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Memory CD8$^+$ T cells require CD8 coreceptor engagement for calcium mobilization and proliferation, but not cytokine production

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Summary

Memory T-cell responses are faster and more robust than those of their naïve counterparts. The mechanisms by which memory T cells respond better to subsequent antigenic exposure remain unresolved. A portion of the more rapid response is undoubtedly the result of the increased frequency of antigen-specific cells. In addition, there are also differences in the cells themselves with respect to their requirements for costimulation and the apparent avidity of the T cells. We used major histocompatibility complex (MHC) class I tetramers to stimulate T cells to focus on the interaction of T-cell receptor (TCR)/MHC and CD8 in the absence of other molecules that are present on cell surfaces and so contribute to the activation of T cells by undefined mechanisms. Mutated MHC class I tetramers that are unable to engage CD8 were used to investigate the role of CD8 engagement in memory cell activation. Either wild-type tetramers or tetramers carrying the mutation were used to stimulate both memory and naïve TCR transgenic T cells *in vitro*. Surprisingly, like naïve cells, memory CD8$^+$ T cells required CD8 engagement for calcium mobilization and optimum proliferation. In contrast, the requirements for cytokine production differed. Unlike naive cells, memory cells were able to produce cytokine in the absence of CD8 engagement. This suggests both a CD8-dependent pathway for early events and a CD8-independent pathway for cytokine production in memory cells.

Keywords: CD8 coreceptor; major histocompatibility complex tetramer; memory

Introduction

The requirement of CD8 coreceptor engagement in the activation of CD8$^+$ T cells has been an area of recent interest. CD8 has been proposed to have two distinct functions in T-cell activation: stabilization of peptide-major histocompatibility complex/T-cell receptor (pMHC/TCR) complexes, as well as a direct role in T-cell signalling via p56^{lck}.[1–4] Work carried out by our laboratory and others has shown that CD8 engagement is required for full CD8$^+$ T-cell activation during interaction of moderate- to low-affinity pMHC/TCR in naïve CD8$^+$ T cells.[4–9] Furthermore, increased pMHC/TCR affinity can partially compensate for the absence of CD8 binding to pMHC.[4–8,10–13] While the work described above concentrates on CD8 coreceptor requirements in naïve CD8$^+$ T cells, very little work has been conducted to directly measure CD8 coreceptor requirements in memory cells.

There are at least two distinct memory populations: effector memory cells and central memory cells.[14–16] Each population is defined by the expression of a set of cell surface molecules. Effector memory cells are rapidly activated and turned over. They express lower levels of L-selectin that allow homing to non-lymphoid tissues. Central memory cells are characterized by a lower activation state, and localize to lymph nodes.

Memory cells were historically defined by a faster response to antigen than naïve cells. Indeed, as described above, effector memory cells are constitutively activated and react almost immediately to stimulation.[14] Even though central memory cells have a lower activation state, responses for proliferation, cytokine production and cytotoxic T-lymphocyte activity are faster than for naïve cells.[14] For example, memory cells can become cytolytically activated and eliminate targets within 1–4 hr, compared to the approximately 2–3 days *in vitro* for

naïve cells.[17] However, the mechanism for this increased responsiveness remains elusive. Certainly the precursor frequency of antigen-specific cells is higher in immune mice.[14] Thus, more cells are present to respond to a specific antigen. However, memory cells respond by producing more cytokine, more rapidly and at lower antigen concentrations than naïve cells.[18–21]

While it has been shown that memory T cells do require less costimulation through CD80/86 and CD28 than their naïve counterparts,[22] very little has been done to study the coreceptor requirements of memory $CD8^+$ T cells, in contrast to active cytotoxic T lymphocytes. Bachmann *et al.* showed that down-regulation of CD8 occurred on memory cells more effectively than on naïve cells.[23] They argued further that a higher fraction of CD8 molecules are preassociated with $p56^{lck}$ than in naïve cells, suggesting that the enhanced association of $p56^{lck}$ was a possible cause of enhanced responsiveness. In the same paper they note that memory CD8 T cells proliferate less when treated with anti-CD8 antibody than naïve T cells when stimulated with high-affinity peptide KAVYNFATM (C9M) presented on thioglycollate-induced macrophages.[23] This is different from what we reported when stimulating naïve T cells with C9M/D^b tetramer.[4] Using a D^b tetramer that is defective in CD8 engagement, we found that naïve cells proliferated well when stimulated with C9M, but much less with wild-type gp33 tetramer.[4] This led us to hypothesize that the requirements for CD8 engagement would be reduced for memory T cells as compared to naïve T cells.

Soluble MHC tetramers provide the ability to examine specific pMHC/TCR interactions without costimulation. Tetramers can be used to track specific cells[24–26] and elicit T-cell responses similar to those elicited by antigen-containing antigen-presenting cells (APCs).[9,27,28] The D227K mutation of MHC has been extensively used as a means to abrogate CD8 interaction with MHC.[4,7,29–32]

Here, we examine the requirements of CD8 coreceptor engagement in the stimulation of $CD8^+$ memory T cells. Surprisingly, although $CD8^+$ memory cells produce cytokine in a shorter time than naïve cells, their requirements for coreceptor engagement remain virtually the same as naïve cells for proliferation and early events, independent of pMHC/TCR affinity. However, the rapid production of interferon-γ (IFN-γ) and interleukin-2 (IL-2) by memory cells is independent of CD8 engagement, suggesting clear differences in the memory cells themselves.

Materials and methods

Mice

B6.D2 TgN(*Tcr-Lcmv*)327Sdz/Fre (P14) mice were bred at the University of North Carolina as previously described.[9] B6-GFP mice (C57BL/6-Tg(*H2K^b-GFP*)/Fre with the GFP gene under control of the H-$2K^b$ promoter have been previously described.[33] P14-GFP mice were produced by breeding P14 TCR transgenic mice to GFP mice and selecting for the transgenic TCR and GFP expression. B6.SJL-*ptprc^a* (formally designated B6-*Ly5^a*) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). C57BL/6Tac mice were purchased from Taconic Laboratories (Germantown, NY).

Tetramers and cellular purification

Tetramers were produced as previously described.[4,9] All batches were routinely assayed for lipopolysaccharide contamination. Naïve $CD8^+$ splenocytes were purified by magnetic activation cell sorting (MACS) negative selection using MHC II and CD4 microbeads (Miltenyi Biotec, Auburn CA) as previously described.[4] Experiments used both KAVYNFATM (D^b/C9M) and KAVYNFATC (D^b/gp33). Many publications incorrectly refer to C9M as the wild-type gp33 epitope, but it is the lymphocytic choriomeningitis virus (LCMV) encoded sequence that is KAVYNFATC.[34] The avidity of P14 T cells for D^b/C9M tetramers is 6 nM while that of the D^b/gp33 tetramers is 19 nM, the avidity of the D227K mutant tetramers is approximately 100 nM.[4] The affinity measured by surface plasmon resonance of D^b/C9M and D227K/C9M monomers is identical.[4] Figure 1(a) shows the binding of the four tetramers used in this study to purified P14 T cells. As can be seen, all the tetramers show saturable binding. The absolute magnitude of the binding cannot be directly compared because of differences in the streptavidin used to form the tetramers. All experiments reported here were performed with concentrations far above the K_d (the K_d is the concentration at which 50% of the sites are occupied) and as the tetramers are continuously present, this ensures substantial occupancy, focusing the results on CD8 engagement.

Purification of memory cells

Ten to 20 million naïve $CD8^+$ splenocytes from either P14 or P14-GFP mice were purified and injected intraperitoneally into B6-*Ly5^a* or B6 mice, respectively. After 2 days, the recipient mice received an intraperitoneal injection of approximately 50 plaque-forming units of LCMV (Armstrong strain). After 30 days, $CD8^+$ memory cells were purified from spleens by MACS purification. For all assays except calcium mobilization, $CD8^+$ memory cells were stained with fluorescein isothiocyanate-conjugated anti-CD45.1 (Ly5.1) antibody and sorted using a MoFlo flow cytometer (Cytomation, FT Collins, CO). Alternatively, when P14 GFP cells were transferred, $CD8^+$ cells were purified by MACS and sorted by GFP expression. All cells were examined by flow cytometry before use and their purity exceeded 95%.

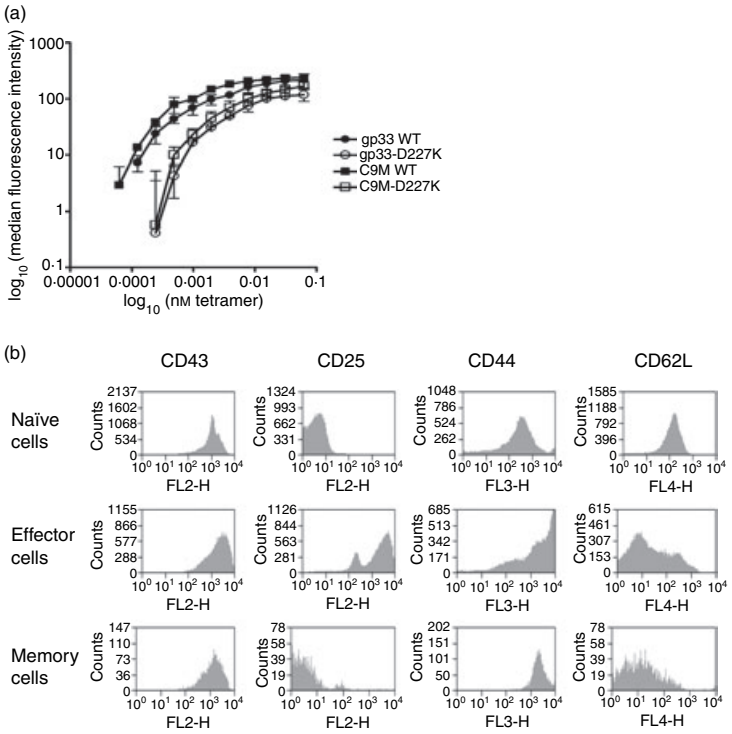

Figure 1. (a) Binding of tetramers to P14 T cells. The graph shows the binding of each tetramer to P14 T cells. Data are the raw data from ref. 4. (b) Expression of surface markers on $CD8^+$ T cells. Flow cytometry histograms of cell surface markers on naïve, effector and memory P14 T cells. Cells were gated by size to eliminate dead cell debris, and gated on FL1-positive cells (GFP or congenic marker Ly5b). These cells were then examined for expression of CD43 or CD25, CD44 and CD62L by four-colour FACS analyses. All antibodies were used at a saturation determined empirically (0·5 μg/ml). Effector cells were stimulated for 24 hr prior to staining and analysis. The median fluorescence of each marker is indicated in the top lefthand corner of each histogram. Cells from P14 mice previously infected with LCMV exhibited a $CD43^{low}$, $CD25^{low}$, $CD44^{high}$ and $CD62L^{low}$ phenotype of effector memory cells (shown here as 'memory' cells).

Phenotypic analysis of memory cells

P14 $CD8^+$ splenocytes from infected (memory cells) or naive mice were stained with: anti-CD25 or anti-CD43 phycoerythrin, anti-CD44 CyChrome, or anti-CD62L APC (BD Pharmingen, San Diego, CA) for memory cell phenotyping. All antibodies were used at 0·5 μg/ml final concentration. Transferred cells were identified by GFP or CD45.2 expression. Control effector cells were generated by *in vitro* culture of naïve, $CD8^+$ P14 splenocytes with 500 nM D^b/C9M tetramer for 48 hr. Previous work has determined that cells stimulated in this manner are fully functional $CD8^+$ effector cells.[4,9,28] Samples were analysed on a FACSCalibur flow cytometer (BD Pharmingen) and quantified using SUMMIT software (Cytomation Inc. Denver, CO).

Calcium mobilization

Assays were performed as described previously.[4] Briefly, memory cells, or control naïve cells, were labelled with Indo1-AM. Cells were prewarmed to 37° and then run on a MoFlo flow cytometer, and stimulated by addition of tetramer (10 μM monomer equivalent final concentration). The intracellular Ca^{2+} concentration was determined in real time using the absorbance ratio for 480 : 485. The fluorescence ratio was converted to nM calcium from a standard curve.

Proliferation

[^{3}H]Thymidine incorporation was used to measure proliferation by the method described previously.[4] Cells were cultured at a concentration of 1×10^5/well in 200-μl total volume of complete media (RPMI-1640 + 10% fetal calf serum), and stimulated with tetramer as indicated. Higher cell concentrations can overcome the observed effects, presumably because of increased cytokine production.

Cytokine staining

For intracellular cytokine staining, purified $CD8^+$ splenocytes from infected or naïve P14 and B6 mice were cultured at 1×10^6/ml in a 24-well plate. Cells were cultured at 37° with 10 μM tetramer for 1 hr before addition of 10 μg/ml Brefeldin A, and then incubated for a total of 6 hr in the continuous presence of tetramer. Unstimulated and phorbol 12-myristate 13-acetate/ionomycin controls were included. After incubation, cells were transferred to tubes for FACS staining with Becton Dickinson Cytofix/Cytoperm reagents, according to the manufacturer's protocol (BD Pharmingen). IFN-γ production was measured with anti-IFN-γ phycoerythrin antibody (BD Pharmingen) or isotype control. All cells were also surface-stained with anti-CD8 CyChrome (BD Pharmingen). Samples were run on a FACSCalibur flow cytometer (BD Pharmingen).

For cytokine secretion measurements, cells were cultured with 500 nM tetramer for 36 hr. IFN-γ, tumour necrosis factor-α (TNF-α), IL-2, IL-4 and IL-5 in supernatants were measured using a Cytometric Bead Array (CBA) assay (BD Pharmingen) according to the manufacturer's protocol.

It is important to note that because different tetramers have different abilities to bind to P14 cells, we performed all of the experiments at concentrations that were orders of magnitude higher than the K_D of tetramer binding (nM versus μM) to effectively saturate the signal. This ensured that the occupancy of each TCR was equivalent and that the differences seen were the result of either duration of binding of individual TCR or the engagement of CD8.

Results

LCMV infection induces differentiation of transferred P14 T cells

The cell surface phenotype of transferred P14 T cells was determined to be certain that we had produced memory cells following transfer and infection with LCMV. We looked at the levels of CD43, CD25, CD62L and CD44. CD43 plays a role in T-cell trafficking and contraction of the immune response[35] and is found on effector T cells, but not on naïve or memory $CD8^+$ cells as detected by the glycosylation sensitive antibody 1B11.[36] CD25 is the high-affinity IL-2 receptor heavy chain, which is up-regulated upon naïve T-cell activation and is reduced in memory cells. CD62L, L-selectin, the lymph homing receptor, is down-regulated in effector memory cells. Amounts of CD44 (H-CAM), the integrin β_1 adhesion molecule that binds to hyaluronic acid and is thought to be critical to the effective interaction of T cells with APC,[37] are expected to be high on memory cells. Based on the staining pattern, we conclude that the phenotype of the $CD8^+$ P14 T cells recovered ($CD62L^{lo}$ $CD25^{lo}$ $CD44^{hi}$ $CD43^{int}$) is an effector memory cell phenotype (Fig. 1, Table 1). Surprisingly few cells with a $CD62L^{hi}$ central memory phenotype were found. We had expected that spleens from these mice would be a mixture of central memory and effector memory cells as had been previously described.[16]

CD8 engagement is necessary for Ca^{2+} mobilization in memory cells

Our earlier results showed that CD8 engagement was required for Ca^{2+} mobilization in naïve cells on stimulation with tetramer.[4] To determine whether memory cells also require CD8 engagement for early signalling events, we stimulated memory cells with wild-type or D227K tetramer, and measured the ability of the cells to mobilize calcium. As with naïve cells, lack of CD8 engagement resulted in no increase in intracellular calcium when stimulated with either the moderate-affinity tetramer (19 nM) assembled with gp33 peptide or the high-affinity tetramer (6 nM) assembled with C9M peptide (Fig. 2). Thus, memory cells also required CD8 engagement for calcium mobilization, although the shapes of the curves suggest that there might be differences in the kinetics of the response between high-affinity and moderate-affinity ligands, the requirement for CD8 engagement is clear. This indicates that the signalling pathways that lead to calcium mobilization are similar for naïve and memory cells.

Table 1. Phenotyping of T cells by cell surface markers

	Naïve	Effector	Effector memory	Central memory
CD43 (1B11)	Low	High	Low	Low
CD25	Low	High	Low	Low
CD44	Low	High	High	High
CD62L	High	Low	Low	High

Memory $CD8^+$ T cells from LCMV-infected mice were distinguished from naïve and effector T cells by the surface markers indicated.

CD8 engagement in cellular proliferation of memory cells

Naïve $CD8^+$ T cells require CD8 engagement for proliferation when stimulated with moderate-affinity tetramer, but not when high-affinity tetramer is used.[4] Since memory cells might have a lower threshold for activation, we wondered if they might respond to D^b/gp33 without CD8 engagement. To determine whether proliferation of memory cells is independent of CD8 engagement, we next performed proliferation experiments by stimulation of memory cells with wild-type or D227K tetramers. Not surprisingly, memory $CD8^+$ T cells did not require CD8 engagement for efficient proliferation with high-affinity D^b/C9M tetramers, the same as naïve cells (Fig. 3a). Surprisingly, the moderate-avidity tetramer assembled with gp33 still required CD8 engagement for optimum proliferation, just as the naïve cells did (Fig. 3b), although the relative response was somewhat higher in memory cells than naïve cells. Thus, even with this relatively small change in avidity (fourfold) the T cells maintained the requirement for CD8 engagement, suggesting that the requirement did not change in memory cells. This CD8 dependence based on affinity is in agreement with Bachmann *et al.*, who showed that blocking the proliferation stimulated by C9M peptide presented by thioglycollate-elicited macrophages could not be blocked by anti-CD8 antibody in memory cells, while stimulation with the lower affinity Y4A peptide was blocked.[19]

Cytokine production by memory cells does not require CD8 engagement

To test the requirements of CD8 engagement in memory T-cell function, we measured IFN-γ production following tetramer stimulation. We first measured the

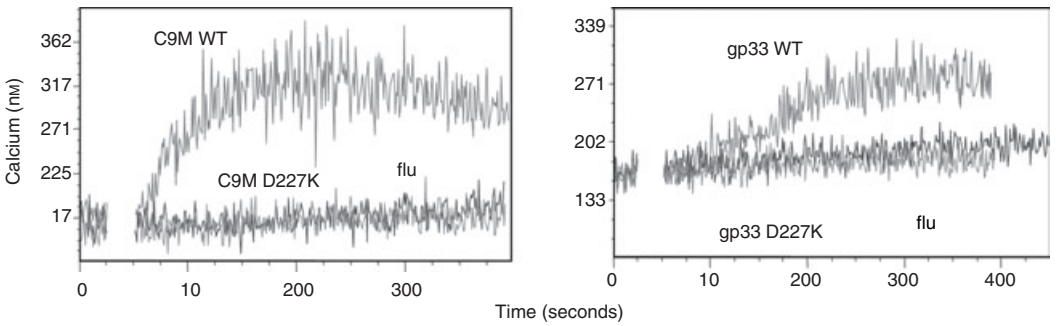

Figure 2. Calcium mobilization in memory CD8$^+$ T cells requires CD8 engagement. The ability of memory CD8$^+$ T cells to mobilize calcium with and without CD8 engagement was measured by stimulation with wild-type (upper dark line) and D227K (lower dark line) tetramers. Similarly to naïve CD8$^+$ T cells,[4] memory cells must engage CD8 for calcium mobilization in response to tetramers containing both high-affinity C9M (left) and moderate-affinity gp33 (right) peptides. Calcium responses were specific, as wild-type tetramers containing an irrelevant influenza peptide did not cause calcium flux (lower light line). All tetramers were used at 10 μM final concentration (monomer equivalents).

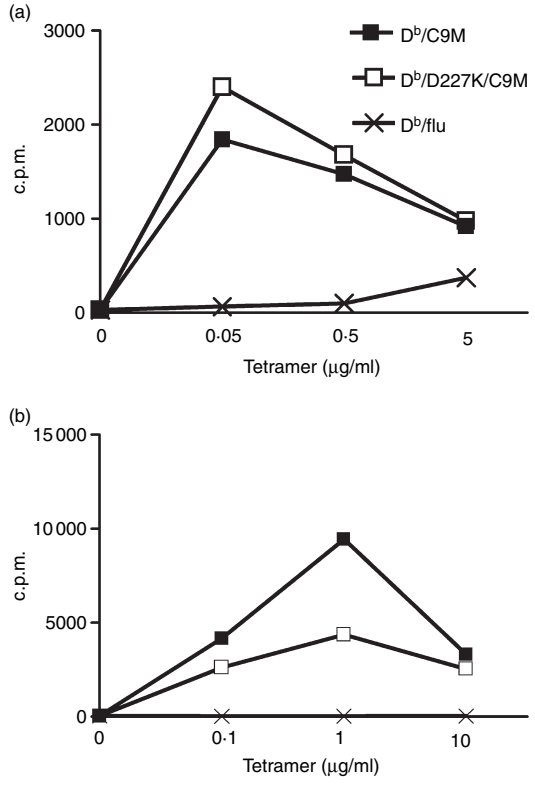

Figure 3. Moderate-affinity pMHC/TCR interaction requires CD8 binding for proliferation. Memory cell proliferative response was measured in response to high-affinity C9M (a) and gp33 (b) wild-type or D227K tetramers. Similarly to naïve cells,[4] cells proliferate equally with and without CD8 engagement in high-affinity pMHC/TCR interactions (a). However, in moderate-affinity pMHC/TCR interactions such as with D^b/gp33, cells require CD8 engagement for efficient proliferation. Data are representative of three independent experiments for D^b/gp33 tetramers and two independent experiments for D^b/C9M tetramers. The tetramer dose is in peptide equivalents (approximately equivalent to μM monomer). Data plotted are the means of duplicate samples, error bars represent the range. Where no bars are shown the range was less than the size of the symbol.

frequency of IFN-γ-producing cells by intracellular cytokine staining. Cells were stimulated by tetramer directly *ex vivo*. Similar frequencies of IFN-γ-producing memory cells were detected when stimulated with either wild-type or D227K tetramers at both high (Fig. 4a) and moderate (Fig. 4b) affinities. Since naïve T cells did not produce any detectable IFN-γ during the 6 hr *in vitro* stimulation performed to compare the frequency of IFN-γ-secreting cells, we stimulated naive P14 cells with either D227K or wild-type D^b tetramer bound to either C9M or gp33 for 36 hr and determined the frequency by intracellular cytokine staining. An equal fraction of cells stimulated with each tetramer produced IFN-γ (Fig. 4c,d). Unlike proliferation, where CD8 engagement is required for a response to gp33, the production of IFN-γ does not require CD8 binding to D^b/gp33.

Interestingly, this contrasts with our previous report in naive cells, where we noted that the amount of IFN-γ produced depended on CD8 engagement.[4] However, in those studies we measured the amount of IFN-γ secreted not the frequency of IFN-γ-producing cells as reported above. We measured cytokine accumulation in cultures of memory cells stimulated by tetramers either with or without CD8 engagement. We measured the amounts of IFN-γ, TNFα, IL-2, IL-4 and IL-5 produced following stimulation with each of the four tetramers. As seen in Fig. 5, both C9M and gp33 tetramers were able to induce production of TNF-α, IFN-γ and IL-2. No IL-4 or IL-5 was detected. When we tested the D227K tetramers they were also able to induce synthesis of these same cytokines regardless of whether we used high- or moderate-avidity tetramers. This was different than previously reported in naïve cells, when CD8 engagement was required to induce cytokine secretion.[4] In addition, we reproducibly found a small amount of IL-2 produced by memory cells.

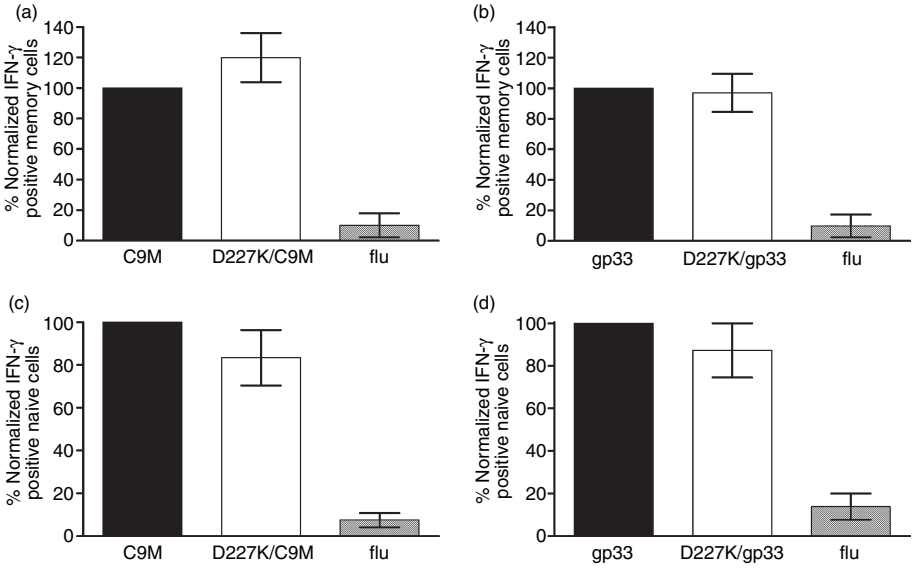

Figure 4. Memory CD8 T cells do not require CD8 binding to pMHC to produce cytokines. Percentage of memory (a,b) or naïve (c,d) cells producing IFN-γ in response to tetramers containing C9M (a,c) and gp33 (b,d) peptides. By *t*-test, no significant differences in IFN-γ production between memory or naïve cells stimulated with wild-type or D227K tetramers were seen, regardless of affinity. In each experiment the maximum response of wild-type D^b tetramer was set to 100%. Means were 43% for C9M memory cells; 42% for gp33 memory cells; 39% for C9M naïve cells and 26% for gp33 naïve cells. Error bars are ± SEM. Each experiment was performed at 0·5 and 5 μM with no significant differences observed.

Discussion

In this communication, we investigated the requirement of CD8 coreceptor engagement in memory $CD8^+$ T-cell activation. Since memory cells have a more rapid response and show reduced requirements for costimulation, we thought that there might also be a change in their requirements for coreceptor engagement. We were particularly intrigued because T cells show functional affinity maturation.[38] Specifically, we hypothesized that CD8 requirements would be relaxed for memory cells because the cells were hyperresponsive compared to naïve T cells. Our previous work on naïve cells had demonstrated that for high-avidity C9M/D^b tetramers, there was a reduced requirement for CD8 engagement.[4,7,10,12] We were surprised to see that like naïve cells, $CD8^+$ memory cells still required CD8 engagement for most T-cell events, suggesting that the molecular machinery involved in activation was not fundamentally changed. Indeed the ability to rescue a requirement for CD8 engagement was still accomplished by a modest increase in the avidity of the tetramers. Interestingly, Kersh *et al.* reported little difference in the early phosphorylation events between memory and naïve T cells when tested with altered peptide ligands presented on APC.[15] These data are consistent with our experiments where we found little difference in calcium mobilization (Fig. 2).

The previous experiments reported by Bachmann *et al.* attempted to address the same issue of CD8 engagement using peptide-pulsed APCs and anti-CD8 antibody.[19] In their system, the involvement of costimulatory molecules could have masked the role of CD8 engagement. Using class I tetramers we were able to address this issue directly without the complication of other cell surface molecules. We could show directly that the engagement of CD8 is required for the production of proliferation and calcium mobilization in memory cells (Figs 2 and 3). These requirements appear to be identical to those in naïve cells, where a high-avidity interaction could obviate the need for CD8 for proliferation but not for calcium signalling.

Two reports of peptide representation from tetramers have appeared[39,40] and they suggest that the mechanism responsible for stimulation of CD8 T cells by class I/peptide monomers is by peptide representation, not direct stimulation by the complexes. It is important to note that in these experiments we have used different TCR and MHC complexes, tetramers, and not monomers, and that stimulation was used for both very short times (i.e. calcium mobilization) as well as longer times. We have previously shown that about 50% of $CD8^+$ T cells responded to tetramer when cultured as single cells.[9] This is similar to the fraction of CD8 T cells that divide when cultured at 1×10^5/well, as used in these experiments when examined by carboxyfluorescein diacetate succinimidyl ester (CFSE) labelling (data not shown). We have also reported a 40-fold shift in the dose–response curve using KAVYN-FATM peptide complexes compared to free peptide.[9] Thus, while the experiments reported above show that peptide representation can occur, it cannot account for the results in this manuscript.

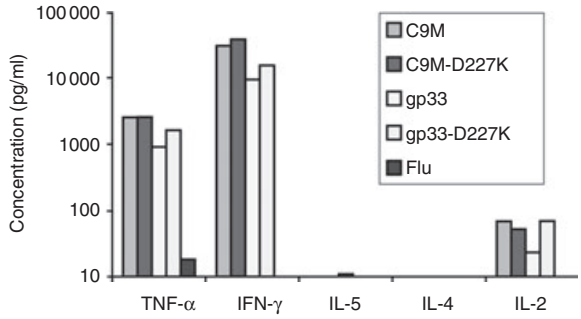

Figure 5. Cytokine secretion by memory cells does not require CD8 engagement. Cytokines secreted by memory cells in response to wild-type or D227K tetramers containing C9M (a) or gp33 (b) peptides. Stimulation with C9M-containing tetramers was performed three times, stimulation with gp33-containing tetramers was performed twice.

Our data on IFN-γ production in CD8 memory cells are in striking contrast to those in naïve cells.[4] Here we show that IFN-γ, as well as TNF-α and IL-2, are all independent of CD8 engagement in memory cells (Fig. 5). In naïve cells only IFN-γ production was observed and then only when CD8 was engaged. Even provision of high-affinity pMHC could not overcome the need for CD8 engagement. With memory cells, we showed that either C9M (high-avidity) or gp33 (moderate-avidity) tetramers were able to induce secretion of all three cytokines (Fig. 5). Thus the avidity threshold for CD8 independence, which remains in place for proliferation and Ca^{2+} mobilization is at least lowered and perhaps gone for cytokine production. This suggests that the signalling pathway through TCR has at least two distinct arms, one that controls Ca^{2+} mobilization and proliferation and another that regulates cytokine production. IFN-γ is not stored in granules in the same way as RANTES,[41] thus the cytokine production described here occurs from new transcription. A mechanism that could account for the observations is that the threshold required for transcription is different from that required for proliferation. We postulate two pathways, one requires signalling through CD8 and $p56^{lck}$ and the other is independent of this signalling. This is surprising considering the dependence of IL-2 synthesis on the NFAT and Ca^{2+} signalling. It has been observed that the promoter for IFN-γ is hypomethylated in memory T cells and that this phenotype is inherited.[42,43] This would suggest that this promoter on memory cells is more sensitive to the apparently smaller signals that are generated in the absence of CD8 binding.

All of the experiments reported here were performed with tetramer continuously present at levels far above the apparent avidity on T cells. While most experiments were performed at several concentrations, we have reported only the highest (typically above 10 μM compared to 1–100 nM) to ensure that the TCR are saturated. Thus, occupancy is not an issue because it is driven by the high concentration of tetramer. The difference we see must be the result of either the stability of individual complexes (not occupancy) or of CD8 engagement. While the concentrations used are far higher than would normally be experienced by a T cell confronting a pMHC complex on an APC, the use at high concentrations of wild-type and mutant tetramer allows us to separate the roles of CD8 engagement and its role in increasing the apparent avidity of binding.[4] It is our belief that CD8 engagement seems much more likely to be important, especially given our previous finding that CD8 orientation with TCR changes as a result of engagement with a tetramer that can bind both CD8 and TCR, but not with one that cannot.[4] Indeed, a similar impact on CD8 engagement is seen with the AH3 TCR and its binding to both mouse and human MHC.[10]

Our data indicate that CD8 engagement is required for most memory T-cell activation events. Memory cells respond similarly to naïve cells in their requirements for CD8 engagement. However, one distinct change is the ability of memory cells to secrete high amounts of IFN-γ (as well as some TNF-α and IL-2) without CD8 engagement, no matter what the affinity of pMHC/TCR. This is striking, as naïve cells require CD8 engagement for efficient IFN-γ production. This indicates that memory cells may be metabolically preactivated, as suggested by Kersch *et al.*,[15] but they are not more sensitive to TCR engagement itself, because the overall number of memory cells making IFN-γ did not change, yet the amount of IFN-γ did increase.

Acknowledgements

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