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Modulation of Memory CD4 T Cell Function and Survival Potential by Altering the Strength of the Recall Stimulus

Deepa S. Patke and Donna L. Farber

Optimization of long term immunity depends on the functional persistence of memory T cells; however, there are no defined strategies for promoting memory T cell function and survival. In this study, we hypothesized that varying the strength of the recall stimulus could modulate the function and survival potential of memory CD4 T cells. We tested the ability of peptide variants of influenza hemagglutinin (HA) exhibiting strong and weak avidity for an HA-specific TCR, to modulate HA-specific memory CD4 T cells in vitro and in vivo. In vitro stimulation with a weak avidity peptide (L115) uncoupled memory CD4 T proliferation from effector cytokine production with low apoptosis, whereas stimulation with a strong avidity peptide (Y117) fully recalled memory T cell functions but triggered increased apoptosis. To determine how differential recall would affect memory T cells in vivo, we boosted BALB/c hosts of transferred, CFSE-labeled HA-specific memory CD4 T cells with native HA, Y117, and L115 variant peptides and found differences in early Ag-driven memory T cell proliferation and IL-7R expression, with subsequent changes in memory T cell yield. High avidity boosting resulted in rapid proliferation, extensive IL-7R down-regulation, and the lowest yield of HA-specific memory cells, whereas low avidity boosting triggered low in vivo proliferation, maintenance of IL-7R expression, and the highest memory T cell yield. Our results indicate that memory CD4 T cell function and survival can be modulated at the recall level, and can be optimized by low level stimulation that minimizes apoptosis and enhances responses to survival factors. The Journal of Immunology, 2005, 174: 5433–5443.

The goal of an effective vaccine is to prime for and maintain significant numbers of functionally proficient memory T lymphocytes that mediate rapid recall responses upon Ag challenge. Most vaccination strategies involve prime/boost regimens, to initially generate a population of Ag-specific memory T cells by priming, and further enhance memory T cell numbers and/or persistence via boosting. For vaccines directed against pathogens, the prime/boost strategy must generate pathogen-specific immunity in the absence of infection, and with minimal inflammatory responses, thus avoiding vaccine-induced immunopathology. However, although vaccines designed to generate long-lived T cell immunity against intracellular pathogens such as malaria and HIV show good priming of T cell responses, there is gradual attrition of memory T cells (1), particularly in peripheral sites (2). It is not known how best to maintain memory T cell numbers and function in the absence of infection, and there is a great need for immunological strategies to boost existing memory responses.

An important consideration in optimizing immune memory is to enhance memory T cell survival and homeostasis, while reducing memory T cell attrition by apoptosis. Cytokines such as IL-7 have been shown to promote survival of both memory CD4 and CD8 T cells (3–6), and expression of the IL-7 receptor (IL-7R) marks memory T cells for survival (7). Although memory T cells have been shown to be resistant to apoptosis (8) due to enhanced expression of antiapoptotic molecules (9, 10), they are also known to undergo apoptosis in vivo in response to viral infections (11) and antigenic recall in certain systems (12). Identifying conditions that differentially control memory T cell apoptosis and responses to survival factors is important for manipulating memory immune responses in vivo.

Recent findings on the heterogeneous nature of memory T cells in phenotype, function, and tissue distribution suggest that memory heterogeneity should also be a consideration in optimizing memory immune responses. Two subsets of Ag-specific memory T cells have been identified in humans and mice based on homing to lymphoid vs nonlymphoid tissue (13–17). It has been shown that tissue-specific memory T cells can provide protection at the site of infection (18), suggesting that strategies for manipulation of memory T cell tissue distribution may help promote immunity toward a specific pathogen.

A third consideration in regulating memory T cell responses is their functional capacity. Memory T cells have been shown to arise from effector cells, with the recall effector function of the resultant memory T cell population reflecting that of the precursor effector T cells (19, 20). However, we have previously shown that a clonotypic population of Ag-specific memory T cells exhibits functional plasticity as demonstrated by the change in cytokine profile from predominantly IFN-γ to IL-4 when the recall stimulus was altered by using anti-CD3 or increased Ag dose (21). On the basis of these results, we hypothesized that altering the strength of the recall stimulus may likewise affect memory CD4 T cell functions, with possible implications for modulating the survival, turnover, and tissue distribution of existing memory T cells in vivo.

In this study, we have directly tested the role of antigenic signal strength in modulating T cell memory by using altered antigenic peptide ligands (APL)3 to differentially engage the TCR of a...
population of memory CD4 T cells both in vitro and in vivo. APLs represent single amino acid substitutions of a native antigenic peptide that exhibit differential avidity for an Ag-specific TCR (22). These analog peptides can serve as partial agonists or antagonists of T cell activation, leading to uncoupling or selective activation of T cell functions such as cytokine production and proliferation (23, 24), anergy induction (25), or alteration of effector function (26); however, their ability to selectively modulate memory CD4 T cell functions has not been addressed.

We demonstrate here that altered peptide variants of influenza hemagglutinin (HA) exhibiting strong and weak avidity for an HA-specific TCR clone designated 6.5, can differentially recall HA-specific (6.5) memory T cells in vitro and in vivo. In vitro, we found that the weak avidity APL could stimulate memory T cell activation and proliferation, but not effector cytokine production even at high doses, demonstrating uncoupling of memory CD4 T cell function by weak antigenic stimulation. Conversely, stimulation with increasing doses of a strong agonist peptide yielded decreased Ag responses and increased apoptosis. In vivo, differential boosting of HA-specific memory T cells with native, strong, and weak avidity HA peptides resulted in alterations in early memory T cell proliferation and the extent of IL-7R down-regulation, with subsequent changes in memory T cell yield in lymphoid and nonlymphoid tissues. Low avidity boosting resulted in the highest memory T cell yields, whereas high avidity boosting gave the lowest overall memory T cell yields, particularly in nonlymphoid tissue. Our results demonstrate that functional maintenance of memory CD4 T cells can be regulated on the level of recall responses and have important implications for boosting immunity in vaccines and abrogating memory in autoimmune diseases.

Materials and Methods

Mice

BALB/c mice (8–16 wk of age) were obtained from the National Cancer Institute Biological Testing Branch. Influenza HA-TCR-transgenic mice (27) bred as heterozygotes onto BALB/c (Thy-1.2) or BALB/c (Thy-1.1) hosts, and RAG2−/− mice (28) on BALB/c backgrounds (Taconic Farms) were maintained in the Animal Facility at the University of Maryland Medical Center (Baltimore, MD) under specific pathogen-free conditions.

Abs and peptides

The following Abs were purified from bulk culture supernatants and purchased from BioExpress: anti-CD8 (TIB 105), anti-CD4 (OK1-5), anti-I-A^d (212.A1), and anti-Thy-1 (TIB 238). The 6.5 anti-clonotype Ab directed against the HA-TCR (27) was purified and conjugated to biotin (Pierce). PE-conjugated anti-IFN-γ, CD25, IL-7R, and PerCP-conjugated anti-CD4 were purchased from BD PharMingen.

HPLC-purified influenza HA peptide (110–120, SFERFEIYPKE) and APLs SFERFEIYPKE (Y117) and SFERFLIFPKE (L115) were synthesized by the Biopolymer Laboratory at the University of Maryland School of Medicine.

Generation of effector and memory CD4 T cells

For generation of HA-specific effector CD4 T cells, CD4 T cells were purified from spleens of HA-TCR mice as described (29) and cultured with 5.0 μg/ml HA peptide and mitomycin C-treated APCs prepared from BALB/c splenic APC (27). These antigen-presenting cells were prepared from spleens of BALB/c mice (8–16 wk of age) purchased from the National Cancer Institute Biological Testing Branch. Influenza HA-TCR-transgenic mice bred as heterozygotes onto BALB/c (Thy-1.2) or BALB/c (Thy-1.1) hosts as previously described (21). Briefly, memory CD4 T cells were cultured with APC and HA peptide or APL for a total of 12–45 h, and monensin (Golgistop; BD PharMingen) was added 6 h before harvesting. Specific time points for each experiment are indicated in the figure legends. Cells were stained for surface markers with PerCP-conjugated anti-CD4, biotinylated anti-6.5 TCR, and PE-conjugated anti-CD25, IL-7R, and PerCP-conjugated anti-CD4 were purchased from BD PharMingen.

ELISPOT

The relative frequencies of IFN-γ and IL-4 secreting memory CD4 T cells in response to stimulation with HA and APLs were determined using ELISPOT as described (33, 34). Briefly, 96-well MAHA nitrocellulose plates (Millipore) were coated overnight at 4°C with rat anti-mouse IFN-γ (AN18; Mabtech) or rat anti-mouse IL-4 Abs (11B11; Mabtech), before the addition of 20,000 purified memory T cells, 60,000 BALB/c APC, and HA, Y117, or L115 peptide (5 μg/ml) per well. Plates were incubated at 37°C for 12 h, washed, and developed using biotinylated detection Abs (anti-mouse IFN-γ, mAb R4–6A2-Biotin and anti-mouse IL-4, BVD6, both from Mabtech) followed by streptavidin-HRP (Vectastain ABC kit (Elite); Vector Laboratories) and AEC substrate (Vector Laboratories). Spots were counted using the ImmunoSpot ELISPOT reader (CTL, BD Biosciences).

In vivo boosting of memory CD4 T cells

To monitor the recall proliferation of HA-specific memory CD4 T cells to native HA and APLs in vivo, HA-specific memory CD4 T cells derived from HA-TCR (Thy-1.1) effector cells transferred into RAG2−/− hosts as described above (Fig. 2A) were harvested 8 wk posttransfer from spleens of 3–6 adoptive hosts, enriched for CD4 cells, labeled with 2.5 μM CFSE,
The resultant memory CD4 T cells were recovered from control (Fig. 2). The generation of HA-specific memory CD4 T cells from in vitro priming of naive 6.5 T cells with native HA peptide and APC to generate effector cells that are subsequently transferred to RAG2−/− adoptive hosts. HA-specific 6.5 memory CD4 T cells were recovered from adoptive hosts 8–16 wk posttransfer and recalled in vitro with native HA, the Y117 or L115 APL, or no peptide as a control, and activation, proliferation, and effector cytokine production (prod.) were monitored. B, Experimental strategy for differential boosting of HA-specific memory CD4 T cells in vivo. Spleen-derived memory CD4 T cells generated as in A were labeled with CFSE, transferred in equal numbers into BALB/c secondary hosts, and boosted i.v. 4 days later with 100 μg of HA, Y117, or L115 peptides in PBS, with PBS alone as a negative control. After boosting (2, 4, and 7 days), memory CD4 T cells were isolated from the spleen, liver, and lung and analyzed.

FIGURE 2. Experimental design for in vitro and in vivo recall of HA-specific memory CD4 T cells with HA and HA APL. A, Schematic for generation of HA-specific memory CD4 T cells from in vitro priming of naive 6.5 CD4 T cells with native HA peptide and APC to generate effector cells that are subsequently transferred to RAG2−/− adoptive hosts. HA-specific 6.5 memory CD4 T cells were recovered from adoptive hosts 8–16 wk posttransfer and recalled in vitro with native HA, the Y117 or L115 APL, or no peptide as a control, and activation, proliferation, and effector cytokine production (prod.) were monitored. B, Experimental strategy for differential boosting of HA-specific memory CD4 T cells in vivo. Spleen-derived memory CD4 T cells generated as in A were labeled with CFSE, transferred in equal numbers into BALB/c secondary hosts, and boosted i.v. 4 days later with 100 μg of HA, Y117, or L115 peptides in PBS, with PBS alone as a negative control. After boosting (2, 4, and 7 days), memory CD4 T cells were isolated from the spleen, liver, and lung and analyzed.

Results

Use of HA APL to vary the strength of the antigenic stimulus

To test the hypothesis that altering the avidity of the antigenic stimulus would differentially recall memory CD4 T cells, we used influenza HA-specific CD4 T cells derived from HA-TCR-transgenic mice exhibiting the 6.5 clonotype (27) to generate memory CD4 T cells as previously described (21, 29), and we analyzed their ability to respond to peptide variants of HA exhibiting different avidities for the 6.5 TCR. We chose single amino acid substitutions of HA that exhibited strong (peptide Y117) and weak (peptide L115) avidity for the 6.5 TCR relative to native HA peptide, based on their ability to stimulate a 6.5+ CD4 T cell clone (Table I; Ref. 35).

We first assessed how these different avidity peptides would stimulate primary 6.5+ T cells by assaying proliferative dose response of naive HA-TCR (6.5+) CD4 T cells derived from HA-TCR mice to HA, Y117, and L115 peptides in vitro. In response to the weak avidity L115 peptide, we found decreased proliferation that was 40% of the level seen with a similar dose of native HA peptide (Fig. 1). By contrast, proliferative responses to the high avidity Y117 peptide were comparable with that of native HA at 0.5- and 5.0-μg/ml doses but inhibitory at high doses (Fig. 1). These results demonstrate differential responses of primary HA-TCR CD4 T cells to L115 and Y117 peptide variants that are consistent with their being weak and strong avidity peptides, respectively.

Proliferation and apoptosis of memory CD4 T cells stimulated with HA peptide and APL

We generated HA-specific memory CD4 T cells as previously described (21, 29) using in vitro priming of HA-TCR (6.5+) CD4 T cells with HA peptide and APC followed by in vivo transfer into RAG2−/− adoptive hosts (Fig. 2A). The resultant memory CD4 T cells harvested 2–6 mo posttransfer from RAG2−/− hosts bear all of the phenotypic and functional attributes of memory T cells and are also indistinguishable from memory CD4 T cells similarly generated in BALB/c hosts (21, 29, 30). We assessed the ability of peptide variants of influenza HA to differentially recall HA-specific memory T cells in vitro and in vivo as outlined in Fig. 2, with results presented below.

We assessed proliferative dose responses of CFSE-labeled HA-specific memory CD4 T cells to native, strong (Y117), and weak (L115) avidity HA variants. We analyzed proliferation qualitatively by examining the CFSE profile (Fig. 3A) and quantitatively by calculating the percent of dividing cells and the proliferative response of HA-TCR CD4 T cells to L115 and Y117 peptide variants that are antigenic, low, and high affinity peptide, they exhibit suboptimal proliferative responses (~40% of native HA response) to low doses of a low avidity peptide and to high doses of a high avidity peptide.

We investigated whether the decline in proliferative capacity of memory T cells to high doses of the strong avidity Y117 peptide...
FIGURE 3. Proliferative responses of HA-specific memory CD4 T cells to HA variant peptide ligands in vitro. A, Proliferative dose response of CFSE-labeled HA-specific memory CD4 T cells. Memory CD4 T cells generated by adoptive transfer of HA-TCR effector cells (see Materials and Methods) were isolated >2 mo posttransfer from spleens of RAG2−/− adoptive hosts, CFSE labeled, and cultured with BALB/c APC and the indicated concentrations of HA, Y117, or L115 peptides. Proliferation was monitored after 72 h and analyzed by flow cytometry. Results are shown as histograms of peptide-stimulated (bold lines) compared with APC alone (thin line), gated on 6.5+/CD4+ cells, and are representative of three experiments. Numbers in each graph indicate the percent of dividing cells (see Materials and Methods). B, Proliferative index of differentially stimulated memory CD4 T cells. The number of events at each division delineated by markers in each histogram represented by M1–M8 (inset) was used to calculate the undivided cohort (see Materials and Methods), and the proliferative index was calculated by dividing the total number of events by the total undivided cohort. Results are representative of four separate experiments with similar indices.

(Fig. 3) was due either to increased apoptosis, which is known to occur with high avidity stimulation (36), or to decreased responsiveness via down-modulation of the TCR. For determination of the extent of apoptosis induced with each stimulus, we measured the percentage of Ag-specific 6.5+ annexin V+ T cells in response to different peptide doses after 3 days of activation (during the peak proliferative response; Fig. 4A). Stimulation of memory CD4 T cells with the high avidity Y117 peptide gave the highest percentage of apoptotic cells at all doses (Fig. 4B), whereas stimulation with the weak avidity or native HA peptide yielded similar percentages of apoptotic cells at all doses, with increased proportions of 6.5+ apoptotic cells observed with increased peptide doses (Fig. 4B). Similar distinctions in the percent apoptotic cells between the three peptides were observed after 2 days of stimulation (data not shown). These results demonstrate that although high avidity Y117 peptide stimulation yielded the highest level of memory T cell apoptosis, increased apoptosis was also observed with the high doses of HA and L115 stimulation.

To assess whether different avidity stimulation was also affecting memory T cell responses via modulation of TCR expression, we measured the extent of TCR down-regulation triggered by different doses of the three peptides. As shown in Fig. 5 (top), the level of 6.5 TCR expression decreased with increasing peptide dose, although the extent of TCR down-regulation at each dose was less for L115 stimulation than for HA and Y117 stimulation, each of which induced a similar extent of 6.5 TCR down-regulation. The maintenance of a higher level of TCR expression from L115 stimulation compared with Y117 stimulation at all doses is depicted in the pooled analysis in Fig. 5 (bottom). We observed this lower level of TCR down-regulation with L115 stimulation, compared with Y117 and HA stimulation, at both early (2–12 h) and late times (48–72 h) after stimulation (data not shown). These results suggest that L115-stimulated memory CD4 T cells maintain a higher level of antigenic responsiveness than do Y117-stimulated cells, due to maintenance of a higher level of Ag-specific TCR expression.

Uncoupling of memory CD4 T cell function by HA APL

Given the different dynamics of stimulation induced by the HA APL, we asked whether memory CD4 T cell effector function could be differentially recalled by these peptides. We initially compared the ability of HA-specific memory to produce IFN-γ, proliferate and up-regulate CD25 (as an additional measure of activation) with the abilities of HA, Y117, and L115 at the optimal
We asked whether the low level of IFN-γ production observed with L115 stimulation reflected either suboptimal stimulation that could be overcome with increased peptide dose, or alterations in cytokine profile from IFN-γ to IL-4 production, as seen with anti-CD3 stimulation of this memory T cell population (21). To address the first question, we compared IFN-γ production from 6.5+ memory CD4 T cells stimulated with low (0.5 μg/ml), medium (5.0 μg/ml), and high (50 μg/ml) doses of native HA, Y117, or L115 APL using ICS and ELISPOT, with pooled results shown in Fig. 6B. Fig. 6B shows that stimulation with high doses of the weak avidity L115 peptide still resulted in suboptimal IFN-γ production compared with stimulation with native HA peptide. Stimulation with low and medium doses of the high avidity Y117 peptide yielded comparable IFN-γ production as HA stimulation and decreased IFN-γ production at high doses (Fig. 6B). These results demonstrate that IFN-γ production by memory T cells is inhibited by low avidity stimulation, which cannot be overcome by increased peptide dose. The complete dose and kinetic responses of HA-specific memory vs naive CD4 T cells are shown in Fig. 7, demonstrating that although the hierarchy of stimulation by HA APL is similar in both primary and memory responses, HA-specific memory cells respond with more rapid kinetics and lower peptide concentration (5 μg/ml; Fig. 6A). We found that 6.5+ memory CD4 T cells exhibited comparable up-regulation of the activation marker CD25 and comparable cell division, in response to stimulation with HA, Y117, or L115 (Fig. 6A, top row); however, although both HA- and Y117-stimulated memory CD4 T cells exhibited high levels of IFN-γ production, L115-stimulated memory CD4 T cells produced low levels of IFN-γ production, similar to background levels observed with APC alone (Fig. 6A, bottom row). These results indicate that the low avidity stimulus delivered via L115 was capable of inducing memory CD4 T cell proliferation but not effector cytokine production. To address whether L115-mediated stimulation triggered a switch in memory CD4 T cell cytokine profile, we assessed the production of the Th2 cytokine IL-4 from peptide-stimulated memory CD4 T cells using ELISPOT, with the mean differences in percent MFI of differentially restimulated cells were measured relative to that of HA expressed as the mean ± SD from three separate experiments.

peptide doses and exhibit more sustained responses to all three peptides, compared with naive CD4 T cells. To address whether L115-mediated stimulation triggered a switch in memory CD4 T cell cytokine profile, we assessed the production of the Th2 cytokine IL-4 from peptide-stimulated memory CD4 T cells using ELISPOT, with the mean differences in cytokine secretion for Y117- and L115-stimulated memory CD4 T cells relative to HA-stimulated memory cells shown in Table II. Both HA- and Y117-stimulated memory CD4 T cells show a preponderance of IFN-γ production, with low levels of IL-4 (Table II, Columns 5 and 6 and “Ratio Y117:HA”). L115-stimulated memory CD4 T cells also exhibited greater numbers of IFN-γ secretors compared with IL-4 secretors (Table II, Columns 5 and 8), although at a greatly decreased (>6-fold) proportion compared with HA-stimulated memory CD4 T cells (Table II). A similar trend was observed with IL-2 production, with a 4-fold decrease between the numbers of IL-2 secreting cells in response to restimulation with L115 compared with HA (data not shown). We did not detect significant levels of IL-10 or IL-5 from native, Y117-, or L115-stimulated memory CD4 T cells (data not shown). These results ascertain that low avidity (L115) stimulation of memory CD4 T cells does not promote a shift in cytokine profile but rather...
results in functional uncoupling of proliferation from cytokine production.

In vivo proliferative responses of differentially boosted HA-specific memory CD4 T cells

Our in vitro results showed that weak and strong avidity HA-APL exhibited profound differences in their ability to trigger memory T cell effector cytokine production, apoptosis, and modulation of TCR expression. We therefore hypothesized that in vivo recall of memory T cells with these peptide ligands would likewise result in differential activation that could potentially affect survival in vivo. To follow the fate of differentially recalled HA-specific memory CD4 T cells in vivo, we recovered persisting memory CD4 T cells from the spleens of RAG2−/− adoptive hosts 2–4 mo after transfer of primed cells, labeled them with CFSE, and transferred these memory CD4 T cells in equal numbers to intact BALB/c hosts bearing a Thy-1 allelic difference that were subsequently immunized with 100 μg/ml HA, Y117, or L115 peptide in PBS or with PBS alone as a control (Fig. 2B). The effects of this differential in vivo peptide boosting on memory T cell proliferation, yield, and precursor frequency were analyzed as described below.

We assessed in vivo proliferation two days after peptide boosting to assess the immediate effects of in vivo stimulation of HA-specific memory CD4 T cells with different HA peptide ligands. We found increased numbers of HA-specific memory CD4 T cells in response to boosting with all three peptides, compared with PBS-boosted controls indicating rapid memory CD4 T cell expansion in vivo (Fig. 8A, top row). Consistent with these increased numbers, we observed a substantial level of in vivo memory cell division in response to boosting with all three peptides, with similar proliferative responses to native HA peptide (46% of cells underwent division), and Y117 (50% dividing cells) and lower proliferation in response to L115-boosting (23% dividing cells) (Fig. 8A, second row). These results establish that HA-specific memory CD4 T cells exhibit rapid proliferative responses to all three peptide ligands in vivo, with decreased proliferation from cells boosted with the weak avidity peptide. We also measured the kinetics of in vivo proliferation to the different peptides 2, 4, and 8 days after peptide boosting with pooled results shown in Fig. 8B. In vivo proliferation peaked at 2–4 days for each peptide boost and declined by 8 days, and the overall percent of proliferating cells was similar for HA- and Y117-boosted and lowest for L115-boosted memory T cells at all time points (Fig. 8B).

Because we found differences in apoptosis triggered by the three peptides in vitro, we also tested for the generation of annexin V+ cells 2 and 4 days after in vivo peptide boosting. We were unable to detect differences in the proportion of 6.5+ annexin V+ cells from any of the boosting groups (data not shown), most likely due to the rapid uptake of apoptotic cells by scavenger macrophages in vivo (37, 38). As an alternate approach to assess in vivo memory CD4 T cell activation and potential for survival, we measured expression of the IL-7R that has been correlated with memory T cell survival (7). After 2 days, we found a lower level of IL-7R expression on L115-boosted (MFI = 30) and Y117-boosted (MFI = 29) memory CD4 T cells, compared with PBS-boosted controls (MFI = 89); however, the level of IL-7R expression on L115-boosted memory CD4 T cells

Table II. Cytokine secretion from memory T cells restimulated in vitro with HA, Y117, or L115

<table>
<thead>
<tr>
<th>Experiment</th>
<th>HA</th>
<th>Y117</th>
<th>L115</th>
<th>HA</th>
<th>Y117</th>
<th>L115</th>
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<th>IL-4:HA Ratio</th>
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<td>320</td>
<td>1.0</td>
<td>0.86</td>
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</table>

Mean ± SD 0.95 ± 0.04 0.9 ± 0.3 0.15 ± 0.07 0.18 ± 0.07

a Number of cytokine-secreting cells per 1 × 10⁶ memory CD4 T cells measured by ELISPOT 12–24 h after in vitro recall with 5 μg/ml HA peptide and BALB/c APC. b Ratio obtained by dividing the number of IFN-γ or IL-4-producing cells obtained from Y117 or L115 stimulation, respectively, by the number obtained by stimulation with native HA peptide.
and the highest proportion of IL-7R peptide-stimulated memory T cells in vivo; 4 days after peptide stimulation of IL-7R expression on Y117-boosted cells (14% IL-7R A).

Differential boosting of memory CD4 T cells alters yield in lymphoid and nonlymphoid tissues

We asked how the variations in early proliferative responses and IL-7R down-regulation as a result of differential boosting would affect the subsequent distribution and yield of Ag-specific memory CD4 T cells in both lymphoid and nonlymphoid tissues. We thus harvested memory CD4 T cells from spleen, lung, and liver of secondary hosts 8 days after in vivo boosting and analyzed them by flow cytometry (Fig. 9). In control PBS-boosted mice, the persisting 6.5+ memory CD4 T cells were nondividing and present in spleen, liver, and lung, demonstrating migration of memory CD4 T cells to nonlymphoid tissue independent of cell division (Fig. 9A, Column 1). HA-boosted memory T cells were also present in these three tissues, with nondividing and dividing cells present in spleen and a preponderance of maximally dividing 6.5+ cells present in lung and liver (Fig. 9A, Column 2). By contrast, the 6.5+ memory CD4 T cells persisting in Y117-boosted mice comprised cells that had undergone only one or two divisions and were present in low numbers in lung and liver tissue (Fig. 9A, Column 3). In L115-boosted mice, substantial numbers of 6.5+ memory CD4 T cells were found in spleen, lung, and liver, with the majority of cells exhibiting less than two divisions. These results show that differential boosting of memory CD4 T cells affects their in vivo turnover and distribution in nonlymphoid tissue. Substantial proportions of maximally dividing memory CD4 T cells persisted only with HA boosting, whereas persisting cells resulting from Y117 and L115 boosting consisted primarily of minimally dividing cells.

To address whether the differential boosting affected overall memory CD4 T cell yields, we calculated the absolute numbers of 6.5+ Thy-1.1+ T cells in each tissue from differentially boosted mice and present results as yield relative to PBS-boosted control mice (Fig. 9B). We found that in all tissues, the relative yield of memory CD4 T cells boosted with the weak avidity L115 was highest (Fig. 9B), particularly in the spleen, where an average of 1.5-fold more 6.5+ memory CD4 T cells were found compared with PBS-boosted mice (p < 0.023). By contrast, Y117-boosted memory CD4 T cells were present in low numbers in all tissues, with the most striking deficiencies found in nonlymphoid lung tissue (50% of control in lung, p < 0.0006; and 60% of control in liver tissue, p < 0.428) (Fig. 9B). Boosting with HA gave yields that were close to PBS-boosted memory T cells in the spleen, with higher numbers in nonlymphoid lung and liver tissue (Fig. 9B). The increased yield induced by L115 boosting and decreased numbers seen with Y117 boosting persisted up until 30 days in vivo (data not shown). These results demonstrate that boosting with peptides of varying avidity can differentially affect memory T cell yield in different tissues; boosting with a low avidity peptide gave the highest yields in all tissues; boosting with the high avidity peptide resulted in the lowest yields particularly in nonlymphoid tissue; and boosting with the native peptide gave higher yields in nonlymphoid tissues.
We further examined the functional precursor frequency of HA-specific memory CD4 T cells after differential boosting by measuring early IFN-γ and IL-2 production of splenic memory CD4 T cells after tertiary reactivation with HA peptide in vitro by ELISPOT. Consistent with our findings that L115-boosting resulted in the highest yield of HA-specific memory T cells, the greatest number of Ag-specific IFN-γ and IL-2 producers was also observed (Fig. 9C). By contrast, both HA- and Y117-boosted memory CD4 T cells showed decreased IFN-γ and IL-2 producers relative to PBS- and L115-boosted mice (Fig. 9C, black and white bars), consistent with their overall lower yields in spleen (Fig. 9B). These results demonstrate that the increased memory T cell yield found in L115-boosted mice represents an increase in functionally competent memory CD4 T cells.

FIGURE 9. Tissue distribution and functional yield of differentially boosted memory CD4 T cells. Memory CD4 T cells were harvested from spleen, lung, and liver of secondary hosts 1 wk after boosting and analyzed. A. In vivo proliferative response of 6.5⁺ memory CD4 T cells after differential boosting. Results are shown as the CFSE profile from an individual mouse representative of five separate experiments. B. Relative yield of 6.5⁺ memory CD4 T cells in the spleen, lung, and liver of secondary hosts after differential boosting with HA peptide ligands. Relative yield is expressed as (6.5⁺ cells in peptide-boosted mice/6.5⁺ cells in PBS-boosted mice) × 100. Results are shown from individual mice and pooled from five separate experiments for spleen, and three separate experiments for lung and liver. Matched symbols in all tissues were derived from individual mice. C. Frequency of IFN-γ and IL-2 producers from differentially boosted memory CD4 T cells. Splenic memory CD4 T cells were harvested from secondary hosts as in A and B and cultured with 5 μg/ml HA peptide and BALB/c APC for 12 h; the frequency of IFN-γ and IL-2 producers was determined by ELISPOT (see Materials and Methods). Results are representative of three separate experiments and shown as the mean of duplicate cultures with variance <10%. Controls containing memory T cells cultured with APC alone and no peptide showed <10 spots (data not shown).
Discussion

We demonstrate here that alterations in the recall signal strength mediated by antigenic peptides differing in TCR avidity can modulate Ag-specific CD4 T cell memory in vitro and in vivo. In vitro, stimulation of HA-specific memory CD4 T cells with a weak avidity peptide ligand uncouples memory CD4 T proliferation from effector cytokine production and triggers a low level of apoptosis. By contrast, stimulation with a strong avidity HA peptide fully recalls memory T cell functions yet triggers a high level of apoptosis and is inhibitory at high concentrations. In vivo recall/boosting with these HA peptide variants led to differences in early Ag-driven memory T cell proliferation and down-regulation of IL-7R expression, with subsequent changes in memory cell yield in lymphoid and nonlymphoid tissues. Boosting with the high avidity Y117 peptide led to a similar high level of Ag-specific memory CD4 T proliferation in vivo as native HA yet resulted in the lowest level of IL-7R expression and the lowest yields in lymphoid and nonlymphoid tissues. By contrast, boosting with the weak avidity L115 peptide triggered less Ag-driven proliferation in vivo compared with native HA and Y117 but resulted in maintenance of IL-7R and the highest overall functional yields. Our data suggest that memory T cell function and survival can be selectively modulated by varying the avidity of the recall or boosting stimulus.

Our finding that altered peptide ligands of distinct TCR avidity can differentially recall effector and proliferative responses of HA-specific memory CD4 T cells indicates that memory T cell activation can be regulated by changes in antigenic signal strength. The weak avidity L115 APL acts as a "partial agonist" in its ability to uncouple memory T cell proliferation and effector cytokine production. Whereas partial agonist activation of T cell clones and primary T cells with a weak avidity peptide resulted in lymphokine secretion in the absence of proliferation (41), activation of memory CD4 T cells with L115 gave the converse response: proliferation but no effector cytokine production. Given recent findings that memory CD4 T cells express high constitutive levels of proteins for rapid entry into the cell cycle (41), it is possible that the thresholds for proliferation vs cytokine production differ in memory CD4 T cells. We also found a lower level of TCR down-regulation on memory CD4 T cells when stimulated with L115, compared with HA (Fig. 5), which indicates alterations at the level of TCR signaling (42). Although it is not known whether the multiple functions of memory T cells (43, 44) are controlled by diverse signaling pathways, it is possible that functional uncoupling of memory T cells results from differential triggering of TCR-coupled intracellular signaling pathways.

Our in vivo results demonstrate that the survival potential and eventual yield of memory CD4 T cells can be modulated by different avidity stimuli. We obtained the highest yield from boosting with the low avidity L115 peptide and the lowest yield from boosting with the strong avidity Y117 peptide. Several mechanisms may account for these results. First, the potency of TCR signaling can directly affect memory T cell survival. We demonstrate here that stimulation of memory CD4 T cells with a strong avidity peptide induces the highest level of apoptosis compared with stimulation with the native or weak avidity peptide, consistent with reports showing that a strong agonist can trigger death of activated T cells (45). Moreover, we found that high avidity stimulation led to extensive TCR down-regulation that likely results in reduced Ag responsiveness, as has been previously demonstrated for CTL clones (46). Conversely, low avidity stimulation is associated with a high association/dissociation kinetics (47) that may account for the maintenance of TCR expression in L115-boosted memory T cells.

Another factor affecting memory T cell survival and yield concerns their ability to respond to survival factors. Certain cytokines such as IL-7 and IL-15 have been shown to promote survival and/or homeostatic turnover of memory T cells in vivo (4–6). Furthermore, expression of the IL-7R is correlated to memory CD8 T cell survival (7) and homeostasis of endogenous memory CD4 T cells (48). We found that the extent of early IL-7R down-regulation and the level of IL-7R re-expressed on boosted memory CD4 T cells correlated with their survival in vivo; L115 boosting yielded the highest level of IL-7R expression and showed the highest survival and yield in vivo, and Y117-boosted memory cells exhibited the lowest IL-7R expression, and the lowest in vivo survival. These results indicate that the boosting stimulus which promoted the lowest Ag-driven proliferation also promoted the greatest survival potential via homeostatic cytokines.

Finally, the effector functions elicited by memory T cells can also affect survival. It has been found that IFN-γ production by effector cells results in their rapid attrition (49), suggesting that a booster stimulus that promotes IFN-γ production (such as native HA and Y117) may also promote memory cell loss, whereas a booster stimulus that promotes turnover but not IFN-γ production and redifferentiation to effector cells (as shown with L115 stimulation) will ultimately promote survival.

When taken together, our results indicate that there are multiple parameters that contribute to memory T cell longevity in vivo, including Ag-driven and Ag-independent factors. In Fig. 10, we present a model for the dynamics of memory T cell expansion and survival after differential boosting with antigenic peptides of varying avidity. Reactivated or boosted memory T cells pass through three distinct kinetic phases: in phase 1 (1–2 days after challenge),...
antigenic activation triggers rapid proliferation and down-regulation of both the TCR and IL-7R, with the extent of their down-regulation directly correlating to the avidity of the recall Ag; in the second phase (3–5 days), cells continue to divide via antigen stimulation and undergo apoptosis depending on the strength of the signal (optimum signals allow prolonged periods of cell cycling, whereas high avidity signals reduce Ag responsiveness via extended TCR down-regulation). In the final phase (>1 wk), Ag-driven proliferation is substantially decreased, and the cells now maintain their survival by homeostasis and their ability to respond to survival factors. In this phase, memory CD4 T cells that express a higher level of IL-7R expression (e.g., from low avidity boosting) have an advantage.

The ability to modify an existing memory T cell response with differential boosting has important implications for optimization of memory in vaccines and abrogation of memory in autoimmunity. In vaccines, it has proved difficult to maintain a strong memory T cell response to pathogens such as malarial parasites (50, 51), HIV, and hepatitis C (52). Our results showing augmentation of memory T cell numbers by low affinity stimulation in the absence of adjuvants or infection suggest that a similar strategy could be incorporated into pathogen-specific prime/boosting regimens to increase memory T cell yield and protective immunity in vaccines. Conversely, our findings that high avidity peptide boosting may trigger memory T cell apoptosis and a decreased propensity for survival suggest that a similar strategy may be incorporated into therapies for autoimmune diseases. Altered peptide ligands have been used to modify autoreactive T cell responses in animal models of autoimmune diseases such as multiple sclerosis (53–56), experimental allergic encephalomyelitis (57), and type I diabetes (58, 59). However, clinical trials using peptide therapy in human experimental allergic encephalomyelitis (57), and type I diabetes mediated TCR down-regulation). In the final phase (>1 wk), Ag-driven proliferation is substantially decreased, and the cells now maintain their survival by homeostasis and their ability to respond to survival factors. In this phase, memory CD4 T cells that express a higher level of IL-7R expression (e.g., from low avidity boosting) have an advantage.

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