Thymic Selection of CD8+ Single Positive Cells with a Class II Major Histocompatibility Complex-restricted Receptor

By Jörg Kirberg, Agnes Baron, Sylvia Jakob, Antonius Rolink, Klaus Karjalainen, and Harald von Boehmer

From the Basel Institute for Immunology, 4005 Basel, Switzerland

Summary

We describe mice that express a transgenic T cell receptor α/β (TCR-α/β) specific for peptide 111-119 from influenza hemagglutinin presented by I-Eα class II major histocompatibility complex (MHC) molecules. The transgenic TCR is expressed on CD4+8- as well as CD4-8+ mature T cells even in mice that are deficient in rearrangement or do not express endogenous TCR-α/β genes. The CD4+8+ T cells require I-Eα class II MHC molecules for positive selection and can be activated to proliferate and to kill by I-Eα molecules presenting the relevant peptide. Full maturation of these cells, however, also requires the presence of class I MHC molecules. The results are compatible with the notion that T cell maturation requires multiple receptor-ligand interactions and establish an exception to the rule that class II-restricted TCRs are exclusively expressed by mature CD4+8- cells.

There were also reports on the existence of CD4+ T cells that could recognize class I MHC antigen (10-12). Unfortunately it was not clear whether this was simply due to some crossreactivity of class II MHC-selected cells, or whether these cells were selected by class I MHC molecules.

Here we present another apparent exception to the rule of "matched" specificity of coreceptor and TCR-α/β on mature T cells. We have analyzed TCR transgenic mice with a TCR specific for peptide 111-119 from influenza hemagglutinin presented by I-Eα class II MHC molecules. In these mice, we have detected a small but significant portion of mature CD4-8+ T cells bearing a class II MHC-restricted TCR-α/β, even when the transgenic TCR required I-Eα class II MHC molecules for positive selection in the thymus. Full maturation of these cells, however, also required the presence of class I MHC molecules.

In accord with previous data, our results indicate that downregulation of the CD4 coreceptor does not require binding of the TCR-α/β to thymic class I MHC molecules, at least in a subset of developing cells; yet class I MHC molecules are required for full maturation of these CD4-8+ cells bearing a class II MHC-restricted TCR.

Materials and Methods

Mice. BALB/c and C56BL/6 (B6) mice were obtained from IFFA-Credo (France). B10.GD mice, of H-2d haplotype (13),
Figure 1. Thymus (A) and lymph nodes (B) of TCR transgenic mice on H-2\(^d\) and H-2\(^b\) MHC haplotype. Single cell suspensions of thymus or lymph nodes from transgenic BALB/c and B6 (F2) mice were used for three colour immunofluorescence. Lymphocytes were depleted of slg\(^+\) cells before staining. Absolute numbers were \(8.3 \times 10^7\) and \(10.7 \times 10^7\) thymocytes and \(6.5 \times 10^7\) and \(7.0 \times 10^7\) lymphocytes in H-2\(^d\) and H-2\(^b\) mice, respectively. All CD4\(^+\)8\(^-\) and CD4\(^-\)8\(^+\) mature T cells stained homogeneously positive with TCR V\(_{\beta}\)-specific mAbs.
nabeads (Milan Analytica, La Roche, Switzerland). In some experiments T cells were depleted by staining with Thy-1-specific mAbs followed by complement (Low-Tox Rabbit Complement; Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) treatment, as recommended by the manufacturer.

**Antibodies and FACS® Analysis.** Hybridoma supernatants containing mAbs were purified by protein A or protein G (Pharmacia LKB, Uppsala, Sweden) affinity chromatography. mAbs were labeled using biotin- or fluoresceine-succinimidyl-ester (FLUOS) (Boehringer Mannheim, Mannheim, Germany) or FITC (Nordic Immunology, Tilburg, Netherlands) according to the manufacturers instructions. The following mAbs have been used: T19.191 (anti-D^d^) (Hämmerling, G. J., unpublished results), AF6-88.5.3 (anti-K^k^) (16), MKD6 (anti-I-A^d^) (17), AF6-120.1.2 (anti-I-A^k^) (16), T24 (anti-Thy-1) (18), M5/114 (anti-I-A^D^k^, anti-I-E^D^k^) (19), M1/69 (anti-heat-stable antigen [HSA]) (20), F23.1 (anti-TCR V_{mm} (21), and 6.5 (anticlonotypic TCR). The 6.5 mAb was produced in rats immunized with the soluble TCR obtained as described in (22).

Biotinylated or FITC-labeled F23.1 and FLUOS-labeled 6.5 mAbs were used for TCR detection. Anti-CD8-biotin (Becton Dickinson & Co.), anti-CD8-PE (Caltag Laboratories, S. San Francisco, CA), anti-CD4-PE (Becton Dickinson & Co.) and anti-CD8-Red613 (GIBCO BRL, Gaithersburg, MD) conjugates were obtained commercially. Class II MHC expression was determined by staining with M5/114 mAbs; HSA-specific mAbs M1/69 were biotinylated. With these and second step reagents streptavidin-allophycocyanin (APC, Molecular Probes Inc., Eugene, OR), streptavidin-PE (Southern Biotechnology, Birmingham, AL) or goat anti-mouse Ig-F(ab)_2-FITC (Silenus Laboratories, Pty Ltd., Hawthorne, Victoria, Australia) one-, two-, three-, and four-color flow cytometry was performed on FACScan®, FACStar+® or FACS-Vantage® (Becton Dickinson & Co.) instruments equipped appropriately.

Staining was done in 96-well plates (10^6 cells/well) in 100 µl
Results

**TCR Transgenic Mice.** The productively rearranged TCR-α and β genes were cloned from a T cell hybridoma 14.3.d (derived from a T helper clone V2-15) specific for peptide 111–119 of influenza hemagglutinin (24) presented by class II I-E^d^ MHC molecules as described previously (22, 25). The receptor is encoded by V_{β}2.3, J_{β}2.1 as well as V_{α}1, J_{α}2B4 variable TCR gene segments. Genomic DNA was injected into fertilized (B6 × DBA/2)F2 eggs. Since initially the transgenic offspring showed little expression of the transgenic TCR-α gene, the TCR-β enhancer was introduced downstream of the Cα enhancer. The resulting transgenic mice were backcrossed for four to five generations to mice of the BALB or B6 background.

Expression of TCR-α and β Genes. As expected from previous results, the transgenic mice exhibited an increased ratio of CD4^+ to CD8^+ single positive cells in the thymus, even though <20% of the CD4^+ cells stained with the clonotypic 6.5 antibody. In fact, similarly skewed ratios were already seen in mice that expressed only the TCR-β transgene, irrespective of whether the mice were of the b/b or d/d MHC haplotype (our unpublished results). It is noteworthy that in the thymus of mice with the d/d but not the b/b MHC haplotype, a significant portion not only of the CD4^+ but also of the CD4^+ cells, bear high levels of the transgenic TCR (Fig. 1 A). In lymph node cells, the proportions of CD4^+ T cells with high levels of the transgenic TCR are not really different in MHC b/b vs. d/d mice, presumably because of peripheral selection of cells with endogenous TCR-α chains in both types of mice such that most CD4^+ express either low or only intermediate levels of the transgenic TCR-α chain (Fig. 1 B).

CD4^-8^- T Cells with the Transgenic TCR Are Stimulated to Proliferate and to Kill by Class II MHC Presented Peptide. Spleen cells from the TCR transgenic mice were first stimulated by BALB/c spleen cells in the presence or absence of peptide and tested for cytotoxicity on LPS blasts (class II MHC^+) and Con A blasts (class II MHC^-) with or without peptides as targets. While both targets were equally lysed by B6 anti-BALB/c killer cells, only peptide-pulsed LPS targets but not peptide-pulsed Con A targets were lysed (fig. 2, A and C). We also obtained effector cells by stimulating cells from TCR transgenic mice with spleen cells from a mouse that expresses hemagglutinin as a transgene under the control of Igκ promoter and enhancer (Rolink, A., manuscript in preparation). From the same mice LPS targets were prepared that were lysed by various effector cells (Fig. 2 C). These data, and other data (see Fig. 2, legend) with LPS blasts from I-E^d^ and I-E^d^- mice, showed that all cytolytic T cells were specific for the class II MHC presented peptide 111–119. In Fig. 2 B, CD4^-8^- effector cells were separated from CD4^-8^- effector cells before the killer assay. It can be seen that CD4^-8^- cells give rise to much more potent effector cells than CD4^-8^- cells.

CD4^-8^- T Cells with the Class II MHC-restricted Transgenic TCR in RAG-2^-/- or TCRα-β^−/- Mice. To rule out the possibility that CD4^-8^- T cells with the class II MHC-restricted, transgenic TCR were positively selected because of additional receptors composed of endogenous TCR α chains associated with transgenic TCR-β chains (26–28), the TCR transgenic mice were crossed with RAG-2^-/- and TCRα-β^-/- mice. From the intercrosses we obtained TCR transgenic RAG-2^-/- and TCRα-β^-/- mice that were either of the d/d or b/b MHC haplotypes. It can be seen in
spleen cells and tested either unseparated or after sorting into CD4⁺8⁻ or CD4⁺8⁺ cells. The effector/target ratio of sorted effectors was adjusted to equal the ratio of that population in the unseparated effectors. The increased lysis by CD4⁺8⁺ sorted effectors compared to unseparated effectors might be due to removal of cold target cells carried over from the primary MLR in case of the latter. Contamination of CD4⁺8⁺ effectors by CD4⁺8⁻ cells can not explain the extent of CD4⁺8⁻-mediated killing as it should exceed 5% to explain the killing. However, cell-sorter reanalysis showed that the actual contamination was well below this level. We also purified CD4⁺8⁻ and CD4⁺8⁺ cells before the primary MLR and obtained similar results (data not shown).

Fig. 3 A that significant numbers of mature CD4⁺8⁺ cells with high levels of the transgenic TCR were present in the thymus of d/d but not b/b transgenic mice. The few CD4⁺8⁻ cells in the RAG-2⁻/⁻ b/b mice were mostly HSAhigh and had lower levels of the TCR, and were thus, by these criteria, very immature cells, not affected by positive selection. Thus, both CD4⁺8⁻ as well as CD4⁺8⁺ cells with high levels of the transgenic TCR required positive selection on H-2b MHC molecules (Fig. 3 A). I-Ea⁺ class II MHC molecules were required for the selection of

Figure 2. Transgenic effector cells are specific for peptide presented by class II I-Ea MHC molecules (A and C) and present among both CD4⁺8⁻ and CD4⁺8⁺ cells (B). Spleen cells of TCR transgenic mice were stimulated with BALB/c spleen cells in presence or absence of peptide 110-119 (A-C) or with BALB/c hemagglutinin transgenic spleen cells (C). Effector cells were analyzed on day 4 or 5 on LPS (A-C) and Con A (A and C) blasts, some of them loaded with peptide 110-119 or obtained from hemagglutinin transgenic mice. LPS blasts were >90% class II positive, whereas Con A blasts contained <5% class II positive cells as determined by flow cytometry (not shown). Spleen cells of TCR transgenic mice stimulated in the absence of antigen generated no effector cells (at highest effector/target ratio below 5 and 20% release in A and C, respectively; not done in B). B6 anti-BALB/c effector cells killed LPS and Con A BALB/c targets (+/- peptide) to a similar extent, e.g., >50% at an effector/target ratio of 10:1 in A, >80% at 1:1 in C; not done in B. Peptide pulsed LPS blasts from I-Ea−/− negative strains (see Table 1) were not killed. In B effector cells were generated from transgenic mice and tested either unseparated or after sorting into CD4⁺8⁻ or CD4⁺8⁺ cells. The effector/target ratio of sorted effectors was adjusted to equal the ratio of that population in the unseparated effectors. The increased lysis by CD4⁺8⁺ sorted effectors compared to unseparated effectors might be due to removal of cold target cells carried over from the primary MLR in case of the latter. Contamination of CD4⁺8⁺ effectors by CD4⁺8⁻ cells can not explain the extent of CD4⁺8⁻-mediated killing as it should exceed 5% to explain the killing. However, cell-sorter reanalysis showed that the actual contamination was well below this level. We also purified CD4⁺8⁻ and CD4⁺8⁺ cells before the primary MLR and obtained similar results (data not shown).
Figure 3. Thymus and lymph nodes in TCRoeE-/- and RAG-2-/- TCR transgenic mice. Thymocytes (A) and peripheral cells (from lymph nodes, B) of various mice were prepared and used for four-color flow cytometry. CD4+8+ 6.5+ cells in lymph nodes of H-2b mice repeatedly had lower levels of CD8 as CD4+8+ 6.5+ cells in H-2d mice.

Thymic Selection of CD8+ Class II MHC-restricted T Cells


Table 1. H-2 Haplotypes and Effect on Selection in TCR Transgenic Mice

<table>
<thead>
<tr>
<th>Alleles at H-2 loci:</th>
<th>Positive selection of CD4+8− and CD4−8+ 6.5hi cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K  Aβ  Aα  Eβ  Eα  D</td>
</tr>
<tr>
<td>H-2d</td>
<td>d    d  d    d  d    d</td>
</tr>
<tr>
<td>H-2d×b</td>
<td>d    d  d    d  d    d</td>
</tr>
<tr>
<td>H-2b</td>
<td>b    b  b    b  b    b</td>
</tr>
<tr>
<td>H-2d×g2</td>
<td>d    d  d    d  b    b</td>
</tr>
<tr>
<td>H-2d</td>
<td>d    d  d    d  b    b</td>
</tr>
</tbody>
</table>

The crossover in Eβ2 has been localized to the intron between the Eβ1 and Eβ2 exons (35). The peptide presenting domain of the Eβ2 molecule is therefore of Eα origin and Eβ2 is likely to behave like Eα as shown in this table. Positive selection of CD4−8+ 6.5hi cells was never observed in the absence of positive selection of CD4+8− 6.5hi cells.

With regard to lymph nodes of the various mice, the picture is slightly different from that of the thymus; whereas

Figure 4. Thymocytes from βm+ and βm− TCR transgenic mice of H-2d or H-2b MHC haplotype. Single cell suspensions of thymocytes were prepared from 8-10-wk-old mice and stained and analyzed by four-color flow cytometry. Total numbers of thymocytes (× 10³) were 7.5 and 9.0 for H-2b and 14.4 and 11.6 for H-2d mice expressing or lacking β2m, respectively.
there are very few CD4⁺8⁻ cells in the lymph node of RAG-2⁻/⁻ H-2b TCR transgenic mice, there are few cells with low CD8 levels that express the transgenic receptor (2 \times 10⁶) (Fig. 3 B). These cells may represent a different lineage of T cells that do not require positive selection and are more abundant in TCR transgenic than normal mice (31).

**Full Maturation of Class II MHC-restricted CD4⁺8⁺ Cells Requires Class I MHC Molecules.** To test whether CD4⁺8⁺ T cells with a class II MHC-restricted TCR could be generated in the absence of class I MHC molecules, we analyzed the thymus of TCR transgenic, β₂m⁻/⁻ mice of the d/d or b/b MHC haplotype (Fig. 4). CD4⁺8⁺ cells with high levels of the transgenic TCR were detected in both β₂m⁻/⁻ or β₂m⁻⁻/⁻ H-2d mice, but not in β₂m⁺ or β₂m⁻⁻/⁻ H-2b mice. However, there was a clear-cut difference between the CD4⁺8⁺ T cells with high levels of the transgenic TCR in H-2d β₂m⁻/⁻ and β₂m⁻⁻/⁻ H-2b mice. These results indicate that the CD4⁺8⁺ T cells with class II MHC-restricted transgenic TCR were undergoing positive selection when their receptor binds to class II MHC molecules in the thymus, but that the full maturation into CD4⁺8⁺ HSA negative cells requires the presence of class I MHC molecules.

For that reason CD4⁺8⁺ cells with high levels of the transgenic TCR were virtually absent in lymph nodes of H-2b β₂m⁻⁻/⁻ mice, as were cells with additional receptors containing endogenous TCR α chains (Table 2). In H-2b mice we are able to detect very few CD4⁺8⁺ HSA⁺⁺⁺⁺ cells. These cells may have been selected through receptors containing endogenous TCR α chains (H-2b β₂m⁻/⁻ mice) or may belong to an abnormal subset as described above (H-2b β₂m⁻⁻/⁻ mice).

**Discussion**

Our results document the existence of mature CD4⁺8⁺ cells with a class II MHC-restricted TCR. These cells require positive selection by class II MHC molecules. Because CD4⁺8⁺ thymocytes with a class II MHC-restricted TCR are also present in β₂m⁻⁻/⁻ mice, our data indicate that downregulation of CD4 coreceptors on developing CD4⁺8⁺ thymocytes does not necessarily require ligation of the TCR-α/β to class I MHC molecules, at least in a fraction of developing cells. These data are in accord with the previously published data that demonstrated the existence of CD4⁺8⁺ cells with a class I MHC-restricted TCR as well as CD4⁺8⁺ cells in class II MHC-deficient mice (5). It has previously been assumed that the full maturation of single positive T cells requires coengagement of MHC molecules by the TCR-α/β as well as the respective coreceptor (3-7, 32-34). Our data suggest that there may be exceptions to this rule; we find that the presence of class I MHC molecules can induce the full maturation of CD4⁺8⁺ HSA⁺⁺⁺⁺ T cells into CD4⁺8⁺ HSA⁻ cells, even though they express a class II MHC-restricted TCR. One reason for this might be that the transgenic class II MHC-restricted TCR has some affinity for both class I and class I MHC molecules. We think that this is not a very likely explanation for several reasons. First, positive selection of cells with the transgenic TCR necessarily requires class II MHC molecules. Neither K⁺ nor D⁺ MHC molecules were sufficient for positive selection of cells with the transgenic TCR. Second, in very sensitive binding assays the soluble transgenic TCR failed to bind to class I MHC molecules, although binding to class II MHC molecules, even in the absence of the specific peptide, could easily be visualized (22, Karjalainen, K., unpublished results). It could thus be that for full maturation of some α/β T cells, it suffices when the TCR-α/β binds to one class of MHC molecules (in our case class II) and the coreceptor to the other class (in our case I). This may be sufficient with TCRs of relatively high affinity that can induce activation of mature T cells in the absence of coreceptors, as has been reported for the transgenic TCR studied here (Karjalainen, K., unpublished results). One might assume that some of these receptors also have higher affinity for self MHC molecules in the absence of the specific peptide. Our data are consistent with the notion that the full maturation of α/β T cells requires more than a single interaction of the TCR-α/β or

<table>
<thead>
<tr>
<th>Tg β₂m⁺ H-2d</th>
<th>22.2</th>
<th>20.0</th>
<th>1.30</th>
<th>2.06</th>
<th>0.28</th>
<th>0.07</th>
<th>0.21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg β₂m⁺ H-2b</td>
<td>27.1</td>
<td>26.8</td>
<td>1.75</td>
<td>0.04</td>
<td>0.01</td>
<td>0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Tg β₂m⁻ H-2b</td>
<td>25.8</td>
<td>22.8</td>
<td>0.39</td>
<td>1.81</td>
<td>0.14</td>
<td>n.d.*</td>
<td>0.13</td>
</tr>
<tr>
<td>Tg β₂m⁻ H-2b</td>
<td>23.5</td>
<td>22.7</td>
<td>0.30</td>
<td>0.05</td>
<td>0.03</td>
<td>n.d.</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* n.d., not detectable as too few cells were acquired to give an exact number.

Absolute cell numbers of indicated cell populations in lymph nodes were calculated from total cell numbers and percentages of cells determined by four-color immunofluorescence as shown in Fig. 4.
CD4/CD8 coreceptors with intrathymic ligands (5). They also provide an exception to the rule that class II and class I MHC-restricted receptors are exclusively expressed by mature CD4⁺ and CD8⁺ single positive T cells, respectively. It remains to be seen how frequent these exceptions are which can only be rigorously tested in TCR transgenic mice that do not produce endogenous TCR proteins. Our results show further that CD4⁻8⁻ T cells can be activated by class II MHC-presented peptides to become potent killer cells. One could imagine that the activation of CD4⁻8⁻ lytic T cells by class II MHC-presented peptides could be of significance in regulating immune responses, for instance by destroying class II positive antigen-presenting cells. Such cells could also be of pathophysiological importance either in protective immune responses or in autoimmunity.

We thank Mark Dessing and Stefan Meyer for expert help with flow cytometry, cell sorting, and handling huge amounts of data; the animal caretakers for making possible a large breeding program; Hans-Peter Stahlberger for art work; and Nicole Schoepflin for typing various versions of this manuscript. The invaluable TCR clonotype-specific mAb 6.5 was produced by B. Riwar and H. Kishi (unpublished). TCRα⁻/⁻, β⁻/⁻, βγ⁻/⁻, and B10.GD mice were kindly provided by Drs. M.J. Owen, F. Alt, R. Jaenicke, and van Mourick, respectively.

The Basel Institute for Immunology was founded and is supported by F. Hoffmann-La Roche Ltd., Basel, Switzerland.

Address correspondence to Jörg Kirberg, Basel Institute for Immunology, Grenzacherstrasse 487, CH-4005 Basel, Switzerland.

Received for publication 7 February 1994 and in revised form 22 March 1994.

References


