and ATM−/− mice were CD19 depleted using a magnetic separation column (Miltenyi Biotec). Cells were then labeled with 1 μM CFSE (Molecular Probes) as previously described. CFSE-labeled cells (30 × 10^6) were cultured for 72–96 h with either 500 ng/ml anti-CD3 Ab (2C11), 60 × 10^6 syngeneic 129 splenocyte APCs, or 60 × 10^6 allogeneic BALB/c splenocyte APCs in a six-well tissue culture plate (BD Falcon). APCs were exposed to 500 rad irradiation prior to culture. Cells were harvested, washed in tissue culture medium, and stained for flow cytometric analysis.

Statistical analysis

All statistical analysis was done using a Student two-tailed t test.

Results

Trans-rearranged TCR loci are transcriptionally active in peripheral T cells

Expressed TCR chains are well characterized as the products of rearrangement between V, D, and J elements encoded in cis at each of the four TCR loci. We have recently reported that trans-rearrangements between different TCR loci, including Vγ2-Jβ and Vγ2-Dβ-Jβ trans-rearrangements, also occur during the DN2/DN3 stage of T cell development and are dramatically increased in the absence of ATM (9). Sequence analysis revealed that some of these trans-rearrangements preserved the reading frame of the Vγ2-Dβ-Jβ hybrid locus. It was not established, however, whether these trans-rearranged genes were in fact expressed. It was possible that trans-rearrangement would disrupt the configuration of cis-acting regulatory elements within the TCRβ and TCRγ loci and/or interfere with splicing of immature transcripts to generate mature mRNA transcripts. To determine whether trans-rearrangements are transcribed, we isolated RNA from WT and ATM−/− splenocytes and prepared cDNA. Real-time PCR with primers specific for the Vγ2 and Cβ gene segments revealed that a trans-rearranged transcript was clearly detectable in WT splenic T cells and at ~20–30-fold higher abundance in ATM−/− splenic T cells (Fig. 1A). Subsequent sequencing confirmed the identification of Vγ2-Cβ hybrid transcripts that were both in-frame and out-of-frame (Table I). PCR amplification and sequencing revealed additional hybrid transcripts resulting from trans-rearrangement between Vβ6 and Jγy genes (Supplemental Table I). These data indicate that trans-rearranged hybrid loci can be transcribed and spliced into mature transcripts.

Peripheral T cells expressing cell surface Vγ2Cβ hybrid receptor chains are detectable in WT and ATM−/− mice

The existence of hybrid products such as Vγ2-Cβ transcripts raised the possibility of an expressed TCR chain containing a Cβ constant domain and a hybrid variable domain encoded by a Vγ2-Dβ-Jβ trans-rearrangement. However, changes in the variable domain structure could potentially render the product unstable and/or unable to pair in expressed TCR dimers, preventing expression on the cell surface. To examine the presence of a cell surface–expressed, trans-rearranged TCR chain on peripheral T lymphocytes, we used a flow cytometric strategy involving available reagents specific for multiple cell surface TCR determinants. Our staining parameters allowed us to distinguish between cells expressing the Vγ2-Jβ-Cβ hybrid receptor and conventional αβ or γδ T cells. Total splenocytes from WT and ATM−/− mice were stained with Abs for B220, Cβ TCR, Cβ TCR, Vγ2 TCR, and a pool of anti-Vβ TCR Abs that, when combined, stained ~60–70% of αβ TCRs in peripheral T cells. Cells were gated on B220+, Cβ−, and Vβ− populations to exclude B cells, γδ T cells, and most Vβ-expressing αβ T cells, respectively. A subset of trans-rearranged receptors was predicted to be double-positive for Cβ and Vγ2. Our results revealed the presence of a population of Vγ2-Cβ cells representing 0.001% of WT and 0.03% of ATM−/− T cells (Fig. 1B, 1C). Expression levels of TCR Cβ on the surface of the Vγ2-Cβ population were similar to those of conventional H57+ αβ T cells (Fig. 1B).

To determine whether the cells detected by this strategy are indeed expressing a hybrid Vγ2-Cβ product, the Vγ2-Cβ and Vγ2 Cβ populations from WT or ATM−/− spleens were sorted by flow cytometry, and RNA was isolated and cDNA synthesized.
Real-time PCR revealed that the Vγ2-Cβ+ cells contained high levels of Vγ2-Cβ transcripts compared with the absence of detectable transcripts in the Vγ2-Cβ population (Fig. 1D). Sequence verification of the amplified products from both WT and ATM−/− splenocytes revealed multiple unique in-frame Vγ2-Jβ-Cβ rearrangements (Table II).

To address the possibility that cells expressing a Vγ-Cβ hybrid TCR also express an intact TCRβ-chain that could be responsible for development and selection, we repeated our flow cytometry analysis without negatively gating on the pool of Vβ Abs. We found that the Vγ-Cβ-expressing cells were almost universally negative for surface Vβ expression, suggesting that the Vγ2-Cβ chain is capable of mediating development and selection, as well as TCRβ allelic exclusion (Fig. 1E). These data indicate that not only can immature thymocytes with trans-rearranged chromosomes survive thymic selection and be released into the periphery, but also that the trans-rearranged locus is transcribed and expressed as a surface TCR chain that can mediate selection, allelic exclusion, and, potentially peripheral function of mature T cells.

Vγ2* cells are developmentally dependent on expression of the TCRα-chain

Pairing of TCR receptor chains is mediated through a disulfide bond between cysteine residues located between the C domain and
Table I. Vγ2-(Dbj)-Jβ trans-rearrangement sequences from WT and ATM⁻/⁻ splenocytes

<table>
<thead>
<tr>
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<th>Dβ</th>
<th>Jβ</th>
<th>In Frame</th>
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<tr>
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<td>CGG</td>
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<td>1.4</td>
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<td>GGTCA</td>
<td>AGTCAAAAC</td>
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<tr>
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</table>

Peripheral T cells contain Vγ2-(Dbj)-Jβ trans-rearranged transcripts. Sequences derived from Vγ2-Cβ PCR-amplified cDNA from sorted WT (bold) and ATM⁻/⁻ splenocytes are shown. All sequences are unique Vγ2-(Dbj)-Jβ trans-rearrangements utilizing multiple Jβ gene segments. Jβ segment number is indicated in the sixth column. The far-right column indicates whether the rearrangement was in-frame or out-of-frame. A schematic diagram of the amplified locus is shown below. Arrows indicate the locations of the Vγ2 and Cβ specific primers used for amplification.

transmembrane domain of each receptor chain (18). To test whether Vγ2-Cβ hybrid TCR chains pair with TCRα-chains we analyzed the expression of TCRα on the surface of Vγ2+Cβ⁺ T cells using Abs to the Vα3.2 and Vα8 domains, which are

Table II. Vγ2-(Dbj)-Jβ trans-rearrangement sequences from sorted Vγ2+Cβ⁺ splenocytes

<table>
<thead>
<tr>
<th>Vγ2</th>
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<th>Dβ</th>
<th>Jβ</th>
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</table>

Vγ2+Cβ⁺ cells express in-frame Vγ2-(Dbj)-Jβ trans-rearrangements. Sequences derived from Vγ2-Cβ PCR-amplified cDNA from sorted WT (bold) and ATM⁻/⁻ Vγ2+Cβ⁺ splenocytes are shown. All sequences are unique in-frame Vγ2-(Dbj)-Jβ trans-rearrangements utilizing multiple Jβ gene segments. Jβ segment number is indicated in the last column. A schematic diagram of the amplified locus is shown below the table. Arrows indicate the locations of the Vγ2 and Cβ specific primers used for amplification.
The development of V\textsubscript{γ}2-\textbeta{2} T cells with an intact V\textgamma{γ}\textbeta{2} expression of a surface-expressed TCR complex consisting of an \textgamma{γ}- and CD8 expression on splenic T cells bearing a V\textgamma{γ}\textbeta{2} C\textalpha{2} gene that are unable to express a functional TCR\alpha-chain (15). The populations of V\textgamma{γ}2-\textbeta{2} cells in the spleens of WT and ATM\textsuperscript{−/−} mice were absent in TCR\alpha−/− mice and in mice double deficient for ATM and TCR\alpha (Fig. 2B). In contrast, the numbers of V\textgamma{γ}2-\textbeta{2} γδ T cells were unaffected by the absence of TCR\alpha (Fig. 2C). Thus, similar to αβ T cells expressing a \textalpha{2}-rarranged TCR\beta-chain, TCR\alpha expression is required for the development of V\textgamma{γ}2-\textbeta{2} TCR-expressing cells. These findings suggest that selection and survival of V\textgamma{γ}2-\textbeta{2} cells requires expression of a surface-expressed TCR complex consisting of an intact V\textgamma{γ}C\textalpha{2} chain paired with the V\textgamma{γ}C\textbeta{2} hybrid chain. Thus, we have identified a unique, naturally occurring T cell population in the mouse with an αβ-like TCR containing V\gamma instead of V\beta domains.

V\textgamma{γ}2-\textbeta{2} cells are CD4 or CD8 SP and contain αβ-like subpopulations

The identification of a population of T cells expressing a TCR\alpha-chain paired with the V\textgamma{γ}C\textbeta{2} hybrid chain allowed assessment of the role of V region expression in determining lineage fate and critical properties of TCR specificity. The selective ligands guiding γδ T cell development in the thymus are poorly understood, but γδ T cells do not have a strict developmental dependence on classical MHC I and MHC II molecules. Unlike γδ T cells, the selection of αβ T cells is overwhelmingly dependent on classical MHC I and MHC II molecules through interactions with TCR and the cognate CD8 and CD4 coreceptors, respectively. V\textgamma{γ}C\textbeta{2} hybrid TCR-expressing cells provide a unique natural model with which to study the structural determinants of the TCR that are critical for lineage specification and selection. Analysis of CD4 and CD8 expression on splenic T cells bearing a V\textgamma{γ}2-\textbeta{2} hybrid TCR revealed that, like conventional αβ T cells (and unlike γδ T cells, which are primarily CD4\textsuperscript{−}CD8\textsuperscript{−}), this population is a mix of CD4 and CD8 SPs (Fig. 3A). CD4 SP (but not CD8 SP) αβ T cells express the transcription factor ThPOK, which is critical for CD4 SP lineage choice (19). To determine whether V\textgamma{γ}2-\textbeta{2} hybrid TCR-expressing cells displayed a similar pattern of ThPOK expression, we used ThPOK-YFP reporter mice (20). Similar to conventional αβ T cells, V\textgamma{γ}2-\textbeta{2} hybrid TCR-expressing cells showed almost universal expression of ThPOK among the CD4\textsuperscript{+} population but not in the CD8\textsuperscript{+} population (Fig. 3B), suggesting similar transcriptional requirements for CD4 lineage choice.

Ag-experienced memory αβ T cells in the periphery upregulate the receptor CD44 and downregulate the homing molecule L-selectin (CD62L). It was of interest to determine whether V\textgamma{γ}2-\textbeta{2} T cells in vivo were similarly capable of differentiation into cells expressing a memory phenotype. To determine whether a subset of cells expressing the V\textgamma{γ}2-\textbeta{2} hybrid TCR chain in the spleen displayed a memory phenotype, we stained splenocytes from WT and ATM\textsuperscript{−/−} mice with Abs for CD44 and CD62L. Similar to conventional αβ T cells, we found that the V\textgamma{γ}2-\textbeta{2} subset contained a mix of CD62L\textsuperscript{hi}CD44\textsuperscript{hi} naive phenotype cells and CD62L\textsuperscript{lo}CD44\textsuperscript{lo} memory cells, suggesting that the V\textgamma{γ}2-\textbeta{2} hybrid receptor is capable of mediating activation and differentiation into memory cells (Fig. 3C).

T regulatory cells (Tregs) are a subpopulation of αβ T cells defined by the expression of the transcription factor Foxp3 (21). To investigate the presence of Foxp3-expressing Tregs within the V\textgamma{γ}2-\textbeta{2} T cell population, we used Foxp3-GFP reporter mice (16). We found that the V\textgamma{γ}2-\textbeta{2} subset contained a similar or higher frequency of Tregs in comparison with total C\textbeta{2} T cells, in contrast to the much lower frequency in GL3\textsuperscript{+} γδ cells (Fig. 3D). Taken together, the subpopulations identified within V\textgamma{γ}2-\textbeta{2} T cells suggest that patterns of differentiation are similar to those of conventional αβ T cells.

**FIGURE 2.** V\textgamma{γ}2-\textbeta{2} cells express V\alpha and are developmentally dependent on TCR\alpha expression. (A) Flow cytometric analysis of pooled V\alpha3.2 and V\alpha8 expression on V\textbeta{8}11\textsuperscript{+}, GL3\textsuperscript{+}, and V\textgamma{γ}2-\textbeta{2} ATM\textsuperscript{−/−} splenocytes. Leu\textsuperscript{4} isotype control is shown in gray. Results are representative of three independent experiments. (B and C) Splenocytes from WT, TCR\alpha−/−, ATM−/−, and ATM−/− TCR\alpha−/− mice were analyzed for the presence and frequency of (B) V\textgamma{γ}2-\textbeta{2} cells and (C) V\textgamma{γ}2GL3\textsuperscript{+} cells. Data are a summary of three independent experiments.
Vγ2Cβ⁺ cells express CD4 or CD8 and contain αβ-like subsets. (A) CD4 and CD8 expression were analyzed by flow cytometry on Cβ⁺ (H57), γδ (GL3⁺), and Vγ2Cβ⁺ splenocytes from WT and ATM⁻/⁻ mice. Data are representative of four independent experiments with 4–10 mice pooled for each experiment. (B) αβ (H57⁺), γδ (GL3⁺), and Vγ2Cβ⁺ cells were analyzed for ThPOK expression in ThPOK-YFP reporter mice. Results are representative of three independent experiments with two to six mice pooled per experiment. (C) Naïve and memory T cell subsets were analyzed on ATM⁻/⁻ Cβ⁺ (H57) and Vγ2Cβ⁺ T cells by double staining with CD4 and CD62L. Data are representative of three independent experiments. (D) Foxp3-GFP reporter mice were used to analyze the presence of Tregs within the Cβ⁺ (H57⁺), γδ (GL3⁺), and Vγ2Cβ⁺ populations of splenocytes. Data are representative of three independent experiments.

FIGURE 3. Vγ2Cβ⁺ cells express CD4 or CD8 and contain αβ-like subsets. (A) CD4 and CD8 expression were analyzed by flow cytometry on Cβ⁺ (H57), γδ (GL3⁺), and Vγ2Cβ⁺ splenocytes from WT and ATM⁻/⁻ mice. Data are representative of four independent experiments with 4–10 mice pooled for each experiment. (B) αβ (H57⁺), γδ (GL3⁺), and Vγ2Cβ⁺ cells were analyzed for ThPOK expression in ThPOK-YFP reporter mice. Results are representative of three independent experiments with two to six mice pooled per experiment. (C) Naïve and memory T cell subsets were analyzed on ATM⁻/⁻ Cβ⁺ (H57) and Vγ2Cβ⁺ T cells by double staining with CD4 and CD62L. Data are representative of three independent experiments. (D) Foxp3-GFP reporter mice were used to analyze the presence of Tregs within the Cβ⁺ (H57⁺), γδ (GL3⁺), and Vγ2Cβ⁺ populations of splenocytes. Data are representative of three independent experiments.

To assess the ability of Vγ2Cβ⁺ T cells to respond to in vitro stimulation, we measured proliferation of splenocytes by CFSE dilution in response to anti-CD3 and in an MLR with irradiated allogeneic stimulator cells. The Vγ2Cβ⁺ subset proliferated in response to anti-CD3 to a similar extent as did the conventional αβ and γδ subsets (Fig. 4A). In MLR, Vγ2Cβ⁺ cells were also responsive to allogeneic stimulation.

Vγ2Cβ⁺ cells are selected by MHC I and MHC II interactions

The CD8 and CD4 coreceptors on DP thymocytes interact with MHC I and MHC II, respectively, on thymic stromal cells. These interactions, along with TCR-pMHC interactions, facilitate positive selection of DPs and progression from the DP to SP stage. The importance of MHC interactions is evident in MHC I– and MHC II–deficient mice in which there is a drastic reduction in the percentage of mature CD8⁺ and CD4⁺ TCRαβ T cells, respectively. Unlike αβ cells, γδ cells are predominantly CD4⁺ CD8⁻ and developmentally unaffected by the loss of MHC I and MHC II (22, 23). To determine whether TCRs bearing the Vγ2-Cβ hybrid chain undergo thymic selection by the same MHC-mediated mechanism as conventional αβ T cells, we analyzed the dependence of Vγ2-Cβ⁺ cell development on MHC expression in β2M⁻/⁻ (deficient in MHC I) and MHC II⁻/⁻ mice. WT and ATM⁻/⁻ Vγ2Cβ⁺ cells are a mix of CD4 and CD8 SPs (Fig. 5). Absence of MHC I resulted in selective loss of the CD8 population of Vγ2Cβ⁺ cells whereas absence of MHC II resulted in loss of CD4⁺ Vγ2Cβ⁺ cells, precisely paralleling the MHC dependence of conventional αβ T cells (Fig. 5). The absolute numbers of Vγ2Cβ⁺ cells per spleen are indicated in Supplemental Fig. 1. γδ Cells were primarily negative for CD4 and CD8 in all genotypes analyzed. These results indicate that interactions between the Vγ2-Cβ hybrid TCR and MHC molecules in the thymus are necessary for selection and maturation, similar to conventional TCRs bearing Vβ-Cβ chains and despite the absence of Vβ sequences.

Discussion

We have described a novel population of naturally occurring T cells expressing a hybrid TCR chain encoded by interchromosomal trans-rearrangement between the TCRγ and TCRβ loci. These T cells express a Vγ-Cβ hybrid TCR, coexpressed with TCRα, in the absence of intact Vβ-containing TCRβ-chains, and thus provided a naturally occurring model in which to study the determinants of TCR lineage choice and MHC restriction. αβ T cells, which express the αβ TCR heterodimer, express CD4 or CD8 coreceptors on cells that are highly MHC II or MHC I dependent for their development, whereas γδ T cells do not express CD4 or CD8 and develop independently of MHC interaction. We found that cells expressing the Vγ2-Cβ hybrid TCR differentiate as CD4⁺ or CD8⁺ cells that are MHC II– or MHC I–restricted, respectively, demonstrating that lineage choice and MHC restriction are not dependent on TCRβ expression.

The developmental determinants that enforce MHC restriction on αβ T cells during thymic selection have been a subject of considerable controversy (24). It has been proposed that conserved, germline-encoded amino acids within the CDRI and
CDR2 loops of Va and Vb domains have been selected through the coevolution of the Vaβ TCR with MHC I and MHC II molecules to allow Ag recognition only in the context of MHC (3, 25). An alternative model proposes that Vaβ TCRs do not have an inherent specificity for recognition of MHC, but that MHC restriction is imposed on developing thymocytes by CD4 or CD8 coreceptor interactions during positive selection (7, 26, 27). In this study, we have described a naturally occurring population of T cells expressing a hybrid TCR chain with a variable domain encoded by a trans-rearrangement between the Vγ2 gene and DB or JB genes and a constant domain encoded by Cβ genes. Vγ2Cβ+ cells, in which the germline CDR1 and CDR2 loops are encoded by the Vγ2 gene instead of Vβ genes, offered a unique natural model in which to study factors critical for MHC restriction and selection. TCRs are coexpressed on the surface of Vγ2Cβ+ cells, and in the absence of TCRα, Vγ2Cβ+ cells fail to develop, suggesting that the selecting TCR complex consists of the Vγ2-Cβ hybrid chain and an intact TCRα-chain. The developmental phenotype of Vγ2Cβ+ cells suggests that they likely progressed through β selection by pairing with pre-To and functioning in the pre-TCR complex. The lack of an intact TCRβ-chain on the surface as measured by Vβ expression further indicates that the pre-TCR signal mediated by the Vγ-Cβ hybrid chain effectively suppresses rearrangement and expression of the non–trans-rearranged TCRβ allele.

Crystallographic studies of conventional Vaβ TCRs in complex with peptide-MHC ligands have revealed a common diagonal docking orientation with conserved interactions between specific residues on the CDR1 and CDR2 loops of the V domains with specific residues on the α helices of MHC I and MHC I (3, 28). Furthermore, mutation of these conserved residues within the Vβ CDR2 region impairs positive selection and transition from the DP to SP stage, supporting the theory that Vaβ TCRs have a germline-encoded proclivity for MHC recognition (25). However, it has recently been reported that mutation of these same conserved residues on MHC-unrestricted TCR had a similar effect on positive selection and SP differentiation, indicating that the importance of these residues in ligand recognition is not specific for MHC (29). The CDR1 and CDR2 of Vγ2 lack any of the putative conserved contact residues that are present within many of the Vβ genes. Although γδ T cells are not typically MHC restricted, certain γδ TCRs that recognize MHC-like molecules have been described (30, 31). Crystallization of one such γδ TCR in complex with the nonclassical MHC I molecule T22 revealed that the large CDR3 loop and, to a lesser extent, CDR1, CDR2, and CDR3γ are in contact with T22. Little or no direct interaction between CDR1γ or CDR2γ and the T22 molecule was observed and thus, in this very limited context, there is no evidence of germline-encoded recognition of MHC by the Vγ domain (31). Our observation that the Vγ2Cβ+ population invariably expresses either CD4 or CD8 coreceptors in the periphery, and that selection of these populations corresponds to the presence of MHC II or MHC I, is not supportive of a germline Vβ-encoded basis for the MHC restriction of αβ TCRs. It instead suggests that MHC restriction can be imposed on T cells lacking conventional MHC-restricted Vβ domains provided that the MHC–coreceptor interactions are intact. These results are consistent with recent work showing that replacement of Vβ CDR1 and CDR2 sequences with those of a Vγ domain does not significantly alter the dynamics of positive selection or CD4/CD8 lineage commitment (32).

The presence of Vγ2Cβ+ cells in the spleen with a CD4 restore CD62L+ memory phenotype suggests that these cells are capable of recognizing and responding to antigenic stimulation in vivo and thus may be participating in protective immune responses. The
presence of Foxp3+ Tregs within the Vγ2+Cb+ population indicates additional lineage differentiation potential. Additional work is needed to characterize the specificity of the receptors and to determine what, if any, functional difference exist between them and the typical αβ TCR repertoire. Additionally, it remains to be seen whether the presence of large chromosomal abnormalities in these Vγ2+Cb+ cells has any effect on responsiveness or proliferative capacity.

The Vγ2+Cb+ population represents a novel mouse T cell subset with a generally αβ-like phenotype, including CD4+ or CD8+ lineage choice, memory cell phenotype differentiation, and Foxp3+ Treg development. Despite lacking the conventional Vβ domain of the TCR, these cells are strictly selected on classical MHC molecules during development, indicating that the entire Vβ domain is dispensable for MHC recognition and lineage choice. These data support a model of MHC recognition and T cell selection in which factors including MHC–coreceptor interactions direct MHC restriction of the αβ T cell repertoire rather than a germline-encoded structural bias of TCR for MHC.

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Disclosures
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References


