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Peptide Antigen Priming of Naive, But Not Memory, CD8 T Cells Requires a Third Signal That Can Be Provided by IL-12[1]

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Challenge with peptide Ag in the absence of adjuvant results in tolerance of CD8 T cells specific for the Ag. In contrast, administration of IL-12 along with peptide results in massive clonal expansion, development of effector function, and establishment of a long-lived memory population. Using adoptive transfer of TCR-transgenic CD8 T cells, this effect of IL-12 is shown to be independent of CD4 T cells and to require costimulation provided by CD28 and possibly LFA-1. IL-12 supports responses when IL-12Rβ1-deficient mice are used as recipients for the adoptively transferred CD8 T cells, demonstrating that the IL-12 is acting directly on the T cells rather than on host APC. These results provide strong support for a three-signal model for in vivo activation of naive CD8 T cells by peptide Ag, in which the presence or absence of the third signal determines whether tolerance or activation occurs. In contrast, memory CD8 T cells are effectively activated by peptide Ag in the absence of IL-12 or adjuvant. ***The Journal of Immunology*****, 2002, 168: 5521–5529.**

For CTL to contribute to the killing and clearance of virus-infected cells and tumors, the naive Ag-specific $CD8^+$ T cells must first be activated to undergo clonal expansion and develop effector functions. Following an effective CTL response, some of the cells survive to form a long-lived memory population that can be rapidly reactivated to provide protection against subsequent infections or tumor recurrence. A two-signal model accounts for many aspects of T cell activation (1–3), with signal 1 resulting from TCR engagement of MHC class I/peptide Ag complexes and signal 2 provided by costimulation leading to generation of IL-2. CD8 T cell activation usually involves cross-priming, and evidence is accumulating to show that tolerance also frequently results from the indirect presentation pathway; i.e., cross-tolerance occurs (4). This has usually been interpreted as being due to immature dendritic cells (DC)[4] delivering signal 1 in the absence of signal 2. However, a recent study has suggested that DC maturation is also required for cross-tolerization to occur, and that tolerance can be induced even when signal 2 is available (5). Based on this, it was suggested that the presence or absence of an undefined third signal determined whether priming or tolerance was induced.

With the ability to readily identify potential CD8 T cell epitopes in viral and tumor proteins, tremendous interest has developed in how to use these to effectively immunize for CD8-mediated protection and therapy. In most cases, effective in vivo activation of T cell responses with protein or peptide Ag requires that the Ag be delivered along with adjuvants (6–8). Adjuvants act, at least in part, to optimize delivery of signals 1 and 2 to T cells by activating DC to increase expression of MHC/Ag complexes and costimulatory ligands (9, 10), and migration to lymphoid organs where the Ag can be presented to T cells (11, 12). Adjuvants also stimulate APC to produce a variety of cytokines that can shape T cell responses once they have been initiated. However, recent evidence has suggested that cytokines may not only shape the response but may also be critical for initiating productive T cell responses by providing a third signal that must be present along with TCR engagement and costimulation. Studies of the minimal requirements for in vitro activation of CD8 T cells demonstrated that naive cells require this third signal to clonally expand and develop effector functions, while memory cells do not (13). Memory CD8 T cells from TCR-transgenic mice could be effectively stimulated with IL-2 and microspheres having class I/peptide Ag complexes on the surface, or with microspheres having class I/peptide Ag and B7 ligands on the surface. In contrast, naive CD8 T cells from the same mice could not respond to these stimuli, but responded strongly if IL-12 was also added to the cultures. Because the CD8 T cells were the only cells present in these cultures, it could be concluded that IL-12 was acting directly on the T cells to provide an essential signal for activation. Although numerous studies have demonstrated that IL-12 can augment or enhance CTL responses (14, 15), the critical dependence of naive cells on this third signal had not been appreciated. This was due in part to the fact that even small numbers of APC in cultures can obscure the dependence of the naive cells on this signal. Furthermore, in most studies it could not be determined whether the IL-12 was acting directly on the T cell or whether it was acting on other cells in the culture, e.g., to enhance Ag presentation or costimulation.

The dependence of naive CD8 T cells on IL-12 for an in vitro response raised the possibility that one of the contributions of adjuvants to in vivo CD8 T cell priming by Ag was through stimulating DC to produce IL-12 or some alternate third signal. In fact, we found that administration of peptide Ag and IL-12 was as effective as peptide and CFA for stimulating clonal expansion, development of effector function, and establishment of a long-lived memory population by adoptively transferred CD8 T cells having a TCR specific for the peptide (16). In contrast, administration of

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[4] Abbreviations used in this paper: DC, dendritic cell; KO, knockout; FSC, forward scatter; LN, lymph node; DLN, draining LN.

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just the peptide resulted in only weak clonal expansion, and the cells were rendered tolerant. Thus, IL-12 was sufficient to convert a tolerizing dose of peptide Ag to an immunizing dose. These results were consistent with the possibility that IL-12 was providing a third signal to the CD8 T cells in vivo but did not directly demonstrate that this was the case or rule out the possibility that IL-12 was having its effect on host APC. In this report we describe the results of experiments that provide strong evidence that in vivo activation of naive CD8 T cells by peptide Ag does, in fact, require three signals: Ag, costimulation (IL-2), and IL-12 (or other cytokine). This three-signal model for CD8 T cell activation has important implications for understanding whether cross-priming by DC leads to tolerance vs priming for effective immunity, and whether tolerance or autoimmunity result from recognition of self-Ag. In contrast to naive cells, peptide Ag can effectively activate memory CD8 T cells in vivo without a third signal, i.e., without addition of IL-12 or adjuvant, suggesting one way in which a memory response can be mounted more rapidly than a primary response.

Materials and Methods

Mice

2C TCR-transgenic mice (17) were a kind gift from Dr. D. Loh (Washington University, St. Louis, MO) and were bred to wild-type C57BL/6 mice to generate mice heterozygous for the 2C TCR transgene. $CD8^+$ T cells from these mice were used as donors in all adoptive transfer experiments, and C57BL/6 mice (Charles River Breeding Laboratories, Wilmington, MA), ICAM-1 knockout (KO) mice (C57BL/6J-$Icam1^{tm1Bay}$), CD4 KO mice (C57BL/6J-$CD4^{M1Mak}$), and IL-12Rβ1 KO mice (B6.129S1-$Il12rb1^{tm1Jm}$; The Jackson Laboratory, Bar Harbor, ME) (18) were used as recipients. All mice were housed in a specific pathogen-free environment at all times.

Antibodies

The 1B2 mAb, specific for the transgenic 2C TCR, was from the 1B2 hybridoma (19) (a gift from Dr. H. Eisen, Massachusetts Institute of Technology, Boston, MA) grown in complete RPMI medium (RPMI 1640; Cellgro, Herndon, VA) with 10% FCS (Tissue Culture Biologicals, Tulare, CA), 0.2% L-glutamine, 0.1% penicillin/streptomycin, 0.1% HEPES (BioWhittaker, Walkersville, MD), 0.1% nonessential amino acids, 0.1% sodium pyruvate (Cellgro), and 0.05% 2-ME. The 1B2 mAb was purified from supernatants using a protein A-Sepharose column. Anti-mouse IL-2 (clone S4B6; American Type Culture Collection, Manassas, VA) and CTLA4-Ig (a gift from Dr. R. J. Evans, Searle, St. Louis, MO) cultures were also grown in complete RPMI medium, and Abs were purified using a protein G-Sepharose column with elution by 50 mM glycine at pH 2.5. ChromPure whole molecule rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as an in vivo Ab control. Other Abs used were anti-CD44-FITC (Pgp1), anti-CD25-FITC, anti-CD8α-CyChrome, streptavidin-allophycocyanin, and rat IgG2a κ-FITC isotype control (all from BD PharMingen, San Diego, CA).

Adoptive transfer of 2C-transgenic cells

Pooled lymph node (LN) cells (axillary, brachial, cervical, inguinal, periaortic, and mesenteric) from 2C-transgenic mice were ammonium chloride-treated to remove RBC and adherence-depleted for 90 min. The nonadherent cells were washed in PBS and enriched for $CD8^+$ cells by negative selection using the $CD8^+$ Cellect column purification kit (Biotex Laboratories, Edmonton, Canada) or MACS magnetic separation columns (Miltenyi Biotec, Auburn, CA). Before transfer, the purified population was analyzed by flow cytometry to determine the percentage of $1B2^+CD8^+$ cells and their phenotype with respect to CD25, CD44, and forward scatter (FSC) to insure that the transferred population was not activated. A total of $3–5 \times 10^6$ $1B2^+CD8^+$ cells in 500 μl PBS was transferred via tail vein injection into age- and sex-matched naive 6- to 8-wk-old recipients. Recipient mice were then rested for 24 h before immunizations.

Immunizations

The synthetic peptide SIYRYYGL (20) (Chiron Mimotopes, Clayton, Victoria, Australia) was prepared in PBS and injected alone or emulsified in CFA (Sigma-Aldrich, St. Louis, MO). All peptide immunizations were done on day 0 by s.c. injection of 50 μg peptide in 300 μl in two sites on the back. Recombinant murine IL-12 (Genetics Institute, Cambridge, MA) was administered i.p. at 1 μg ($2.7 \times 10^3 \pm 1.2 \times 10^3$ U/μg) in 100 μl PBS with 0.1% sterile mouse serum on days 0, 1, and 2. Anti-mouse IL-2 Abs (clone S4B6; American Type Culture Collection) were administered i.p. at 1 mg in 100 μl PBS on days -1, 0, and 1. CTLA4-Ig was administered i.p. at 200 μg in 100 μl PBS 6 h before day 0 and then on days 0, 1, and 2. As controls, transferred animals were also immunized with PBS alone (transfer only) or whole molecule rat IgG (Jackson ImmunoResearch Laboratories).

Flow cytometric analysis of in vivo populations

Transferred and immunized mice were sacrificed at varying times after priming or rechallenge, and the LN and spleen were removed, homogenized, and ammonium chloride-treated to lyse RBCs. PBL was drawn from the heart using a heparin-loaded syringe, and the RBCs were lysed by ammonium chloride treatment. Brachial, axillary, and inguinal LNs were pooled as draining LN (DLN). Each cell population (DLN, spleen, and PBL) was counted for total cell number using trypan blue to exclude dead cells. Isolated cells ($1–2 \times 10^6$) from each site were stained with 1B2-biotin mAb, anti-CD8α-CyChrome, and either anti-CD44-FITC (Pgp1) or anti-CD25-FITC. After 20–30 min of incubation, cells were washed and streptavidin-allophycocyanin was added for detection of 1B2-biotin. Stained cells were fixed with 1% formaldehyde and analyzed by flow cytometry using the CellQuest software package (BD Biosciences, San Jose, CA) as described (21). All cytometer settings were identical for all time points within a given experiment. A total of $35–40 \times 10^3$ lymphocyte-gated events were collected and analyzed, and the percentage of $1B2^+CD8^+$ cells was multiplied by the total number of cells recovered from the site to determine the total number of $1B2^+CD8^+$ cells. All determinations were done in duplicate mice for each condition and time point. Values shown in figures are averages and error bars represent the range of the duplicates. Phenotypes of the 2C cells from various sites were determined by gating on the $1B2^+CD8^+$ cells and collecting 200 events examining the FITC fluorescence of the various phenotype marker mAbs. Gates denoting high and low expression of each surface marker were set based on the phenotype of naive T cells. Replacing the specific mAbs with isotype control mAbs resulted in almost no events ($<1\%$) falling into the high gate for each marker.

Results

The IL-12 adjuvant effect on $CD8^+$ T cells is independent of $CD4^+$ T cells

Using adoptive transfer of 2C TCR-transgenic $CD8^+$ T cells that recognize the synthetic peptide SIYRYYGL in the context of $H\text{-}2K^b$ (17, 20, 21), we previously showed that administration of IL-12 along with peptide was as effective as administration of peptide in CFA for activating 2C cells to undergo clonal expansion, develop effector function, and establish a long-lived responsive memory population (16). In contrast, administering peptide alone resulted in only weak clonal expansion and no detectable effector function. Furthermore, these cells were rendered tolerant; they persisted in small numbers for at least 60 days but did not expand when the mice were rechallenged with peptide and either CFA or IL-12.

This same adoptive transfer system was used in the experiments described in this work to further study the adjuvant effects of IL-12 and determine whether the properties of this response support a three-signal model to explain in vivo CD8 T cell activation. Activation of adoptively transferred 2C cells clearly requires signal 1; without peptide immunization the cells do not up-regulate activation markers or undergo cell division (13, 16, 21). However, it was possible that IL-12 might be bypassing or substituting for a requirement for signal 2. One way in which this might occur is by IL-12 activating recipient $CD4^+$ T cells or skewing endogenously activated $CD4^+$ T cells to a Th1 phenotype (15), so that they can then provide help to the CD8 cells by providing IL-2 or by some other means. We examined this possibility in experiments using CD4 KO mice as recipients of the adoptively transferred 2C cells. 2C T cells ($3–5 \times 10^6$) were injected i.v. into naive age- and

sex-matched C57BL/6 or CD4 KO recipients, and the mice were immunized 1 day later. Responding 2C populations were monitored using the anti-clonotypic 1B2 mAb (19) and anti-CD8 mAb for flow cytometric analysis. The $1B2^+CD8^+$ 2C population comprises <1% of the LN cells 1 day after transfer and before immunization (16, 21). Day 3 postimmunization was chosen for the analyses based on time course experiments showing that maximal clonal expansion of 2C cells in the DLN (inguinal, brachial, and axillary) occurs at this time (Ref. 16 and data not shown). To confirm that the 2C $CD8^+$ T cell response to IL-12 and peptide immunization was specific for the SIYRYYGL peptide, adoptively transferred mice were also immunized with an equal amount of SIINFEKL, an OVA peptide that is also bound by $H\text{-}2K^b$ (22). The 2C $CD8^+$ T cells in these mice remained essentially the same in both absolute numbers and phenotype as those found in transferred mice that received just PBS (data not shown).

One day after transfer, mice were immunized with PBS (transfer only group) or with SIYRYYGL peptide (50 μg/mouse s.c. distributed between two sites on the back) in the presence or absence of IL-12. This amount of SIYRYYGL resulted in optimal 2C responses as shown by dose response studies (data not shown). IL-12 (1 μg/day, $2.7 \times 10^3 \pm 1.2 \times 10^3$ U/μg) was administered i.p. on days 0, 1, and 2 in 100 μl PBS with 0.1% sterile mouse serum. As shown in Fig. 1, immunization with peptide alone resulted in weak

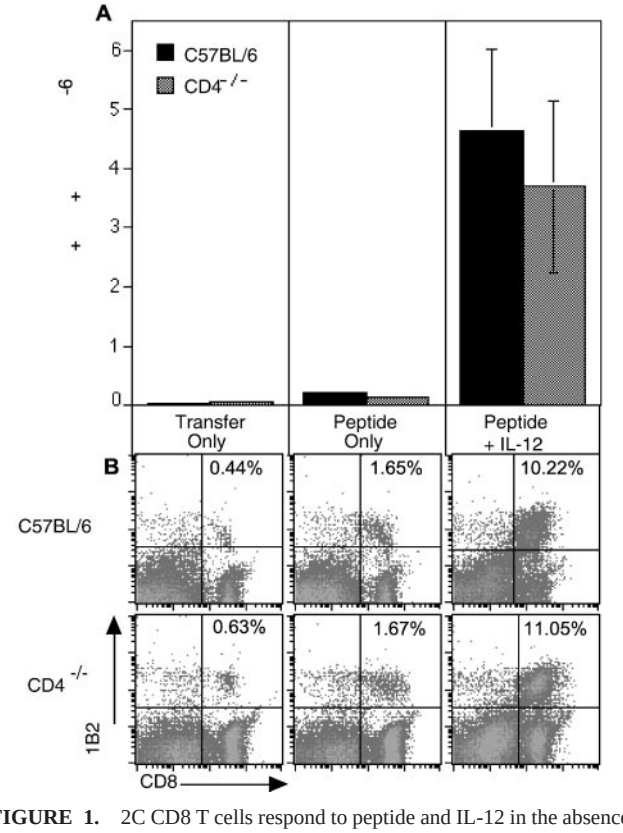

FIGURE 1. 2C CD8 T cells respond to peptide and IL-12 in the absence of CD4 T cells. *A*, C57BL/6 or $CD4^{-/-}$ mice received 2C cells by adoptive transfer and were challenged on day 0 with PBS (Transfer Only) or with SIYRYYGL peptide alone (Peptide Only; 50 μg in 0.3 ml s.c.) or along with IL-12 (Peptide + IL-12; 1 μg/mouse i.p. on days 0, 1, and 2). Cells were harvested from the DLN on day 3 and analyzed by flow cytometry to determine the number of 2C cells ($1B2^+CD8^+$ cells) as described in *Materials and Methods*. Values shown represent the average for duplicate mice and error bars represent the range. *B*, Flow cytometry dot plots showing detection of $1B2^+CD8^+$ cells from DLN of mice immunized in *A*. The percentage of total lymphocytes accounted for by 2C cells is shown.

but reproducible clonal expansion of the 2C cells in the DLN by day 3, and the magnitude of the expansion was comparable in normal and $CD4^{-/-}$ recipients. The majority of 2C cells in these mice exhibited an activated phenotype, including increased CD44 levels and high FSC indicative of blast transformation (data not shown). In contrast, immunization with peptide in the presence of IL-12 resulted in massive clonal expansion, and this was also comparable in normal and CD4 KO recipients (Fig. 1). Again, the majority of the 2C cells expressed high CD44 levels and high FSC (data not shown). Administration of IL-12 without peptide had no detectable effects on adoptively transferred 2C cells (Ref. 13 and data not shown). Thus, host $CD4^+$ T cells are not required for the adjuvant effect of IL-12 in the response of 2C cells to peptide Ag and make no detectable contribution to the response.

The response to IL-12 and peptide Ag depends on costimulation

To determine whether costimulation to provide signal 2 is required for the 2C response to peptide and IL-12, experiments were done to examine the effects of blocking two of the major costimulatory pathways for CD8 T cells, CD28/B7 and LFA/ICAM-1. These costimulatory interactions are required, at least in part, for the production of IL-2 that is then used in an autocrine and/or paracrine fashion (3). Either the CD28 or LFA-1 pathway can costimulate Ag-dependent IL-2 production by CD8 T cells, and engagement of both receptors synergistically increases the level of IL-2 produced by the cells in vitro (23).

The contribution of CD28/B7 interactions to the response to peptide Ag and IL-12 was examined by administering CTLA4-Ig to block these interactions. CTLA4-Ig binds to B7-1 and B7-2 with high affinity and prevents CD28 from binding to them (24). Normal C57BL/6 mice were adoptively transferred with 2C cells and immunized with PBS (transfer only), SIYRYYGL peptide, SIYRYYGL and IL-12, or SIYRYYGL and IL-12 along with CTLA4-Ig. Peptide alone caused a small increase in the number of 2C cells in the DLN, spleen, and PBL on day 3, and a large clonal expansion was seen at all sites when IL-12 was given along with peptide (Fig. 2). One group of mice received CTLA4-Ig (200 μg/dose in 100 μl PBS injected i.p.) 6 h before immunization and again on days 0, 1, and 2 for a total of 800 μg per mouse. Clonal expansion was reduced 5- to 6-fold at all sites in these mice in comparison to mice that received just peptide and IL-12 (Fig. 2). Although clonal expansion was greatly reduced by CTLA4-Ig treatment, some expansion still occurred in comparison to that in mice that received just peptide. The 2C cells that did expand in the CTLA4-Ig treated mice had essentially the same phenotype as those in the mice that received just peptide and IL-12, having high CD44 expression (56% CD44 with CTLA4-Ig and 65% CD44 without), high FSC (59% with CTLA4-Ig and 70% without) and high CD25 (62% with CTLA4-Ig and 56% without). Thus, blocking CD28/B7 interactions greatly reduces 2C T cell clonal expansion in response to peptide and IL-12, strongly suggesting that this response remains dependent on signal 2 being provided by this costimulatory pathway.

Responses were also examined in ICAM-I-deficient adoptive transfer recipients to assess potential contributions of LFA-1/ICAM-I interactions. Clonal expansion of 2C cells in response to peptide was also greatly augmented by IL-12 in these mice (Fig. 2*B*), but the magnitude of the expansion was substantially reduced in comparison to that in normal mice. The extent of clonal expansion at all sites was further reduced when CTLA4-Ig was administered to $ICAM\text{-}1^{-/-}$ mice immunized with peptide and IL-12 (Fig. 2*B*). The phenotypes of the 2C cells were essentially identical in normal C57BL/6 and $ICAM\text{-}1^{-/-}$ recipients with respect to CD44, CD25, and FSC. Reduced responses in the $ICAM\text{-}I^{-/-}$

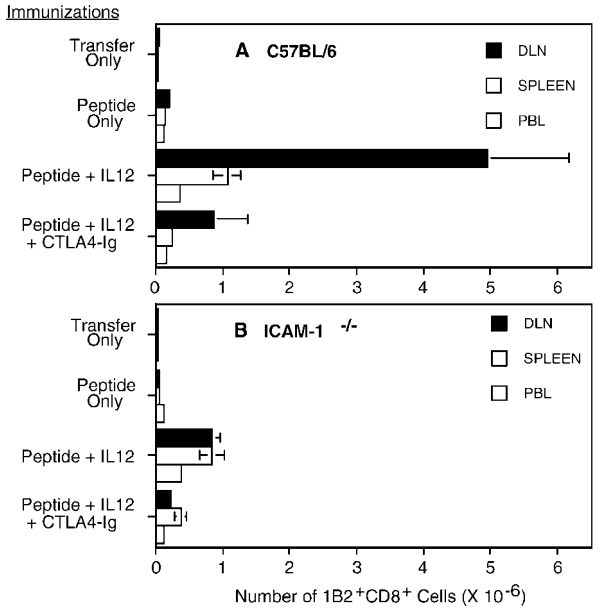

FIGURE 2. Response of 2C CD8 T cells to peptide and IL-12 requires costimulation. *A*, C57BL/6 mice received 2C cells by adoptive transfer and were challenged on day 0 with PBS (Transfer Only) or with SIYRYYGL peptide alone (Peptide Only; 50 μg in 0.3 ml s.c.) or along with IL-12 (Peptide + IL-12; 1 μg/mouse i.p. on days 0, 1, and 2). One group immunized with peptide and IL-12 also received CTLA4-Ig (200 μg/mouse) 6 h before day 0 and on days 0, 1, and 2 (Peptide + IL-2 + CTLA4-Ig). Cells were harvested from the DLN, spleen, or PBL on day 3 and analyzed by flow cytometry to determine the number of 2C cells ($1B2^+CD8^+$ cells) as described in *Materials and Methods*. Values shown represent the average for duplicate mice and error bars represent the range. *B*, ICAM-$I^{-/-}$ mice received 2C cells by adoptive transfer and were then immunized and analyzed as in *A*. Values shown are averages for duplicate mice and error bars represent the range. Essentially identical results were obtained in two independent experiments.

mice suggests that the LFA-I/ICAM-I pathway may make a significant costimulatory contribution to the response to peptide and IL-12. Furthermore, the large reduction seen when either the CD28 (Fig. 2*A*) or LFA-1 (Fig. 2*B*) pathways are blocked, and the further reduction that occurs when both are blocked (Fig. 2*B*), suggest that these pathways may synergize in providing costimulation for the 2C response, as has been observed in vitro (23). However, the results obtained using ICAM-$1^{-/-}$ mice must be interpreted with caution, because absence of this ligand may also affect lymphocyte trafficking. Together, these results demonstrate that the adjuvant effect of IL-12 does not bypass a need for costimulation. Rather, the IL-12-dependent response to peptide Ag clearly depends upon costimulation provided by the CD28/B7 pathway, and the LFA-1/ICAM-I pathway may make a synergistic contribution to the costimulation.

Endogenous IL-2 contributes to the response to peptide Ag and IL-12

Costimulatory interactions between T cells and APC stimulate production of IL-2 by T cells, which then supports proliferation and development of effector function. The dependence of the response to peptide and IL-12 on costimulation suggested that the response also depends upon IL-2, and this was examined in experiments using neutralizing anti-IL-2 mAb. Mice were adoptively transferred with 2C cells and immunized, with some groups receiving anti-mouse IL-2 mAb (1 mg/day in 100 μl PBS i.p.) on days -1, 0, and 1, and the DLN and spleen were analyzed on day 3 to determine 2C cell numbers and phenotypes. The administration of anti-IL-2 mAb caused a significant reduction in total 2C $CD8^+$ T cell numbers in the day 3 DLN when either CFA or IL-12 was used as the adjuvant (Fig. 3*A*). When CFA is used as the adjuvant, the 2C response is largely confined to the DLN and little clonal expansion is seen in the spleen on day 3 (Fig. 3*B*), probably due to the sequestration of Ag to the local site by the mineral oil in CFA. In contrast, a large clonal expansion is seen in the spleen on day 3 when IL-12 is used as the adjuvant (Fig. 3*B*), and mice treated with anti-IL-2 mAb also exhibited a substantial decrease in clonal expansion at this site (Fig. 3*B*). Administration of control rat IgG to mice had no detectable effects on clonal expansion at any sites (data not shown).

Administration of anti-IL-2 mAb only partially blocked the 2C responses (Fig. 3) and was less effective than blocking CD28/B7 or LFA-1/ICAM-I interactions (Fig. 2). It is difficult to completely block IL-2 effects with Ab in vivo, probably due to the autocrine nature of the response making it difficult to neutralize the IL-2 produced by a cell before it binds the IL-2R of the same cell. Phenotypic analysis revealed no significant differences in the 2C cells that responded in the absence or presence of the anti-IL-2 mAb (data not shown). These results demonstrate that IL-2 contributes to the 2C $CD8^+$ T cell response when IL-12 is the adjuvant. Thus, signal 2 is clearly required for a 2C cell response to IL-12/peptide (Fig. 2), and this second signal involves, at least in part, the production and use of IL-2.

IL-12 provides a third signal to naive $CD8^+$ T cells

In vitro studies demonstrated that IL-12 could act as a distinct third signal for activating CD8 T cells to proliferate and develop effector function by acting directly on the T cells (13). The adjuvant effects of IL-12 are consistent with its having this role in vivo, but the in vivo effects could potentially result from other mechanisms such as up-regulation of class I MHC proteins or costimulatory molecules on host APC. To examine this, the responses of adoptively transferred 2C cells were examined in IL-12Rβ1 KO mice (18). In this situation host cells lack functional IL-12Rs, and effects of IL-12 on the response must result from IL-12 binding to the IL-12Rs expressed by the 2C cells themselves.

For these experiments 2C LN cells were adherence cell-depleted and enriched for $CD8^+$ cells using negative selection columns

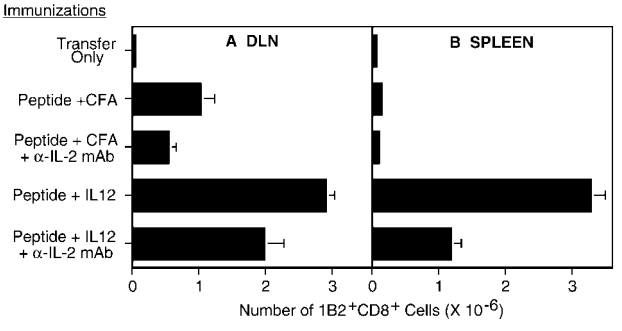

FIGURE 3. Response of 2C CD8 T cells to peptide and IL-12 depends upon IL-2. C57BL/6 mice received 2C cells by adoptive transfer and were challenged on day 0 with PBS (Transfer Only), SIYRYYGL peptide and CFA (Peptide + CFA), or peptide and IL-12 (Peptide + IL-12). Some groups, as indicated, also received anti-IL-2 mAb (1 mg/mouse i.p.) on days -1, 0, and 1. Cells were harvested from the DLN (*A*) and spleen (*B*) on day 3 and analyzed by flow cytometry to determine the number of 2C cells ($1B2^+CD8^+$ cells) as described in *Materials and Methods*. Values shown represent the average for duplicate mice and error bars represent the range. Essentially identical results were obtained in two independent experiments.

(85–90% $CD8^+$) before the transfer into normal C57BL/6 or IL-12β1 KO recipients. Groups of recipient mice were immunized on day 0 with PBS (transfer only), peptide only, or peptide and IL-12, and the number of 2C cells in the DLN and spleen was determined on day 3. As expected, peptide alone stimulated weak clonal expansion in the normal mice, and coadministration of IL-12 resulted in strong clonal expansion in both DLN and spleen (Fig. 4). The response to peptide alone was comparable in the IL-12β1 KO recipients, and there was a small but significant reduction in the response to peptide and IL-12 (Fig. 4). Essentially identical results were obtained in two independent experiments. The responding 2C cells in C57BL/6 and IL-12Rβ1 KO mice were indistinguishable with respect to CD44, CD25, and FSC levels (data not shown). Thus, the major effect of IL-12 in supporting clonal expansion of Ag-specific $CD8^+$ T cells appears to occur via provision of a third signal directly to the T cell.

$CD8^+$ memory T cells do not require a third signal for activation

In vitro studies of CD8 T cell activation using latex microspheres with class I/peptide Ag on the surface as the stimulus demonstrated that naive ($CD44^{low}$) cells could only proliferate and develop lytic effector function when both IL-2 (or B7 costimulation) and IL-12 were provided (13). In contrast, memory cells ($CD44^{high}$) were effectively stimulated to proliferate and develop lytic effector function in response to just Ag and IL-2 (25), and addition of IL-12 caused only about a 2-fold increase in the response (data not shown). Thus, naive cells required a third signal provided by IL-12 while memory cells responded well in the absence of a third signal, and the response was only weakly augmented when IL-12 was present.

The in vitro results predicted that the in vivo response of memory cells to peptide Ag might not require adjuvant or IL-12, and this was tested in the 2C adoptive transfer system. Mice received 2C cells by adoptive transfer and were then primed with either PBS, peptide plus CFA, or peptide plus IL-12. After at least 60 days the mice were rechallenged as indicated (Fig. 5), and 3 days later the numbers and phenotypes of 2C cells in the DLN were determined. 2C cells in control mice given just PBS during priming and rechallenge were present in small but detectable numbers and the majority retained a naive, resting phenotype ($CD44^{low}CD25^{low}FSC^{low}$). As was previously shown (16), priming with peptide along with either CFA or IL-12 resulted in establishment of a long-term memory population; 2C cells were present in larger numbers than in mice that had received just PBS during priming and the majority had a resting memory phenotype ($CD44^{high}CD25^{low}FSC^{low}$). When 2C cells are labeled with PKH26 lipophilic dye before adoptive transfer and challenge with peptide and CFA or IL-12, the fluorescence of all of the cells decreases greatly within 8 days, demonstrating that all of the cells have undergone multiple rounds of division (Ref. 16 and data not shown). Thus, no detectable naive 2C cells remain following the priming protocol used in this study to establish memory populations.

Memory cells in the Ag-primed mice exhibit no detectable changes in number or phenotype upon rechallenge with just PBS. Rechallenge of the primed mice with just peptide resulted in substantial clonal expansion of the 2C cells in the DLN (Fig. 5), and a large fraction of these cells had high FSC on day 3, indicating that they were proliferating (data not shown). Rechallenge with peptide plus CFA resulted in comparable expansion of the 2C population, and rechallenge with peptide plus IL-12 resulted in only about twice as many 2C cells (Fig. 5). Thus, unlike primary responses where CFA or IL-12 dramatically increase clonal expansion in comparison to peptide alone (Figs. 1 and 2 and Ref. 16), these adjuvants have little or no effect on the secondary response to peptide.

While a third signal is not necessary for activating memory cells in vitro (25) or in vivo (Fig. 5), the in vitro response does require signal 2, which can be provided by IL-2. Neutralizing anti-IL-2 mAb was used to determine whether IL-2 is also required for the in vivo response of memory cells to peptide. Adoptively transferred mice were immunized with peptide plus CFA, rested for >60 days, and rechallenged, and the number of 2C cells in the DLN was examined on days 1–4 of the response. As expected,

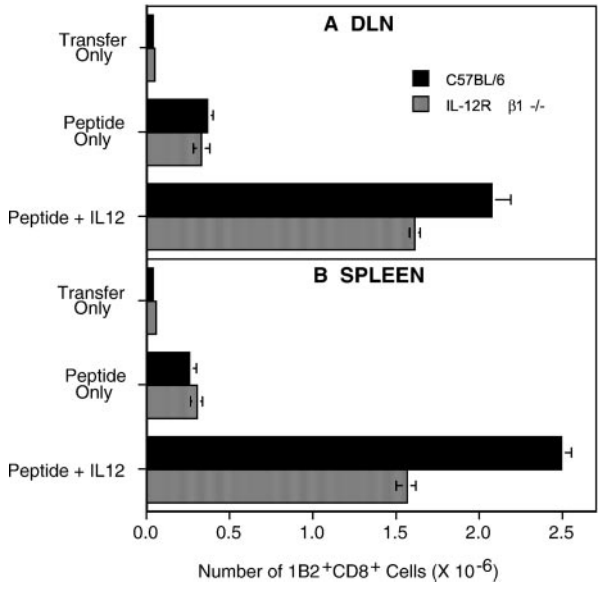

FIGURE 4. 2C CD8 T cells respond to peptide and IL-12 in IL-12Rβ1-deficient recipients. C57BL/6 and IL-12R$\beta1^{-/-}$ mice received purified $CD8^+$ 2C T cells by adoptive transfer and were challenged on day 0 with PBS (Transfer Only), SIYRYYGL peptide alone (Peptide Only), or peptide and IL-12 (Peptide + IL-12). Cells were harvested from the DLN (*A*) and spleen (*B*) on day 3 and analyzed by flow cytometry to determine the number of 2C cells ($1B2^+CD8^+$ cells) as described in *Materials and Methods*. Values shown represent the average for duplicate mice and error bars represent the range. Essentially identical results were obtained in two independent experiments.

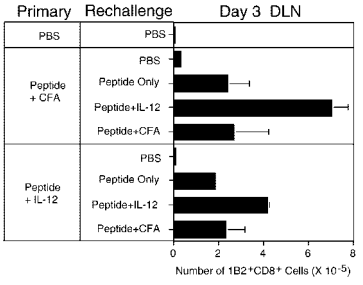

FIGURE 5. 2C CD8 memory T cells respond to peptide in the absence of adjuvant or IL-12. C57BL/6 mice received 2C cells by adoptive transfer and groups were primed with PBS, SIYRYYGL peptide and CFA, or peptide and IL-12 as indicated. After at least 60 days the mice were rechallenged with PBS, peptide (Peptide Only), peptide and IL-12 (Peptide + IL-12), or peptide and CFA (Peptide + CFA) as indicated. Cells were harvested from the DLN on day 3 after the rechallenge and analyzed by flow cytometry to determine the number of 2C cells ($1B2^+CD8^+$ cells) as described in *Materials and Methods*. Values shown represent the averages for duplicate mice and error bars represent the range. Essentially identical results were obtained in two independent experiments.

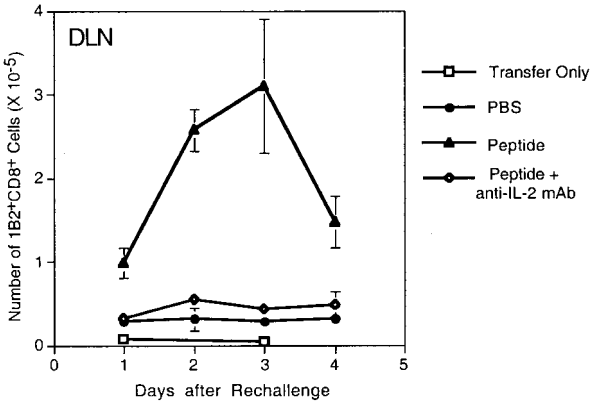

FIGURE 6. The response of 2C CD8 memory T cells depends upon IL-2. C57BL/6 mice received 2C cells by adoptive transfer and were primed with SIYRYYGL peptide and CFA; one group (Transfer Only) was left unprimed. After at least 60 days the mice were rechallenged with PBS or peptide only (Peptide). One group rechallenged with peptide on day 0 also received anti-IL-2 mAb (1 mg/mouse i.p.) on days 0, 1, and 2, peptide and IL-12 (Peptide + IL-12), or peptide and CFA (Peptide + CFA) as indicated. Cells were harvested from the DLN on the indicated days after rechallenge and analyzed by flow cytometry to determine the number of 2C cells ($1B2^+CD8^+$ cells) as described in *Materials and Methods*. Values shown represent the averages for duplicate mice and error bars represent the range. Essentially identical results were obtained in four independent experiments.

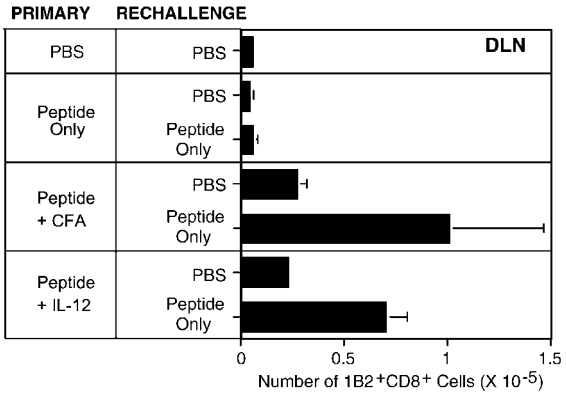

FIGURE 7. Priming with adjuvant or IL-12 is required to generate 2C CD8 memory T cells capable of responding to peptide. C57BL/6 mice received 2C cells by adoptive transfer and groups were primed with PBS, SIYRYYGL peptide only (Peptide Only), peptide and CFA (Peptide + CFA), or peptide and IL-12 (Peptide + IL-12) as indicated. After at least 60 days the mice were rechallenged with PBS or peptide (Peptide Only). Cells were harvested from the DLN on day 3 after the rechallenge and analyzed by flow cytometry to determine the number of 2C cells ($1B2^+CD8^+$ cells) as described in *Materials and Methods.* Values shown represent the averages for duplicate mice and error bars represent the range. Essentially identical results were obtained in four independent experiments.

mice primed with peptide plus CFA had larger numbers of 2C cells than adoptively transferred mice that had not been primed (Fig. 6), and these cells remained quiescent when rechallenged with PBS. Rechallenge with peptide resulted in a large expansion of the 2C population that peaked in the DLN on day 3, and this response was almost completely eliminated in mice that received anti-IL-2 mAb before and during rechallenge with peptide (Fig. 6). Thus, while a third signal does not appear to be required for memory cells to respond to Ag, their response remains dependent on signal 2.

While memory cells can respond to peptide Ag in the absence of adjuvant, establishment of this peptide-responsive memory population does depend upon adjuvant during priming. When primary immunization is done using just peptide, a population of 2C cells having a memory phenotype (data not shown) is present at day 60 and beyond (Fig. 7). However, these cells are tolerant, in that they do not expand in response to rechallenge with peptide (Fig. 7), nor do they respond when rechallenged with peptide and adjuvant (16). In contrast, strong memory responses were obtained when mice were primed with peptide plus either CFA or IL-12 and rechallenged with peptide (Fig. 7). IL-12 and CFA appear to be equally effective as adjuvants in priming for a memory response to peptide (Figs. 5 and 7).

Discussion

Lafferty and Cunningham (26) initially suggested that lymphocyte activation may require two signals to become activated, with signal 1 provided by the Ag receptor and signal 2 by some other surface receptor. Support for this concept in T cell activation was provided by Jenkins and Schwartz (27) with the demonstration that stimulation by Ag alone resulted in induction of anergy rather than a productive response. A wealth of data has subsequently accumulated to support a two-signal model for T cell activation, with signal 2 being provided by costimulatory receptors on the T cell, most notably the CD28 receptor that binds B7 ligands on APC (1, 3, 28–30). It has also become increasingly clear that inflammatory cytokines can enhance T cell responses, and this is usually interpreted in terms of the ability of these cytokines to increase Ag processing and expression of costimulatory ligands on APC, thus enhancing the levels of signals 1 and 2 available to the T cells (9, 10, 31, 32). However, there is also evidence to suggest that some of these cytokines may contribute more directly to T cell clonal expansion and survival (33–38) independently of effects on the CD28 costimulation pathway.

A direct requirement for an inflammatory cytokine to stimulate naive CD8 T cells became apparent in studies using artificial APC as a stimulus in vitro (13). Microspheres having class I/peptide Ag complexes and B7-1 and/or ICAM-1 on the surface were not sufficient to stimulate proliferation or development of effector function by highly purified TCR-transgenic CD8 T cells, although the same microspheres effectively stimulated memory CD8 T cells bearing the same TCR. However, when IL-12 was added to the cultures, naive cells responded strongly to the artificial APCs. IL-12 was clearly acting directly on the T cells, because they were the only cells present in the cultures. In addition, all three stimuli were clearly required, because high levels of signal 1 or signal 2 could not overcome the need for IL-12. These results strongly suggested that IL-12 was acting as a distinct third signal and not simply as an alternate signal 2.

As predicted by the in vitro findings, the results presented in this report demonstrate that in vivo activation of naive CD8 T cells by peptide Ag also requires three signals. Adoptively transferred CD8 T cells responded to peptide Ag by clonally expanding and developing effector function, but only if IL-12 was administered at the same time (Fig. 1 and Ref. 16). This response did not require CD4 T cells (Fig. 1) but did depend upon costimulation provided by CD28/B7 interactions (Fig. 2*A*) and possibly by LFA-1/ICAM-1 interactions (Fig. 2*B*). This dependence on signal 2 probably results, at least in part, from its being required to induce production of IL-2 that is needed to support the response (Fig. 3). IL-12 was effective in supporting the response to peptide when IL-12Rβ1-deficient mice were used as the adoptive transfer recipients, although the response was somewhat reduced in comparison to that in normal recipients (Fig. 4). Thus, IL-12 can act directly on the CD8 T cells because these were the only cells able to respond to

the cytokine in the IL-12Rβ1-deficient recipients. This result demonstrates that sufficient costimulation is present to provide effective signal 2 for peptide Ag-dependent activation, even when the expression of costimulatory ligands on host APC is not being upregulated. The small but reproducible reduction in the response in the IL-12Rβ1-deficient recipients suggests that IL-12 might also contribute to responses in normal mice through effects on APC, perhaps increasing expression of cytokines (including IL-12) or costimulatory ligands. TCR-transgenic mice deficient in IL-12Rβ1 are being generated to examine this possibility. Together with the previous findings (13, 16), these results argue strongly for a three-signal model for productive activation of naive CD8 T cells by peptide Ag both in vitro and in vivo. The third signal, in the form of IL-12, does not bypass the need for signal 2. Further evidence against redundancy in the signaling requirements is provided by the inability of high levels of Ag, signal 2, or IL-12 to overcome the need for all three signals (13).

Numerous studies have shown that IL-12 can augment or enhance generation of CTL responses (14, 15, 39), but its critical role of providing a requisite third signal to naive CD8 T cells has not been appreciated. In most cases, generation of lytic activity was measured, and the experiments did not distinguish effects on clonal expansion vs effector function. In addition, direct effects of IL-12 on the CD8 T cells were not distinguished from effects on APC. Furthermore, most experimental models have not exhibited an absolute dependence on IL-12 but rather an augmentation of response. This may result in part from the fact that even small numbers of APC in in vitro cultures can obscure the requirement for a third signal (J. M. Curtsinger and M. F. Mescher, unpublished results), and from the fact that memory cells do not depend on a third signal for effective activation (Figs. 5 and 6). In addition, IL-12 does not appear to be the only thing that can provide the required third signal. Adoptive transfer recipients that are deficient in IL-12 production are still able to respond to challenge with peptide Ag in CFA (16), and anti-IL-12 mAb does not completely block in vitro responses to peptide-pulsed splenic APC from normal mice (J. M. Curtsinger and M. F. Mescher, unpublished results). A number of cytokines have been tested and fail to replace IL-12 in providing signal 3, including IL-1, -4, -5, -6, -7, -10, -15, -18, TNF-α, and IFN-γ (J. M. Curtsinger and M. F. Mescher, unpublished results). Preliminary results are consistent with the alternative signal 3 being a soluble factor, and experiments are in progress to identify this factor.

Although naive CD8 T cells have a strict requirement for a third signal to respond to peptide Ag, this is not the case for memory cells. In vitro studies showed that Ag-bearing microspheres and IL-2 are sufficient to stimulate the $CD44^{high}$ memory population of cells that is present in transgenic mice (13). Two signals are also sufficient in vivo; memory cells generated by in vivo priming with peptide Ag and either CFA adjuvant or IL-12 respond by clonal expansion upon rechallenge of the mice with just peptide (Fig. 5). Including adjuvant or IL-12 during rechallenge does not substantially increase the extent of clonal expansion (Fig. 5 and 6), while the response remains dependent on IL-2 (Fig. 6). The ability of memory cells to respond in the absence of a third signal is likely to contribute to the ability of these cells to respond rapidly upon reappearance of Ag, in that they can respond before a significant inflammatory response develops. In the parlance of the danger model (40), they have already learned that the Ag they are specific for is dangerous by having responded to IL-12 (or the alternate third signal) during the initial priming.

Accumulating evidence regarding Ag presentation in vivo for effective CD8 T cell activation is consistent with a model in which IL-12 can provide a requisite third signal. DC are the principal cells involved in presenting Ag to T cells to initiate responses and can provide for cross-presentation of Ags to CD8 T cells (4, 41–43). Two subclasses of DC can be distinguished based on the expression of CD8α on their surface (44), and these subsets differ with respect to the cytokines they produce. Recent studies have demonstrated that $CD8^-$ DC are more efficient for presentation of class II-restricted Ag to CD4 T cells, while $CD8^+$ DC are more efficient for cross-presentation of Ag to CD8 T cells (43, 45). $CD8^+$ DC also produce high levels of IL-12 upon stimulation, while $CD8^-$ DC produce little (46). Thus, it appears likely that at least one property of $CD8^+$ DC that makes them effective for CD8 T cell activation is their ability to provide the requisite third signal needed to support responses by the naive cells. DC produce IL-12 upon stimulation with microbial products (47), and in response to CD40 ligation (48). Several studies have demonstrated that CD4 T cells can provide help to initiate CD8 T cell responses via a CD40/CD40 ligand-dependent mechanism that conditions APC to make them effective activators of CD8 T cells (49–51). This conditioning may involve, at least in part, CD40 ligand-dependent stimulation of IL-12 production by the DC so that they can provide signal 3 to the naive CD8 T cells. This would be consistent with recent results from Albert et al. (5) showing that mature DC are able to provide signal 1 and 2 to CD8 T cells but still induce cross-tolerance unless a third signal is also present, and this third signal could be provided by a CD40/CD40 ligand-dependent interaction of the DC with CD4 T cells.

We have shown that IL-12 can fully support the response of naive CD8 T cells to peptide Ag, and that this effect is primarily at the level of the T cell and not host APC (Fig. 4). Thus, when the Ag used is a class I-restricted peptide epitope, activation of host APC does not appear to be required for effective Ag presentation or costimulation as long as signal 3 is provided. However, use of peptide as the Ag bypasses requirements for DC to take up and process Ag and migrate to draining LN where the Ag can be presented to T cells. It may also bypass a need to up-regulate expression of class I or costimulatory ligands on DC due to relatively high levels of class I/peptide Ag complex being achieved. Thus, our results argue that one critical function of activated DC is the production of a cytokine(s) to provide signal 3 but do not suggest that other functions of activated DC are not critical during viral or microbial infections.

The ability of IL-12 to support generation of responses and memory upon immunization with peptide may have practical applications in using virus- and tumor-specific peptides for protective or therapeutic immunization. Indeed, Weber et al. (52) have recently shown that including IL-12 upon immunization of melanoma patients with peptides from gp100 and tyrosinase significantly increased the numbers of Ag-specific tetramer-positive and IFN-γ-producing cells in peripheral blood. We have obtained preliminary results suggesting that such immunization can significantly reduce growth of established, progressing tumors in a murine model. IL-12 replaces the need for adjuvant in supporting a response to peptide Ag by adoptively transferred TCR-transgenic OT-1 CD8 T cells specific for $Kb/OVA_{257–264}$ (22), with essentially identical effects as in the 2C responses described in this report (J. Goldberg and M. F. Mescher, unpublished results). B-16 melanoma cells transfected with OVA grow as colonies in the lungs following i.v. injection into mice having adoptively transferred OT-I cells; visible colonies are detectable by day 7 after tumor injection, and tumor-bearing mice die around day 25 with a large tumor burden in the lungs. In preliminary experiments, we have found that immunization of tumor-bearing mice on day 15 with $OVA_{257–264}$ peptide and IL-12 results in activation of the OT-I cells and a dramatic reduction in tumor burden in the lungs

when assessed on day 22 (F. Popescu and M. F. Mescher, unpublished results). Thus, it appears that IL-12 supports development of antitumor immunity in this model, and additional experiments are in progress to determine whether this results in therapeutic benefit.

Immunization with a class I-restricted peptide alone is often seen to result in tolerance. When adoptively transferred mice are given peptide alone, weak clonal expansion of the Ag-specific CD8 T cells occurs (Fig. 1), but these cells do not develop lytic effector function (16) or production of IFN-γ (J. M. Curtsinger and M. F. Mescher, unpublished observations). A small population of the Ag-specific cells persists long term and has a memory phenotype, but these cells are nonresponsive to rechallenge with peptide alone (Fig. 7) or peptide with either CFA or IL-12 (16). These results suggest that the presence or absence of signal 3 at the time of priming can determine whether exposure to Ag results in functional activation and formation of long-term memory or instead results in tolerance. Thus, Ag recognition and costimulation in the absence of signal 3 (i.e., in the absence of inflammation) may be one of the mechanisms by which CD8 T cells are rendered tolerant to self Ags. Alternatively, a concomitant inflammatory response (and resulting production of signal 3) at the time of exposure to self Ag could potentially result in an autoimmune response.

It has become increasingly clear that inflammatory cytokines produced by the innate response have important roles in supporting development of productive T cell responses in vivo, but the basis for their effects are poorly understood. A three-signal model for productive activation of naive CD8 T cells suggests a direct link between the innate and adaptive responses, with the innate response providing the essential third signal in the form of IL-12 or an as yet unidentified cytokine. As discussed above, this model suggests the basis for a number of observations regarding the in vivo requirements for effective CD8 T cell activation. That CD4 T cells may have a similar requirement for a third signal is suggested by several observations. IL-1 can substantially increase in vitro responses of CD4 T cells by acting directly on the T cell (33–36, 38) and IL-1 can replace the need for adjuvant in supporting an in vivo response of CD4 T cells to peptide Ag (53). If these findings do, in fact, reflect a three-signal requirement for CD4 T cell activation, which will need to be determined in vivo, they suggest that different cytokines provide the third signal for CD4 and CD8 T cell subsets; CD4 responses are supported by IL-1 but not IL-12 (53), while CD8 responses are supported by IL-12 but not IL-1 (16).

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