This information is current as of April 14, 2017.

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J Immunol 2012; 189:3299-3310; Prepublished online 27 August 2012; doi: 10.4049/jimmunol.1102495
http://www.jimmunol.org/content/189/7/3299

Supplementary Material
http://www.jimmunol.org/content/suppl/2012/08/28/jimmunol.1102495.DC1

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CD8 T Cell Priming in the Presence of IFN-α Renders CTLs with Improved Responsiveness to Homeostatic Cytokines and Recall Antigens: Important Traits for Adoptive T Cell Therapy

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Previous mouse and human studies have demonstrated that direct IFN-α/β signaling on naive CD8 T cells is critical to support their expansion and acquisition of effector functions. In this study, we show that human naive CD8 T cells primed in the presence of IFN-α possess a heightened ability to respond to homeostatic cytokines and to secondary Ag stimulation, but rather than differentiating to effector or memory CTLs, they preserve nature-like phenotypic features. These are qualities associated with greater efficacy in adoptive immunotherapy. In a mouse model of adoptive transfer, CD8 T cells primed in the presence of IFN-α are able to persist and to mediate a robust recall response even after a long period of naturally driven homeostatic maintenance. The long-lasting persistence of IFN-α-primed CD8 T cells is favored by their enhanced responsiveness to IL-15 and IL-7, as demonstrated in IL-15−/− and IL-7−/− recipient mice. In humans, exposure to IFN-α during in vitro priming of naive HLA-A2+ CD8 T cells with autologous dendritic cells loaded with MART126−35 peptide renders CD8 T cells with an improved capacity to respond to homeostatic cytokines and to specifically lyse MART1-expressing melanoma cells. Furthermore, in a mouse model of melanoma, adoptive transfer of tumor-specific CD8 T cells primed ex vivo in the presence of IFN-α exhibits an improved ability to contain tumor progression. Therefore, exposure to IFN-α during priming of naive CD8 T cells imprints decisive information on the expanded cells that can be exploited to improve the efficacy of adoptive T cell therapy. The Journal of Immunology, 2012, 189: 3299–3310.

In response to infection or effective immunization strategies, Ag-specific naive CD8 T cells undergo massive expansion and subsequent differentiation into either short-lived effector CTLs, equipped with the capacity to kill and secrete cytokines, or long-lived memory CTLs, which persist in an Ag-independent, cytokine-driven lifestyle providing long-term protection (1, 2). The mechanisms underlying the differentiation of naive CD8 T cells into effector or memory cells remain a matter of debate (1, 3). It is becoming apparent that the signals integrated during the priming phase, including cognate Ag recognition, costimulation, and direct effects of innate inflammatory cytokines, determine the quality, persistence, and heterogeneity of the ensuing CD8 T cell response (4).

Help from CD4 T cells (5–7) has been asserted to be necessary for the development of an optimal CTL response, particularly in the context of mild inflammatory conditions, such as those found in tumors, dendritic cell (DC)-based vaccination, immunizations with proteins or peptides, or in certain virus infections, such as HSV or influenza virus infection (8–10). In these settings, the lack of CD4 T cell help during priming results in defective memory CTLs that mediate effector functions but do not undergo a second round of clonal expansion on Ag rechallenge (7, 11, 12). However, several studies in mice have indicated that the requirement for CD4 T cell help is not absolute and can be bypassed by the intense inflammatory environment elicited by certain pathogens (13–18) or several TLR ligands (13, 19–21).

The activation of innate immune cells through TLR and other innate pattern recognition receptors induces the production of inflammatory cytokines, such as IL-12, and type I IFNs (IFNs-I), among others. Elegant experiments in mice have shown that these two cytokines directly provide signals to naive CD8 T cells, acting
in synergy with Ag and costimulation, to support their expansion, acquisition of effector functions, and differentiation into memory CTLs (22–24).

We and others have shown that IL-12 and IFN-α also act directly on human naive CD8 T cells promoting the proliferation whereas favoring the acquisition of effector functions (25, 26). However, there is scant information about the effects of innate inflammatory cytokines on the differentiation of human naive CD8 T cells into effector or memory CTLs. Previous data from Farrar’s group suggest that IL-12 and IFN-α behave as nonredundant signals that differentially regulate the development of memory CTLs with T effector memory cell (TEM; CD62LlowCCR7hi) or T central memory cell (TCM; CD62LhighCCR7high) phenotypes, respectively (25). Frequently, specific surface markers have been used to ascribe functional attributes to memory CTLs, but phenotype cannot be directly translated into functional properties (27).

It is currently unknown whether the exposure to inflammatory cytokines during CD8 T cell priming may affect the responsiveness to homeostatic cytokines and recall Ags of the ensuing CTLs. These pieces of knowledge may allow the formulation of new adjuvants to enhance the protective efficacy of human vaccines, as cytokines during CD8 T cell priming may affect the responsiveness to homeostatic cytokines and recall Ags of the ensuing CTLs. Previous data from Farrar’s group suggest that IL-12 and IFN-α behave as nonredundant signals that differentially regulate the development of memory CTLs with T effector memory cell (TEM; CD62LlowCCR7hi) or T central memory cell (TCM; CD62LhighCCR7high) phenotypes, respectively (25). Frequently, specific surface markers have been used to ascribe functional attributes to memory CTLs, but phenotype cannot be directly translated into functional properties (27).

In this study, we show that IFN-α promotes the expansion and acquisition of effector functions of human naive CD8 T cells while maintaining them in an early differentiation state that is less differentiated than that of effector or memory CTLs. Interestingly, expanded cells possess a heightened ability to respond to homeostatic cytokines and to homeostatic cytokines and recall Ags of the ensuing CTLs. This hypothesis.

Importantly, this is also true for MART1-specific CD8 T cells raised in vitro from the repertoire of naive CD8 T cells. The features bestowed by IFN-α on human naive CD8 T cells might be exploited to improve the antitumor efficacy of adoptive T cell therapy. We provide data from in vivo studies in mice supporting this hypothesis.

**Materials and Methods**

**CTL isolation and culture**

Human CD45RA+CD27high (naive), CD45RA−/−CD27− (effector), CD45RA+CD27+ (memory) CD8 T cells were purified from PBLs eluted from leukocyte filters as Hervas-Stubbs and colleagues described previously (26). All blood donors gave written informed consent. This study was approved by the Medical Ethics Committee from the University Clinic of Navarra (013/2009). Samples of sorted cells and RNA extracted from them were frozen to be further analyzed, and naive CD8 T cells were cultured as follows. For polyclonal stimulation, CD8 T cells were cultured in vitro from the repertoire of naive CD8 T cells. The features bestowed by IFN-α on human naive CD8 T cells might be exploited to improve the antitumor efficacy of adoptive T cell therapy. We provide data from in vivo studies in mice supporting this hypothesis.

**FACS assays**

Fluorochrome-conjugated mAbs to the following human Ags were used: anti-human CD8 (RPA-T8), CXC-3 (IC6/CXCXR3), CD27 (M-T271), CD28 (CD28.2), CD62L (DERG-56), CCR7 (G043H7), IFN-γ (4B3), granzyme B (GB11), CD45RA (HI100), and killer cell lectin-like receptor G1 (KLRG1; 13F12F2) (29). IL-15Rα (20 ng/ml; Miltenyi) and poly I:C (20 μg/ml; Ampligen Bioclonex) were added directly to the culture (without washing), and cells were cultured for 1 additional day. For peptide loading, FASTDCs were harvested and incubated (37 °C) at 5 × 105 cells/ml in AIMV medium (without the supplementary system) using 10ng/mL MART126–35 peptide for 2 h. Subsequently, cells were irradiated (30 Cy), extensively washed, and resuspended in CTL medium at 2 × 106 cells/ml.

**Real-time RT-PCR**

Total RNA was extracted using the Nucleic Acid Purification lysis solution and the semiautomated ABI Prism 6100 Nucleic Acid PrepStation system (Applied Biosystems). Total RNA was treated with DNase before RT with FlowJo (Tree Star).

**Cytotoxicity assays**

Cytotoxicity activity of MART1-26–35-specific CD8 T cells was assayed against 51Cr-labeled Me275 (A2+/MART1+) and Na8 (A2+/MART1–) melanoma cell lines (3000/well) that were not or not pulsed with MART126–35 peptide. [3]Cr release in the 4-h culture supernatants was counted in a Topcount (Packard). Percentage lysis was calculated as follows: [experimental release − spontaneous release]/(maximum release − spontaneous release) × 100.

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[Invitrogen] and 1% penicillin/streptomycin (Invitrogen) supplemented with IL-7 (5 ng/ml) for 48 h. Afterward, IL-7 was removed and cells were maintained in culture for 24 h longer. Then naive CD8 T cells were specifically primed in vitro following a protocol based on an in vitro method to generate CTLs from the naive repertoire (28). For this purpose, CD8 T cells were cocultured in CTL medium with autologous irradiated-monoocyte-derived fast DCs (FASTDCs) (see later) loaded with MART126–35 peptide (Proimmune) at 5 × 106 CD8+ T cells (T/DC ratio = 5:1), in the presence and in the absence of IFN-α (500 IU/ml). On day 3 of stimulation, the medium was supplemented with IL-7 (5 ng/ml) and IL-2 (13 IU/ml), and on day 5, FASTDCs were removed by transferring nonadherent cells (mainly CD8+ T cells) to a fresh well. CD8 T cells were fed at days 5 and 7 with fresh medium containing IL-7 (5 ng/ml), IL-2 (13 IU/ml), and IL-15 (10 ng/ml), and at day 10 of culture, the cells were harvested for analysis.

**Generation and maturation of DCs**

Monocytes were isolated from PBL by positive selection using anti-CD14-conjugated paramagnetic beads (Miltenyi). Purified monocytes were cultured (1.5 × 106 cells/ml) for 2 d in AIM-V serum-free media (Life Technologies-BRL), supplemented with 5% AB human serum, GM-CSF (500 IU/ml; Miltenyi), and IL-4 (IL-4, 1000 IU/ml; Miltenyi). These DCs, derived from monocytes with GM-CSF and IL-4 over a 2-d culture, are referred to henceforth as FASTDCs (28). At day 2, maturation stimuli (TNF-α [20 ng/ml; Miltenyi] and poly I:C [20 μg/ml; Ampligen Bioclonex]) were added directly to the culture (without washing), and cells were cultured for 1 additional day. For peptide loading, FASTDCs were harvested and incubated (37 °C) at 5 × 105 cells/ml in AIMV medium (without the supplementary system) using 10ng/mL MART126–35 peptide for 2 h. Subsequently, cells were irradiated (30 Cy), extensively washed, and resuspended in CTL medium at 2 × 106 cells/ml.

**Cytotoxicity assays**

Cytotoxicity activity of MART1-26–35-specific CD8 T cells was assayed against 51Cr-labeled Me275 (A2+/MART1+) and Na8 (A2+/MART1–) melanoma cell lines (3000/well) that were not or not pulsed with MART1-26–35 peptide. [3]Cr release in the 4-h culture supernatants was counted in a Topcount (Packard). Percentage lysis was calculated as follows: [experimental release − spontaneous release]/(maximum release − spontaneous release) × 100.

**Real-time RT-PCR**

Total RNA was extracted using the Nucleic Acid Purification lysis solution and the semi-automated ABI Prism 6100 Nucleic Acid PrepStation system (Applied Biosystems). Total RNA was treated with DNase before RT with FlowJo (Tree Star).
TGTG-3'), B cell lymphoma 2 protein (BCL2; 5'-CTGCACCTGAGG-3') and a: 5'-ACAGCAGAGGAAATCAAACAG-3'), telo-merase reverse transcriptase (TERT: 5'-CTCAGAGGACCAACAGA-3' and a: 5'-TACACCTCATCAGGCG-3'), and hypoxanthine phospho-ribosyltransferase 1 (HPRT1; 5'-GATGATGAGCTAGGTTAGGAC-3' and a: 5'-CTATCATTCTCTTTAATCGTC-3'). Results were normalized to HPRT1. The amount of each transcript was expressed by the formula: 2^-ΔΔCt (where ΔΔCt = Ct(transcript) - Ct(control)).

**Mouse experiments**

C57BL/6 mice were from Harlan (Barcelona, Spain), OT-1 TCR transgenic mice (C57BL/6-Tg [Tcra/Tcrb] 1100 Mjb/J) and Ly5.1 (CD45.1) transgenic mice (C57BL/6-Tg [Ly5.1]) were obtained from Jackson Laboratory (Bar Harbor, ME). IL-15-/- mice (C57BL6/6Tc78-/-IL15tm1Imx N5) were from Taconic (Laven, Denmark). IL-7 (IL-7Rα-/-mice) were coated with DimerX H-2Kb:Ig (2.5 μg/10^7 beads; R&D Systems) fusion proteins. Nonadherence of beads was examined throughout the culture on cells that had undergone division (PKH26low: primed daughter cells). To determine whether IFN-α prompts the differentiation of human naive CD8 T cells into fully differentiated effector or memory CTLs, FACS-sorted human naive CD8 T cells (CD45RA+CD27-CD28-CD62L-CCR7-) were examined throughout the culture on cells that had undergone division (PKH26low; primed daughter cells). Except for CXCR3, whose expression was upregulated by CD3/CD28 triggering, the level of expression of the other markers remained similar to those on the starting naive population (Fig. 1, Supp. Fig. 1B). IFN-α hardly altered the expression pattern of these molecules. However, beads+IFN-α+primed daughter cells showed a tendency to lower levels of CD45RA molecule and higher levels of expression for CXCR3, CD62L, and CCR7 that were counterparts primed only with beads (Fig. 1, Supp. Fig. 1B). Similar tendencies were observed when naive CD8 T cells were cocultured with total CD4 T cells, either in the presence or in the absence of IFN-α (data not shown).

To assess in depth the state of differentiation of beads+IFN-α+primed cells, we measured the expression of two terminal differentiation markers, KLRG1 and CD57, both expressed in memory and effector CTLs, but not in naive CD8 T cells (Fig. 1C). As depicted in Fig. 1, both markers were undetectable or expressed at very low level in daughter cells resulting from the CD3/CD28 triggering (Fig. 1C), and IFN-α did not alter the expression of these molecules. Coculture with total CD4 T cells did not alter the expression of these markers either (data not shown). The low expression of KLRG1 was also confirmed by RT-PCR (Fig. 1D). The preservation of daughter cells at a minimal state of differentiation was further confirmed by measuring the expression of genes coding for EOMES, BCL2, and TERT. All these molecules are closely related to the differentiation state of CD8 T cells. As depicted in Fig. 1D, beads ± IFN-α+primed daughter cells exhibited an expression pattern of EOMES, BCL2, and TERT genes more similar to that showed by naive CD8 T cells than by effector or memory CTLs. Treatment with IFN-α alone (in the absence of beads) did not alter the expression of any of these markers (data not shown). Similar results were found for IFN-α in and IFN-α- (Fig. 1, Supp. Fig. 1, and data not shown). In summary, even though IFN-α promotes the expansion and acquisition of effector functions, human naive CD8 T cells primed in the presence of IFN-α remain at an early state of differentiation.

**Results**

**IFN-α favors the expansion and acquisition of effector functions of CD8 T cells whereas precluding terminal differentiation**

We have proved that IFN-α supports human naive CD8 T cell proliferation and survival, whereas favoring the acquisition of effector functions (26). Data in mice support that an adequate inflammatory response may bypass the requirement for CD4 T cell help in the induction of a productive CTL response (13–18, 30). CD4 T cells can provide help to CD8 T cells through interplays between CD4 T cells and DC and CD4 and CD8 T cells. This takes place via cell-to-cell interactions, mediated by CD40/ CD40L (5, 7), and by means of paracrine cytokines, such as IL-2 and IL-21 (6). IFNs-I may bypass the requirement of CD4 T cell help for the induction of a CTL response by delivering maturation signals to DC, influencing the degree of Ag presentation and co-stimulation (31). Working with highly purified total CD4 and naive CD8 T cells, we show that, in concert with CD3/CD28 stimulation, direct IFN-α signaling on human naive CD8 T cells bypasses, to some extent, the direct CD4 T cell help to CD8 T cells for proliferation and expression of effector molecules (IFN-γ and granzyme B; Supplemental Fig. 1A).

To determine whether IFN-α prompts the differentiation of human naive CD8 T cells into fully differentiated effector or memory CTLs, FACS-sorted human naive CD8 T cells (CD45RA+ CD27low) were labeled with a fluorescent dye (PKH26) and stimulated with anti-human CD3 and CD28 Ab-coated beads (henceforth beads) either alone or together with IFN-α (IFN-α2b or IFN-α; henceforth beads-primed or beads+IFN-α+primed cells, respectively; Fig. 1A). The expression of different phenotypic surface markers dictates the activation/differentiation status of CD8 T cells (CD45RA, CXCR3, CD27, CD28, CD62L, and CCR7) was examined throughout the culture on cells that had undergone division (PKH26low; primed daughter cells). Except for CXCR3, whose expression was upregulated by CD3/CD28 triggering, the level of expression of the other markers remained similar to those on the starting naive population (Fig. 1, Supp. Fig. 1B). IFN-α hardly altered the expression pattern of these molecules. However, beads+IFN-α+primed daughter cells showed a tendency to lower levels of CD45RA molecule and higher levels of expression for CXCR3, CD62L, and CCR7 that were counterparts primed only with beads (Fig. 1, Supp. Fig. 1B). Similar tendencies were observed when naive CD8 T cells were cocultured with total CD4 T cells, either in the presence or in the absence of IFN-α (data not shown).

To assess in depth the state of differentiation of beads+IFN-α+primed cells, we measured the expression of two terminal differentiation markers, KLRG1 and CD57, both expressed in memory and effector CTLs, but not in naive CD8 T cells (Fig. 1C). As depicted in Fig. 1, both markers were undetectable or expressed at very low level in daughter cells resulting from the CD3/CD28 triggering (Fig. 1C), and IFN-α did not alter the expression of these molecules. Coculture with total CD4 T cells did not alter the expression of these markers either (data not shown). The low expression of KLRG1 was also confirmed by RT-PCR (Fig. 1D). The preservation of daughter cells at a minimal state of differentiation was further confirmed by measuring the expression of genes coding for EOMES, BCL2, and TERT. All these molecules are closely related to the differentiation state of CD8 T cells. As depicted in Fig. 1D, beads ± IFN-α+primed daughter cells exhibited an expression pattern of EOMES, BCL2, and TERT genes more similar to that showed by naive CD8 T cells than by effector or memory CTLs. Treatment with IFN-α alone (in the absence of beads) did not alter the expression of any of these markers (data not shown). Similar results were found for IFN-α2b and IFN-α- (Fig. 1, Supp. Fig. 1, and data not shown). In summary, even though IFN-α promotes the expansion and acquisition of effector functions, human naive CD8 T cells primed in the presence of IFN-α remain at an early state of differentiation.

**Cells primed in the presence of IFN-α are more responsive to homeostatic cytokines**

To investigate the ability of expanded CD8 T cells to respond to homeostatic stimuli, we first analyzed the expression of the high-
FIGURE 1. Human naive CD8 T cells primed in the presence of IFN-α remain at an early state of differentiation. (A) Total human CD8 T cells were FACs-sorted into CD45RA−CD27high (naive), CD45RA+/−CD27− (effector), and CD45RA−CD27+ (memory) cells. Naive cells were labeled with PKH26 and stimulated with beads either alone or together with IFN-α2b. At day 6, those cells that had undergone division (PKH26low cells, namely, primed daughter cells) were analyzed by flow cytometry (B, C) or were FACs-sorted to subsequently extract their RNA to be analyzed by RT-PCR (D). (B and C) Surface expression of CD45RA, CXCR3, CD27, CD62L, CCR7 (B), CD57, and KLRG1 (C) at day 6 of culture on cells that had undergone division (primed daughter cells) in comparison with that exhibited by the starting naive population (B, C) and by memory and effector peripheral CTLs from the same subject (C). (C) Quadrants were set up using appropriate isotype control mAbs. (D) Expression of KLRG1, EOMES, BCL2, and TERT genes analyzed by quantitative RT-PCR in FACs-sorted daughter CD8 T cells and in the naive, effector, and memory CD8 T cell populations from the same subject. (A–C) Cells gated on live total CD8 T cells (A) or PKH26+ cells (B, C). One donor representative of at least three identically tested. APC, Allophycocyanin; B, beads; N.D., nondetected.
affinity receptor chains for IL-7 and IL-15, which are considered the two most important homeostatic cytokines for CD8 T cells. As depicted in Fig. 2, the expression of IL-7Rα in cells undergoing division dropped during the first 4 d of culture and subsequently rose again (Fig. 2A, 2B), with the IL-7Rα expression at days 6 and 8 of culture being brighter on beads+IFN-α–primed cells (Fig. 2B). In a previous study, we showed that IFN-α–derived signals upregulated the expression of IL-15RA transcript in human naive CD8 T cells (26). This was confirmed when we measured the surface expression of IL-15RA (Fig. 2A, 2B), which peaked at day 4 of culture (Fig. 2B).

Next, we studied the capacity of CD8 T cells developed in the presence or absence of IFN-α to proliferate in response to homeostatic cytokines. CFSE-labeled human naive CD8 T cells were stimulated with beads either alone or together with IFN-α. On day 6 of culture, daughter cells (CFSE<sub>low</sub> cells) were FAC-sorted and stimulated for another 4 d with IL-15 or IL-7 (Supplemental Fig. 2A). Sorting out the CFSE<sub>low</sub> cells ensured that these cells are those that have been activated and differentiated under the stimuli applied during the primary culture. We found that beads+IFN-α–primed daughter cells proliferated more efficiently in response to IL-15 and, to a much lesser extent, to IL-7 than their counterparts developed only with beads (Fig. 2C). Beads+IFN-α–primed daughter cells also showed an increased responsiveness to IL-2 (Supplemental Fig. 2B), probably because of the enhanced expression of CD25 as induced by IFN-α (26). Strikingly, IL-21 per se did not promote proliferation of primed cells but enhanced proliferation prompted by IL-15 and IL-7 (Supplemental Fig. 2B). Similar results were found for IFN-α<sub>2b</sub> and IFN-α<sub>4</sub> (Fig. 2, Supplemental Fig. 2).

IFN-α–derived signals program human naive CD8 T cells to become highly responsive to secondary Ag stimulation

Freshly isolated daughter (CFSE<sub>low</sub>) cells generated in the presence of beads alone or together with IFN-α were further stimulated for another 4 d with beads to investigate the ability of CD8 T cells primed in our experimental conditions to proliferate in response to an early restimulation with Ag (Supplemental Fig. 2A). Strikingly, only beads+IFN-α–primed daughter cells were able to proliferate after an early-onset second re-encounter with Ag (mimicked by beads) (Fig. 2D).

Because this enhanced proliferation in response to an early secondary antigenic stimulation might be influenced by the recent triggering of the IFN-αR, we decided to culture sorted daughter cells in the presence of IL-15 for several days before the Ag re-encounter (Fig. 3A). After a 15-d resting culture with IL-15, the expression of CD62L, CCR7, and IL-7Rα was similar in cells derived from beads-primed and beads+IFN-α–primed daughter cells (Supplemental Fig. 2C). After this period of IL-15–driven survival/proliferation, cells were deprived of IL-15 for 36 h, labeled again with CFSE, and stimulated in the presence or absence of beads in IL-2–conditioned medium (Fig. 3A). IL-2 supplementation was absolutely necessary for proliferation and survival of cells at this phase (data not shown). On re-encounter with the Ag, those cells that were initially primed with beads+IFN-α downregulated more intensely CD62L, CCR7, and IL-7Rα than their counterparts developed only with beads (Supplemental Fig. 2D), suggesting an enhanced capacity to differentiate to effector cells.

During the recall response, cells derived from beads+IFN-α–primed cells proliferated more efficiently than those derived from beads-primed cells (Fig. 3B), and importantly, cells resulting from the secondary stimulation of those cells initially primed with beads+IFN-α expressed higher levels of IFN-γ and granzyme B.
than their counterparts derived from cells initially primed only with beads (Fig. 3C). It is important to consider that no other stimuli (most commonly PMA/ionomycin) were used to facilitate the detection of intracellular IFN-γ. Therefore, exposure to IFN-α during priming of human naive CD8 T cells imprints some sort of information that results not only in enhanced readiness to proliferate during recall response but also in an enhanced capacity to differentiate to effector cells on Ag re-encounter. No differences were found between IFN-α2b and IFN-α5 subtypes in these effects (Fig. 3, Supplemental Fig. 2).

**FIGURE 3.** IFN-α–derived signals program human naive CD8 T cells to become highly responsive to second Ag stimulation. (A) Schematic representation of the experiment. CFSE-labeled purified human naive CD8 T cells were stimulated with beads either alone (beads-primed cells) or together with IFN-α (IFN-α2b or IFN-α5; beads+IFN-α–primed cells). At day 6, CFSElow cells (daughters) were sorted and cultured with IL-15 for 15 d. Then cells were deprived of IL-15 (36 h), labeled again with CFSE, and stimulated (4 d) in the presence or absence of beads in IL-2–conditioned medium (beads or medium, respectively). (B) Extent of proliferation upon re-encounter with the Ag monitored by CFSE dilution. Percentages of divided cells are indicated. Tinted histograms correspond to cell cultures with medium alone. Striped dot-headed segments indicate daughter cells resulting from the division of the original beads- or beads+IFN-α–primed population during the secondary stimulation. (C) Histograms show MIF of IFN-γ and granzyme B immunostaining on daughter cells from the second stimulation, as indicated in (B). To detect intracellular IFN-γ, we cultured cells in the presence of brefeldin A for the last 6 h of culture. Dashed lines correspond to cells stained with isotype-matched control mAb. (B and C) One donor representative of five.

**IFN-α–induced long-term programming of mouse CD8 T cells to strongly respond to in vivo Ag rechallenge**

To ascertain whether IFN-α–primed CD8 T cells maintained the ability to mount a strong secondary response after a long period of naturally driven homeostasis, we moved to a mouse model using OT-1 TCR transgenic cells stimulated in vitro (3-d culture) with beads coated with OVA257–264 peptide-loaded H2-Kb and B7-1 molecules (24) (from here on referred to as beads), together with recombinant mouse IFN-α4-containing supernatant from NSO IFN-α4 gene-transfected cells or control supernatant from
identically treated untransfected cells. In this mouse setting, we confirmed (Supplemental Fig. 3A) that during the primary response, IFN-α promotes the expression of effector molecules (IFN-γ and granzyme B; as previously reported in Ref. 24). Similar results were found when instead of IFN-α-containing supernatant, we used recombinant mouse IFN-α from different subtypes (Supplemental Fig. 3A). Control supernatant did not modify the stimulatory properties of beads alone, as is shown in Supplemental Fig. 3A.

OT-1 cells primed in the presence of IFN-α-containing supernatant (henceforth beads+IFN-α–primed cells) also showed higher expression of surface markers, such as CD44, CD69, ly6C, CD25, IL-15Rα, and IL-7Rα, than their counterparts developed with beads plus control supernatant (henceforth beads-primed cells; Supplemental Fig. 3B). However, regardless of the enhanced activation, IFN-α–primed cells maintained high levels of CD62L expression and did not express KLRG1 (Supplemental Fig. 3B). This expression pattern of CD62L and KLRG1 exhibited by IFN-α–primed OT-1 cells was maintained even when the culture was prolonged up to day 6 (data not shown).

Beads+IFN-α–primed OT-1 cells also responded more efficiently to IL-15, IL-7, and IL-2 stimulation than beads-primed cells (Supplemental Fig. 3C, 3D). Unlike what happened in humans, IL-21 induced the proliferation of expanded cells, mainly in the case of beads+IFN-α–primed OT-1 cells, although to a much lesser extent than IL-15, IL-7, and IL-2 did (Supplemental Fig. 3C, 3D). IL-21 enhanced the proliferation induced by IL-7 (Supplemental Fig. 3C, 3D), in a similar way to what happened with human CD8 T cells.

To test the response of in vitro expanded cells on adoptive transfer, daughter cells derived from OT-1 cells primed in vitro with beads or beads+IFN-α were transferred into recipient congenic mice, and the percentage of OT-1 cells in PBL was monitored thereafter (Fig. 4A). Around days 2–4 posttransfer, a sudden increase in the percentage of OT-1 cells was observed in mice receiving in vitro primed OT-1 cells, but not in mice receiving naive OT-1 cells (Fig. 4B). The numeric increase was more pronounced in those mice receiving beads+IFN-α–primed daughter cells (Fig. 4B). The peak coincided with a strong in vivo BrdU incorporation into OT-1 cells (data not shown). Importantly, cell expansion after transfer was dependent on IL-7, because the increase in the cell number was much less pronounced when recipient mice were deficient for IL-7 (Fig. 5). The initial expansion was followed by a decline phase (Fig. 4B), with the attrition rate being sharper in IL-15–/– recipient mice (Fig. 5). No transferred cells could be detected beyond day 10.

**FIGURE 4.** IFN-α–induced long-term programming of mouse CD8 T cells to highly respond to in vivo Ag rechallenge. (A) Schematic representation of the experimental settings. CFSE-labeled purified OT-1 transgenic CD8 T cells were cultured with OVA peptide-loaded H-2Kb/B7-1–coated beads together with recombinant mouse IFN-α–containing supernatant (beads+IFN-α) or control supernatant (beads). At day 3, CFSElow cells were sorted and transferred to Ly5.1 congenic mice. As a control, a group of mice received naive OT-1 cells (B). At day 90 (C, D) posttransfer, recipient mice received a single i.v. injection of control or OVA peptide. (B and C) Percentage of OT-1 cells in PBL of Ly5.1 recipient mice upon adoptive transfer (B) or upon i.v. rechallenge with peptide (C). Numbers reflect the mean ± SEM (B, n = 9; C, n = 5). (D) IFN-γ and granzyme B expression in OT-1 cells upon in vivo challenge. Ten hours 30 min after peptide boosting, mice received brefeldin A i.p. Ninety minutes later, splenocytes were harvested, and expression of IFN-γ and granzyme B was determined on gated OT-1 cells by flow cytometry. Numbers reflect the MFI ± SEM (n = 4). Dashed lines correspond to cells from OVA peptide-boosted mice stained with isotype-matched control mAb. (B–D) Transferred OT-1 cells were from OT-1 mice (CD45.2+) and were identified by staining with anti-CD45.2 mAb. One experiment representative of three independent experiments is shown. B, Beads.
To assess the functionality of OT-1 cells on in vivo Ag rechallenge, 10 h after boost, we injected brefeldin A i.v. into recipient mice and 90 min later, splenocytes were stained for granzyme B and IFN-γ.

FIGURE 5. Enhanced responsiveness to IL-15 and IL-7 extends the in vivo persistence of IFN-α–primed OT-1 cells. CFSE-labeled, purified OT-1 transgenic CD8 T cells were cultured as in Fig. 4. At day 3, CFSE<sup>+</sup> cells were sorted and transferred to WT, IL-15<sup>−/−</sup>, or IL-7<sup>−/−</sup> recipient mice. Transferred OT-1 cells were from OT-1xCD45.1 mice (CD45.1<sup>+</sup>), and all congenic recipient mice were CD45.2<sup>+</sup>. At day 52 posttransfer, recipient mice received a single i.v. injection of OVA peptide. Panels show absolute numbers of OT-1 cells per milliliter of blood upon adoptive transfer of OT-1 cells into WT, IL-15<sup>−/−</sup>, and IL-7<sup>−/−</sup> mice (left y-axis), and upon i.v. rechallenge with peptide (right y-axis). Transferred cells were beads-primed (upper panel) or beads+IFN-α–primed (lower panel) OT-1 cells. Arrows under the left and right x-axes indicate the time point when OT-1 cells and OVA peptide were injected, respectively. Numbers reflect the mean ± SEM (n = 7). Transferred OT-1 cells were identified by staining with anti-CD45.1 mAb. One experiment representative of two independent experiments is shown.

FIGURE 6. Exposure to IFN-α during in vitro priming of naive HLA-A2* CD8 T cells with autologous FASTDCs loaded with MART1<sub>26–35</sub> peptide rendered highly functional specific CD8 T cells. HLA-A2<sup>+</sup> naive CD8 T cells were cocultured with autologous irradiated-FASTDCs pre-loaded with MART1<sub>26–35</sub> peptide in the absence or in the presence of IFN-α (<sup>−</sup>IFN-α or +IFN-α, respectively), as indicated in Materials and Methods, and at day 10, cells were harvested for analysis. (A and B) Percentage of HLA-A2/MART1<sub>26–35</sub>–pentamer<sup>+</sup> cells among CD8 T cells in freshly purified cells (basal values) and at day 10 of culture with or without IFN-α. (C) Fold cell expansion (day 10) in terms of absolute numbers of MART1<sub>26–35</sub>–specific CD8 T cells detected by specific pentamer immunostaining. (D) Homeostatic proliferation, measured by CFSE dilution, of MART1<sub>26–35</sub>–specific CD8 T cells in response to IL-15 and IL-7 stimulation. At day 10, total cells were labeled with CFSE and cultured for additional days with medium or medium containing IL-15 or IL-7. The code for each cytokine is indicated only in the upper panel. (E and F) Granzyme B expression in MART1<sub>26–35</sub>–specific CD8 T cells (day 10). (F) ∆MIF = MIF values of cells stained with anti-granzyme B mAb subtracted from those stained with isotype-matched control mAb. Cells are gated on CD8 T cells (A, B) or HLA-A2/MART1<sub>26–35</sub>–pentamer<sup>+</sup> cells (D–F). Each line represents an anonymous individual donor (D represents donor). Numbers in italics indicate p value (Wilcoxon matched-pairs signed-rank test). (A and E) D8, who is representative of 10 donors. (D) D11, who is representative of two tested.
Strikingly, OT-1 cells that had been originally primed with beads+IFN-α showed much more expression of granzyme B and IFN-γ (Fig. 4D). Altogether, these results clearly show that IFN-α exposure during priming of naive CD8 T cells enhances their responsiveness to homeostatic cytokines, favoring the long-term persistence in adoptive transfer settings, and improves the performance of primed cells in the recall response.

**Exposure to IFN-α during in vitro priming of human naive CD8 T cells rendered highly functional tumor-specific CD8 T cells against a shared melanoma Ag**

There is increasing evidence that adoptive T cell therapy might be enhanced by the infusion of less-differentiated CD8 T cells that possess greater proliferative potential than differentiated CTLs (33, 34). The fact that the direct IFN-α signaling on naive CD8 T cells renders cells with an enhanced responsiveness to homeostatic cytokines and secondary Ag stimulation, without leading them to a terminal differentiation state, suggests that the use of IFN-α during the in vitro stimulation of naive CD8 T cells may potentially improve their efficiency in adoptive T cell therapy of tumors.

Moreover, we examined the effects of including IFN-α in a prototype in vitro method to generate CTLs for immunotherapy from the naive T cell repertoire (28). Following the scheme depicted in Supplemental Fig. 4A, MART126–35–specific CD8 T cells were generated from healthy HLA-A2* donors. As depicted in Fig. 6, exposure to IFN-α during the in vitro stimulation of naive CD8 T cells not only increased the percentage of MART126–35–specific CD8 T cells in the culture (Fig. 6A, 6B), but also promoted their expansion (Fig. 6C). Strikingly, priming in the presence of IFN-α remarkably enhanced the ability of MART126–35–specific CD8 T cells to proliferate on stimulation with IL-15 and IL-7 (Fig. 6D), and the expression of granzyme B (Fig. 6E, 6F). Moreover, MART126–35–specific CD8 T cells stimulated in the presence of IFN-α exhibited a superior capacity to produce IFN-γ on recognition of HLA-A2* target cells loaded with cognate peptide (Supplemental Fig. 4B). Regardless of the intense expansion and the enhanced effector functions, MART126–35–specific CD8 T cells derived from naive cells in the presence of IFN-α maintained high levels of expression of CD27, CD62L, and CCR7, slightly downregulated CD45RA, and did not express KLRG1, resembling very closely the starting naive population (Supplemental Fig. 4C).

Interestingly, CD8 T cells derived in the presence of IFN-α killed HLA-A2* melanoma cell lines loaded with MART126–35–peptide more efficiently than their counterparts derived in the absence of IFN-α (Supplemental Fig. 4D). Most importantly, IFN-α–primed CD8 T cells exhibited an enhanced capacity to kill a HLA-A2* melanoma cell line naturally expressing MART1 Ag (Me275; Fig. 7A, 7B, Supplemental Fig. 4E). This lysis was Ag dependent because expanded cells were completely unable to kill a HLA-A2* melanoma cell line that does not express MART1 Ag (Na8; Fig. 7A, 7B, Supplemental Fig. 4E).

However, the percentage of lysis against Me275 exhibited by the bulk culture after the first round of cell expansion (Supplemental Fig. 4A) did not reach meaningful values (Fig. 7A, 7B, Supplemental Fig. 4E). In the processes of in vitro expansion of specific CD8 T cells to be used in adoptive therapy, cultured cells frequently undergo several rounds of expansion. To assess whether a second round of expansion (in other words, a secondary Ag stimulation) would enhance the cytotoxic activity of expanded cells, 10 d after the first stimulation, we added irradiated autologous CD8-depleted PBL pulsed with MART126–35–peptide to the cultures together with IL-2, and cells were analyzed 5 d later (Supplemental Fig. 4F). As control, cells were also cocultured with nonpeptide-loaded PBL in the presence of IL-2. As depicted in Supplemental Fig. 4G, the percentage of MART126–35–specific CD8 T cells decreased when cells were restimulated with nonpeptide-loaded PBL+IL-2, although the drop was less pronounced if the cells had been initially primed with IFN-α. Restimulation with peptide-loaded PBL+IL-2 efficiently expanded MART126–35–specific CD8 T cells, and the percentage of this population was higher when cells had been originally primed with IFN-α (Supplemental Fig. 4G). Expression of granzyme B also increased on the second round of expansion, with the expression levels being higher in IFN-α–primed CD8 T cells (Supplemental Fig. 4H). More remarkably, the second expansion round enhanced the capacity of expanded cells to specifically kill naturally expressing MART1 melanoma cells. This increase in killing was much more
noticeable when CD8 T cells had been initially primed in the presence of IFN-α (Fig. 7C, 7D). The improved ability exhibited by MART1-specific CD8 T cells after the second expansion to kill naturally expressing MART1 melanoma cells is likely due to the increase in the number of specific T cells and/or to the selection of TCRs with enhanced affinity/avidity for the cognate peptide. Further experiments are necessary to elucidate this. Almost identical effects were found with both IFN-α25 and IFN-α5 (data not shown).

Adoptively transferred tumor-specific CD8 T cells generated ex vivo with IFN-α exhibited a superior capacity to contain tumor progression in vivo

To assess the immunotherapeutic profile of CD8 T cells primed in vitro with IFN-α, we moved to a mouse model of established melanoma. Beads- and Beads+IFN-α–primed daughter OT-1 cells generated in vitro as described earlier were transferred at two different doses (5 × 10^5 and 2 × 10^6) into BL6 mice bearing established B16OVA tumors. As depicted in Fig. 8, infusion of 2 × 10^5 beads+IFN-α–primed CD8 T cells significantly delayed tumor growth and prolonged survival of recipient mice, whereas CD8 T cells primed without IFN-α exerted a much weaker therapeutic effect. Of note, the tumor growth and survival curves of recipient mice that received 5 × 10^5 beads + IFN-α–primed CD8 T cells were similar to those of the group receiving the highest doses of beads-primed cells. Interestingly, the beneficial effect of the adoptive transfer of the CD8 T cells primed in vitro with IFN-α was observed without any other concomitant treatment, such as therapeutic vaccination, exogenous administration of cytokines, or chemotherapy or radiotherapy.

Discussion

This study offers an outline of the effects of IFN-α on the shaping of human CD8 T cell response. Indeed, direct IFN-α effects on human naive CD8 T cells during the priming phase promote their expansion and acquisition of effector functions (26) and, more importantly, confer on the ensuing CD8 T cell progeny an enhanced capacity to respond to homeostatic cytokines and to secondary Ag stimulation (this study). Significantly, this is also true when an Ag-specific CD8 T cell response is generated in vitro from the repertoire of human naive CD8 T cells. The enhanced responsiveness to IL-15 and IL-7 allows CD8 T cells primed in the presence of IFN-α to extend their persistence upon adoptive transfer, as we show in a parallel mouse model of adoptive transfer. Interestingly, such adoptively transferred IFN-α–primed CD8 T cells are able to undergo a robust recall response even after a long period (90 d) of naturally driven homeostatic maintenance in vivo. Furthermore, CD8 T cells primed in the presence of IFN-α exhibit an improved performance in adoptive immunotherapy of established B16OVA melanoma. Therefore, IFN-α direct effects on naive CD8 T cells hold promise to enhance the efficacy of adoptive T cell therapy.

Interestingly, although the CD8 T cell progeny derived from naive CD8 T cells primed in the presence of IFN-α exhibits many functional properties of memory CTLs (enhanced ability to persist and to mount a robust recall response), it also displays phenotypic features classically astride naive and TCM CD8 T cells. However, the lower KLRG1 and CD57 expression exhibited by IFN-α–primed CD8 T cells reveals their less differentiated state, in comparison with TCM CTLs (35). CD8 T cells primed in the presence of IFN-α also express lower levels of the transcript coding Eomes, a T-box transcription factor that promotes full effector functions in CD8 T cells (36). The low differentiation profile of IFN-α–primed CD8 T cells is also supported by their high expression levels of Bcl2 and telomerase encoding genes, both involved in preventing apoptosis and in maintaining the replicative potential of naive CD8 T cells (37, 38). Nicholas Restifo’s group has coined the term “memory T cells with stemlike properties” for a similar lymphocyte state observed in humans (39).

It has been described that naive CD8 T cells have a natural resistance to reach terminal differentiation when they are primed in vitro (34). It is possible that the conditions necessary to differentiate effector or memory CTLs from the naive repertoire were difficult to recreate in vitro, and that IFN-α may also support the differentiation of human naive CD8 T cells into effector and/or memory CTLs in in vivo settings, as seems to happen in mice (22). However, the interesting point is that, in concert with TCR/CD28 stimulation, direct IFN-α signaling during the priming of naive CD8 T cells manages to stamp important features for the performance of the ensuing CD8 T cells, without leading them to a terminal state of differentiation. Retrospective analysis of clinical trials has revealed that T cells associated with clinical efficacy possess higher levels of CD27 and longer telomeres (40–42). Furthermore, studies in mouse models have revealed that optimal T cells for adoptive therapy exhibit low levels of KLRG1 and, unexpectedly, low levels of EOMES, diminished IFN-γ production, and minimal specific cytolysis (43–45). The superior anti-tumor function of less differentiated cells is attributed to their
greater proliferative potential (43–46). Consistently, in adoptive tumor immunotherapy, self-renewable TCM CTLs exhibit a performance superior to terminally differentiated effector CTLs (33) and, in the same way, adoptively transferred activated CD8 T cells derived from naive rather than from TCM CD8 T cells mediate superior antitumor immunity (43). The fact that human naive CD8 T cells primed in the presence of IFN-α exhibit important functional features of memory CTLs (enhanced ability to persist and to mount a robust recall response) whereas preserving a low differentiation profile, as that shown by T cells associated with clinical efficacy, strongly suggests that the exposure to IFN-α during the in vitro priming of naive CD8 T cells may be beneficial for adoptive T cell therapy.

How the features conferred during the priming phase are remembered by the ensuing CD8 T cell progeny is an open question that suggests epigenetic changes. Chromatin remodeling has been demonstrated for several genes expressed during CD8 T cell activation (47–49). In fact, memory CTLs exhibit increased histone acetylation levels for genes, such as EOMES, IFNG, PRF1, and GZMB, which are expressed more rapidly in memory than in naive CD8 T cells after activation (50–53). In vitro experiments with murine CD8 T cells have revealed that IFN-α/β, as well as IL-12, promote chromatin remodeling of critical genes such as GZMB and EOMES (49). Although experimental evidence in human T cells is still missing, a similar effect of IFN-I in chromatin remodeling might explain the readiness of IFN-α–primed human CD8 T cells to respond to a secondary stimulation.

Another feature imprinted by IFN-α on CD8 T cells that is beneficial for adoptive therapy is the heightened responsiveness to IL-15 and IL-7. Our data in the mouse model show that the promptness to respond to IL-15 and IL-7 mainly occurs on IFN-α–primed CD8 T cells (26). Our results suggest the promotion of IL-2 in CD8 T cells (26), IFN-α might shape the program of the primed CD8 T cells, at least in part, by the autocrine effect of IL-2. Interestingly, IL-21 acts as a cofactor for the homeostatic proliferation induced by IL-15 and IL-7 mainly on CD8 T cells primed in the presence of IFN-α. Our data suggest that transfer-based immunotherapy with IFN-α–primed cells may benefit from the subsequent administration of cytokines, such as IL-2, IL-15, and IL-21, which either alone or in combination promote the antitumor functions of infused CD8 T cells (54, 55).

Our results with MART1-specific CD8 T cells generated in vitro from the repertoire of human naive CD8 T cells indicate that exposure to IFN-I during the first Ag stimulation makes the most of these cells, which after a second Ag stimulation mediate important levels of cytotoxicity against a melanoma cell line naturally expressing the cognate Ag.

In addition, our data in vivo with the B16OVA model offer a proof of concept for the more efficacious antitumor performance of CD8 T cells generated ex vivo in the presence of IFN-α. Several reasons may account for such superiority: 1) low-differentiation state with naive-like features of the transferred CD8 T cells, which may favor their replication on infusion; 2) heightened capacity to mount a recall response; 3) enhanced responsiveness to IL-15 and IL-7, which extends the in vivo persistence; and 4) readiness to respond to IL-2 and IL-21, which may enable transferred cells to receive help from CD4 T cells (6). Even though in our experimental approach the adoptive T cell transfer per se only delayed tumor growth, we believe that IFN-α–primed CD8 T cells, in combination with other supportive treatments (e.g., therapeutic vaccination and/or administration of cytokines together with non-myeloablative chemotherapy or irradiation), would lead to an enhanced therapeutic effect. Ongoing experimentation will elucidate this hypothesis.

Interestingly, the effects of human IFN-α2b and IFN-α5 subtypes on human CD8 T cells are identical. This is true even when they were compared at multiple concentrations (26). In addition, the effects on mouse CD8 T cells of the different sources and subtypes of murine IFN-α tested are also quite similar. Although more detailed comparative experiments would be necessary, our data strongly suggest that in terms of CD8 T cell activation, there is a clear redundancy in the IFN-α system.

All in all, our human in vitro and murine in vivo studies demonstrate that IFN-α can be used to in vitro program naive CD8 T cells into a state with excellent potential for adoptive T cell immunotherapy. The availability of GMP-grade IFN-α facilitates the translation of our findings to the clinic. Translational possibilities are envisioned not only for tumor therapy, but also for the treatment of chronic or latent viral diseases. It remains to be seen whether these effects of IFNs-I can be exploited in vivo to formulate adjuvants that improve CD8 T cell priming after vaccination.

Acknowledgments
We are grateful to the staff of the Blood Bank Service of the Hospital of Navarra (Spain), to Dr. Romero (University of Lausanne) for kindly providing Mc275 and Na8 melanoma cell lines, and to Dr. Vieira (Institute Pasteur) for generously providing IL-7+/- mice. We also thank E. Ciordia and E. Elizalde for animal care husbandry and Dr. P. Miller for English-language editing. We acknowledge Drs. Juan Ruiz and Pablo Ortiz from Digna-Biotech for continuous support.

Disclosures
The authors have received grant support and reagents from Digna-Biotech (Madrid, Spain). I.G. and U.M. are full-time employees of Digna-Biotech.

References


