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Inflammation can have both positive and negative effects on development of CD8 T cell memory, but the relative contributions and cellular targets of the cytokines involved are unclear. Using CD8 T cells lacking receptors for IL-12, type I IFN, or both, we show that these cytokines act directly on CD8 T cells to support memory formation in response to vaccinia virus and Listeria monocytogenes infections. Development of memory to vaccinia is supported predominantly by IL-12, whereas both IL-12 and type I IFN contribute to memory formation in response to Listeria. In contrast to memory formation, the inability to respond to IL-12 or type I IFN had a relatively small impact on the level of primary expansion, with at most a 3-fold reduction in the case of responses to Listeria. We further show that programming for memory development by IL-12 is complete within 3 days of the initial naive CD8 T cell response to Ag. This programming does not result in formation of a population that expresses killer cell lectin-like receptor G1, and the majority of the resulting memory cells have a CD62L$^{\text{high}}$ phenotype characteristic of central memory cells. Consistent with this, the cells undergo strong expansion upon rechallenge and provide protective immunity. These data demonstrate that IL-12 and type I IFN play an essential early role in determining whether Ag encounter by naive CD8 T cells results in formation of a protective memory population. The Journal of Immunology, 2009, 182: 2786–2794.

Formation of a CD8 T cell memory population following an initial response to pathogen infection or vaccination is critical for protection against subsequent reinfecion, but the signals required to program activated CD8 T cells to survive and become memory cells have not been fully defined. Help from CD4 T cells can be important (1, 2), and IL-2 signals during priming contribute to establishment of a responsive memory population (3). The size of the memory population that persists following a primary response is influenced by numerous factors, including dose and duration of Ag, levels of costimulatory signaling through CD28 and other receptors, and various cytokines (4, 5). Distinguishing between those signals that are fundamentally required to initiate the differentiation program leading to survival and memory formation, vs those that influence the magnitude of the response, has been difficult.

IL-12 and IFN-αβ can provide a third signal, along with Ag and costimulation, to support CD8 T cell clonal expansion and development of effector functions (6–11), thus coupling innate immunity to acquisition of effector functions as naive CD8 T cells respond to Ag. The role that signals from these cytokines play in CD8 T cell memory formation following infections with pathogens is less clear. Administering IL-12 along with peptide Ag replaces the requirement for an adjuvant to generate a memory response, with the IL-12 acting directly on the responding CD8 T cells, suggesting a possible role in programming the cells to persist as memory cells (7, 11, 12). Conversely, recent reports suggest that IL-12 can hinder development of long-term memory by promoting formation of short-lived fully activated effector cells that express killer cell lectin-like receptor G1 (KLRG1$^+$) on the surface (13, 14). The role of type I IFNs in programming responding CD8 T cells to become memory cells is also unclear. The primary response of CD8 T cells to lymphocyte choriomeningitis virus (LCMV) infection is reduced by over 99% when the cells lack the type I IFNR, but the small numbers of cells that do expand are able to transition to memory (6, 10, 15). In contrast, type I IFNR-deficient cells are only slightly impaired for responses to vaccinia virus (VV) and Listeria monocytogenes (LM) (16, 17).

In vitro experiments have shown that IL-12 and IFN-αβ act similarly as signal 3 cytokines for inducing effector functions (9), and the gene regulation that occurs in response to signals from these cytokines is quite similar (P. Agarwal, A. Raghavan, S. Nandiwada, J. Curtsinger, P. Bohjanen, D. Mueller, and M. Mescher, manuscript in preparation). This suggests that they may have largely redundant roles in vivo, and that this might include roles in programming for memory development. If so, this could explain why examination of CD8 responses to various infections in mice that are deficient in either IL-12 or the type I IFNR has usually not revealed critical roles for these cytokines. To evaluate this potential redundancy and assess the roles of IL-12 and IFN-αβ in memory formation, and to determine whether these are the only cytokines that support memory programming, we have examined responses to VV and LM by CD8 T cells lacking receptors for one or both of these cytokines. The results demonstrate that either can provide a signal needed to program for memory development, and that one or the other is required in a normal host. Furthermore,
cytokine-dependent programming of naive CD8 T cells to develop memory occurs early during the initial phase of primary expansion.

**Materials and Methods**

**Mice, cell lines, and reagents**

OT-I mice having a transgenic TCR specific for H-2Kb and OVA257–264 (18) were crossed with Thy1-congenic B6.PL-Thy1a/Cy (Thy1.1) mice (Jackson ImmunoResearch Laboratories) and bred to homozygosity. OT-I mice deficient for single cytokine receptors were obtained by crossing OT-I mice with mice deficient for the IL-12 β1 or IFNAR1 receptor and breeding to homozygosity, and mice deficient for both receptors were obtained by crossing OT-IL-12R/−/− mice (OT-IL-12RKO) with OT-IFNAR1−/− (OT-IFNARKOKO) and breeding to obtain OT-1−/−/IL-12Rβ1−/−/IFNAR1−/− mice (OT-ILDKO). CD8 T cell development in all strains appeared normal with respect to numbers, distribution, and phenotype (data not shown). Mice were maintained under specific pathogen-free conditions at the University of Minnesota, and these studies have been reviewed and approved by the Institutional Animal Care and Use Committee. C57BL/6NCr mice were purchased from the National Cancer Institute. Mice deficient for the IL-12 receptor and breeding to homozygosity, and mice deficient for both receptors were obtained by crossing OT-I mice having a transgenic TCR specific for H-2Kb and OVA257–264 (18) were bred with receptor-deficient mice to generate OT-I.DKO mice (OT-I.IL12RKO) with OT-I.IFNAR1−/− mice (OT-I.DKO).

**Viruses and bacteria**

Recombinant VV-GFP-JAW-OVA (VV-OVAp) expressed the OVA257–264 epitope fused C terminally to GFP and the transmembrane region of JAW-1 (provided by J. Yewdell, National Institutes of Health, Bethesda, MD). Viral titers were determined by plaque assays using JAWS-Kel-2 cells, and mice were infected i.p. with 5–10^5 PFUs. Recombinant LM expressing full-length secreted OVA (LM-OVA; a gift from Hao Shen, University of Pennsylvania, Philadelphia, PA) was used for infection at 10^5/mouse i.p. for primary challenge, and 10^5 i.v. for rechallenge of primed mice.

**Naive T cell purification**

Inguinal, axillary, brachial, cervical, and mesenteric lymph nodes (LN) were harvested in white-type (WT) or receptor-deficient OT-I mice, pooled, and disrupted to obtain a single-cell suspension. CD8^+CD44^− cells were enriched by negative selection using MACS magnetic cell sorting (Miltenyi Biotech). In brief, cells were coated with FITC-labeled Abs specific for CD4, B220, I-A^b, and CD44. Anti-FITC magnetic MicroBeads (Miltenyi Biotech) were then added, and the suspension was passed over separation columns attached to a MACS magnet. Cells that did not bind were collected, and were >95% CD8^+ and <0.5% CD4^+CD8^−. The numbers of CD8 T cells obtained from WT and receptor-deficient OT-I mice were comparable.

**Adoptive transfer and flow cytometric analysis**

Purified naive WT or receptor-deficient cells were adoptively transferred into normal C57BL/6NCr mice by i.v. (tail vein) injection at the numbers indicated for each experiment, and rested for 1 day before infection. Mice were sacrificed at the indicated times, and spleens, pooled LN, and lungs were harvested. Single-cell suspensions were prepared, viable cell counts were done (trypan blue), and the percentage of OT-I cells in the sample was determined by flow cytometry. For some experiments, adoptive transfer recipients were C57BL/6.Ly5.2 (CD45.1) mice that received either just WT or just receptor-deficient OT-I cells. In this case, OT-I cells were identified as CD8^+CD45.2^+ cells. In other experiments, WT OT-ILP and one of the receptor-deficient cells were cotransferred into the same C57BL/6.Ly5.2 (CD45.1) recipients. In this case, OT-I cells were identified as CD8^+CD45.2^− cells. In other experiments, WT OT-ILP and one of the receptor-deficient cells were cotransferred into the same C57BL/6.Ly5.2 (CD45.1) recipients. In this case, OT-I cells were identified as CD8^+CD45.2^− cells, and staining with anti-Thy1.1 was used to distinguish WT OT-ILP (Thy1.1^+?) from receptor-deficient (Thy1.1^−?) recipient cells. Determining OT-I cell numbers was determined by identical staining of cells from normal C57BL/6 mice (no adoptive transfer). Analysis was done using a FACScalibur flow cytometer and CellQuest software (BD Biosciences) to determine the percentage and total OT-I cells in the samples.

**Intracellular cytokine staining after in vitro rechallenge**

Spleen cells from adoptively transferred mice were incubated at 2 × 10^6 cells/ml in RP-10 with 0.2 μM OVA257–264 peptide and 1 μl of GolgiPlug (BD Biosciences) for 3.5 h at 37°C. Cells were then fixed in Cytofix buffer (BD Biosciences) for 15 min at 4°C, permeabilized in saponin-containing Perm/Wash buffer (BD Biosciences) for 15 min at 4°C, and stained with FITC-conjugated Ab to IFN-γ for 30 min at 4°C. Cells were then washed once with Perm/Wash buffer and once with PBS containing 2% FBS, and analyzed by flow cytometry.

**Results**

**Both IL-12 and type 1 IFN contribute to development of memory in response to LM infection**

OT-I cells expressing a transgenic TCR specific for H-2Kb/OVA257–264 (18) were bred with receptor-deficient mice to generate strains lacking the IL-12 β1-chain (OT-IL12RKO), the type 1 IFN receptor (OT-IFNARKOKO), or both receptors (OT-ILDKO).

When stimulated in vitro with artificial APC (uAPC) presenting H-2Kb/OVA257–264 and B7.1, naive OT-IL-12RKO cells developed cytolytic activity in response to IFN-α, but not IL-12, and the reciprocal was true for OT-IFNARKOKO cells (9). IL-21 can also provide a third signal to support development of cytolytic function in vitro (21). OT-ILDKO cells stimulated with uAPC did not develop cytolytic activity in response to IL-12 or IFN-α, but did in response to IL-21 (Fig. 1). Thus, the double-receptor-deficient cells can respond when they receive appropriate signals. To assess responsiveness in vivo, purified naive (CD44^−) OT-I cells from WT or receptor-deficient strains were adoptively transferred into normal C57BL/6 recipients. In some experiments, WT and one of the receptor-deficient OT-I cells were transferred into the same recipients to allow comparison in the same mouse. Transferred cells were detected using Thy1 and CD45 congenic markers, as described in Materials and Methods.
Both IL-12 and type I IFNs are produced in response to infection with LM (22–25), and Ag-specific CD4 and CD8 T cells undergo clonal expansion and develop effector functions. When OT-I T cells are adoptively transferred and the mice then infected with LM that expresses OVA (LM-OVA), the OT-I cells undergo strong clonal expansion that peaks at days 7–8 and a large fraction of the cells produce IFN-γ upon restimulation, paralleling the response to endogenous Ag-specific CD8 T cells. To examine the contributions of IL-12 and type I IFN to the response, mice were adoptively transferred with WT OT-I cells along with an equal number of either OT-I.IL12RKO, OT-I.IFNARKO, or OT-I.DKO cells. The mice were then infected with LM-OVA (i.p.), and the responses of the transferred cells were assessed. The OT-I cells lacking the receptor for either IL-12 or type I IFN underwent strong primary clonal expansion, but expansion was significantly reduced when the OT-I cells lacked both receptors (OT-I.DKO cells; Fig. 2A). For these cells, the peak numbers were reduced ~60% in comparison with WT OT-I cells, and this was the case when either high (1 × 10^6) or low (5 × 10^5) numbers of OT-I cells were adoptively transferred. The ability of the cytokine receptor-deficient cells to produce IFN-γ was somewhat reduced, particularly for the OT-I.DKO cells (Fig. 2B).

When the memory populations remaining at day 30 following LM-OVA infection were examined, dramatic effects of cytokine-receptor deficiency were found. In comparison with WT OT-I cells, the number of remaining OT-I.IFNARKO cells was reduced ~4-fold, OT-I.IL12RKO cells were only marginally detectable, and there were no remaining OT-I.DKO cells detectable above background (Fig. 2C). Differences in the numbers of WT vs receptor-deficient cells in the LN and lungs paralleled those in the spleen (Fig. 2D), indicating that differences in the lymphoid organs were not a result of differential trafficking and localization. It should be noted that the OT-I.IL12RKO cells we have used lack the IL-12Rβ1 chain, which is shared with the IL-23R. Thus, it is formally possible that the response of WT cells might involve IL-23, and be lost in the OT-I.IL12RKO cells. However, in vitro experiments using naive OT-I cells have failed to detect any signal 3 activity of IL-23 (our unpublished results). These results suggest that whereas signals from IL-12 and/or type I IFN are not required to support primary clonal expansion in response to LM-OVA infection (Fig. 2A), they do contribute to development of effector function (Fig. 2B) and are necessary to program for development of a memory population (Fig. 2, C and D).

Development of memory in response to VV requires IL-12 or type I IFN

Ag-specific CD8 T cells that lack the type I IFN receptor make almost no response to LCMV (6, 15), but respond well to VV (6, 10, 15), suggesting that IL-12 may be the major signal 3 cytokine that supports the response to VV. To examine this, WT and cytokine-receptor-deficient OT-I cells were adoptively transferred and the mice were infected (i.p.) with VV expressing the OVA_{257–264} epitope (VV-OVAp). Following VV-OVAp infection, WT OT-I cells reach peak clonal expansion at day 5. The numbers then decline over the next 2 wk, and a memory population is established by day 30 (20), paralleling the response of the endogenous CD8 T cells (20, 26). Receptor-deficient OT-I cells also expanded vigorously, and their numbers were similar to those of WT cells on day 5 in the spleen (Fig. 3A). The decline in numbers over the next week, however, was much more precipitous for the OT-I.IL12RKO cells, and the memory population at day 30 was reduced by more than 90% compared with WT cells. OT-I.DKO cells declined even more dramatically, and became undetectable by day 30. OT-I.IFNARKO cells were least affected, consistent with reports that IFNRI-deficient CD8 T cells make a primary response to VV (6, 15), and the number of memory cells at day 30 was not significantly different from that of WT cells. Differences in the numbers of WT vs receptor-deficient cells in the LN and lungs paralleled those in the spleen (Fig. 3B).

Thus, despite strong primary expansion, OT-I.DKO cells that cannot receive signals from IL-12 or IFN-αβ do not survive to become memory cells following VV-OVAp infection. Their function is also compromised at the peak of expansion, as evidenced by reduced IFN-γ production (Fig. 3C). OT-I.IL12RKO cells are also severely compromised for memory formation in response to VV-OVAp (Fig. 3, A and B), because they are following a response to LM-OVA (Fig. 2, C and D). This is not due to an absolute requirement for IL-12 to signal for memory programming, however, as shown by the response to challenge with OVA_{257–264} peptide Ag and poly(I:C), an adjuvant that induces high levels of IFN-αβ. In this study too, primary expansion by WT and OT-I.IL12RKO cells was comparable, but in this...
case the memory population of OT-I.LIL12RKO cells was also comparable to that of WT cells (Fig. 3D). Similarly, the OT-I.DKO cells fail to form a detectable memory population (data not shown). Thus, IL-12 signaling is not required for memory formation when sufficient IFN-αβ is available to program the cells.

To confirm that the numbers of WT and receptor-deficient OT-I cells reflect the status of protective memory, mice were adoptively transferred with either WT or receptor-deficient OT-I cells and challenged with VV-OVAp. Thirty days later, they received a heterologous challenge with LM-OVA (27) and bacterial titer in the spleen was determined 3 days later. As predicted by the numbers obtained for the three or four mice in each group, the bars are the average. Based on Student’s t test, the number of WT cells was significantly greater than DKO cells (p < 0.001) or IFNARKO cells (p < 0.001), but did not differ significantly from IFNARKO cells (p = 0.68).

IL-7 contributes to formation and homeostatic maintenance of memory CD8 T cells. The high-affinity IL-7R chain, IL-7Rα, is down-regulated upon stimulation of naive cells and is expressed on a variable fraction of effector cells (20, 28). The effector cells that express IL-7Rα at the peak of response to LCMV were shown to be the cells capable of forming a memory population (28). It was possible, therefore, that IL-12- and IFN-αβ-dependent priming for memory might involve regulation of IL-7Rα expression, and that OT-I.DKO cells might not survive due to impaired expression of the receptor. This was not the case, however. By day 5 of the response to VV-OVAp, expression of IL-7Rα was similar on WT and receptor-deficient OT-I cells, and at day 7 a larger fraction was IL-7Rα high for all of the cells (Fig. 5). In fact, a
greater fraction of the OT-I.DKO cells was IL-7Rα high at this time, suggesting that within this rapidly declining population the cells that express IL-7Rα may have some survival advantage, but that this is not sufficient to rescue the population from contraction. Thus, regulation of the receptor for IL-7 cannot account for the inability to form a memory population when CD8 T cells do not receive signals from IL-12 or IFN-α/β.

Memory to VV can develop in the absence of IL-12 or type I IFN signals in IL-12-deficient host mice

The results described above demonstrate that OT-I cells that cannot receive signals from either IL-12 or type I IFN fail to develop a memory population in response to LM-OVA or VV-OVAp infections in a normal host, and that the memory population is greatly reduced in the absence or presence of IL-12. However, Orgun et al. (29) recently reported that mice deficient in both IL-12 p40-deficient, and IL-12 p55-deficient mice received 10^5 WT or receptor-deficient OT-I cells and were infected with VV-OVAp. Spleen cells were analyzed on day 30, and the numbers of OT-I cells were determined. A, Day 30 OT-I cell numbers in C57BL/6 (B6) and IL-12 p40-deficient hosts. Results shown are average and SEM for three mice/group. B, Day 30 OT-I cell numbers in C57BL/6 (B6) and IL-12 p35-deficient hosts. Results shown are average and SEM for four mice/group. C, CD62L expression on day 30 of WT and receptor-deficient cells from B. Too few OT-I.DKO cells were detected in C57BL/6 hosts to determine CD62L expression. Histograms shown are representative for each group.

IL-12 programs naive CD8 T cells to develop memory during the first 3 days of response to Ag and costimulation

Inflammatory cytokines are produced early in response to infections, and levels usually decline within a few days. Previous work showed that in vitro responses of naive CD8 T cells required that IL-12 be present along with Ag during the first 2–3 days to support the signal 3-dependent differentiation necessary for development of effector functions (19). This suggested that the IL-12 and/or type I IFN signals that CD8 T cells receive as they initially proliferate in response to Ag may be sufficient to program the cells to form a memory population. Consistent with this possibility, Malek and coworkers (30, 31) have shown that CD8 T cells that have been activated in vitro for 3 days, under conditions in which all three signals would be expected to be present, rapidly transition to a memory phenotype upon adoptive transfer into naive hosts and persist as memory cells. We used a similar approach to determine whether IL-12-dependent signaling for memory development occurs during the initial period of stimulation.

Naive (CD44^lo^) OT-I T cells were placed in microtiter wells coated with H-2Kb/OVA257–264 and B7-1 and cultured for 3 days in the absence or presence of IL-12, referred to as 2SigOT-I and 3SigOT-I, respectively. IL-2 was added in all cases to insure that levels of this cytokine were not limiting and that comparable clonal expansion occurred. Clonal expansion at 72 h was equivalent in the absence or presence of IL-12 and, as expected (9), cells stimulated in the presence of IL-12 expressed much higher levels of grzB and produced more IFN-γ upon Ag restimulation (data not shown). The in vitro stimulated cells were then harvested, washed, and adoptively transferred by i.v. injection into naive mice (10^6 cells/mouse), and their fates were determined.

Engraftment in the spleens at 12 h posttransfer was comparable for naive OT-I, 2SigOT-I, and 3SigOT-I cells, and the number of naive OT-I cells remained stable for 4 days (Fig. 7). The number of 3SigOT-I cells increased, and had expanded ~100-fold at the peak on day 4. This is in agreement with the results of Rolle et al. (31), who showed that OT-I cells adoptively transferred after 3 days of in vitro stimulation continued to divide and expand in number over several days following transfer. Between days 4 and 15, the number of 3SigOT-I cells contracted 5- to 6-fold and then remained stable to day 30. In contrast, the number of 2SigOT-I cells declined rapidly following engraftment, and had fallen to undetectable levels by day 30.

These results demonstrate that IL-12 signals present during the initial 3-day period of stimulation with Ag program the cells to form a population that survives long-term. That these were responsive memory cells was suggested by the finding that ~50% of the 3SigOT-I cells present at day 30 produced IFN-γ upon rechallenge with OVAp for 3.5 h in vitro, comparable to the memory population of OT-I cells that persists 30 days after...
infection with VV-OVAp (data not shown). To further determine memory status of these cells, mice were challenged with LM-OVA, and the bacterial titer and numbers of OT-I cells in the spleen were determined 3 days later. The 3SigOT-I cells expanded $\approx$200-fold in response to the LM-OVA infection (Fig. 7B), and reduced the bacterial titer by several logs in comparison with that in mice that had received naive OT-I cells 30 days earlier (Fig. 7C). In contrast, 2Sig OT-I cells remained below the level of reliable detection 3 days after challenge with LM-OVA (Fig. 7B) and provided no protection (Fig. 7C). Thus, signals received from IL-12 during the first 3 days as naive cells respond to Ag and costimulation are necessary and sufficient to program the cells for survival and development of a protective memory population.

**IL-12-dependent programming results in CD62L$^{high}$ and CD62L$^{low}$ memory populations without development of a KLRG1$^{high}$ population**

OT-I cells that are stimulated in vitro for 3 days with Ag/B7-1 and IL-12 and then transferred into normal mice exhibit changes in CD62L expression levels similar to those of OT-I cells responding to VV-OVAp infection (Fig. 8). Eight days after the initial stimulation, both CD62L$^{high}$ and CD62L$^{low}$ populations are present, with the majority being CD62L$^{low}$. At longer times, there is a shift to a greater proportion of CD62L$^{high}$ cells. This shift was more pronounced for the in vitro stimulated cells, so that $>80\%$ of the Sig3OT-I cells were CD62L$^{low}$ by day 30, characteristic of central memory cells, whereas $\approx50\%$ of the OT-I cells from VV-OVAp-infected mice were CD62L$^{high}$. The higher proportion of CD62L$^{high}$ cells in the Sig3OT-I memory population may account for the greater re-expansion that occurs upon rechallenge with LM-OVA (Fig. 7B). Thus, despite presumably uniform delivery of signals to the cells programmed in vitro to develop memory, there is heterogeneity in CD62L expression levels and the population distribution evolves over time in vivo.

The above results strongly support the conclusion that IL-12 or IFN-γβ is required to program responding CD8 T cells to develop a memory population. At the same time, however, there are recent reports suggesting that IL-12 can hinder development of long-term memory.
memory by promoting formation of short-lived fully activated effector cells, and that expression of KLRG1 on the surface provides a marker for these cells (13, 14). OT-I T cells responding to challenge with VV-OVAp develop a KLRG1high population beginning about day 5, at the peak of clonal expansion. At day 8, when total numbers of OT-I cells are declining, about one-third of the cells have become KLRG1high (Fig. 9A), and this population selectively declines at longer times (data not shown). OT-I DKO cells undergo similar clonal expansion, and a KLRG1high population appears to develop somewhat more slowly, but the fraction of cells that are KLRG1high by day 8 does not differ significantly from that of OT-I cells (Fig. 9A). Thus, eliminating the ability of the cells to receive signals from IL-12 or IFN-αβ reduces, but does not eliminate the development of a KLRG1high population during a response to VV-OVAp.

We also examined KLRG1 expression on OT-I cells stimulated with Ag and B7-1 for 3 days in vitro in the absence or presence of IL-12 following transfer of the cells into normal mice. Although cells stimulated for 3 days in the presence of IL-12 are potent effector CTL (9), they are KLRG1low (data not shown), and few, if any, KLRG1high cells develop following transfer into mice (Fig. 9B). Thus, IL-12 signaling can be sufficient to program cells to develop a memory population under conditions in which it does not induce a KLRG1high population of effector cells.

**Discussion**

The results described in this study using cytokine receptor-deficient OT-I T cells demonstrate that both IL-12 and type I IFNs contribute to programming for memory in CD8 T cells responding to VV or LM, and that signals from one or the other must be available to obtain a memory population in a normal host. For vaccinia infection, the predominant cytokine that supports memory is IL-12, with IFN-αβ making a relatively small contribution (Fig. 3). For LM infection, the memory population is substantially reduced when the receptor for either cytokine is absent (Fig. 2), suggesting that when the OT-I T cells are initially responding to Ag, the levels of IL-12 and IFN-αβ may be relatively low, and that many of the cells only receive sufficient signaling to program for memory when they can respond to both cytokines. Thus, IL-12 or IFN-αβ signals are not only important for differentiation of naive CD8 T cells to effectors (8, 9), they are required for development of a memory population following responses to pathogens in a normal host. Although Ag and costimulation levels, as well as numerous cytokines and surface ligands, can influence the magnitude of primary and memory CD8 T cell responses, it appears that IL-12 and IFN-αβ are uniquely able to act as the switch that determines whether the outcome of a response to Ag is memory vs tolerance in a normal host.

Although required for memory development, signals from IL-12 and IFN-αβ made only modest contributions to the magnitude of primary clonal expansion to vaccinia or LM, with at most a 3-fold reduction in expansion in response to LM when the cells lacked both cytokine receptors. This contrasts with LCMV infection, in which primary expansion of TCR transgenic P14 CD8 T cells deficient for type I IFNR1 is reduced by more than 99% compared with WT cells (6, 10, 15). Because primary expansion was severely compromised, the importance of IFN-αβ signals for memory programming could not be distinguished in those studies. However, a small number of type I IFNR1-deficient cells did persist long-term following LCMV infection (<1%), and were most likely cells that received an IL-12 signal. Consistent with this, type I IFNR1-deficient mice produce more IL-12 in response to LCMV infection than do WT mice (6, 10, 15), and can make a strong CD8 T cell response to the virus (16).

It is not clear why primary expansion of OT-I CD8 T cells to VV-OVAp (Fig. 3) is affected relatively little when signals from these cytokines are absent (Figs. 2 and 3), whereas the expansion to LM-OVA is substantially reduced (Fig. 2), and the expansion of P14 cells to LCMV is severely compromised in the absence of IFN-αβ signaling (6, 10). In a peptide immunization model, coadministration of IL-12 was shown to strongly enhance primary clonal expansion at low
peptide doses by enhancing survival, but had less effect at high peptide doses in which clonal expansion was strong in the absence of the cytokine (7). It may be the case that signal 3 cytokines can promote survival to enhance primary expansion when Ag levels are low, but contribute less when strong early survival signals are available due to high levels of Ag, high TCR affinity, or possibly high levels of costimulatory ligands. Differences in these parameters for VV, LM, and LCMV infections may account for the varied dependence of primary expansion on IL-12 and type I IFN signals. In all cases, however, formation of a responsive memory population is critically dependent on IL-12 or IFN-αβ signals.

Although CD8 T cells require IL-12 or IFN-αβ signals to develop memory in a normal host environment, in which both IL-12 and IFN-αβ are present and the endogenous host cells express receptors for the cytokines, this is not the case in an IL-12-deficient host. In the absence of IL-12, both OT-I-LFNARKO and OT-I.DKO cells form memory populations following infection with VV-OVAp (Fig. 6), consistent with the results of Orgun et al. (29), showing that mice deficient in both IL-12 p40 and type I IFNRI expression develop CD8 T cell memory populations comparable to those of WT mice following infection with LM AcAcA, an attenuated strain of LM. Host deficiency for a given cytokine can have multiple effects, including altered production of other cytokines. For example, CD8 T cell responses to LCMV infection in WT mice depend almost completely upon IFN-αβ (6, 10, 15), but a strong CD8 T cell response occurs in type I IFNRI-deficient mice in which IL-12 production is increased (16). Our results suggest that an alternate third signal is present in the IL-12-deficient environment, one that is not normally produced in sufficient amounts in an intact host to support a memory response. Further work will be needed to determine the identity of this alternate signal, but IL-21 is a candidate, given its ability to support development of cytolytic function in vitro (21).

The ability of OT-I.DKO cells to form memory in IL-12-deficient hosts makes the important point that most of the experiments described in this study examine responses of WT and receptor-deficient CD8 T cells responding in a normal host environment, in which both IL-12 and IFN-αβ can be present and the endogenous host lymphocytes express receptors for the cytokines. Thus, conclusions can be drawn regarding the signals directly needed by the transferred cells to respond and develop memory. However, the results cannot be extrapolated to what might be seen upon infection of mice that are deficient in the cytokine or receptor, in which there may be effects on numerous lymphocyte and dendritic cell subsets, levels of production of other cytokines, rates of Ag clearance, etc.

IL-12 and type I IFN, and other inflammatory cytokines, are produced early in response to infections, and levels decline within a few days, suggesting that early IL-12 and IFN-αβ signaling, during the time the cells are initially responding to Ag, may program the CD8 T cells to subsequently form a memory population. Consistent with this, in vitro stimulation with Ag, B7-1, and IL-12 for 72 h was sufficient to program development of a functional memory population upon transfer into a normal host mouse (Fig. 7). Thus, during the period that the signal 3 cytokines are supporting differentiation to develop effector functions (19), they also initiate the gene regulation program required for survival and formation of long-lived memory cells. Furthermore, despite presumably uniform delivery of signals in vitro, the memory cells include both CD62L<sup>high</sup> and CD62L<sup>low</sup> populations, a phenotype consistent with both effector and central memory cells being present.

Although our results show that IL-12 can provide a critical third signal, along with Ag and B7-1, to program memory development, there is also some evidence that IL-12 can hinder development of long-term memory by promoting formation of relatively short-lived, fully activated effector cells (13, 14). Joshi et al. (13) characterized a short-lived effector population that arises during a response to LCMV that could be identified based on increased expression of KLRG1 in the surface, and suggested that formation of this population was driven by high T-bet expression induced by high levels of inflammatory cytokines, including IL-12. In contrast, a more recent report by Sarkar et al. (32), also examining responses to LCMV, has suggested that formation of this KLRG1<sup>hi</sup> terminal effector population is driven by continuing Ag stimulation during the late stages of infection. Our results clearly show that IL-12 can provide a critical third signal to program for development of memory under conditions in which a KLRG1<sup>hi</sup> population is not induced (Fig. 9B). The system described in this study, using in vitro stimulation under well-defined conditions, followed by adoptive transfer to monitor the transition to memory, should provide a means of determining how signals present during the postprogramming phase will affect the size and phenotype of the memory pool, and such experiments are in progress.

CD4 Th cells can be necessary for development of CD8 T cell memory, and one way in which they are likely to contribute is by stimulating DC to produce IL-12 and/or IFN-αβ that can then program the CD8 T cells to develop memory. CD4 T cell help to condition or license DC to effectively activate CD8 T cells requires CD40 engagement on the DC by CD40L on the CD4 T cell (33–35), and ligation of CD40 induces DC to produce IL-12 (36, 37). In an ectopic heart transplant model requiring CD4 T cell help for CD8-mediated rapid graft rejection, Filatenkov et al. (38) demonstrated that CD4 T cells stimulated IL-12 production by DC in a CD40-dependent manner, and that IL-12 was necessary for the CD8 T cells to develop effector functions and mediate rejection. In responses to pathogens, this role for CD4 help may not be critical because viral or bacterial components provide TLR ligands that can activate DC and induce inflammatory cytokines, including IFN-αβ and IL-12 (36). CD4 T cells may still be important, however, for long-term maintenance of CD8 memory T cells (39), and/or for producing IL-2 that can play an important early role in programming for memory (3).

There are reports in the literature of a number of proteins whose expression by CD8 T cells is regulated by IL-12, including Bcl-3, which can promote survival of activated CD8 T cells (40, 41); cellular FLIPs, which may protect against fas-mediated apoptosis (42); and CD25, which can increase sensitivity to IL-2 signaling (43). In fact, it is likely that regulation of expression of numerous proteins is involved in IL-12- and IFN-αβ-dependent programming for memory. Oligonucleotide microarray analysis of IL-12 and IFN-α gene regulation in naive CD8 T cells responding in vitro to Ag and B7-1 have revealed that each cytokine initiates a complex program of altered gene expression during the first 72 h of the response as effector functions develop and programming for memory occurs. Over 350 genes, including those for numerous transcription factors, are regulated in common by the two cytokines (P. Agarwal, A. Raghavan, S. Nandi-wada, J. Curtisinger, P. Bohjanen, D. Mueller, and M. Mescher, manuscript in preparation). The realization of the critical role that these cytokines play in determining whether Ag encounter leads to CD8 T cell memory, when they are present, or tolerance, in their absence, and the further elucidation of the molecular pathways that determine the differentiation process should contribute substantially to the development of improved strategies for optimizing vaccines.

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Disclosures

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