Peripheral T Cell Survival Requires Continual Ligation of the T Cell Receptor to Major Histocompatibility Complex–Encoded Molecules

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Summary
In the thymus, T cells are selected according to their T cell receptor (TCR) specificity. After positive selection, mature cells are exported from primary lymphoid organs to seed the secondary lymphoid tissue. An important question is whether survival of mature T cells is an intrinsic property or requires continuous survival signals, i.e., engagement of the TCR by major histocompatibility complex (MHC) molecules in the periphery, in a similar way as occurring during thymic positive selection. To address this issue we used recombination-activating gene (Rag)-deficient H-2b mice expressing a transgenic TCR restricted by I-Ed class II MHC molecules. After engraftment with Rag2−/− H-2d fetal thymi, CD4+8− peripheral T cells emerged. These cells were isolated and transferred into immunodeficient hosts of H-2b or H-2d haplotype, some of the latter being common cytokine receptor γ chain deficient to exclude rejection of H-2b donor cells by host natural killer cells. Our results show that in the absence, but not in the presence of selecting MHC molecules, peripheral mature T cells are short lived and disappear within 7 wk, indicating that continuous contact of the TCR with selecting MHC molecules is required for survival of T cells.

Materials and Methods
Mice. BALB/c and BALB/c nu/nu mice were from IFFA-Credo (Orléans, France). BLACK nu/nu mice were from Bom-holtgart (Ry, Denmark) and screened for H-2b homozygosity. Rag-2−/− mice were from Bom-holtgart (Ry, Denmark) and screened for H-2b homozygosity. R ag-2−/− and Rag-1−/− (19) deficient mice were from Bom-holtgart (Ry, Denmark) and screened for H-2^d homozygosity.

1 Abbreviations used in this paper: dGuo, 2'-deoxyguanosine; Rag, recombination activating gene; slg, surface Ig; HSA, heat stable antigen.
gous. Hemagglutinin-specific TCR transgenic mice (ABII TCR) on R ag-2-/- background have been described (8, 20). H-2b R ag-2-/- mice were obtained from Drs. Antonius Rolink and Shunichi Takeda (Bazal Institute for Immunology, Basel, Switzerland; reference 15). These mice were crossed with IL-2R γ-/- mice to obtain H-2b R ag-2-/- IL-2R γ-/- mice. All breeding was done in the animal colonies at the Basel Institute for Immunology (Basel, Switzerland) and at the Netherlands Cancer Institute (Amsterdam, The Netherlands).

B Cell Depletion, Cell Sorting, and FACS Analysis. Single cell suspensions of thymus, lymph nodes, and/or spleen (RBCs lysed or removed by Ficoll density gradient centrifugation) were prepared in PBS with 2% FCS. Where applicable, surface immunoglobulin-positive (sIg+) cells were depleted using Dynabeads (Milenyi, Switzerland).

6.5 (anti-ABII-TCR; reference 8) and MKD6 (anti-I-Aβ; reference 21) mAbs were biotinylated. FITC-labeled 104.2.1 mAbs (Boehringer Mannheim, Mannheim, Germany). Heat stable antigen (HSA)-specific reference 21) mAbs were labeled with FLUOS (Boehringer Mannheim, Mannheim, Germany).

CD4-PE, anti-CD8-Red613 (GIBCO BRL, Gaithersburg, MD), and streptavidin-allophycocyanin (Molecular Probes Inc., Eugene, OR) conjugates were obtained commercially.

Results

H-2d-restricted CD4+8- T cells in T hyms Graft 2 ABII TCR R ag-2-/- H-2d Mice. In our studies, we used ABII TCR transgenic mice that express a transgenic TCR specific for peptide 111-119 of influenza hemagglutinin presented by the percentage of cells within the region of interest as determined by FACS® analysis. In case of staining before and after depletion of sIg+ cells, the average of both determinations, taking into account sIg+ cell depletion, was used for calculation.

Lymphocyte Proliferation Assay. Cell sorter purified responder cells were cultured with 5 x 10^5 X-irradiated (2,200 rad) stimulators in 200 µl Iscove’s modified Dulbecco’s medium supplemented with FCS (10%), β-mercaptoethanol (5 x 10^-5 M), penicillin (100 IU/ml), and streptomycin (100 mg/ml). To some cultures, peptide 107-119 of influenza hemagglutinin (SVSS-FERFEIPFK) was added at a final concentration of 5 µM. Cultures were kept in a water-saturated atmosphere of 6% CO₂ in air at 37°C. After 48–60 h, 1 µCi [³H]thymidine (Amersham Corp., Arlington Heights, IL) was added and cells were cultured for a further 12–24 h after they were harvested. Incorporated radioactivity was measured by standard liquid scintillation counting.

Figure 1. Thymic development of CD4+8- T cells in H-2d fetal thymus-grafted H-2b ABII TCR R ag-2-/- H-2d Mice. In our studies, we used ABII TCR transgenic mice that express a transgenic TCR specific for peptide 111-119 of influenza hemagglutinin presented by
I-E<sup>d</sup> class II MHC molecules. These mice were crossed onto the R<sup>ag</sup>-/- background to exclude the interference of TCRs with unknown specificity due to lack of allelic exclusion of the TCR-α locus (25, 26). To obtain T cells that are selected in the thymus but not able to encounter the selecting MHC molecules in peripheral lymphoid tissue, we transplanted H-2<sup>d</sup> ABII TCR R<sup>ag</sup>-/- mice with fetal thymus of H-2<sup>d</sup> haplotype that had previously been transplanted with fetal thymus lobes from H-2<sup>d</sup> R<sup>ag</sup>-/- mice. Cells were analyzed by four-color flow cytometry as shown.

To confirm that the CD4<sup>+</sup> T cells in grafted mice were functionally mature, cells from such mice as well as from H-2<sup>d</sup> ABII TCR R<sup>ag</sup>-/- mice were isolated and stimulated with antigen presented by H-2<sup>d</sup> APCs. As shown in Table 1, both populations of cells gave proliferative responses that were in the same order of magnitude, indicating that they had acquired functional competence.

There was, however, a clear difference in the absolute number of CD4<sup>+</sup> T cells in the thymus-grafted H-2<sup>d</sup> ABII TCR R<sup>ag</sup>-/- mice and the H-2<sup>d</sup> ABII TCR R<sup>ag</sup>-/- mice in that the former contained far fewer cells than the latter (2.9 x 10<sup>6</sup>; n = 3); and ranging between 10<sup>5</sup> to 10<sup>5</sup>, respectively). This difference could be due to the fact that fewer CD4<sup>+</sup> T cells are produced/exported from the grafted thymus and/or the fact that the lack of I-E<sup>d</sup> class II MHC molecules in the peripheral lymphoid organs of grafted H-2<sup>d</sup> ABII TCR R<sup>ag</sup>-/- mice resulted in a shorter life span of these cells. Further, the few CD4<sup>+</sup> T cells might be continuously renewed and/or depend on few I-E<sup>d</sup>-expressing cells originating from the fetal thymus graft. To address the issue of whether peripheral expression of MHC molecules the CD4<sup>+</sup> T cells were selected on in the thymus was required for their peripheral survival, we performed transfer experiments into immunodeficient hosts expressing different MHC molecules.

| Table 1. Proliferation of CD4<sup>+</sup> T Cells from H-2<sup>a</sup> ABII TCR R<sup>ag</sup>-/- Mice | Stimulators (2,200 rad) |
|-----------------------------------------------|---------------------|
| CD4<sup>+</sup> responders of                  | BALB/c nu/nu       |
|                                               | BALB/c nu/nu + peptide |
| ABII RAG-2/-/- H-2<sup>d</sup>                 | 600                 | 15,000                |
| ABII RAG-2/-/- H-2<sup>b</sup> H-2<sup>d</sup> | 260                 | 23,000                |
| N one                                         | 160                 | 170                   |

CD4<sup>+</sup> lymphocytes were cell sorted purified from H-2<sup>a</sup> ABII TCR R<sup>ag</sup>-/- and H-2<sup>b</sup> ABII TCR R<sup>ag</sup>-/- mice that had previously been transplanted with an H-2<sup>R</sup> R<sup>ag</sup>-/- fetal thymus. 5 x 10<sup>5</sup> responder cells were cultured with irradiated BALB/c nu/nu stimulators. To some cultures, peptide had been added as source of antigen.
Peripheral T Cell Survival and TCR–MHC Interaction

As shown in Fig. 4, 6.5<sup>1</sup>CD4<sup>18<sup>2</sup></sup> cells survived only in H-2<sup>d</sup> recipients, whereas 6.5<sup>1</sup>CD4<sup>28<sub>low</sub></sup> cells survived both in H-2<sup>b</sup> and H-2<sup>d</sup> recipients when analyzed 7 wk after transfer. Taken together, these results show that in H-2<sup>b</sup> recipients, NK cells do not reject the transferred cells as 6.5<sup>1</sup>CD4<sup>28<sub>low</sub></sup> cells survive, but 6.5<sup>1</sup>CD4<sup>18<sup>2</sup></sup> cells vanish due to the lack of H-2<sup>d</sup> MHC molecules. In contrast, 6.5<sup>1</sup>CD4<sup>18<sup>2</sup></sup> cells survive long term in the presence of H-2<sup>d</sup> MHC molecules once not rejected by NK cells. 6.5<sup>1</sup>CD4<sup>28<sub>low</sub></sup> cells survived then as well.

In other experiments, we have followed the fate of CD4<sup>18<sup>2</sup></sup> and CD4<sup>28<sub>low</sub></sup> cells from thymus-grafted H-2<sup>d</sup> ABII TCR Rag<sup>2/2</sup> mice upon transfer into either H-2<sup>b</sup> or H-2<sup>d</sup> immunodeficient recipient mice. (The H-2<sup>d</sup> ABII TCR Rag<sup>2/2</sup> mice themselves cannot select the ABII TCR in the thymus due to preferential pairing of E<sup>a</sup> with Eβ<sup>b</sup> chains; references 8, 31, 32.) 3 d after transfer, both populations were present in H-2<sup>d</sup> nu/nu and H-2<sup>d</sup> nu/nu recipients. 7 wk later, both subsets were present in H-2<sup>d</sup> nu/nu recipients but could no longer be found in H-2<sup>b</sup> nu/nu recipients (data not shown, made available to reviewers). This might indicate that the shared class I MHC molecule expression between H-2<sup>d</sup> hybrid donor and homozygous host protected the donor cells from NK cell-mediated lysis in H-2<sup>d</sup> homozygous hosts. Such differences might become evident only upon transfer of relatively small numbers of cells as performed here. An inverse correlation between the level of class I expression and NK cell lysis has indeed been observed (33), and NK cell-mediated lysis of hybrid cells did not always occur to the same extent in each of the parental strains (34). Further, H-2<sup>b</sup> cells expressing transgene-encoded D<sub>d</sub> MHC class I molecules were rejected by NK cells in otherwise syngenic H-2<sup>b</sup> hosts (35).

**Discussion**

Our data show that appropriate MHC molecules are required to support the survival of mature αβ T cells in peripheral lymphoid tissue; a conclusion in line with previously published experiments (15). In the latter study, however, polyclonal CD4<sup>18<sup>2</sup></sup> T cells in thymus-grafted RAG class II MHC double deficient mice appeared to survive much longer since significant numbers of cells could still be found 16 wk after export from the thymus ceased. This may be due to the fact that in these experiments, CD4<sup>18<sup>2</sup></sup> T cells with class I MHC–restricted TCRs (36–38) could interact with class I MHC molecules expressed in the peripheral lymphoid tissue or due to the fact that some class II MHC–positive cells had migrated from the transplanted thymus into the periphery. In both of these cases, T cells could have been stimulated by antigen. The high proportion of proliferating CD4<sup>18<sup>2</sup></sup> T cells at various points in time after thymus transfer could indicate that this was indeed the case, and some CD4<sup>18<sup>2</sup></sup> T cells might have rather disappeared because of exhaustion (39, 40). These possibil-

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Figure 3. CD4<sup>18<sup>2</sup></sup> lymphocytes from H-2<sup>d</sup> thymus-grafted H-2<sup>b</sup> ABII TCR Rαg<sup>−/−</sup> mice disappear in H-2<sup>b</sup> adoptive hosts. 7.5 × 10<sup>6</sup> CD4<sup>18<sup>2</sup></sup> and 5 × 10<sup>6</sup> CD4<sup>18<sup>2</sup></sup> fetal thymus-transplanted H-2<sup>b</sup> ABII TCR Rαg<sup>−/−</sup> mice were adoptively transferred into H-2<sup>b</sup> and H-2<sup>d</sup> nu/nu mice. 7 wk later, cells from lymph nodes and spleen (RBCs lysed) were isolated, depleted of sIg<sup>1</sup> cells, and analyzed by four-color flow cytometry. Data from lymph nodes and spleen gave similar results. Calculated numbers of 6.5<sup>1</sup>HSA<sup>2</sup> cells were 5,600, 6,500, and 1,800 for CD4<sup>18<sup>2</sup></sup> and 1,800, 463,600, and 400 for CD4<sup>18<sup>2</sup></sup> cells in unmanipulated H-2<sup>b</sup> (one mouse), injected H-2<sup>b</sup> (average of two mice), and injected H-2<sup>d</sup> (average of two mice) nu/nu mice, respectively. (Note that numbers also contain the calculated background values of mice not injected.) Another experiment and two experiments using H-2<sup>b</sup> Rαg<sup>−/−</sup> and H-2<sup>d</sup> Rαg<sup>−/−</sup> recipients gave similar results.
mature CD4+ T cells were of known antigen specificity and MHC restriction. Any potential class II MHC molecule–expressing cells originating from the thymus graft were removed by cell sorting before transfer into adoptive recipients. Any potential class II MHC molecule–expressing cells were of known antigen specificity and MHC restriction, whereas peripheral T cell survival requires TCR interaction with selecting MHC molecules on any type of cell. The latter would be analogous to the requirement of TCR–MHC molecule interaction on thymic epithelial cells for thymic positive selection (3). Interestingly, RelB-deficient mice that lack dendritic cells have an increased proportion of activated T cells, whereas absolute numbers of T cells are reduced (52, 53). The former might be due to limited self-censorship in the thymus followed by peripheral expansion of CD4+8− T cells expressing the naïve phenotype that label with BrdU (42).

Irrespective of the MHC environment, CD4+8low cells that are not dependent on positive selection in the thymus, did expand after transfer. Presently, the biology of these cells is not well understood and it has been speculated that these cells represent γδ lineage T cells expressing the transgenic αβ TCR (27).

While it becomes established that T cells require the interaction of their TCRs with selecting MHC molecules for survival in the peripheral lymphoid tissue, the mechanism behind this requirement is unknown. In the thymus, immature CD4+8− cells express low levels of the cell death-repressing bcl-2 protein. They have a half life of 3 d (43) unless their TCR binds with sufficient affinity to self-MHC molecules resulting in maturation that is accompanied by bcl-2 upregulation (44–47). One might then speculate that the level of bcl-2 expression and, hence, survival is (indirectly) regulated by TCR ligation with selecting MHC molecules in the absence of antigen. In that respect, peripheral survival could be similar to thymic positive selection. The data reported on T cells from bcl-2-deficient mice are compatible with such a hypothesis (48–51). We have investigated bcl-2 expression by intracellular staining of CD4+8− cells from thymus-grafted H-2b ABII TCR Rag−/− mice and H-2d ABII TCR Rag−/− mice. However, the differences we observed were far less dramatic than during thymic positive selection (mean fluorescence reduced to 74 compared to 98 in controls, whereas in the thymus, a threefold difference was detectable: 44 versus 120 in an independent experiment). This could be due to the fact that cells with low bcl-2 expression are rapidly dying and eliminated and escape detection.

Further, it will be of interest to determine whether TCR contact with selecting MHC molecules on any type of cell is sufficient for T cell survival or whether the selecting MHC molecules have to be encountered on a specific cell type. The latter would be analogous to the requirement of TCR–MHC molecule interaction on thymic epithelial cells for thymic positive selection (3). Interestingly, RelB-deficient mice that lack dendritic cells have an increased proportion of activated T cells, whereas absolute numbers of T cells are reduced (52, 53). The former might be due to limited self-censorship in the thymus followed by peripheral activation by self-antigens the T cells were not tolerized for in the thymus (52–55). The latter, however, could indicate that (naive) peripheral T cell survival requires TCR interaction with selecting MHC molecules on dendritic cells. The data of DeKoning et al. on transfer of naive TCR transgenic T cells into RelB−/− mice support this theory (53).
We thank M. Deesing, S. Meyer, and E. Noteboom for expert help with flow cytometry and cell sorting; the animal care takers (especially E. Wagner and W. Metzger, and L. Tolkamp in Basel, Switzerland and Amsterdam, The Netherlands, respectively) for making possible these experiments; H.-P. Stahlberger for art work; P. Krumpenfort for providing the IL-2R γ-chain mice; and John D. Allen, Thomas Brocker, and Hergen Spits for reading the manuscript. The TCR clonotype specific mAb 6.5 was produced by B. Riwai and H. Kishi. The Basel Institute for Immunology was founded and is supported by F. Hoffmann-La Roche Ltd. (Basel, Switzerland). J. Kirberg receives a fellowship from the Boehringer Ingelheim Foundation (Stuttgart, Germany).

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Received for publication 2 May 1997 and in revised form 30 July 1997.

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