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*J Immunol* 2003; 171:1278-1284; doi: 10.4049/jimmunol.171.3.1278
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Chronic Exposure to Low Levels of Antigen in the Periphery Causes Reversible Functional Impairment Correlating with Changes in CD5 Levels in Monoclonal CD8 T Cells

Panagiota Stamou,† James de Jersey,* Danielle Carmignac,‡ Clio Mamalaki,‡ Dimitris Kioussis,* and Brigitta Stockinger†*

This study describes a double-transgenic model in which monoclonal CD8 F5 T cells are chronically exposed to self Ag (nucleoprotein) in the periphery, but are not affected during thymic development. Chronic exposure of CD8 T cells to their cognate Ag rendered them unable to proliferate or produce cytokines such as IL-2 and IFN-γ in response to antigenic stimulation in vitro. However, the cells still retained some killer function in vivo and continuously eliminated APC expressing high levels of Ag. In addition, when crossed with mice expressing Ag in the anterior pituitary gland (triple-transgenic mice), F5 T cells migrated to this site and killed growth hormone producing somatotrophs. The anergic state was reversible upon transfer into Ag-free recipients, resulting in full recovery of in vitro responsiveness to Ag. Anergic CD8 T cells express higher levels of CD5, a negative regulator of T cell signaling, whereas after transfer and residence in Ag-free hosts, CD5 levels returned to normal. This suggests that up-regulation of negative T cell regulators in peripheral T cells exposed to chronic stimulation by Ag may prevent full functionality and thus avoid overt autoreactivity. *The Journal of Immunology, 2003, 171: 1278–1284.

Peripheral tolerance mechanisms are thought to account for avoidance of autoreactivity in T cells that have for one reason or another escaped central tolerance induction during thymic development. Mechanisms underlying peripheral tolerance induction are diverse and in many cases not fully understood (1). Depending on how peripheral Ag is expressed, T cells may react in a variety of different ways. In some cases contact with self Ag is avoided because T cells may not have access to its site of expression (e.g., eye, brain, and testis) and thus T cells remain ignorant of the presence of self Ag (2, 3). In other cases excessive amounts of Ag encountered in the periphery can cause deletion following activation (4–6), whereas Ag expressed in low amounts or on nonprofessional APC is thought to cause T cell anergy, also termed adaptive tolerance (7–10). The functional activity and life span of so-called anergic T cells are compromised to different extent depending on the degree of antigenic stimulation they have been subjected to (11, 12).

We have used a double-transgenic model in which monoclonal CD8 F5 T cells are chronically exposed to self Ag in the periphery, but are not affected during thymic development. Chronic exposure of CD8 T cells to their cognate Ag rendered them unable to proliferate or produce cytokines such as IL-2 and IFN-γ in response to antigenic stimulation in vitro. However, the cells still retained some killer function in vivo. Dendritic cells (DCs)2 expressing high levels of nucleoprotein (NP) were absent in double-transgenic mice, but detectable in lymphocyte-deficient Rag−/− mice expressing NP. In addition, when crossed with mice expressing Ag in the anterior pituitary gland (triple-transgenic mice) (13), F5 T cells migrated to this site and killed growth hormone producing somatotrophs. The anergic state was reversible upon transfer into Ag-free recipients, resulting in full recovery of in vitro responsiveness to Ag. Anergic CD8 T cells express higher levels of CD5, a negative regulator of T cell signaling (14), whereas after transfer and residence in Ag-free hosts, CD5 levels returned to normal, suggesting that chronic stimulation by Ag may result in induction of negative T cell regulators that prevent full functionality.

Materials and Methods

Mice

Rag1−/− mice expressing a fragment encoding influenza A/NT/60/68 NP under control of the MHC class I H-2Kb promoter (termed NP47) (15) were crossed to F5 Rag1−/− mice harboring CD8 T cells with specificity for peptide 366–374 encoded by the NP transgene. The double-transgenic mice were termed NP47F5. Rag1−/− F5 mice expressing green fluorescent protein (GFP) under control of the CD2 promoter in all T cells (GFPF5) (16) were used to distinguish CD8 T cells from single-transgenic mice from those of double-transgenic mice. Syngeneic Rag1−/− H-2b mice were used as adoptive hosts. Mice expressing influenza NP in the anterior pituitary gland, termed strain 48 Rag−/− (13) were crossed to NP47F5 mice to obtain triple-transgenic mice, termed NP47F5 × 48 (all Rag−/−). All mice were bred in the animal facilities of the National Institute for Medical Research in accordance with established guidelines.

Determination of absolute CD8 T cell numbers

Absolute numbers of peripheral T cells were calculated as the number of CD8 T cells in the spleen plus two times the number of CD8 T cells in the mesenteric and inguinal lymph nodes which represent about one-half of the lymph nodes (17).

Flow cytometry and cell sorting

Cells were stained with fluorescent or biotin-labeled mAbs (all from BD Pharmingen, San Diego, CA): anti-CD4PE (H129.19), anti-CD8α-APC (53-6.7), anti-Vβ11-Bio (RR3-15), anti-CD44-PE and biotin (IM7), anti-
CD62-L-biotin (MEL-14), anti-CD5-PE and FITC (53-7.3), and anti-HSA-FITC (M1/69). CyChrome 7-PE-streptavidin (Caltag Laboratories, Burlingame, CA) was used as a secondary reagent for biotin. Single-cell suspensions were preincubated with unlabeled mAb to FcγRII/III (2.4G2) to minimize unspecific staining. Stainings with individual reagents were performed for 30 min on ice in FACS buffer (PBS, 2% FCS, 0.1% azide) and washed with FACS buffer. Mature CD8 single thymocytes were isolated for in vitro activation assays in two steps. First, NP47F5 or F5 thymus preparations were depleted of CD4, CD8 double-positive thymocytes using CD4 Dynabeads (Dynal, Oslo, Norway). Second, the remaining thymocytes were FACS sorted from CD4-negative-HSA<sup>+</sup> cells using anti-CD4-PE and anti-HSA-FITC mAbs. Four-color cytometry was done on a FACSCaliber flow cytometer (BD Biosciences, San Jose, CA) and the results were analyzed with CellQuest software. Cell sorting was done on a MoFlo cell sorter (Cytomation, Fort Collins, CO).

**In vitro T cells activation assays**

T cell activation assays were either performed with FACS-sorted T cells or total splenocytes adjusted to the same percentage of T cells as in F5 spleens by adding Rag<sup>−/−</sup> H-2<sup>b</sup> spleen cells with GM-CSF as described (18), in 96-well bottom plates with different concentrations of NP peptide. Triplicate cultures were plated of each T cell population tested. Cells were incubated at 37°C in 5% CO2 for 48 h. Proliferation of the responding cells was measured as incorporation of \(^{3}H\)thymidine, given for the last 16 h. IL-2 production was assessed on day 2 by transferring culture supernatants to fresh wells with 5 × 10<sup>3</sup> IL-2-dependent CTLL cells and proliferation was assessed by uptake of \(^{3}H\)thymidine or by fluorescence measurement of the dye Alam blue (19) (Bio-source, Nivelles, Belgium) (19). IFN-γ production was assayed on day 2 using sandwich ELISA with anti-IFN-γ mAb AN-18 and biotinylated R4-6A2. All assays were set up in triplicates and data are represented as mean absorbance values with SD.

**In vivo cytotoxicity assay**

Target cells were prepared for in vivo evaluation of cytotoxic activity as previously described (20). Briefly, cells from C57B10/10 spleen suspensions were pulsed with 1 μM NP peptide for 90 min at 37°C, washed, and labeled with CFSE (21) at a density of 10<sup>6</sup> cells/ml in a final concentration of 5 μM for 10 min. At 37°C. Uncoated control target cells were labeled at a lower CFSE concentration of 0.5 μM. For i.v. injection 5 × 10<sup>6</sup> cells of each population were mixed in 200 μl PBS. Specific in vivo cytotoxicity was determined by collecting spleen and lymph node cells from mice 19 h after injection of the target cells. Detection of the differentially labeled fluorescent target cell population was analyzed by flow cytometry. The ratio R between the numbers of uncoated vs NP coated (CFSE<sub>low</sub>/CFSE<sub>high</sub>) was calculated to target cell population was analyzed by flow cytometry. The ratio R between the numbers of uncoated vs NP coated (CFSE<sub>low</sub>/CFSE<sub>high</sub>) was calculated to obtain a numerical value of cytotoxicity.

**In vitro cytotoxicity assay**

Spleen cells from NP47F5, F5 and F5→NP47 donors (2.5 × 10<sup>6</sup>/ml) were cultured for 4 days in vitro in the presence of syngeneic bone marrow culture-derived DC (2.5 × 10<sup>6</sup>/ml). They were then tested for cytotoxic activity in the Jam test (22), using Con A-activated B10 spleen cells pulsed with NP peptide or unpulsed as target cells.

**Generation of BM chimeras**

Two-month-old GFP F5 or NP47F5 mice were treated with 1.0 Gy of ionizing radiation (\(^{60}Co\)), and then were reconstituted with 10 × 10<sup>6</sup> BM cells from NP47F5 or GFPF5 mice, respectively. After 8 wk the degree of chimerism was analyzed by FACS for the presence of endogenous or transferred T cells that can be differentiated by the expression or not of GFP. At this time point most of the peripheral T cells were of donor origin (>95%) for the NP47F5→GFPF5 chimeras and (>80%) for the GFPF5→NP47F5 chimeras.

**Generation of memory F5 CD8 T cells**

A total of 5 × 10<sup>6</sup> T cells from F5 Rag<sup>−/−</sup> hosts were transferred with 5 × 10<sup>6</sup> influenza virus-pulsed bone marrow culture-derived DC into syngeneic Rag<sup>−/−</sup> hosts. Resting memory T cells were obtained from 4 wk after transfer.

**In vitro Ag presentation assays**

Isolation of splenic APCs for in vitro Ag presentation assays was performed by enzyme digestion of whole spleens with 1.6 mg/ml collagenase type IV (Sigma-Aldrich, St. Louis, MO) and 0.1% deoxyribonuclease (DNase-I, fraction IX, Sigma-Aldrich) in Iscove’s modified Dulbecco medium (Sigma-Aldrich) for 45 min. Subsequently, DCs were positively selected by AutoMACS using anti-mouse CD11-c Microbeads (Miltenyi Biotec, Auburn, CA). The negative fraction was stained with biotinylated Ab F4/80 and macrophages were positively selected using streptavidin Microbeads (Miltenyi Biotec). A total of 1 × 10<sup>7</sup> F5 lymph node cells per well were cultured in triplicates in round bottom 96-well plates with a mixture of CD11-c- and F4/80-positive spleen cells isolated from either NP47, NP47F5, or Rag<sup>−/−</sup> H-2<sup>b</sup> mice. After 6 days of culture, IL-2 production in the culture supernatant was assessed with an Alamar blue-based CTLL assay. At the same time point (day 6) cultured cells were stained with anti-CD8-APC, CD44-BIO, CD69 FITC and CD25-PE mAbs and analyzed by FACS for the expression of activation markers on F5 CD8 T cells.

**Radioimmunoassays**

Mouse growth hormone in pituitary extracts was assayed by RIA, as previously described for the rat (23), using mouse reagents kindly provided by the National Institute for Diabetes and Digestive and Kidney Diseases.

**Results**

**CD8 T cells in double-transgenic NP47F5 mice develop normally in the thymus, but become tolerized in the periphery**

Although the NP transgene under control of a MHC class I promoter would be expected to be expressed ubiquitously on all cells in the body, line NP47 failed to express NP in a functional manner in the thymus. As a consequence, F5 T cells specific for NP undergo thymic development in double-transgenic NP47F5 mice indistinguishable from that of normal F5 mice, as indicated by the absolute number (Table I) and percentage of single-positive CD8 T cells (Fig. 1A), their level of TCR, and low expression of the activation marker CD44 (Fig. 1B).

Furthermore, purified single-positive CD8 thymocytes from double-transgenic mice proliferated in response to cognate NP peptide in vitro to the same degree as F5 CD8 single-positive thymocytes (Fig. 1C). In contrast, peripheral CD8 T cells from double-transgenic mice, while present in similar numbers as in F5 control mice (Table I), express lower levels of TCR (Fig. 1D) and have up-regulated the activation marker CD44 compared with peripheral F5 T cells (Fig. 1E). In addition, NP47F5 T cells failed to proliferate, secrete IL-2, or IFN-γ in response to NP peptide in vitro, resembling an anergic phenotype (Fig. 1F). Nevertheless NP47F5 T cells had comparable cytotoxic activity in vitro to F5 T cells. It should be emphasized that all mice used in this study were crossed onto a Rag1<sup>−/−</sup> background excluding any T cells of other specificities.

The anergic phenotype develops by interaction with NP-expressing bone marrow-derived cells

To establish whether the functional impairment of F5 T cells in NP47F5 mice is the result of interactions with nonprofessional, e.g., epithelial cells or with bone-marrow-derived DCs and macrophages, bone marrow chimeras were generated. NP47F5 mice were lethally irradiated, followed by injection of bone marrow

**Table I. Absolute T cell numbers in F5 and NP47F5 mice**

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SE (number × 10&lt;sup&gt;6&lt;/sup&gt;)</th>
<th>NP47F5</th>
<th>F5</th>
<th>p Value</th>
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<td>Total thymocytes</td>
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<td>77 ± 20</td>
<td>87 ± 17</td>
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<td>CD8 SP thymocytes</td>
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<td>14 ± 3.7</td>
<td>20 ± 5.9</td>
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<td>9.76 ± 1.2</td>
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</table>

<sup>a</sup> Total peripheral CD8 T cells were calculated as the absolute number of CD8 T cells in the spleen plus two times the absolute number of CD8 T cells in the lymph nodes.
from GFPF5 mice. In the resulting chimeras Ag expression is restricted to radioresistant, non-bone marrow-derived cells. As shown in Fig. 2A (group I), in such mice GFPF5 T cells developed normally, did not up-regulate CD44, and proliferated, as well as secreted cytokines in response to NP peptide in vitro (Fig. 2B). In contrast, bone marrow chimeras in which Ag expression was restricted to bone marrow-derived cells after lethal irradiation of GFPF5 mice followed by reconstitution with bone marrow from NP47F5 (Fig. 2A, group II), showed the phenotype and impaired function (Fig. 2B) observed in NP47F5 T cells.

Peripheral T cells from NP47F5 mice are heterogeneous, but a homogenous “anergic” phenotype is achieved by T cell transfer

The phenotype of peripheral T cells from NP47F5 mice indicated a degree of heterogeneity with some cells retaining high levels of TCR and low levels of CD44. This could be due to the continuous thymic output of naive T cells in F5 mice that do not undergo significant thymic involution (24). Recent thymic emigrants may be Ag responsive for a period of time before they are tolerized. To obtain a more homogenous population of “anergic” T cells, F5 T cells were transferred into NP47 (Rag1<sup>-/-</sup>/H<sup>11002</sup>/H<sup>11002</sup>) hosts and analyzed 20 days after transfer. At early points after transfer, F5 T cells expanded and then underwent a contraction phase (not shown), but by 20 days after transfer the number of recovered cells stabilized and they exhibited a homogenous CD44<sup>high</sup> phenotype (Fig. 3A). Their function was compromised as seen with NP47F5 T cells and likewise they exhibited cytotoxic activity in vitro (Fig. 3B). To be able to attribute functional behavior to a homogenous cell population, some of the experiments were conducted with NP47 mice that had received transfer of F5 mice at least 20 days before. These were termed F5<sup>3</sup>NP47 to distinguish them from T cells isolated directly from double-transgenic NP47F5 mice.

Tolerized F5 T cells retain some functionality in vivo

The results from the bone chimera experiments suggested that F5 T cells are chronically exposed to Ag presented by bone marrow-derived DCs, and macrophages which are responsible for the reduced in vitro functionality of these T cells. Nevertheless, their cytotoxic activity in vitro suggests that they might still retain some functionality. To determine whether F5 T cells chronically exposed to Ag have cytotoxic activity in vivo, a sensitive in vivo killing assay was used. Spleen cells from H-2<sup>b</sup> mice were labeled

**FIGURE 1.** Phenotypic characterization and in vitro function of thymic and peripheral NP47F5 CD8 T cells. Total F5 (A, left panel) or NP47F5 (A, right panel) thymocytes were stained for CD4, CD8, and TCR or for CD4, CD8, and CD44 mAbs. Percentages of individual thymic subpopulations are given in the contour plots. Histogram overlays compare expression of TCR (B, left panel) or CD44 (B, right panel) between F5 (gray filled histograms) and NP47F5 (black line) on the SP CD8 thymic subpopulation. Proliferation of SP mature CD8<sup>+</sup> cells was stained for CD8, TCR, and CD44 mAbs. Percentages of CD8 populations with different levels of expression of TCR are given in the contour plot. Histogram overlays (E) compare expression of CD44 on F5 (gray filled histogram) and NP47F5 (black line) on peripheral CD8 cells. Proliferation, IL-2 and IFN-γ production as well as cytotoxicity of peripheral F5 (●) and NP47F5 (■) T cells after stimulation with NP peptide (F).

**FIGURE 2.** Impact of NP expression on BM cells or on radioresistant cells in the phenotype and function of developing F5 T cells. A, Histogram overlay compares expression of CD44 surface marker on donor peripheral GFP<sup>+</sup> CD8<sup>+</sup> TCR<sup>+</sup> cells from type I chimeric mice (gray filled histogram), against donor peripheral GFP<sup>+</sup> CD8<sup>+</sup> TCR<sup>+</sup> cells from type II chimeric mice (black line). B, Proliferation and IL-2 production of peripheral T cells from type I (●) or type II (○) chimeras after stimulation with NP peptide. The results shown are representative for five mice of each type of chimera.

**CHIMERAS**

<table>
<thead>
<tr>
<th>IRR. Recipient</th>
<th>Type I</th>
<th>Type II</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM cells injected</td>
<td>GFPF5</td>
<td>NP47F5</td>
</tr>
<tr>
<td>Ag expression:</td>
<td>Non-BM derived cells</td>
<td>BM-derived cells</td>
</tr>
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</table>

**A**

CD44 ON DONOR CD8

**B**

Proliferation

**C**

Proliferation

**D**

Periphery

**E**

IL-2

**F**

IFN-γ

**G**

Cytotoxicity

**H**

% specific lysis

**I**

Effector/target ratio

**J**

NP µM
T cells in vitro. Fig. 4 shows that DC and macrophages isolated from NP47 mice activated naive F5 T cells to up-regulate CD25 expression compared with F5 T cells transferred into syngeneic Rag-/- mice. While biochemical defects in anergic T cells maintained in vivo "anergized" T cells and the biochemical pathways underlying functional impairment seem to be different. In many experimental studies, it has been described previously that the "anergic" phenotype of in vivo "anergized" T cells and the biochemical pathways underlying functional activity and resets CD5 levels.

**Peripheral T cells in NP47F5 mice express high levels of CD5**

It is thought that the developmentally controlled expression of CD5 is important for setting the TCR response threshold for p:MHC complexes encountered during thymic development and the strength of signaling through the TCR (26, 27). More recently it has been suggested that some degree of sensory adaptation of CD5 levels is continued in the periphery during T cell interaction with p:MHC survival ligands (28). We analyzed whether CD5 levels in T cells from NP47F5 mice differ from those in F5 mice that develop in the absence of cognate Ag or memory T cells that had encountered a transient antigenic stimulus. Staining of single-positive CD8 thymocytes from NP47F5 and F5 mice revealed similar levels of CD5 expression. However, peripheral T cells from NP47F5 mice continued to express the high levels of CD5 found on thymocytes, whereas peripheral F5 T cells had down-regulated levels of CD5 (Fig. 5A). Fig. 5B gives a comparison of CD5 levels on naive F5 T cell, anergized NP47F5 or F5→NP47 T cells and memory F5 T cells.

**Transfer of tolerated F5→NP47 T cells into Ag-free recipients restores functional activity and resets CD5 levels**

It has been described previously that the "anergic" phenotype of in vivo-tolerized T cells is dependent on continuous contact with Ag, but can be reversed upon transfer into Ag-free hosts. We transferred F5→NP47 T cells as well as F5 control T cells into Ag-free syngeneic Rag-/- hosts and assessed functional activity of the T cells 3 and 6 wk after transfer. While 3 wk after transfer F5→NP47 T cells still showed impaired proliferation and cytokine production compared with F5 T cells transferred into syngeneic Rag-/- hosts (Fig. 6A, functional activity was restored 6 wk after transfer (Fig. 6B). Fig. 6C shows that CD5 levels on F5→NP47 T cells 2 days after transfer into Ag-free recipients are higher than those of transferred naive F5 T cells (left panel), similar to what was observed in peripheral T cells of the original mice (see Fig. 5). Little change of CD5 levels was observed after 3 wk (Fig. 6C, middle panel). However, 6 wk after transfer T cells from F5→NP47 mice had down-regulated CD5 levels so that both groups expressed similar levels of CD5 (Fig. 6C, right panel).

**Discussion**

It is well established in several experimental models that CD4 as well as CD8 T cells can be functionally impaired if they are chronically exposed to Ag (reviewed in Ref. 1). CD8 T cells have been shown to be either deleted or anergized depending on the Ag dose, (15, 29–31). The commonly observed defect concerns failure to proliferate and produce cytokines in response to antigenic stimuli in vitro. While biochemical defects in anergic T cells maintained in vitro are relatively well characterized, less is known about in vivo "anergized" T cells and the biochemical pathways underlying functional impairment seem to be different. In many experimental
models, unresponsiveness is “set” during thymic development (32) and maintained by continuous presence of the Ag in the periphery. We have concentrated on a model in which thymic differentiation of T cells proceeds normally despite the fact that the transgene encoding the cognate Ag was expressed under the MHC class I Kb promoter that in principle should direct Ag expression to all cells in the body. It is clear, however, that this construct lacks the necessary locus control regions which would ensure copy number dependent, and cell type specific expression. Therefore, position effect variegation resulting in incomplete and variable expression in different cell types is to be expected (33). It proved impossible to detect Ag expression by any other means than a functional readout of T cell activation, suggesting that the overall levels of Ag are very low. We have no explanation why expression in the thymus is particularly suboptimal, but it is evident both phenotypically and functionally that mature CD8 thymocytes in mice expressing the F5 TCR as well as the NP transgene look indistinguishable from normal F5 thymocytes. Therefore the impaired functional phenotype only develops once thymocytes have emigrated to the periphery. The phenotypic heterogeneity observed in peripheral T cells of NP47F5 mice, as manifested by differential levels of expression of CD44, indicates that induction of nonresponsiveness is a gradual process presumably preceded by a responsive phase (29, 34–36). In contrast to F5 T cells which are anergized in the periphery, transgenic OT-I T cells emigrating from the thymus were shown to be gradually deleted (37). Furthermore, it is clear that at least cytotoxic activity is never completely abolished in NP47F5 mice since T cells were shown to kill Ag-pulsed target cells not only in vitro but also in vivo, and are able to destroy growth hormone producing somatotrophs in mice expressing the Ag in the pituitary gland. In addition, double-transgenic NP47F5 mice lack DCs and macrophages with high stimulatory potential for naive F5 T cells in vitro when compared with DC and macrophages isolated from mice expressing Ag only. This suggests ongoing cytotoxic activity against bone marrow-derived APC expressing the cognate Ag at high levels. In that respect CD8 T cells from NP47F5 mice seem to undergo activation induced nonresponsiveness, which was described as a part of the differentiation program of CD8 T cells (38). However, in contrast to T cells undergoing activation induced nonresponsiveness they do not secrete IFN-γ. The residual functional activity seen in NP47F5 mice could be due to recent thymic emigrants that have not yet been tolerized, especially since F5 mice do not undergo significant thymic involution (24) and continue to export thymocytes even in old age. Nevertheless, the functional impairment of peripheral T cells is apparent early on as indicated for instance by the reduced autoimmune phenotype in triple-transgenic mice that also express Ag in the pituitary gland. Mice that contain normal F5 T cells and only

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**FIGURE 4.** Peripheral CD8 T cells from F5→NP47 mice retain cytolytic capacity in vivo. A, Untreated F5 (top left panel), F5 mice immunized with 10⁶ PFU of A/NT/60/68 virus 4 days before cytotoxicity test (bottom left panel), NP47 mice that had been injected with 5 × 10⁵ F5 CD8 T cells 20 days before cytotoxicity test (top right panel) and NP47F5 mice (bottom right panel) received a mixture of 5 × 10⁵ CFSE high labeled NP peptide-pulsed target cells and 5 × 10⁶ CFSE low labeled unpulsed target cells. Nineteen hours later, peripheral lymphocytes from mice of each group were examined by FACS to detect CFSE labeled cells. Histograms represent the amount of CFSE-labeled cells of one mouse per group. The ratios R between the numbers of unpulsed vs NP-pulsed targets (CFSE<sup>low</sup>:CFSE<sup>high</sup>) were 1.5 for naive F5 (top left panel), 10 for immunized F5 (bottom left panel), 6.1 for anergized F5 (top right panel) and 2.6 for NP47F5 (bottom right panel). B, In vitro Ag presentation assays with coculture of 1 × 10⁴ responder F5 CD8 lymph node cells with titrated number of a mixture of MACS isolated DC and macrophages as APCs, purified from NP47 (■), NP47F5 (○) or RagNegbb (▲) mice. On day 6 of culture, responder F5 CD8 T cells were stained for CD8, CD25, CD44 and CD69 mAbs and analyzed by FACS. At the same time point culture supernatant was measured for the amount of IL-2 secreted by the responder cells by CTL assay. The response of F5 CD8 T cells is shown as percentage of F5 CD8 T cells positive for CD25, CD44, CD69, and IL-2 secretion. C, Pituitary growth hormone content in 5-mo-old male NP47F5 × 48 compared with NP47F5 age-matched male control mice.
NP47F5 T cells in contrast do not influence the growth rate of mice containing NP in the pituitary gland, although they eventually destroy growth hormone producing somatotrophs as indicated by the drastically reduced levels of growth hormone found in pituitary glands of triple-transgenic mice.

Continuous presence of Ag has been shown to be a prerequisite for the maintenance of an “anergic” phenotype (29, 39, 40). In agreement with this we also find that NP specific CD8 T cells recover their function following transfer into an Ag-free environment, although recovery was not as rapid as observed in a system using transgenic CD4 T cells (1). One of the hallmarks of anergized CD8 T cells was their high expression of CD5. CD5 is a negative regulator of TCR signaling and is thought to be set during thymic development according to the avidity for peptide:MHC molecules encountered during positive selection (26, 27). In contrast, it was suggested that contact with peptide:MHC molecules in the periphery modulates expression of CD5 (28). Naive transgenic F5 T cells in peripheral lymphoid organs, like peripheral T cells from wildtype mice express lower levels of CD5 than thymocytes (27). However, peripheral CD8 T cells in NP47F5 mice fail to down-regulate CD5. Since we previously observed that peripheral CD4 T cells sorted on the basis of high or low expression of CD5 did not change their CD5 profile in response to homeostatic or antigenic signals (41), we assume that normal, transient interactions with cognate ligands do not influence CD5 levels which is also supported by low CD5 levels on memory F5 T cells equivalent to those on naive F5 T cells. In contrast, continuous contact with cognate Ag as experienced by thymic emigrants from NP47F5 mice might prevent down-regulation of CD5 to allow negative regulation of TCR signaling in the face of inescapable exposure to cognate Ag. This effect may be exacerbated by concomitant down-regulation of TCR expression (42).

Increased levels of CD5 expression were also reported for anergic B cells and breeding of mice containing B cells anergized by chronic exposure

![FIGURE 5](image-url) Peripheral NP47F5 CD8 T cells express high levels of CD5 marker. Expression levels of CD5 gated on NP47F5 or F5 mature thymocytes (CD8 SP TCR<sup>high</sup>, top left panel) and on peripheral CD8 T cells (top right panel). The bottom panel gives CD5 expression levels on naive F5, tolerized F5, and memory F5 T cells. The absolute mean fluorescence intensity values are different because of the use of PE- rather than FITC-labeled anti-CD5 Ab in this comparison.

![FIGURE 6](image-url) Recovery of functional activity and reset of CD5 levels upon transfer into Ag-free recipients. A total of 5 x 10<sup>6</sup> peripheral CD8 T cells from F5→NP47 or naive F5 as mice as controls were FACS sorted and transferred separately into syngeneic Rag<sup>−/−</sup> Ag-free hosts. Three weeks (A) and 6 wk (B) after transfer, spleen cells were harvested and equal numbers of CD8 T cells were cultured in vitro with H-2<sup>b</sup> BM cultured DCs and NP Ag for 2 days. Proliferative responses were measured by <sup>3</sup>H incorporation, IL-2 responses were assayed with CTLL assay and IFN-γ cytokine secretion was measured by ELISA. C. Spleen cells from were stained with CD8, TCR and CD5 m Abs. Histogram overlays show expression of CD5 on naive control F5 or tolerized F5→NP47 CD8 T cells 2 days (C, left panel), 3 wk (middle panel), or 6 wk (C, right panel) after transfer into Ag-free Rag<sup>−/−</sup> hosts. Mean fluorescence values of CD5 expression are displayed for two mice of each group.
to transgenic HEL Ag onto a CD5$^{-}/-$ background resulted in loss of the anergic phenotype (43). The assumption that high CD5 expression is related to the anergic phenotype of NP47F5 T cells is supported by the finding that CD5 levels are down-regulated following transfer into Ag-free adoptive hosts. Although these hosts were lymphopenic, therefore allowing homeostatic expansion of transferred cells, it is unlikely that this caused the changes in CD5 levels, as control F5 T cells also subjected to homeostatic expansion after transfer into Rag$^{-/-}$ hosts did not alter their CD5 levels. Thus, it seems that maintaining high levels of CD5 expression resulting in increased thresholds for TCR triggering could be a means to control self specific T cells and avoid overt autoreactivity in the continual presence of self Ag.

Acknowledgments

We thank C. Atkins for cell sorting, and T. Norton and K. Williams for the breeding and welfare of transgenic mice.

References

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