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Autocrine IFN-γ Promotes Naive CD8 T Cell Differentiation and Synergizes with IFN-α To Stimulate Strong Function

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Autocrine IFN-γ signaling is important for CD4 differentiation to Th1 effector cells, but it has been unclear whether it contributes to CD8 T cell differentiation. We show in this paper that naive murine CD8 T cells rapidly and transiently produce low levels of IFN-γ upon stimulation with Ag and B7-1, with production peaking at ∼8 h and declining by 24 h. The autocrine IFN-γ signals for upregulation of expression of T-bet and granzyme B and induces weak cytolytic activity and effector IFN-γ production. IFN-α acts synergistically with IFN-γ to support development of strong effector functions, whereas IL-12 induces high T-bet expression and strong function in the absence of IFN-γ signaling. Thus, IFN-γ is not only an important CD8 T cell effector cytokine, it is an autocrine/paracrine factor whose contributions to differentiation vary depending on whether the response is supported by IL-12 or type I IFN. The Journal of Immunology, 2012, 189: 659–668.

Inflammatory cytokines have a fundamental role in activating T cell responses in that they can provide a third signal that determines whether the response of naive T cells to Ag and costimulation results in tolerance induction, in their absence, versus full activation and memory development if they are present. For naive CD8 T cells, IL-12 and type I IFNs (IFN-α/β) appear to be the major signal 3 cytokines that support responses to pathogens, transplants, tumors, and, IFN-γ; and strong function in the absence of IFN-γ production. Thus, IFN-γ is not only an important CD8 T cell effector cytokine, it is an autocrine/paracrine factor whose contributions to differentiation vary depending on whether the response is supported by IL-12 or type I IFN. The Journal of Immunology, 2012, 189: 659–668.

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Abbreviations used in this article: aAPC, artificial APC; DC, dendritic cell; grzB, granzyme B; IC, intracellular; MFI, mean fluorescence intensity; wt, wild-type.

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Institutional Animal Care and Use Committee. C57BL/6Ncr and C56BL/6Ly5.2 mice were purchased from the National Cancer Institute. Peptide Ags OVA257-264 (SIINFEKL) and hgp10025–33 (KVPRNQDWL) were obtained from New England Peptide (Gardner, MA). Peptide Ag GP33–41 was a gift from Dr. D. Masopust (University of Minnesota, Minneapolis, MN). All directly conjugated fluorescent Abs were purchased from eBioscience (San Diego, CA), BioLegend (San Diego, CA), or BD Biosciences (San Diego, CA).

Purification and in vitro stimulation of naive CD8 T cells
In some experiments, unseparated lymph node or spleen cells from TCR transgenic mice were used. In other experiments, lymph nodes were harvested from mice, pooled, and disrupted to obtain a single-cell suspension. CD8+CD44hi naive cells were enriched by negative selection using magnetic MACS MicroBeads from Miltenyi Biotec (Auburn, CA). In brief, cells were coated with FITC-labeled Abs specific for CD4, B220, I-AK, CD11c, and CD44. Anti-FITC MicroBeads 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% CD44hi.

Artificial APC (aAPC) were prepared by immobilizing DimerX H-2Kb: Ig fusion protein or H-2Dk: Ig fusion protein (BD Biosciences) and murine recombinant B7-1/Fc chimera protein (R&D Systems, Minneapolis, MN) on 5-μm-diameter sulfated polystyrene latex microspheres, loaded with cognate peptide as previously described (9), and used at 2 × 10^6 aAPC/T cells. Alternatively, DimerX and B7-1/Fc were immobilized in flat-bottom microtiter wells and loaded with peptide Ag as described previously (27). A total of 5 × 10^4 purified naive CD8 T cells were stimulated by Ag in microtiter wells in 0.2 ml RP-10 medium with 2.5 U/ml rIL-2 added. When added, murine rIL-12 (R&D Systems) was used at 2 ng/ml and Universal Type 1 IFN (PBL Biomedical Laboratories, Fiscatway, NJ) was used at 1000 U/ml.

Assay of cytotoxic activity and IFN-γ production
Cytolytic activity was determined in a standard 4-h [3H]Cr release assay using E.G7 cells as targets and EL-4 cells as controls. IFN-γ production was determined by intracellular staining of washed, fixed, and permeabilized cells using an allophycocyanin-conjugated anti–IFN-γ mAb, followed by analysis by flow cytometry. In all experiments, 0.6 μl/ml GolgiStop (BD Biosciences) was added for the last 4 h of stimulation. For measuring effector IFN-γ of cells at the end of 3 d of culture, the cells were harvested, washed, resuspended in the presence of 1 μg/ml cognate peptide and incubated for 4 h in the presence of GolgiStop prior to analysis.

Adaptive transfer, tumor growth, and immunization
Adaptive transfer of 3-d in vitro-stimulated cells was done by i.v. injection of the indicated numbers of cells into the tail vein, and OT-1 cells in the host mice were subsequently identified and quantified by flow cytometry, based on CD8 and Thy1 expression as described previously (2, 11). For experiments examining effects of adaptive transfer of in vitro-stimulated OT-1 cells on tumor growth, mice were challenged by s.c. injection of B16. OVA tumor, the tumors were allowed to grow until they were visible and palpable (8–10 d), and OT-1 effector cells were then injected i.v. in the tail veins. Tumor growth was assessed by right-angle measurements of the tumor mass, with results expressed as area (mm²).

Results
Naive CD8 T cells produce IFN-γ early in response to Ag and costimulation
When Ag, B7-1, and either IL-12 or IFN-α/β signals are provided in vitro, naive CD8 T cells expand and differentiate over the course of 3 d to develop potent cytolytic activity and acquire the capacity to produce high levels of IFN-γ upon re-encounter with Ag (9, 28). A report of early production of low levels of IFN-γ by naive human CD8 T cells (23) raised the possibility that this might also occur for murine cells and, if so, that autocrine IFN-γ signaling might contribute to differentiation. To examine this, spleen cells from three CD8 TCR transgenic mouse strains, OT-I (24), pmel (25), and P14 (26), were stained with anti-CD44 mAb and stimulated in vitro with cognate peptide for 4 or 8 h, and IFN-γ production was then determined by intracellular staining. Despite being isolated from naive mice, the CD8 T cell populations included cells having low, intermediate, and high levels of CD44+ by flow cytometry, with homogenous IFN-γ production by all samples at both 4 and 8 h and in all cases greater than 1.5% IFN-γ+ events in the CD44hi gate (Fig. 1). Cells with the CD44hi memory phenotype probably arise from homeostatic expansion, and this is consistent with OT-I cells undergoing stronger homeostatic expansion than pmel cells (29) and having the highest proportion of CD44hi cells.

For all three TCR transgenic cell types, a large fraction of the CD44hi memory population produced IFN-γ within 4 h of stimulation, as expected for memory cells. In addition, however, significant fractions of the CD44int and CD44low populations also produced IFN-γ early in response to Ag.
IFN-γ, albeit levels were lower based on mean fluorescence intensities (MFI), and production appeared to be somewhat slower (Fig. 1). IFN-γ production by the CD44low and CD44int populations was quite comparable. Although readily detectable, the levels of IFN-γ produced early by naive cells were 2–3 logs lower than levels produced by effector cells after 3 d of differentiation and restimulation with Ag (see below).

To better define the stimulation requirements for early IFN-γ production, naive CD44low OT-I cells were purified by negative selection (see Materials and Methods) and stimulated in vitro using aAPC having class I MHC–peptide complexes immobilized on the surface, with or without coimmobilized B7-1/IFc ligand. Although IFN-γ production did not require B7-1–dependent costimulation, the fraction of CD44low OT-I cells producing IFN-γ was increased 2–3-fold when B7-1 was present (Fig. 2A, 2B). The contribution from B7-1–dependent costimulation was not due to more IL-2 being produced, because addition of IL-2 to cultures stimulated with just Ag on aAPC did not increase IFN-γ production (Fig. 2B). The response of the purified naive CD8 T cells to aAPC stimulation strongly argues that Ag and B7-1–dependent costimulation are the necessary and sufficient signals to activate this response and that cytokines or other surface ligands on APCs are not involved.

CD44 levels increase on stimulated cells beyond ~8 h, so the time course for IFN-γ production by naive CD44low cells in a mixed population cannot be determined. This can be determined using purified CD44low cells, however, because the purification yields a population that is predominantly CD44low with only a small number of CD44int and almost no CD44hi cells (Fig. 2C). IFN-γ+CD44low cells are readily detected within 4 h (Fig. 2C, top) and numbers increase by 8 h (Fig. 2C, bottom) when CD44 expression is beginning to increase on the stimulated cells. Beyond 8 h, the response declines (Fig. 2D). Thus, IFN-γ production by the naive CD44low cells is rapid and transient. In nine experiments examining Ag-stimulated IFN-γ production by purified CD44low OT-I cells obtained from mice ranging in age from 4 to 14 wk, the percentage of cells that were IFN-γ+ at 8 h was 23.8 ± 5.8% (average ± SD), and there was no correlation between age of the mice and percent of IFN-γ producers.

A large fraction of CD44int memory cells rapidly produce IFN-γ upon restimulation with Ag, and we therefore compared the Ag dose response for early IFN-γ production by the OT-I cells having low, intermediate, and high levels of CD44. OT-I cells from lymph nodes of naive mice have all three populations (Fig. 3A), and cells in each population produce IFN-γ within 8 h of stimulation with aAPC (Ag and B7-1/IFc) (Fig. 3B, 3C). When IFN-γ production was examined at varied peptide Ag concentrations, the fraction of...
IFN-γ+ cells plateaued at ~25% for the CD44low/int cells and ~75% for the CD44hi cells (Fig. 3D). Similarly, the average amounts of IFN-γ produced as measured by MFI plateaued at higher peptide concentrations, and levels were about two to three times higher for memory versus naive cells (Fig. 3E). Thus, although both CD44a and CD44low/int cells produce IFN-γ early after stimulation and the Ag dose responses are very similar (Fig. 3D, 3E), a smaller fraction of the CD44low/int population produces IFN-γ, and they produce distinctly less than do CD44hi cells.

Purified naive cells from OT-I.Rag−/− CD8 T cells produced more early IFN-γ than did OT-I cells, ruling out a potential requirement for dual TCR expression (Fig. 4A–C). IL-12 and type I IFN both stimulate development of strong effector IFN-γ production by cells stimulated with Ag and B7-1 for 3 d (28). To determine whether endogenous levels of these cytokines in normal mice might be responsible for some in vivo priming of naive cells for IFN-γ production, we examined early IFN-γ production by OT-I cells deficient for both the IL-12 and type I IFNRS (Fig. 4D–F). The receptor-deficient cells included CD44low, CD44int, and CD44hi populations (Fig. 4D). None of the cells produced IFN-γ when cultured in the absence of Ag (Fig. 4E), but when Ag was present, they exhibited the same pattern of early IFN-γ production as wt OT-I, with CD44hi cells making the highest levels and a fraction of the CD44low and CD44int cells making somewhat lower levels (Fig. 4F). The peptide dose response for the OT-I-IL-12R−/−IFNAR−/− cells was essential the same as for wild-type (wt) OT-I (data not shown). Thus, in vivo priming by IL-12 and/or type I IFN is not required for naive cells to have the capacity to produce early IFN-γ in response to Ag-dependent stimulation.

**IFN-γ signals for development of weak effector functions but is not required for IL-12-mediated development of effector functions**

Optimal development of effector functions by CD8 T cells requires a third signal that can be provided by IL-12 or IFN-α/β (9, 28). However, some weak cytolytic activity develops in the absence IL-12 or IFN-α/β, and stimulation with just Ag and B7-1 upregulates weak and transient expression of genes for many effector proteins, including granzyme B (grzB) and IFN-γ (22). To determine whether the IFN-γ produced early in response to stimulation through the TCR and CD28 might contribute to this limited differentiation of the cells, OT-I T cells that lacked the gene for either IFN-γ (OT-I-IFN-γ−/−) or the receptor for IFN-γ (OT-I-IFN-γR−/−) were examined.

Stimulation of wt OT-I cells with Ag/B7 results in some Ag-specific cytolytic activity, but at a level that is ~80–90% less (based on comparison of E:T ratios required for a given level of lysis) than the activity of cells stimulated with Ag/B7 in the presence of IL-12 (Fig. 2A, 2B). This weak cytolytic activity is absent, however, in OT-I-IFN-γ−/− or OT-I-IFN-γR−/− cells stimulated with Ag/B7 (Fig. 2A, 2B). Thus, IFN-γ provides a signal necessary for development of weak cytolytic function in response to Ag/B7-dependent signals. However, it is not necessary for development of strong cytolytic activity when IL-12 is present; both OT-I-IFN-γ−/− and OT-I-IFN-γR−/− cells develop potent activity in response to Ag/B7 and IL-12 (Fig. 5A, 5B).

IFN-γ signaling effects on grzB expression paralleled the effects on development of cytolytic activity. Stimulation with Ag/B7 resulted in some upregulation of grzB expression in wt OT-I cells, but upregulation was minimal in OT-I-IFN-γ−/− or OT-I-IFN-γR−/− cells (Fig. 5C, 5D). IL-12 increased grzB expression in OT-I cells 2- to 3-fold, and comparably high levels were expressed in OT-I-IFN-γ−/− or OT-I-IFN-γR−/− cells when IL-12 was present. Thus, IFN-γ signaling supports upregulation of grzB but is not required for IL-12–dependent upregulation. This was further confirmed by examining effects of adding IFN-γ or neutralizing anti–IFN-γ mAb to cultures stimulated with Ag/B7 in the absence of IL-12 (Fig. 5D). Addition of IFN-γ to cultures of OT-I, IFN-γ−/− cells increased grzB expression to levels comparable to those in OT-I cells, and this increase was blocked when anti–IFN-γ mAb was also added. As expected, addition of IFN-γ to OT-I-IFN-γR−/− cells did not increase grzB levels. Addition of neutralizing anti–IFN-γ mAb partially inhibited Ag/B7–dependent upregulation in OT-I cells, whereas addition of IFN-γ increased expression (Fig. 5D). Anti–IFN-γ mAb eliminated the increase resulting from exogenous IFN-γ but some upregulation remained, which is likely due to autocrine signaling being less susceptible to blockade by ligand-neutralizing Ab (30). Consistent with grzB upregulation in response to IL-12 being independent of IFN-γ signaling, addition of anti–IFN-γ mAb to OT-I cells stimulated in the presence of IL-12 did not significantly reduce grzB expression.

Use of OT-I-IFN-γR−/− cells made it possible to also examine early IFN-γ signaling effects on development of the ability to...
produce IFN-γ as an effector cytokine. The receptor-deficient cells cannot receive an IFN-γ signal but retain the ability to produce IFN-γ. Furthermore, the OT-1-LIFN-γR−/− cells produce low levels of early (8 h) in response to stimulation with Ag/B7 (Fig. 6A–C). Over the 3-d course of differentiation, effector CD8 T cells can develop the ability to rapidly produce high levels of IFN-γ upon re-encountering Ag. To assess the signaling requirements for this response, cells were stimulated for 3 d, washed, and incubated with peptide Ag for 4 h in the presence of monensin to block secretion, and the level of IFN-γ production then was determined by intracellular (IC) staining. Stimulating wt OT-I cells with just Ag/B7 results in some effector IFN-γ production by day 3, and this is substantially reduced by addition of anti–IFN-γ mAb during the 3-d culture period (Fig. 6D). Production of IFN-γ by OT-I cells is strongly increased if IL-12 is present during the culture period, and this is not blocked by the presence of anti–IFN-γ mAb during culture (Fig. 6E). In contrast to wt cells, OT-I-LIFN-γR−/− cells do not make effector IFN-γ after stimulation for 3 d with just Ag/B7, although they do produce IFN-γ at 8 h in response to this stimulus. The OT-1-LIFN-γR−/− cells do produce a strong effector IFN-γ response, comparable to that of OT-I cells, when IL-12 is present during the 3 d of culture (Fig. 6G). Thus, as is the case for grzB expression and cytolytic function, early IFN-γ signaling promotes development of weak effector IFN-γ production capacity in cells responding to Ag/B7 but is not necessary for development of a strong response when IL-12 is present.

To determine whether the weak function that develops in vitro in response to IFN-γ results in physiologically relevant in vivo function, cells were tested for their ability to mediate tumor growth control in an adoptive transfer model. OT-I and OT-1-LIFN-γR−/− cells were stimulated for 3 d in vitro with Ag/B7 in the absence or presence of IL-12, washed, and adoptively transferred into mice with progressing B16.OVA tumors growing sc. Mice had been inoculated 10 d earlier with B16.OVA, and all had visible, palpable tumors at the time of effector cell transfer. OT-I effector cells generated in the absence of IL-12 (OT-I/None) caused significant control of tumor growth (p < 0.014 versus control), whereas OT-I-LIFN-γR−/− effector cells (IFN-γRko/None) did not control tumor (Fig. 7). Thus, the weak function that develops in response to IFN-γ signaling can be sufficient to mediate significant in vivo control of Ag-bearing cells.

Consistent with in vitro levels of effector function, OT-I and OT-1-LIFN-γR−/− effector cells generated in the presence of IL-12 (OT-I/IL12 and IFN-γRko/IL12) mediated strong tumor growth control; tumor regression began within a few days of transfer, and growth was controlled for more than 11 d following transfer (Fig 7). OT-I effector cells generated in the presence of IL-12 were >10 times more effective than those generated in its absence. In addition and consistent with in vitro development of strong effector function with IL-12 in the absence of IFN-γ signaling, OT-I-IFN-γR−/− cells generated in the presence of IL-12 mediated strong tumor control and were only marginally if at all less effective than OT-I cells (Fig. 7).

IFN-γ can synergize with IFN-α in supporting development of effector functions

IFN-α acts comparably to IL-12 as a third signal to support development of strong effector functions and long-term survival of naive CD8 T cells responding to Ag and costimulation (7, 28). However, in contrast to IL-12–driven differentiation that is largely independent of IFN-γ signaling, responses to IFN-α exhibit a strong dependence on intact IFN-γ signaling. Cytolytic activity that develops in response to Ag/B7 and IFN-α is reduced >90% in OT-1-LIFN-γ−/− cells in comparison with OT-I cells (Fig. 8A), and this is also the case for OT-1-LIFN-γ−/− cells (Fig. 8C; data not shown). Similarly, IFN-α supports development of strong effector IFN-γ production by OT-I cells, and this is greatly reduced for OT-1-LIFN-γ−/− cells, whereas both cell types produce comparable effector IFN-γ following stimulation in the presence of IL-12 (Fig. 8B).

To rule out the possibility that the IFN-γ dependence observed in these experiments might be due to suboptimal levels of IFN-α, dose responses were examined for OT-I and OT-1-LIFN-γ−/− cells. As shown in Fig. 8C, cytotoxicity (expressed as lytic units), upregulation of grzB expression and effector IFN-γ production were all highly dependent on intact IFN-γ signaling even at very high doses of IFN-α. Thus, signaling through both the type I and type II IFNRs appears to be highly synergistic in driving effector differentiation of CD8 T cells responding to Ag and costimulation.
IFN-γ–dependent upregulation of T-box transcription factors

The T-box transcription factors Eomes and T-bet play central roles in the differentiation of CD8 T cells, influencing both effector and memory development (31–35). In previous studies examining Eomes mRNA, we had found that expression was high in naive CD8 T cells and decreased in cells responding to just Ag/B7 over 3 d in vitro but was maintained at a high level when either IL-12 or IFN-γ was present (22). A somewhat different result was obtained when Eomes protein levels were determined by IC staining. Eomes was marginally detectable in naive cells but was upregulated by stimulation with just Ag and B7-1 within 44 h, and at this time, levels were essentially the same whether IL-12 was present (Fig. 9A). Levels were also the same for OT-I and OT-1IFN-γR−/− cells. By 72 h, Eomes levels had decreased in cells stimulated with just Ag/B7 and remained somewhat higher if IL-12 was present, and in this study too, levels were comparable for OT-I and OT-1IFN-γ−/− cells. Thus, it appears that IFN-γ signaling is not involved in regulation of Eomes protein expression in cells responding to Ag and costimulation.

When intracellular staining was used to examine levels of T-bet protein at day 3, expression was found to be upregulated in wt OT-I in response to Ag/B7. Addition of IFN-γ to the cultures had no effect, but stronger upregulation occurred when IL-12 was added (Fig. 9B). This is consistent with previous results showing a similar pattern of regulation of T-bet mRNA expression in response to stimulation with two or three signals (22). The level of T-bet expression in OT-IIFN-γ−/− and OT-1IFN-γR−/− cells stimulated with Ag/B7 was greatly reduced in comparison with levels in OT-I cells, but the strong upregulation that occurs in the presence of IL-12 was comparable for OT-I and the deficient cells (Fig. 9B). Addition of IFN-γ to cultures of OT-1IFN-γR−/− cells stimulated with Ag/B7 was greatly reduced in comparison with levels in OT-I but comparable if IL-12 was present (Fig. 9C). Thus, IFN-γ–dependent induction of effector functions likely involves, at least in part, stimulation of increased expression of T-bet.

T-bet expression was also examined in OT-I and OT-1IFN-γ−/− cells stimulated over a broad range of IFN-γ concentrations (Fig. 9D). As expected, OT-I cells upregulated T-bet in the absence of IFN-γ, and expression increased with increasing concentrations of IFN-γ. Minimal upregulation occurred for OT-IIFN-γ−/− cells in the absence of IFN-γ, but expression increased with increasing concentrations of IFN-γ.
Your document seems to be an excerpt from a scientific paper discussing the role of IFN-γ in the control of tumor growth and differentiation of CD8 T cells. The authors describe experiments using OT-I T cells, which can be transferred into mice bearing tumors and stimulated in vitro to produce IFN-γ and other cytokines. The results suggest that IFN-γ, when added to the cultures of OT-I cells, enhances their ability to control tumor growth, while IL-12 has a more transient effect.

The paper also discusses the synergistic effects of IFN-γ and IL-12, and the role of IFN-γR2 in supporting the differentiation of CD8 T cells. The authors conclude that IFN-γ can provide a signal that supports development of optimal effector functions and memory, and differentiation in response to these cytokines displays quite distinct dependencies on IFN-γ signaling.

Discussion

The results described in this paper demonstrate that naive CD8 T cells rapidly and transiently produce low levels of IFN-γ, and that this signal is important for the development of weak effector functions, and this is likely mediated in part by upregulation of T-bet expression. Cytolytic activity and effector IFN-γ production are ~10-fold less than that of effector cells that differentiate in response to IL-12 or type I IFN, but the cells can nevertheless have substantial in vivo function as demonstrated by their ability to control tumor growth. Unlike IL-12 or type I IFN, autocrine IFN-γ does not appear to program cells to survive long-term in vivo. When OT-I cells are stimulated in vitro as described in this paper and are adoptively transferred into mice, the cells do not survive long term unless IL-12 was present during the in vitro stimulation (7; i.e., the autocrine IFN-γ signal does not program the cells to survive). Thus, it appears that IFN-γ signaling, along with Ag and costimulation, supports development of a short-lived population of suboptimal effector cells. These are not the equivalent of the short-lived effector cells defined by high KLRG1 expression that arise in response to infections (36, 37), because fewer than 5% of the cells express KLRG1 either in vitro or following transfer into normal mice (data not shown). IL-12 or type I IFN can provide a third signal that supports development of optimal effector functions and memory, and differentiation in response to these cytokines displays quite distinct dependencies on IFN-γ signaling.

FIGURE 7. IFN-γ-dependent differentiation yields effector cells that can mediate tumor growth control. OT-I and OT-LIFN-γR2 (OT-IγRko) cells were stimulated in vitro with Ag/B7 for 3 d with no cytokine addition (none) or with IL-12 added. Cells were then washed and adoptively transferred into mice bearing s.c. B16.OVA tumors that had been growing (none) or with IL-12 added. Cells were then harvested and cytolytic activity (GrzB expression) and IFN-γ production were then determined in a [51Cr] release assay. (A) OT-I (black lines) and OT-I.IFN-γRko (gray lines) cells were stimulated as in (B) OT-I (black lines) and OT-LIFN-γR2 (gray lines) cells were stimulated as in (A) and harvested at day 3. Cells were then harvested and cytolytic activity was determined in a [51Cr] release assay. (B) OT-I (black lines) and OT-LIFN-γR2 (gray lines) cells were stimulated as in (A) and harvested at day 3.

FIGURE 8. IFN-γ and IFN-α synergize to induce development of strong effector functions. A OT-I (solid lines) and OT-LIFN-γR2 (dashed lines) cells were stimulated for 3 d in vitro with Ag/B7 and no cytokine addition (none) or either IL-12 (1 µg/ml) or IFN-α (1000 U/ml) added to the cultures. Cells were then harvested and cytolytic activity was determined in a [51Cr] release assay. B OT-I (black lines) and OT-LIFN-γR2 (gray lines) cells were stimulated as in (A) and harvested at day 3. Cells were then harvested and cytolytic activity was determined in a [51Cr] release assay. C OT-I (black lines) and OT-LIFN-γR2 (gray lines) cells were stimulated for 3 d with Ag/B7, and varying amounts of IFN-α were added to the cultures as indicated. On day 3, cells were harvested, and cytolytic activity (top panel), GrzB expression (middle panel), and IFN-γ production (bottom panel) were determined. Cytolytic activity (top panel) is expressed as lytic units per 1 × 10⁶ cells.
There are successive stages of IFN-γ production by CD8 T cells, with naive cells poised to produce low levels of IFN-γ within hours of TCR and CD28 signaling and signals from the IFN-γ then acting in a feed-forward manner to prime cells to make moderate levels of IFN-γ at later times when they re-encounter Ag (Fig. 6). IL-12 or IFN-α signals support a third stage characterized by very high levels of IFN-γ production in response to Ag (Fig. 6E, 6G). T-bet and Eomes contribute to effector IFN-γ production (30, 31, 33–35, 38), and early IFN-γ signaling upregulates T-bet expression (Fig. 9B). However, regulation of the levels of IFN-γ production do not appear to simply be determined by T-bet and Eomes levels because IFN-γ production in CD8 T cells responding to infections or tumors represents very heterogeneous populations with respect to surface marker expression and functions, including levels of grzB expression and T-bet and IL-12-Stat4-T-bet signaling that act sequentially in an interlinked manner (15). The sequential nature of the pathway is enforced, at least in part, by TCR signals repressing IL-12R expression so that IL-12R is only upregulated upon termination of TCR signaling. Thus, in both CD4 and CD8 T cells, stimulation with Ag results in rapid production of low levels of IFN-γ that upregulate T-bet expression in an autocrine manner. For CD8 T cells, this is sufficient to result in some limited effector functions, including late effector IFN-γ production. Unlike CD4 T cells, IFN-γ signaling to upregulate IL-12R on CD8 T cells is not necessary, because IL-12 can stimulate strong effector functions and long-term survival in its absence. It also appears unlikely that the IFN-γ signaling contribution to IFN-α-dependent responses results from upregulation of the type I IFNR, because the IFN-α dose-response profiles for T-bet upregulation are the same in the absence or presence of IFN-γ signaling (Fig. 9D). IFN-γ can mediate cross-regulation of numerous signaling pathways, including “priming” of macrophages for enhanced type I IFN responses (40, 41).

The results described in this paper predict that the importance of an IFN-γ signal to a CD8 response will vary depending on whether the response is supported by IL-12 or IFN-α/β, and this has been observed. The response to LCMV infection is highly dependent on IFN-α/β signaling directly to the CD8 T cells (1, 5, 6), and Whitton and coworkers (17, 18) showed that P14 TCR transgenic CD8 T cells that lacked the IFN-γR were also strongly impaired in their response to LCMV. In contrast, OT-I cells lacking the IFN-γR made normal responses to immunization with peptide Ag and LPS (20), which induces IL-12 production. Direct IFN-γ signaling to CD8 T cells in vivo may also have effects on proliferation and/or survival that are not apparent in the experiments described in this paper. In addition to synergizing with IFN-α/β, the weak effector functions that develop in response to IFN-γ alone could potentially have a role in helping to initiate a response by mediating killing of cells bearing foreign Ag even in the absence of overt stimulation of APC and in the absence of inflammatory cytokines. This limited cytolysis could then make Ag available to APC for presentation to CD4 helper T cells that, if response occurs, could then provide help to the CD8 T cells to yield full differentiation and optimal effector functions as well as survival signals leading to development of memory. If help is not generated, the short-lived nature of the effectors that arise in response to IFN-γ signaling would ensure that a pathological response to inappropriate Ag does not develop. IFN-γ produced early by CD8 T cells responding to Ag presented on a dendritic cell (DC) may also promote IL-12 production by activated DC to further support the response (42).

The low level of IFN-γ produced early by naive CD8 T cells appears sufficient to provide optimal signaling, at least in vitro. Supplementing cultures with exogenous IFN-γ does not significantly increase responses beyond those reached in response to just the endogenously produced cytokine. It appears likely that IFN-γ signaling to the naive cells occurs in an autocrine manner, and this is consistent with the inability to fully block the endogenous IFN-γ-mediated effects by addition of neutralizing Ab, whereas the same Ab can block IFN-γ effects mediated by exogenous addition of IFN-γ to OT-I IFN-γ−/− cells. The source of IFN-γ to support responses in vivo may vary and include both autocrine and paracrine sources. Stimulated DC can produce IFN-γ, but CD4+ DC produce much more than the CD4+ 8+ DC that play the major role in Ag cross-presentation to CD8 T cells (43). Ag-specific CD8 T cells responding to infections or tumors represent very heterogeneous populations with respect to surface marker expression and functions, including levels of grzB expression and
ability to produce effector IFN-γ, and the basis for this heterogeneity is not well understood. It is likely influenced by many parameters, including TCR affinities for Ag and the extent and duration of Ag exposure. In addition, though, it seems likely that much of the heterogeneity derives from the cells having differing exposure to cytokines, including IFN-γ, IFN-α/β, and IL-12, as they are responding to Ag.

In the classical “two-signal” model for T cell activation, signals from the TCR and a costimulatory receptor, usually CD28, are required to activate the cells and avoid tolerance. A requirement for a third signal for naive CD8 T cells was revealed with the finding that activation through just TCR and CD28 receptors stimulated proliferation of the cells but was not sufficient to support optimal development of effector functions and memory, and the cells were rendered tolerant. Signaling via IL-12 or IFN-α/β could support full activation, and responses still required costimulation through the CD28 receptor. Thus, the cytokines do not provide an alternate form of costimulation but rather a distinct third signal. In support of this “three-signal” model, IL-12, type I IFNs, or both have been shown to support in vivo responses to antigen-specific CD8+ T cells responding to infection. J. Immunol. 174: 6791–6802.


References

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