Differential In Vivo Persistence of Two Subsets of Memory Phenotype CD8 T Cells Defined by CD44 and CD122 Expression Levels

Thierry Walzer, Christophe Arpin, Laurent Beloeil, and Jacqueline Marvel

The existence of distinct subsets of memory CD8 T cells with different characteristics is now well established. In this work, we describe two subsets of mouse CD8 T cells with memory characteristics that coexist in primed thymectomized TCR-transgenic F5 mice and that share some properties with the human central and effector memory cells. The first subset corresponds to CD8 T cells generated following nucleoprotein 68 peptide priming which are CD44intCD122− nucleoprotein 68/H-2Db tetramer+ and express high levels of CCR7 mRNA. In contrast, CD8 T cells in the second subset are CD44highCD122+, are heterogeneous in terms of Ag specificity, and express low levels of CCR7 mRNA. We have studied the functional characteristics and the persistence of these two subsets in thymectomized mice. CD44int CD8 T cells persist like naive cells; i.e., they are slowly lost with time. However, surviving cells maintain their phenotype and memory characteristics for the entire life span of the animal. In contrast, CD44high CD8 T cells are persistent and accumulate in thymectomized but not euthymic mice. This is correlated with an increased in vivo proliferative and survival potential of these cells. These results show that acquisition of enhanced functional characteristics and long-term persistence by memory T cells is independent. This may have important consequences for the design of specific vaccine. The Journal of Immunology, 2002, 168: 2704–2711.

M

emory responses to Ag stimulation are faster and more efficient than primary responses. This qualitative advantage has been shown to result in part from the improved effector functions of memory cells (1, 2). In mice, different transgenic models and Ags have been used to characterize the properties of memory CD8 T cells. In all systems, memory CD8 T cells display some improved functional characteristics. However, based on their surface phenotype and function, memory CD8 lymphocytes seemed heterogeneous, suggesting that different subsets of memory CD8 T cells might be generated under different experimental conditions (3–11). The existence of at least two subsets of memory CD8 T cells was first demonstrated in humans by Sallusto et al. (12), who showed that two populations of memory CD45RA− CD8 T cells could be identified on the basis of the expression of the chemokine receptor CCR7. CD45RA−/CCR7− cells (effector memory T cells (TEM)) display immediate effector function; i.e., they produce high amounts of IFN-γ and contain prestored perforin. In contrast, CD45RA−/CCR7− (central mem-

ory T cells (TCM)) neither contain perforin nor secrete IFN-γ but express lymph node homing receptors and display memory cell characteristics such as response to recall Ags or lower TCR-induced activation threshold. In vitro, TCM differentiate into TEM following anti-CD3+ anti-CD28 activation, suggesting that these cells correspond to different steps in the differentiation pathway from naive to terminally differentiated effector cells (12). These two subsets of memory cells appear to have complementary functions, TEM being capable of immediate effector functions while TCM could sustain the response at later stages. Hence, the generation and maintenance of both subsets could be essential for a long-term protective memory response. However, it has recently been shown that different viruses can lead to differential maturation or maintenance of memory CD8 T cells, resulting in distinct representation of these two subsets among virus-specific memory cells (13). Understanding the conditions leading to the generation and the maintenance of these two subsets is therefore a question of particular interest, especially in vaccinology. Lanzavecchia and Sallusto (14) proposed that because TCM and TEM occupy different niches they may rely on different types of survival signals for their persistence. This possibility is attractive because it fits well with previous findings which showed that memory T cells are heterogeneous in their maintenance requirements (15–17).

In mice, identification of these subsets has been hampered by the lack of a CCR7-specific Ab. We have previously shown that in primed thymectomized (Tx) F5 TCR-transgenic mice two subsets of memory phenotype CD8 memory cells coexist: CD44int CD122− CD8 T cells that are generated following peptide priming and CD44highCD122+ CD8 T cells that are heterogeneous in terms of Ag specificity (8, 18). In this study we show that these two subsets are similar to human TCM and TEM subsets of memory CD8 T cells in terms of CCR7 expression and response to macrophage-inflammatory protein (MIP)-3β. Hence, we have taken advantage of this model to study the persistence of these cells in vivo in the absence of cell transfer.
Materials and Methods

Mice and immunizations

C57BL/10 and F5 TCR-transgenic mice were gifts from D. Kiossis (London, U.K.) (19) and were bred in the animal facility of the Institut National de la Santé et de la Recherche Médicale. C57BL/6 mice were obtained from Iffa Credo (L’Arbresle, France). Thymectomies were performed on 5- to 6-wk-old mice, which were then allowed to recover for at least 4 wk before immunization. F5 mice were immunized i.p. once (once-primed) or twice within a 24-h interval (twice primed), with 50 nmol of the influenza virus matrix (NP)68 peptide: Ala-Ser-Asn-Glu-Asn-Met-Asp-Ala-Met NP 366–373 (Syntem, Nimes, France) in saline. Primed mice were used starting 6 wk after peptide injection. Control F5 mice were not injected.

Purification of CD8 T cell subsets and in vitro proliferation assays

Culture medium is composed of DMEM (Life Technologies, Cergy Pontoise, France) supplemented with 6% FCS, 50 μg/ml gentamicin, 2 mM l-glutamine (Life Technologies), 10 mM HEPES, and 50 μM β-ME (Sigma Chemicals, L’Isle d’Abeau, France). Lymph node or splenic CD8 T cells from F5 mice were purified by magnetic beads using a negative selection strategy. Briefly, a pool of lymph node and spleen cells was applied to Ficoll-Hypaque (Lympholyte-M; Cedarlane Laboratories, Hornby, Ontario, Canada) gradient centrifugation followed by an incubation for 30 min at 4°C with a mixture of culture supernatants containing the following rat mAbs: anti-CD4 (RM4-5), anti-GR-1 (RB6-8C5), anti-Mac-1 (M1/70.15), and anti-I-A^b (M5/114.15.2). A total of 15 μg of purified rat anti-mouse B220 (clone RA3-6B2; Cedarlane Laboratories) were also added. Cells were then washed three times with medium and incubated for 30 min at 4°C with goat anti-rat IgG (H and L)-coupled magnetic beads (Polysciences, Eppelheim, Germany) at a ratio of 20 beads per cell. Positive cells were removed by application to a magnet. Cell population purified by this method contained 92–98% of CDS lymphocytes, as assessed by flow cytometric analysis. For sorting, purified CD8 T cells were incubated for 30 min with fluoro-chrome-coupled anti-CD8 (YTS169.4-PE; BD PharMingen, San Diego, CA) and anti-CD4 (IM-781FITC, made in our laboratory) Abs. After two washes with medium, cells were sorted into CD44^int, CD44^low, or CD44^bright as previously described (18). For proliferation assays, 1 × 10^5 sorted F5 cells were activated in 96-well plates with various concentrations of NP68 peptide in the presence of 2 × 10^5 irradiated (3000 Gy) C57BL/10 spleen cells and 5% of a supernatant containing IL-2. On day 4, cells were pulsed overnight with 0.5 μCi [3H]thymidine/well (2 Ci/mmol; Amersham, Pharmacia Biotech Europe, Saclay, France) and harvested 16 h later.

Induction of cytokine synthesis for intracellular staining assays

For all intracellular cytokine detection assays, staining was preceded by a 5-h in vitro stimulation. Briefly, freshly isolated or cultured F5 splenocytes were applied to Ficoll-Hypaque gradient centrifugation or FACS-sorting, and analyzed on a 6% denaturing polyacrylamide gel. The quantity of protected RNAs was determined using a PhosphoImager and Image-Quant software (both from Molecular Dynamics, Sunnyvale, CA). The length of their respective fragments was used to identify the transcripts.

Results

Two phenotypically and functionally distinct memory phenotype T cell subsets are generated in F5 mice

In F5 mice, in the absence of NP68 immunization most peripheral CDS T cells have the CD44^intLy6C^lo naive phenotype. I.p. injection of NP68 peptide in F5 mice leads to the generation of a memory CD44^brightLy6C^hi CD122^− CD8^+ T cell population that is permissive to antigenic challenge in vitro and has an increased capacity to produce IFN-γ (Fig. 1D and Refs. 8, 18, and 21). These functional characteristics are two features of memory CDS T cells.

In euthymic naive and primed mice a subset (2–5%) of CDS T cells expressing a different memory surface phenotype CD44^highCD122^− naive phenotype, i.p. injection of NP68 peptide in F5 mice leads to the generation of a memory CD44^highLy6C^lo CD122^− CD8^+ T cell population that is permissive to antigenic challenge in vitro and has an increased capacity to produce IFN-γ (Fig. 1D and Refs. 8, 18, and 21). These functional characteristics are two features of memory CDS T cells.

In vitro assay

After surface staining, cells were washed twice in PBS/FCS/NaCl. For cytokine or Bcl-2 staining the Cytofix/Cypermix kit (BD PharMingen) was used according to the manufacturer’s instructions. The following Abs were used: XMGLI.2-FITC (anti-IFN-γ), PE-labeled Armenian hamster anti-Bcl-2, and isotype control (all from BD PharMingen).

Staining for 5-bromo-2-deoxyuridine (BrdU) incorporation was conducted as previously described with minor modifications (8). In brief, cells were stained for surface markers as above, resuspended in cold 0.15 M NaCl solution, and fixed by injection in cold ethanol to a final concentration of 70%. After a 30-min incubation on ice, cells were washed once with PBS and resuspended in PBS, 0.001% Tween 20, and 1% paraformaldehyde.

After a 1-1-h incubation at room temperature, cells were washed with 0.15 M NaCl, and the DNA was partially digested with DNase 1 (Appligene, Illkirch, France) in 25 mM CaCl₂, 5 mM MgCl₂, and 10 mM HEPES (pH 7.4). Cells were then washed with 0.15 M NaCl before adding the anti-BrdU Ab (B44-FITC; BD Biosciences). After overnight incubation, cells were washed with PBS before flow cytometry analysis.

Chemotaxis assays

Naive and primed lymphocytes (2.5 × 10^6 each) were mixed and placed in the upper chamber of a 5-µm pore polycarbonate Transwell culture insert (Costar, Cambridge, MA). Naive or primed cells were labeled with CFSE as previously described (20). Different concentrations of recombinant mouse MIP-3β from R&D Systems (Minneapolis, MN) were added in the lower chamber. The chemotaxis medium consisted of RPMI 1640/10% FCS. After a 90-min migration, cell numbers were evaluated using a set length of acquisition time on the FACSscan. A standard curve was drawn using serial dilutions of the starting cell suspension. The percentage of CDS subsets in the transmigrated cells and the starting population were determined by staining and were used to calculate the number of cells belonging to each subset. These were then used to calculate the migration index that corresponds to the number of transmigrating cells of a given subset divided by the number of cells of that subset in the starting cell suspension multiplied by 100.

Multiprobe RNase protection assays

Cells were applied to Ficoll-Hypaque gradient centrifugation or FACS-sorted, and total cellular RNA was isolated by the RNA Nox method according to the manufacturer’s instructions (Biogentex, Seabrook, TX). Cytokine or apoptosis regulator mRNA levels were measured by RNase protection assays using the Riboquant kit (BD PharMingen) following the instructions of the supplier. In brief, 1–3 μg RNA were hybridized overnight to the 32P-labeled RNA probe, which had been previously synthesized from the supplied template (mapo-2 or a custom probe from BD PharMingen). Single-stranded RNA and free probe were digested by RNase A and T1. Subsequently, protected RNA was phenolized, precipitated, and analyzed on a 6% denaturing polyacrylamide gel. The quantity of protected RNAs was determined using a PhosphoImager and Image-Quant software (both from Molecular Dynamics, Sunnyvale, CA). The length of their respective fragments was used to identify the transcripts.

The Journal of Immunology 2705
Results in are representative of at least three independent experiments.

Characterization of CD44<sup>int</sup> and CD44<sup>high</sup> memory F5 CD8 T cell subsets. Spleen cells from naive, once (1X)- and twice (2X)-primed Tx mice were triple stained for CD8, CD44, and F5 (A). The expression of CD44 and CD122 by gated CD8 T cells is shown and the percentages of memory phenotype CD8 T cells in primed F5 mice to compare memory characteristics but have a distinct persistence in vivo (18). However, although heterogeneous in terms of antigenic specificity, CD44<sup>high</sup> CD8 T cells behave like memory cells and not like anergic cells, as they are able to produce IFN-γ following a 5-h stimulation with anti-CD3 and anti-CD28 Abs (Fig. 1D).

Results in Fig. 1, B and D, indicated that priming F5 mice with a single injection of peptide does not lead to a detectable increase in the percentage of CD44<sup>high</sup>/CD122<sup>−</sup> tetramer<sup>low</sup> cells, suggesting that NP68-specific memory cells with that phenotype are not generated in these priming conditions. However, we have recently observed that following sustained priming (i.e., two injections of NP68 peptide within a 24-h interval: twice-primed) a significant number of CD44<sup>high</sup>/CD122<sup>−</sup> tetramer<sup>low</sup> CD8 T cells are generated (Fig. 1, B and C). CD44<sup>high</sup>/CD122<sup>−</sup> tetramer<sup>low</sup> memory cells generated in twice-primed mice are responsive to peptide stimulation as they produce IFN-γ following a 5-h stimulation with the NP68 peptide (Fig. 1C).

These results indicate that in primed F5 mice at least two subsets of memory phenotype CD8 T cells coexist: CD44<sup>int</sup>/CD122<sup>−</sup> CD8 cells, which are generated only following NP68 peptide priming; and CD44<sup>high</sup>/CD122<sup>−</sup> CD8 T cells, which are heterogeneous in terms of antigenic specificity but can contain a fraction of NP68-specific memory cells.

**CCR7 expression by CD8 T cell subsets**

In humans two subsets of CD8 memory cells that differ by their expression of the chemokine receptor CCR7 have been described (12). To assess the expression of CCR7 by the different subsets of F5 CD8 T cells we have measured the expression of CCR7 mRNA by RNase protection assay. Results presented in Fig. 2A show that F5 CD44<sup>int</sup>/NP68 peptide-specific memory CD8 T cells generated in once-primed mice (or twice-primed mice; data not shown) express high levels of CCR7 mRNA similar to naive F5 CD44<sup>low</sup> CD8 T cells while F5 CD44<sup>high</sup> memory phenotype CD8 T cells express a low level of CCR7 mRNA. To test whether the difference in CCR7 expression at the mRNA level leads to decreased receptor levels at the cell surface, and because we had no access to a mouse CCR7-specific Ab, we have measured the chemotactic response of CD8 cell subsets to the CCR7 ligand MIP-3β. Results in Fig. 2B show that F5 CD44<sup>int</sup> CD8 cells show the same level of response to MIP3β as do CD44<sup>low</sup> CD8 cells. In contrast, CD44<sup>high</sup> CD8 cells show a significantly weaker response to this chemokine, confirming a decrease in CCR7 expression at the cell surface. This is further strengthened by the observation that memory phenotype CD44<sup>high</sup>/CD122<sup>−</sup> CD8 T cells are preferentially found in spleen or peripheral blood and are excluded from the lymph nodes as compared with CD44<sup>int</sup>/CD122<sup>−</sup> CD8 memory cells (Fig. 2C). The differential expression of CCR7 mRNA by these cells suggest that they could be the mouse counterpart of the TEM and TCM subsets of memory phenotype CD8 T cells described in humans.

These results indicate that in Tx primed F5 mice, two subsets of CD8 T cells with memory characteristics coexist: CD44<sup>int</sup>/CD122<sup>−</sup> CCR7<sup>high</sup> CD8 T cells, which are similar to TEM, and CD44<sup>high</sup>/CD122<sup>−</sup> CCR7<sup>low</sup> CD8 T cells, which are similar to TEM.

**Memory phenotype CD8 subsets maintain functional memory characteristics but have a distinct persistence in vivo**

We have taken advantage of the coexistence of these two subsets of memory phenotype CD8 T cells in primed F5 mice to compare
their relative persistence in vivo without cell transfer and in the absence of thymic output. This was done by measuring their number over time in primed Tx animals. The persistence of CD44highCD122+ CD8 T cells in naive Tx mice was also analyzed. Results presented in Fig. 3, A and B, show that the number and the percentage of CD8 T cells in the spleen decrease over time starting 4 mo after thymectomy and that the kinetics are similar in naive and NP68 primed mice. This reduction was due to a steady decline in the percentage of CD44low cells in naive mice or CD44int cells in primed mice (Fig. 3B). These data indicate that CD44int memory cells and CD44low naive CD8 cells have similar life spans in F5 Tx mice. In contrast, the percentage of CD44high CD8 T cells strongly increased in both naive and primed Tx F5 mice. Using twice-primed F5 mice we have also studied the persistence of the small subset of NP68-specific CD44highCD122+ CD8 memory T cells that is generated following sustained priming. We first verified that sustained priming does not modify the persistence of the two subsets of memory CD8 T cells, namely the CD44intCD122− subset and the CD44highCD122− subset (Fig. 3C). Because the same profile of persistence as the one observed in once-primed mice was found for both subsets, we next studied the behavior of CD44highCD122− NP68-specific CD8 T cells by measuring the percentage of CD44highCD122− CD8 T cells producing IFN-γ following a 5-h NP68 stimulation. Results in Fig. 3C show that the persistence profile of these NP68-specific cells is similar to the one observed when the whole CD44highCD122− CD8 subset is monitored. These results suggest that the increased persistence of CD44highCD122+ CD8 T cells is independent of their antigenic specificity. Hence in the next experiments we have studied the CD44highCD122+ CD8 subset as a whole.
The increase we did observe in the percentage of CD44\textsuperscript{high} CD8 T cells was due not only to the loss of CD44\textsuperscript{low} or CD44\textsuperscript{int} CD8 cells but also to an increase in the absolute number of CD44\textsuperscript{high} cells (Fig. 3D). The conversion of CD44\textsuperscript{low/int} CD8 T cells into CD44\textsuperscript{high} CD8 T cells did not contribute significantly to the increase, as the proportion of tetramer\textsuperscript{low} cells among that subset was stable over time. Indeed, if CD44\textsuperscript{low/int}, which are >95% tetramer positive, would convert to CD44\textsuperscript{high}, one would expect an increase in the percentage of tetramer-positive cells among CD44\textsuperscript{high} CD8 T cells. However, this is not the case, as this percentage in old mice (2.5 ± 0.5% Fig. 1C). In euthymic mice, the number of CD44\textsuperscript{high} CD8 cells is relatively stable over 1 year, suggesting that in the presence of a constant thymic output the expansion of CD44\textsuperscript{high} CD8 cells is restricted by newly produced naive cells. This is in agreement with a number of data showing that following transfer memory phenotype CD44\textsuperscript{high} CD8 cells expand in lymphopenic hosts (22–26). To assess whether this was also the case in the absence of cell transfer in thymectomy-induced lymphopenia, the proliferation of these cells was measured in vivo using BrdU labeling. For these experiments we have used euthymic and Tx C57BL/6 mice, which have a similar subset of CD44\textsuperscript{high/CD122}\textsuperscript{+} CD8 T cells that also share functional features with that of F5 mice (Ref. 27 and data not shown). Results presented in Table I show that the percentage of CD44\textsuperscript{high} cells that incorporated BrdU is higher in Tx mice than in non-Tx mice after 1 or 3 wk of BrdU labeling. Moreover, in pulse/chase experiments the BrdU labeling is lost more rapidly by CD44\textsuperscript{high} CD8 T cells in Tx mice than in euthymic mice. This suggests that the expansion of CD44\textsuperscript{high/CD122}\textsuperscript{+} CD8 T cells in Tx animals is due at least in part to increased proliferation.

F5 TCR\textsuperscript{+} CD44\textsuperscript{high/CD122}\textsuperscript{+} CD8 memory T cells were steadily lost with time in Tx but also in euthymic mice (Ref. 8 and data not shown). However, their memory features were retained for the life span of the animal. Indeed, F5 TCR\textsuperscript{+} CD44\textsuperscript{int} CD8 T cells show the same increased proliferation and IFN-γ secretion capacities 2–15 mo after priming (Fig. 4). Similarly, CD44\textsuperscript{high} CD8 T cells also retained their IFN-γ-secreting capacity in response to NP68 peptide stimulation (Fig. 3C).

Altogether, these results indicate that the two subsets of memory phenotype CD8 T cells that coexist in F5 mice show different in vivo persistence. CD44\textsuperscript{high/CD122}\textsuperscript{+} CD8 T cells are selectively maintained and expand in the absence of thymic output while CD44\textsuperscript{int/CD122}\textsuperscript{−} cells, similarly to naive cells, are slowly lost with time. However, both memory phenotype subsets maintain their functional memory characteristics for the entire life span of the mice.

Table I. Thymectomy leads to an increased proliferation rate of the CD44\textsuperscript{high} CD8 T cell subset

| Continuous BrdU Labeling | 1 wk | 3 wk | 1 wk BrdU + 2 wk chase |
|--------------------------|------|------|------------------------|
| C57BL/6                  | 28.8 ± 1 | 45.8 ± 1 | 27.5 ± 1               |
| C57BL/6 Tx              | 48.3 ± 4 | 56 ± 3 | 37.5 ± 5               |

* Eight-wk-old thymectomized or euthymic C57BL/6 mice were given BrdU in the drinking water for 1 wk, 3 wk, or 1 wk followed by a 2-wk chase period. Spleen cells were then triple-stained for CD8, CD44, and BrdU. Results show the percentage of BrdU-positive cells among the CD44\textsuperscript{high} CD8 T cell subset. Mean values ± SD from at least four mice per group are given.

CD44\textsuperscript{high/CD122}\textsuperscript{+} CD8 T cell maintenance correlates with higher expression levels of Bcl-x, A1, and Bcl-2, and with increased resistance to apoptosis

The in vivo persistence of CD44\textsuperscript{high/CD122}\textsuperscript{+} CD8 T cells could also result from improved survival potential compared with the other subsets of CD8 T cells. To evaluate the survival potential of the different F5 CD8 subsets we have first measured their expression of different antiapoptotic genes belonging to the Bcl-2 family. These genes code for proteins that increase cell resistance to a large panel of apoptotic pathways. CD8 T cells were purified respectively from 2-mo-old naive non-Tx mice (>95% of CD44\textsuperscript{low} cells), 3-mo-old primed Tx mice (at least 80% of CD44\textsuperscript{int} cells), and 15- to 20-mo-old Tx mice (90% of CD44\textsuperscript{high} cells). Alternatively, CD44\textsuperscript{low}, CD44\textsuperscript{int}, and CD44\textsuperscript{high} CD8 T cells were sorted by flow cytometry from bulk CD8 populations isolated from naive or immunized age-matched F5 Tx mice. Total RNA was extracted and the level of bcl-2 family members was measured by RNase protection assay. Results presented in Fig. 5, A and B, show that Bcl-x and A1 (mouse homolog of human Bcl-1) mRNA levels are higher in the CD44\textsuperscript{high/CD122}\textsuperscript{+} CD8 T cells than in the other subsets. Although for some unknown reason the level of all RNAs was decreased when the different subsets were sorted (compare scale from upper and lower panels in Fig. 5B), the differential Bcl-x and A1 expression was maintained. This indicates that the difference observed between the subsets is not related to age, because in these experiments the CD8 subsets were obtained from age-matched mice. This differential expression of Bcl-x and A1 was not restricted to F5 mice, as it was also observed in CD8 T cell subsets sorted from C57BL/6 mice (data not shown). The level of Bcl-2 mRNA measured by RNase protection was too low to be accurately quantified. Therefore, the level of Bcl-2 was measured at the protein level using flow cytometry. Results in Fig. 5C show that the CD44\textsuperscript{int/CD122}\textsuperscript{−} and the CD44\textsuperscript{low/CD122}\textsuperscript{−} CD8 T cells both

![FIGURE 4. CD44\textsuperscript{int} CD8 T cells maintain their memory characteristics with higher expression levels of Bcl-x, A1, and Bcl-2, and with increased resistance to apoptosis](image-url)
express the Bcl-2 protein. However, Bcl-2 levels were the highest in CD44<sup>high</sup>CD122<sup>+</sup> CD8 T cells.

To test whether increased Bcl-2, Bcl-x, and A1 gene expression conferred a survival advantage to the CD44<sup>high</sup>CD122<sup>+</sup> subset, we have measured the resistance of these cells to irradiation-induced cell death. Indeed, in cell lines Bcl-2 or Bcl-x, expression correlates with an increased cell survival following irradiation-induced cell death (28–30). For these experiments C57BL/6 mice received three doses of whole-body irradiation. Three days later the survival of CD44<sup>high</sup>CD8 T cells was compared with CD44<sup>low/int</sup>CD8 T cells. Results presented in Fig. 6A show that CD44<sup>high</sup>CD122<sup>+</sup> CD8 T cells are more resistant to irradiation-induced cell death. Indeed, at all doses of irradiation a larger percentage of these cells survived compared with CD44<sup>low/int</sup>CD122<sup>+</sup> CD8 T cells. Similar results were obtained with F5 mice (Fig. 6B).

These results indicate that the increased expression of Bcl-2, Bcl-x, and A1 by CD44<sup>high</sup>CD122<sup>+</sup> CD8 T cells does indeed lead to their increased resistance to apoptosis. This feature of CD44<sup>high</sup>CD122<sup>+</sup> CD8 cells could play a major role in their preferential persistence in vivo.

**Discussion**

We have studied the phenotype, function, and persistence of two subsets of CD8 T cells with memory features. CD8 T cells in the first subset are CD44<sup>high</sup>CD122<sup>+</sup> and are dependent on NP68 priming for their generation. These cells bind the NP68-loaded H-2D<sup>B</sup> MHC tetramers, are hyperresponsive to peptide challenge in vitro, and produce IFN-γ following a short-term peptide stimulation. They express high levels of mRNA coding for the CCR7 protein and show a MIP-3β responsiveness that is similar to naive cells. The increased in vitro proliferation and the expression of CCR7 by these cells could suggest that they are the mouse counterpart of the human TCM subset. However, human CD8 TCM do not produce IFN-γ in response to PMA-ionomycin stimulation (12). This could reflect a difference between CCR7<sup>+</sup> CD8 memory cells in humans and mice. Alternatively, the magnitude of the IFN-γ response could vary according to the method used to activate the T cells. Indeed, in the F5 system stimulation of the CD44<sup>int</sup> subset with anti-CD3 and anti-CD28 Abs leads to a lower levels of IFN-γ staining by a reduced number of cells compared with NP68 peptide stimulation (see Fig. 1C). A third possibility would be that CD44<sup>int</sup> CD8 T cells generated in the F5 system represent an intermediate stage of differentiation between TCM and TEM.

CD8 T cells in the second subset are CD44<sup>high</sup>CD122<sup>+</sup>, the majority of them express Vβ11 but do not bind the NP68 tetramer, and they can be generated independently of NP68 peptide stimulation. However, following sustained priming a subset of tetramer<sup>+</sup>CD44<sup>high</sup>CD122<sup>+</sup> CD8 T cells can be generated. CD8 T cells belonging to the CD44<sup>high</sup> subset express low levels of mRNA coding for the CCR7 protein and show a decreased responsiveness to MIP-3β in vitro. Moreover, in vivo, when compared with CD44<sup>low</sup> naive or CD44<sup>int</sup> memory CD8 T cells CD44<sup>high</sup> CD8 T cells are preferentially distributed in spleen and peripheral blood. This fits well with their decreased expression of CCR7, as this receptor plays an essential role in the migration of immune cells into lymph nodes (31, 32). CD44<sup>high</sup> CD8 T cells produce high levels of IFN-γ following a brief stimulation using a combination of anti-CD3 and anti-CD28 Abs. Similarly, the tetramer<sup>+</sup>CD44<sup>high</sup>CD122<sup>+</sup> CD8 T cells produce IFN-γ in response to stimulation with NP68 peptide. The CD44<sup>high</sup>CD122<sup>+</sup> phenotype of these cells and their functional characteristics are classical hallmarks of memory CD8 T cells in mice. Based on the CCR7 expression and IFN-γ response these CD44<sup>high</sup> CD8 T cells are similar to the TEM described in humans. However, we have been unable to assess the proliferation capacity of the CD44<sup>high</sup> CD8 cells in vitro, as they do not proliferate significantly in response to peptide stimulation (18). This could reflect the low frequency of tetramer-positive cells among the CD44<sup>high</sup> subset. However, these cells also proliferate weakly in response to anti-CD3 plus anti-CD28 stimulation (data not shown). In this respect they are similar to memory phenotype CD8 T cells, which are generated following homeostatic proliferation in other TCR-transgenic systems (6) and to thymus-independent T cells (33). This last point highlights the fact that CD44<sup>high</sup>CD122<sup>+</sup> CD8 T cells in F5 but also in wild-type mice, where they are classically identified as memory cells, could be heterogeneous not only in their antigenic specificity but also in their generation requirements. Indeed, cells with a similar phenotype are generated experimentally by homeostatic pressure (22–26), naturally in elderly patients (34, 35) but...
also after antigenic stimulation (6, 25, 36, 37). Therefore, this subset includes “true” Ag experienced memory cells and T cells masquerading as memory cells. A similar heterogeneity of the human CD8^+ CCR7^+ population has also been described (38, 39).

We took advantage of the in vivo coexistence of two types of memory phenotype CD8 T cells in F5 mice to compare their persistence. In vivo the size of the CD44^{hi} CD8 memory phenotype subset was maintained for the entire life span of the animal. Moreover, these cells preserved their increased responsiveness. Indeed, the small percentage of CD44^{hi} F5 TCR^+ cells producing IFN-γ in response to NP68 peptide stimulation was maintained constant over time. This also suggests that in Tx F5 mice the repertoire of the CD44^{hi}CD122^+ cells is relatively stable and that CD44 phenotype conversion of CD44^{lo/med} tetramer-positive does not contribute to the CD44^{hi} subset increase. In contrast, the size of the CD44^{int} CD8 memory subset showed a slow but steady decrease over time that was similar to the one observed for naive cells. Nevertheless, CD44^{int}CD122^- memory CD8 T cells maintained their improved function in terms of IFN-γ production and in vitro proliferation in response to peptide stimulation. Moreover, although sustained priming does leads to a further increase in the proliferation and IFN-γ secretion capacity of the CD44^{int} CD8 subset, it does not modify their persistence profile. These data also highlight that memory cell characteristics such as long-term persistence and increased responsiveness can be acquired independently. A consequence of this property is that the memory CD8 pool could be even more heterogeneous as a result of differential acquisition of functional or survival properties. Indeed, expression of NK cell inhibitory receptor can drive the preferential accumulation of TM1 cells, a subset of CD44^{hi}CD122^- CD8 memory T cells (27). Moreover, different pathogens could drive the differentiation of naive cells in different subsets of memory cells with different functional properties or survival requirements. In fact, this has recently been found for different viruses or Ags (13, 27).

One important issue is to determine the parameters involved in the preferential persistence of the CD44^{hi}CD122^- CD8 memory subset. The expression of CD122 by CD44^{hi} CD8 T cells could be involved in the preferential survival of CD8 T cells from that subset. Indeed, a recent report where CD122 expression by memory CD8 T cells was induced by addition of cytokines during the priming phase does support this hypothesis (40). Alternatively, environmental Ags that would cross-react with the TCR could drive the persistence of CD44^{hi}CD122^- F5 CD8 T cells. This does not fit with results obtained by others, showing that memory CD44^{hi}CD122^- CD8 T cells persist when transferred in MHC class I knockout mice (41). Moreover, if an endogenous Ag cross-reacting with the F5 TCR did exist, it should react with and drive the persistence of both CD44^{lo/med} and CD44^{hi} NP68-specific CD8 T cells. However, this is not what we observe.

We found that in Tx mice, but not euthymic mice, the size of that subset increases with time. Although CD44^{hi} CD8 T cells contained a high proportion of dividing cell in both types of mice, the size increase observed in Tx mice correlated with a higher percentage of dividing cells in the CD44^{hi} subset. The augmented proliferation could result from the partial lymphopenia caused by the thymectomy and could be driven by the same mechanisms that drive the homeostatic proliferation that occurs when T cells are transferred in lymphopenic hosts (22–26). Alternatively, the thymus, in addition to its role in the production of mature T cells, could be involved in the homeostatic control of that subset. The persistence of the CD44^{hi} CD8 T cells is also associated with increased expression of three antiapoptotic bcl-2 family members: bcl-x, A1, and bcl-2. The elevated expression of these genes is correlated with a higher in vivo resistance to irradiation-induced cell death. Hence we show that the persistence of the CD44^{hi} CD8 T cells could result from increased proliferation and survival capacity of these cells.

In the F5 system the long-term persistence of the CD44^{hi} CD8 memory phenotype cells is associated with the expression of the IL-2Rβ chain (CD122), which is also part of the IL-15R. This fits well with a number of data showing that IL-15 drives the proliferation of CD44^{hi} CD8 T cells and is essential for their maintenance in vivo (for a review see Refs. 42 and 43). Alternatively, we do not know if the IL-15R expression is sufficient for the long-term persistence of memory CD8 T cell it would be important to identify the factors driving its expression. Indeed, long-term CD8 immunity may rely on the acquisition of this receptor by memory cells. Results presented in this paper indicate that for a given Ag, immunization conditions can indeed modulate the type of memory cells that are generated. Defining immunization conditions or adjuvants that would lead to the acquisition of this receptor by CD8 memory cells would be essential in clinical situations such as tumor immunotherapy where peptide immunization against tumor Ag is currently used to induce effector T cells.
Acknowledgments
We thank Dr. Ton Schumacher, who kindly provided PE-labeled tetramers. We also thank Drs. N. Bonnefoy-Bérad and J. Maryanski for critical reading of the manuscript.

References
1. Ahmed, R., and D. Gray. 1996. Immunological memory and protective immunity: understanding their relation. Science 272:54.
2. Dutton, R. W., L. M. Bradley, and S. L. Swain. 1998. T cell memory. Annu. Rev. Immunol. 16:201.
3. Bachmann, M. F., M. Barner, A. Viola, and M. Kopf. 1999. Distinct kinetics of cytokine production and cytolyis in effector and memory T cells after viral infection. Eur. J. Immunol. 29:291.
4. Bruno, L., J. Kirberg, and H. von Boehmer. 1995. On the cellular basis of immunological T cell memory. Immunity 2:37.
5. Cerwenka, A., L. L. Carter, J. B. Reome, S. L. Swain, and R. W. Dutton. 1998. In vivo persistence of CD8 polarized T cell subsets producing type 1 or type 2 cytokines. J. Immunol. 161:97.
6. Cho, B. K., C. Wang, S. Sugawa, H. N. Eisen, and J. Chen. 1999. Functional differences between naive and memory CD8 T cells. Proc. Natl. Acad. Sci. USA 96:2976.
7. Curtsinger, J. M., D. C. Lins, and M. F. Mescher. 1998. CD8+ memory T cells (CD44high, Ly-6C+) are more sensitive than naive cells (CD44low, Ly-6C-) to TCR/CD8 signaling in response to antigen. J. Immunol. 160:3236.
8. Pihlgren, M., P. M. Dubois, M. Tomkowiak, T. Sjogren, and J. Marvel. 1996. Resting memory CD8+ T cells are hyperreactive to antigenic challenge in vitro. J. Exp. Med. 184:2141.
9. Tanchot, C., S. Guillaume, J. Delon, C. Bourgeois, A. Franzke, A. Sarukhan, A. Trautmann, and B. Rocha. 1998. Modifications of CD8+ T cell function during in vivo memory or tolerance induction. Immunity 8:381.
10. Veiga-Fernandes, H., U. Walter, C. Bourgeois, A. McLean, and B. Rocha. 2000. Response of naive and memory CD8+ T cells to antigen stimulation in vivo. Nat. Immunol. 1:47.
11. Zimmermann, C., A. Prevost-Blondel, C. Blaser, and H. Pircher. 1999. Kinetics of the response of naive and memory CD8 T cells to antigen: similarities and differences. Eur. J. Immunol. 29:284.
12. Sallusto, F., D. Leng, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 401:708.
13. Champagne, P., G. S. Ogg, A. S. King, C. Knabenhans, K. Ellefsen, M. Nobile, V. Appay, G. P. Rizzardi, S. Fleury, M. Lipp, et al. 2001. Skewed maturation of memory T lymphocytes. Nature 410:106.
14. Lanzavecchia, A., and F. Sallusto. 2000. Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. Science 290:92.
15. Bunce, C., and E. B. Bell. 1997. CD45RC isoforms define two types of CD4 memory T cells, one of which depends on persisting antigen. J. Exp. Med. 185:767.
16. Toug, D. F., and J. Sprent. 1994. Turnover of naive- and memory-phenotype T cells. J. Exp. Med. 179:1127.
17. Zimmermann, C., K. Brduscha-Riem, C. Blaser, R. M. Zinkernagel, and H. Pircher. 1996. Visualization, characterization, and turnover of CD8+ memory T cells in virus-infected hosts. J. Exp. Med. 183:1367.
18. Pihlgren, M., C. Arpin, T. Walzer, M. Tomkowiak, A. Thomas, J. Marvel, and P. M. Dubois. 1999. Memory CD44high CD8 T cells show increased proliferative responses and IFN-γ production following antigenic challenge in vitro. Int. Immunol. 11:699.
19. Mamalaki, C., T. Norton, Y. Tanaka, A. R. Townsend, P. Chandler, E. Simpson, and D. Kaussius. 1992. Thymic deletion and peripheral activation of class I major histocompatibility complex-restricted T cells by soluble peptide in T-cell receptor transgenic mice. Proc. Natl. Acad. Sci. USA 89:11342.
20. Dubois, P. M., M. Pihlgren, M. Tomkowiak, M. Van Mechelen, and J. Marvel. 1998. Tolerant CD8 T cells induced by multiple injections of peptide antigen show impaired TCR signaling and altered proliferative responses in vitro and in vivo. J. Immunol. 161:5260.
21. Walzer, T., G. Joubert, P. M. Dubois, M. Tomkowiak, C. Arpin, M. Pihlgren, and J. Marvel. 2000. Characterization at the single-cell level of naive and primed CD8+ T cell cytokine responses. Cell. Immunol. 206:16.