Reciprocal responsiveness to IL-12 and IFN-α specifies human CD8+ effector versus central memory T cell fates

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ABSTRACT

Multiple innate signals regulate the genesis of effector and memory CD8+ T cells. In this study, we demonstrate that the innate cytokines IL-12 and IFN-α/β regulate distinct aspects of effector and memory human CD8+ T cell differentiation. IL-12 exclusively promoted the development of IFN-γ- and TNF-α-secreting effector memory TEM cells, whereas, IFN-α drove the development of central memory TCM cells. The development of TEM and TCM was linked to cell division. In rapidly dividing cells, IL-12 programmed TEM through induction of the IL-12 receptor β2 (IL-12Rβ2). In contrast, IFN-α regulated TCM development by slowing the progression of cell division in a subpopulation of cells that selectively expressed elevated IFN-α/β receptor-2 (IFNAR2). The strength of signal delivered through TCR engagement regulated the responsiveness of cells to IL-12 and IFN-α. In the presence of both IL-12 and IFN-α, these cytokine signals were amplified as the strength of the TCR signal was increased, promoting the simultaneous development of both TCM and TEM. Together, our results support a novel model in which IL-12 and IFN-α act in a non-redundant manner to regulate the co-linear generation of both effector and memory cells.
INTRODUCTION

CD8+ T cells are critical mediators of adaptive inflammatory responses to intracellular pathogens. They require a series of signals for efficient expansion and acquisition of effector functions such as cytokine secretion and lytic activity. These signals are delivered by professional antigen presenting cells (APC) and include antigen recognition (“signal 1”), co-stimulatory activation (“signal 2”), and signaling provided by innate inflammatory cytokines (“signal 3”). While signals 1 and 2 prime naïve CD8+ T cells and initiate cell division, signal 3 cytokines program effector functions and ensure clonal survival. A variety of cytokines have the potential to act as signal 3 in CD4+ and CD8+ T cells, and IL-12 and IFN-α/β, in particular, promote efficient induction of innate immunity as well as the development of adaptive type 1 responses to intracellular infection. Thus, IL-12 and IFN-α/β may represent the predominant signal 3 during intracellular infection.

While IL-12 regulates Th1 development in CD4+ T cells, early reports suggested that the induction of IFN-γ secretion and lytic activity in CD8+ T cells was independent of IL-12, STAT4, and T-bet. However, recent studies by Mescher and colleagues found that in vitro priming with IL-12 induced high and sustained secretion of IFN-γ and markedly enhanced lytic activity in murine CD8+ T cells. Further, these effects were dependent on STAT4, indicating that IL-12 signaling provides a necessary third signal for the regulation of CD8+ T cell development. More recent studies have indicated that IFN-α/β can act in a manner similar to IL-12 to provide signal 3 and promote the induction of cytokine secretion, cytolytic activity, and clonal expansion in murine CD8+
Collectively, these studies suggested that IL-12 and IFN-α can act as redundant signals to promote the development of effector responses in murine CD8⁺ T cells.

In addition to enhancing effector cell development, IFN-α/β was implicated in the generation of memory CD8⁺ T cells in vivo. In these studies, IFNAR-deficient, TCR-transgenic (P14) CD8⁺ T cells failed to expand and generate memory populations in response to in vivo LCMV infection despite their ability to proliferate efficiently in vitro. Alternatively, IL-12⁻/⁻ mice displayed defective primary effector responses, whereas development of central memory (T_CM) cells was markedly enhanced compared to wild-type, indicating that IL-12 signaling suppresses T_CM development. Considering that IFN-α/β has been implicated in effector and memory cell development, it is unclear how this signal regulates both events and whether any of these activities operate in human CD8⁺ T cells. Further, it is not clear how IL-12 and IFN-α/β signals are integrated to balance effector and memory cell development, as many intracellular pathogens elicit the secretion of both innate cytokines from professional APCs. In this study, we demonstrate a novel pathway for the variegated programming of human CD8⁺ T cell effector and memory development by IL-12 and IFN-α. Here, we show that IL-12 and IFN-α are not redundant signals in the development of human CD8⁺ T cell responses and instead act in concert in the context of signals 1 and 2 to balance the development of effector and memory cell populations.
MATERIALS AND METHODS

**Human subjects.** Peripheral blood was collected by venipuncture from healthy adult donors with approval of the Internal Review Board (UT Southwestern Medical Center, Dallas, TX). Informed consent was obtained from each donor in accordance with guidelines established by the Internal Review Board and the Declaration of Helsinki.

**Purification and in vitro cultures of human CD8**+** T cells.** CD8**+**CD45RA**+** cells were purified by either flow cytometric sorting or isolated by magnetic bead enrichment. Purified CD8**+**CD45RA**+** cells were cultured at 0.5-1×10⁶ cells/ml on anti-CD3/anti-CD28-coated plates in cIMDM with IL-2 (50 U/ml) with cytokines as follows: “Neutralized” (anti-IL-4, anti-IL-12, anti-IFNAR2, and anti-IFN-γ), “IL-12” (rhIL-12 (10 ng/ml), anti-IL-4, anti-IFNAR2, and anti-IFN-γ), “IFN-α” (rhIFN-α(A) (1000 U/ml), anti-IL-4, anti-IL-12, and anti-IFN-γ), or IL-12 + IFN-α (rhIL-12, rhIFN-α(A), anti-IL-4, and anti-IFN-γ). On day 3, cells were either harvested for analysis or split 1:10 in fresh media supplemented with 50 U/ml rh-IL-2 and cultured to day 7.

**Flow cytometric analysis.** For intracellular staining, cells were activated for 4 hrs with Phorbol 12-myristate 13-acetate (PMA) (80ng/ml) and Ionomycin (1μM) in the presence of Brefeldin A (1μM) or left unstimulated prior to fixation, permeabilization, and staining. For T-bet detection, cells were stained with un-conjugated rabbit anti-human T-bet (Santa Cruz) followed by a anti-rabbit Ig-biotin and either a SA-conjugate to PerCP or Q-dot655 (Invitrogen). For analysis of cell division, cells were resuspended at 1×10⁷ cells/ml in PBS and treated with 1.25μM carboxyfluorescein diacetate succinimidyl ester.
(CFSE, Sigma) or 12μg/ml Pacific-Blue succinimidyl ester (PBSE (Invitrogen)) for 10 min at room temperature. Cells were then activated by culturing on anti-CD3/anti-CD28-coated plates, and cell division was assessed by CFSE/PBSE dilution at either d3 or d5 post activation. All data were collected on an LSRII flow cytometer (BD Bioscience) and analyzed using FloJo software (Tree Star).

Intracellular STAT staining. Purified CD8⁺CD45RA⁺ T cells were polarized under IL-12 + IFN-α conditions for 6 days to generate polarized T₂⁰ and T₅⁰ subpopulations. Cells were either left untreated or treated with IL-12 (10ng/ml) or IFN-α (1000u/ml) for 30 minutes. Detection of tyrosine-phosphorylated (P-Y) STAT1 and STAT4 was performed by intracellular staining as described. Cells were stained with un-conjugated rabbit polyclonal antibodies to STAT1 (clone SC-346), STAT-4 (clone SC-486; Santa Cruz) or anti-phosphotyrosine STAT1 (Upstate) or anti-phosphotyrosine STAT4 (Zymed). A goat anti-rabbit Ig-biotin (Jackson Immunoresearch) was used for secondary detection followed by strepavidin-PerCP (BD Biosciences).

Cytolytic assays. Day 7 polarized cells were rested overnight at 1×10⁶ cells/ml in the absence of IL-2. THP-1 target cells were labeled by culturing in the presence of 150μCi ⁵¹Cr (Na₂(CrO₄)) for 90 minutes. THP-1 cells were left untreated or coated with anti-hCD3 (OKT3) at 1.5 μg/ml in order to provide antigen receptor-dependent lysis. THP-1 targets were incubated with polarized CD8⁺CD45RA⁺ T cells at various effect:target (E:T) ratios for 4 hrs at 37°C, and CTL activity was assessed by measuring release of ⁵¹Cr by scintillation counting.
Live cell chemokine receptor sorting. CD8⁺ CD45RA⁺ sorted cells were polarized for 7 days and rested overnight in the absence of IL-2. Cells were labeled with either CFSE or PBSE and stained with antibodies to human CCR7 and CXCR3. Stained cells were sorted using a FACS Aria (BD Biosciences) for either CCR7^{hi} or CXCR3^{hi} to <90% purity. Sorted cells were rested overnight and subjected to re-directed lysis or were re-activated on anti-CD3-coated plates. Re-activated cells were analyzed for chemokine receptor expression, or lytic activity was assessed by re-directed lysis assays.
RESULTS

IL-12, but not IFN-α, regulates effector CD8+ T cell development

We assessed the development of effector cytokine secretion and lytic activity in purified human CD8+/CD45RA+ T cells in response to IL-12 and IFN-α. In order to strictly control specific cytokine signals, cells were activated with anti-CD3/anti-CD28 in the absence or presence of cytokines or anti-cytokine antibodies as described in the Methods section. As previously reported, IL-12 markedly induced the secretion of IFN-γ and TNF-α compared to neutralizing conditions (Figure 1A,B). In contrast, treatment with IFN-α alone was insufficient to induce either expression or secretion of IFN-γ or TNF-α to levels above the neutralized control. Importantly, IFN-α did not inhibit the ability of IL-12 to induce cytokine expression and secretion (Figure 1A,B), indicating that IL-12 drove effector cytokine expression independently of IFN-α signaling.

We next measured expression of the cytolytic effector molecules perforin and granzyme B. We observed induction of these molecules by both IL-12 and IFN-α, however, the magnitude and pattern by which these cytokines affected this induction was variable among the donors tested (Figure S1). Therefore, to address the precise role of IL-12 and IFN-α on functional cytotoxicity, we assessed the ability of cytokine polarized cells to directly kill target cells in a re-directed lysis assay. Unlike expression of perforin and granzyme B we observed that, IL-12 but not IFN-α promoted strong lytic activity compared to cells activated under neutralizing conditions consistently in all donors (Figure 1C). Although not enhanced by IFN-α alone, IL-12-mediated lytic activity was not inhibited by the presence of IFN-α during priming. The lytic activity observed by these cells was completely inhibited by concanamycin A, demonstrating an exclusive role
for perforin and granzyme as opposed to Fas/FasL-mediated killing in these assays\textsuperscript{18,19} (Figure S2). These data demonstrate that IL-12, but not IFN-\(\alpha\), is sufficient to program both effector cytokine secretion and perforin-mediated CTL activity in human CD8\(^+\) T cells.

**IFN-\(\alpha\) drives the development of human CD8\(^+\) T\(_{CM}\) cells**

Considering recent studies in mice suggesting that IFN-\(\alpha\) could modulate primary expansion and subsequent T\(_{CM}\) development\textsuperscript{20}, we next assessed the ability of IL-12 and IFN-\(\alpha\) to influence the expansion of human CD8\(^+\) effector (T\(_{EM}\)) and central memory (T\(_{CM}\)) cells. T\(_{EM}\) and T\(_{CM}\) are characterized by low expression of CD45RA and differential expression of CCR7 such that T\(_{EM}\) are CCR7\textsuperscript{lo} and T\(_{CM}\) are CCR7\textsuperscript{hi}, whereas naïve cells are CD45RA\textsuperscript{hi}/CCR7\textsuperscript{hi}\textsuperscript{21,22}. As expected, IL-12 enhanced a subpopulation of CCR7\textsuperscript{lo}/CD45RA\textsuperscript{lo} cells that match the proposed T\(_{EM}\) phenotype (Figure 2A,B, upper panels). Interestingly, IFN-\(\alpha\) markedly enhanced the percentage of CCR7\textsuperscript{hi}/CD45RA\textsuperscript{lo} T\(_{CM}\) cells, and the induction of T\(_{CM}\) development by IFN-\(\alpha\) was not inhibited in the presence of IL-12, suggesting a dominant role for IFN-\(\alpha\) in regulating T\(_{CM}\) differentiation. We next assessed two differentially expressed chemokine receptors: CXCR3, which allows for traffic to the periphery and is associated with effector phenotypes\textsuperscript{23-25}, and CCR7, which allows for efficient trafficking to lymph nodes and is associated with the T\(_{CM}\) phenotype\textsuperscript{26,27}. Consistent with the induction of CCR7\textsuperscript{hi}/CD45RA\textsuperscript{lo} T\(_{CM}\) cells, we found that IFN-\(\alpha\) promoted the development of CXCR3\textsuperscript{lo}/CCR7\textsuperscript{hi} populations either in the presence or absence of IL-12 (Figure 2A,B, lower panels). These cells are composed of a subset of the CCR7\textsuperscript{hi}/CD45RA\textsuperscript{lo} cells, and
in agreement with previous studies\textsuperscript{24}, we propose that the CCR7\textsuperscript{hi}/CXCR3\textsuperscript{lo} subset represents a more precisely defined T\textsubscript{CM} population. Together, these analyses reveal that IL-12 acts independently of IFN-\(\alpha\) to program the development of T\textsubscript{EM}, while signaling via IFN-\(\alpha\) promotes the development of T\textsubscript{CM} phenotypes.

**IFN-\(\alpha\)-driven T\textsubscript{CM} cells display functional memory activities**

In order to determine whether the \textit{in vitro} development of T\textsubscript{CM} and T\textsubscript{EM} paralleled their known functional roles, we examined effector molecule expression as a function of T\textsubscript{CM} and T\textsubscript{EM} cell surface phenotypes. Activation of naïve CD8\textsuperscript{+} T cells led to the development of two distinct populations of cells; those which expressed either granzyme B alone, or both granzyme B and perforin (Figure 3A). We assessed expression of CXCR3 and CCR7 within these two populations. The majority of cells expressing both perforin and granzyme B (Figure 3A, right panels, orange gate) uniformly expressed high levels of CXCR3 and low CCR7 regardless of cytokine treatment. In contrast, cells expressing granzyme B alone (Figure 3A, left panels, magenta gate), heterogeneously expressed CCR7 and CXCR3. Here, IFN-\(\alpha\) enhanced a population of CCR7\textsuperscript{hi}/CXCR3\textsuperscript{lo} T\textsubscript{CM} cells, demonstrating that the genesis of the T\textsubscript{CM} cells in these cultures were derived from within the granzyme B single positive population. We also assessed expression of CD127 (IL-7R\(\alpha\)) as T\textsubscript{CM} express relatively high levels of this receptor compared to T\textsubscript{EM}. Here, cells within the CCR7\textsuperscript{hi}/CD127\textsuperscript{lo} gate displayed high frequencies of perforin and granzyme B double positive cells (Figure 3B). Alternatively, cells within the CCR7\textsuperscript{hi}/CD127\textsuperscript{hi} gate expressed granzyme B with very low levels of perforin, demonstrating a direct link between effector potential and decreased expression of the IL-
Finally, cells expressing CXCR3 in the absence of CCR7 overwhelmingly secreted high levels of IFN-γ in response to secondary activation (Figure 3C, population “D”). In contrast, only 50% of cells that co-expressed CXCR3 and CCR7 (Figure 3C, population “C”) and less that 1% of CCR7[^hi]CXCR3[^lo] cells (Figure 3C, population “B”) were capable of secreting IFN-γ upon reactivation. These data directly link T[^EM] phenotypes with secretion of effector molecules and demonstrate that IL-12 and IFN-α differentially regulate both the phenotype and function of T[^EM] and T[^CM], respectively. Expression of T[^bet] has been implicated in the development of effector responses in CD8[^+] T cells. We examined expression of T[^bet] within T[^CM] and T[^EM] subpopulations. Similar to previous reports[^15,28], T[^bet] expression was enhanced within cells which acquired a T[^EM] but not a T[^CM] phenotype (Figure S3).

T[^EM] cells exert immediate effector functions, are considered to be more terminally differentiated, and generally do not expand efficiently upon reactivation. In contrast, T[^CM] cells divide rapidly to secondary challenge giving rise to additional effector cells[^29,30]. Therefore, we sought to determine whether IFN-α-driven T[^CM] cells were capable of rapid proliferation and generation of secondary effector cells. To address this, cells were polarized in the presence of both IL-12 + IFN-α for 7 days and sorted based on the following gates: T[^EM], CCR7[^lo]/CXCR3[^hi], (T[^EM][XR3]) and T[^CM], CCR7[^hi]/CXCR3[^lo] (T[^CM][R7]) (Figure 4A). These purified cells were then examined for direct lytic activity. T[^EM][XR3] cells displayed strong CTL activity, whereas the T[^CM][R7] cells were incapable of immediate lytic activity (Figure 4B). These data are in agreement with our previous observations that T[^EM][XR3] cells expressed higher levels of perforin compared to T[^CM][R7] cells and
demonstrate functional differences in effector capabilities between CXCR3\textsuperscript{hi} and CCR7\textsuperscript{hi} CD8\textsuperscript{+} T cells.

We next assessed the ability of sorted cells to proliferate and expand in response to secondary activation. As expected, activated TEMXR3 cells did not divide, whereas, T\textsubscript{CMR7} cells displayed robust proliferation in response to anti-CD3 stimulation (Figure 4C). The lack of proliferation in the TEMXR3 cells correlated well with a decrease in the total live population of cells as assessed by forward/side scatter analysis and by 7-amino-actinomycin D staining, whereas the T\textsubscript{CMR7} cells maintained a live lymphocyte profile (Figure S4). We examined the ability of sorted cells to expand and give rise to new effector and memory sub-populations in response to secondary activation. Here, the TEMXR3 cells expressed perforin and granzyme B (Figure 4D, right panels), and displayed equivalent lytic activity regardless of whether they were restimulated with anti-CD3 following sorting (Figure 4E, right panel). In contrast, T\textsubscript{CMR7} cells expressed low levels of perforin and granzyme B (Figure 4D, left panels) and displayed poor lytic activity if they were not restimulated following sorting (Figure 4E, left panels). However, in response to reactivation, T\textsubscript{CMR7} cells gave rise to perforin- and granzyme-expressing cells. The induction of perforin and granzyme in these T\textsubscript{CM} cells correlated directly to the acquisition of lytic activity (Figure 4D,E, left panels), demonstrating a high degree of plasticity in their ability to reconstitute effector cell populations. Taken together, these data demonstrate that IL-12-driven TEMXR3 cells function in a more terminally differentiated manner, with poor survival and proliferation and an inability to give rise to heterogenous populations upon secondary activation. In contrast, IFN-\textgreek{a}-regulated T\textsubscript{CMR7}
cells displayed strong survival and division to secondary activation and were endowed with the ability to give rise to functional effector populations.

It was possible that CCR7$^{hi}$/CXCR3$^{lo}$ T$_{CM}$ cells were derived from populations that were not efficiently activated and remained naïve during the primary activation. However, naïve CCR7$^{hi}$ cells derived from cultures, which did not receive primary TCR/costimulatory activation, displayed a resting lymphocyte profile, failed to divide, and did not express granzyme B in response to anti-CD3 alone (Figure S5). In contrast, memory CCR7$^{hi}$ cells, which received primary activation, proliferated and expressed granzyme B as a function of division in response to secondary anti-CD3 stimulation (Figure S5). Therefore, the memory CCR7$^{hi}$ cells are a unique population of memory cells and not simply naïve cells that failed to receive primary activation.

**Reciprocal regulation of the IL-12R and IFNAR in T$_{EM}$ and T$_{CM}$**

In CD4$^{+}$ T cells, Th1 commitment is regulated by IL-12 through the induction of the IL-12R$\beta$2 subunit$^{17}$. However, the IFN-α/β receptor (IFNAR) is thought to be constitutively expressed on all cells, enabling them to respond in an autocrine fashion to IFN-α that is secreted during viral infections$^{6}$. Thus, selective IFNAR expression has not been examined during T cell differentiation. It was possible that differential sensitivities to IL-12 and IFN-α may account for the concomitant development of T$_{EM}$ and T$_{CM}$ in response to combined activation with IL-12 + IFN-α allowing for selective outgrowth or differential programming of these two subpopulations. To address this possibility we measured both IL-12R$\beta$2 and IFNAR2 expression in response to TCR and cytokine activation. As expected, IL-12 + IFN-α stimulation dramatically enhanced IL-12R$\beta$2
expression by day 3 of culture (Figure 5A). Surprisingly, we also found that the IFNAR2 was markedly induced by IL-12 + IFN-α compared to the neutralized control. Further, analysis of co-expression with CCR7 demonstrated the development of a distinct sub-population of cells in which the IL-12Rβ2 was inversely expressed with CCR7 (Figure 5B). In contrast, CCR7 expression was directly correlated with induction of IFNAR2 on a sub-population of cells. These data demonstrate that IL-12 and IFN-α differentially regulate the expression of their surface receptors, implicating a potential role for this response in determining effector or memory development.

Recent studies have suggested that increased proliferation during the primary expansion leads to more terminally differentiated phenotypes of CD8+ T cells that acquire a T_{EM} phenotype\textsuperscript{31,32}. If this observation is related to IL-12 responsiveness, then the development of effector and memory cells may hinge on the differential acquisition of cytokine responsiveness over the course of division. To examine this, we monitored expression of IL-12Rβ2 and IFNAR2 as a function of division on day 3 of culture. First, we observed that IFN-α slowed the progression of cell division compared to activation of cells with either neutralizing conditions or with IL-12, and this effect was evident even in the presence of IL-12 (Figure S6A). Further, IL-12Rβ2 expression was enhanced in response to IL-12 and IFN-α alone at each progressive division and even more dramatically induced in the presence of both IL-12 + IFN-α, indicating a cooperative role for IL-12 and IFN-α in regulating IL-12 responsiveness as a function of cell division (Figure S6A,B). In contrast, expression of IFNAR2 was progressively diminished at each cell division, and this effect was marginally influenced by IL-12 and IFN-α on day 3 of culture.

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At each division, we observed that cells progressively gained expression of IL-12R\textsubscript{β2} while losing expression of IFNAR2. Thus, cells that had progressed through fewer divisions had the potential to be more responsive to IFN-α and less responsive to IL-12. We compared the expression of multiple effector and memory markers in the context of cytokine receptors on day 5 of culture in response to IL-12 + IFN-α stimulation (Figure 5C,D). On day 5 of culture, we observed a greater induction of IL-12R\textsubscript{β2} and IFNAR2 by IL-12 + IFN-α than on day 3 (Figure 5C). Here, the magnitude of modulation of IL-12R\textsubscript{β2} and IFNAR2 was dramatically increased, clearly demonstrating the induction of IL-12R\textsubscript{β2} as a function of division and the retention of IFNAR2 on less divided cells. In addition, IL-12 + IFN-α preserved a subpopulation of cells that remained undivided compared to neutralizing conditions (Figure 5D, left panels). Strikingly, cells that had undergone extensive division displayed a T\textsubscript{EM} phenotype marked by high expression of CXCR3 and IL-12R\textsubscript{β2} and low levels of CCR7 and IFNAR (Figure 5D) and correlated with expression and secretion of perforin, granzyme B, and IFN-γ (data not shown). Those cells that were retained in the undivided population in response to IL-12 + IFN-α displayed characteristic T\textsubscript{CM} phenotypes including low CXCR3 and high CCR7 expression and high IFNAR (Figure 5D) with lower levels of perforin, granzyme B, and IFN-γ (data not shown). Cytokine titration revealed that at lower concentrations of IFN-α (10-100U/ml) the effects of IL-12 signaling dominate, promoting the development of T\textsubscript{EMXR3} cells over that of T\textsubscript{CMR7} cells (Figure S7). However, even in the context of IL-12, as the concentration of IFN-α was increased, the development of T\textsubscript{CMR7} cells was enhanced. This result suggests that IL-12 and IFN-α work independently to induce T\textsubscript{EM} and T\textsubscript{CM} phenotypes. As the ratios of these
cytokines are shifted, the development of $T_{CM}$ and $T_{EM}$ follow accordingly. Collectively, these data demonstrate reciprocal regulation of IL-12R\(\beta\)2 and IFNAR2 on cells that commit to $T_{EM}$ and $T_{CM}$ fates. $T_{EM}$ cells are derived from rapidly dividing cells and are regulated by IL-12 through the progressive acquisition of IL-12 responsiveness at each cell division. In contrast, $T_{CM}$ cells developing in response to IFN-\(\alpha\) are retained at earlier divisions, have the greatest sensitivity to IFN-\(\alpha\), and express the lowest levels of the IL-12R\(\beta\)2.

The reciprocal regulation of the IL-12R and IFNAR suggested that $T_{CM}$ and $T_{EM}$ development was balanced by differential responsiveness to cytokines. Thus, we measured cytokine-driven STAT phosphorylation as a function of division. First, total STAT1 and STAT4 protein was not altered in response to IFN-\(\alpha\) and IL-12, respectively (Figure 5E). Further, we observed strong induction of phospho-STAT1 in response to IFN-\(\alpha\) signaling as well as phospho-STAT4 in response to IL-12 in the total population (Figure 5E) demonstrating a clear responsiveness to cytokine treatment. Interestingly, IFN-\(\alpha\)-mediated STAT1 phosphorylation was observed at various levels in all cells, including cells that had progressed through >4 divisions (Figure 5F). In contrast, IL-12-driven STAT4 phosphorylation was not observed in division 0. However, as cell division progressed, levels of phospho-STAT4 increased 5-6 fold over that of the control cells. Taken together, the ratio of IL-12:IFN-\(\alpha\) responsiveness increased progressively with each cell division, correlating directly with the expression of the respective receptor ratios.

In addition to cell division, the strength of signal delivered through TCR engagement has also been implicated in the regulation of memory cell development\(^31\).
Some studies have suggested that a strong and prolonged antigen signal promotes efficient generation of $T_{\text{EM}}$ leading to the eventual development of $T_{\text{CM}}^{29}$. An alternative view posits that $T_{\text{EM}}$ and $T_{\text{CM}}$ develop in parallel and are balanced by TCR signal strength in which some clones receive a strong signal leading to rapid proliferation and $T_{\text{EM}}$ development, while other clones receive a weaker or less sustained signal leading to $T_{\text{CM}}^{33}$. In the present study, the $\textit{in vitro}$ priming conditions were based on concentrations of anti-CD3 traditionally used to promote efficient proliferation and effector cell development$^{34}$. However, this particular culture condition may only provide a single view of how cells interpret IL-12 and IFN-$\alpha$ signals as they divide and differentiate. We wished to determine whether the strength of TCR engagement altered the balance between $T_{\text{EM}}$ and $T_{\text{CM}}$ as cells develop in response to IL-12 + IFN-$\alpha$. To address this, we examined the effect of increasing concentrations of anti-CD3/anti-CD28 under either neutralizing conditions or with IL-12 + IFN-$\alpha$.

Analysis of cell division revealed that increasing TCR stimulation promoted more efficient and rapid cell division (Figure 6A). However, IL-12 + IFN-$\alpha$ treatment slowed cell division at each concentration of anti-CD3 compared to the neutralized control. Although each culture condition induced proliferation, twice as many cells were retained within divisions 0-3 when activated with the lowest concentration of anti-CD3/anti-CD28 in the presence of IL-12 + IFN-$\alpha$ compared to cells activated under neutralizing conditions (Figure 6A). As described above, cells that remained in the undivided population exhibited all of the cell surface phenotypes and functional characteristics of $T_{\text{CM}}$. Despite the pronounced acceleration of cell division driven by increased TCR
signal strength, IL-12 + IFN-α signaling slowed the progression of cell division and 
enhanced T\textsubscript{CMR7} cells even at the highest concentration of anti-CD3/CD28 (Figure 6B).

We next assessed expression of the IL-12R\textsubscript{β2} and IFNAR2 at each concentration 
of anti-CD3/anti-CD28. Under neutralizing conditions, the IL-12R\textsubscript{β2} was expressed at 
low levels and remained constant at each concentration of anti-CD3/anti-CD28 and at 
each cell division (Figure 7A, top panels). A similar trend was observed with IFNAR2 
expression, although a 2-3 fold increase in expression was observed at 2.5 and 5 \textmu g/ml 
compared to 1 \textmu g/ml of anti-CD3/anti-CD28 (Figure 7B, top panels). However, in the 
presence of IL-12 + IFN-α, we observed a striking regulation of both the IL-12R\textsubscript{β2} and 
IFNAR2 as a function of anti-CD3 concentration. First, IL-12R\textsubscript{β2} remained low on cells 
that did not progress into the first division regardless of anti-CD3/anti-CD28 
concentration (Figure 7A, lower panels). As cells divided, IL-12R\textsubscript{β2} was markedly 
induced up to division 3 in the presence of 2.5 and 5 \textmu g/ml, but not 1 \textmu g/ml of anti-
CD3/anti-CD28. In stark contrast, IFNAR2 was most highly induced in response to IL-
12 + IFN-α on cells that were retained in the undivided population, and this effect was 
amplified in response to increasing anti-CD3/anti-CD28 (Figure 7B, lower panels). 
IFNAR2 levels then declined precipitously at each cell division. Taken together, we 
found that at division 0, IL-12R\textsubscript{β2} remained low while IFNAR2 expression increased 
dramatically in response to IL-12 + IFN-α stimulation and as a function of increased 
TCR signal strength. These T\textsubscript{CM} cells bear the highest potential for IFN-α sensitivity and 
the lowest potential for IL-12 responsiveness. As cells divided in response to TCR 
stimulation, they rapidly induced IL-12R\textsubscript{β2} while simultaneously downregulating the 
IFNAR2. Cells in later divisions lose sensitivity to IFN-α while gaining responsiveness 

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to IL-12, and these cells are characterized phenotypically and functionally as TEM. These results demonstrate that the strength of the TCR signal dictates the level at which cells become responsive to IL-12 and IFN-α. Thus, as the TCR signal threshold is altered, the reciprocal regulation of the IL-12R and IFNAR allow for the simultaneous commitment of precursors to the TEM and TCM fates.
DISCUSSION

In this study, we have systematically examined the independent and combined roles of IL-12 and IFN-α in the regulation of human CD8⁺ T cell differentiation. For the first time, we report non-redundant roles for IL-12 and IFN-α/β in the development of human CD8⁺ T responses. Together, our data support a model in which IL-12 and IFN-α/β act in concert with signals 1 and 2 to promote the variegated development of effector and memory populations of human CD8⁺ T cells (Figure S8).

Recently, IL-12 and IFN-α were proposed to act in a redundant fashion to promote effector cell development in murine CD8⁺ T cells²,³. However, our examination of IL-12- and IFN-α-driven cytokine secretion and lytic activity in human CD8⁺ T cells revealed striking dissimilarities. Here, IL-12, but not IFN-α, drove effector cell development characterized by marked secretion of IFN-γ and TNF-α and enhanced lytic activity. Surprisingly, while IFN-α did not inhibit IL-12-regulated effector cell development, IFN-α signaling was not sufficient to promote this response in the absence of IL-12. Thus, IL-12 remains unique in its ability to drive effector functions and suggests that IL-12 and IFN-α are not redundant signals in this regard.

While human CD8⁺ T cells clearly do not adopt effector functions in response to IFN-α, we found a remarkable role for IFN-α in driving memory cell development. In line with observations that IFNAR⁻ CD8⁺ T cells develop effector phenotypes but lack functional memory¹³,³⁶, we found that IFN-α markedly enhanced human CD8⁺ T cells displaying a TCM surface phenotype. Several models have been proposed to explain the development of effector and memory cells from the same pool of naïve precursors³³. For example, one model proposes that TCM cells develop in a linear manner from a pool of
rested effector cells. Alternatively, multiple studies suggest that effector and memory cells develop from distinct lineages which may arise as early as the first division after Ag encounter. In the present study, we found that cells activated with both IL-12 + IFN-α simultaneously segregated to both the T\textsubscript{CM} and T\textsubscript{EM} fates, suggesting that signaling by both cytokines regulates their parallel development rather than sequential development (Figure S8). The first evidence for this model comes from our observation that signals derived from IFN-α program the development of a population of T\textsubscript{CM} cells. These cells express high levels of the lymphoid homing receptor CCR7, lack immediate effector function, and display the hallmark characteristics of T\textsubscript{CM} cells upon a secondary activation. Importantly, development of T\textsubscript{CM} in response to IFN-α/β occurs concomitantly with the generation of T\textsubscript{EM} cells that develop in response to IL-12 when both cytokines are present.

The data presented here support a model of co-linear commitment to T\textsubscript{EM} and T\textsubscript{CM} that is regulated independently by IL-12 and IFN-α, respectively (Figure S8). Importantly, IL-12 and IFN-α drove these divergent pathways through the reciprocal regulation of the IL-12R and the IFNAR. In the absence of innate cytokines, we clearly observed the outgrowth of cells that phenotypically resembled T\textsubscript{EM} by their selective expression of CXCR3 and low expression of CCR7. However, in the absence of IL-12, these cells were incapable of effector functions. Thus, IL-12 acted in an instructive manner to regulate increased cytokine expression and lytic activity. This correlated precisely with the induction of the IL-12Rβ2, as its expression was markedly increased at each cell division in response to innate cytokines. Further, we observed an even greater enhancement of IL-12Rβ2 expression in response to increasing concentrations of anti-
CD3. In contrast, IFN-α-regulated T_{CM} cells were primarily derived from sub-populations that were either retained in the undivided population or had undergone only 1-2 divisions rather than T_{EM} cells that divided extensively. As the initial TCR signal strength was increased, far fewer cells were retained at earlier divisions giving rise to T_{EM} phenotypes at later divisions. Importantly, IFN-α enhanced the proportion of T_{CM} at every concentration of anti-CD3 tested. These T_{CM} cells expressed low levels of IL-12R and high levels of IFNAR, endowing them with the greatest sensitivity to IFN-α.

TCR signal strength has been implicated as a regulatory component for effector and memory formation\textsuperscript{31}. In some models, low TCR engagement, either through decreased Ag concentration or an acute TCR activation favors the development of memory over effector cells. Whereas, cells receiving strong or prolonged activation develop primarily into effector cells\textsuperscript{39}. These observations have been extended to \textit{in vivo} infection models, in which acute infection promotes stronger memory responses than that of chronic infection\textsuperscript{40,41}. Indeed, our results support this model as cells that map to the T_{CM} phenotype were primarily derived from sub-populations of cells that were either retained in the undivided population or had undergone only 1-2 divisions compared to T_{EM} cells that divided extensively. As the initial TCR strength was increased, far fewer cells were retained at earlier divisions giving rise to T_{EM} phenotypes at later divisions. Importantly, IFN-α enhanced the proportion of T_{CM} by modulating the TCR signal strength and slowing the progression of cell division in some cells. The cyclin dependent kinase family members CDK2 and CDK6 have been implicated in the rapid division of memory cells to secondary activation\textsuperscript{42}. In addition, the CDK inhibitor, p27kip1 has been shown to be highly expressed in cells which do not actively divide\textsuperscript{42,43}. Thus, differential
regulation of these factors by IL-12 and IFN-α may explain the variegated behavior of cells as they commit to TEM and TCM phenotypes.

This study provides new and important insight into the development of effector and memory human CD8+ T cells. Although these responses were derived from cells developing in response to signals 1 and 2 in vitro, this approach allowed us to methodically examine the direct and independent roles of IL-12 and IFN-α/β in the generation of CD8+ T cell effector and memory responses. Our data support a cooperative model in which TCR strength and innate cytokines act as rheostats to fine tune the balance between effector and memory cell development. If the demands are great and antigen levels are high, IL-12 dominates, and effector cells develop at the expense of memory cells. Alternatively, when Ag levels wane, cytokines then act to modulate the development of both effector and memory subpopulations. While this study suggests that TEM and TCM development can occur in parallel, it does not rule out the possibility that TCM can be derived from rested TEM cells as many recent studies have suggested. Nonetheless, the present study has broad implications to the field of CD8+ T cell biology and suggests that optimal memory generation requires a precise balance of TCR signals and innate cytokines. This study marks the first discovery of independent roles for IL-12 and IFN-α/β in the development of human CD8+ T cell responses and underscores the importance of these two cytokines in regulating effector and memory responses to infection.
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AUTHORSHIP

H.J.R. performed research, analyzed data, and wrote the paper; A.M.D. performed research, and analyzed data; A.G.C. performed research; J.D.S. designed research, and contributed reagents; J.F. designed research and analyzed data; J.D.F. designed research, analyzed data, and wrote the paper. The authors declare no conflicts of interest.
REFERENCES

FIGURE LEGENDS

Figure 1. **IL-12 but not IFN-α is sufficient to program human CD8+ T cell effector functions.** (A) Intracellular expression of human IFN-γ, and TNF-α from day 7 *in vitro* polarized human CD8+ T cells. Rested cells were reactivated for 4hrs with PMA and ionomycin in the presence of brefeldin A, and IFN-γ and TNF-α were assessed by intracellular stain and flow cytometric analysis. Data are gated on live, CD8+ cells. (B) Day 7 polarized cells were left un-stimulated or stimulated with anti-CD3 for 24hrs, and supernatants were harvested for ELISA. (C) Characterization of CTL activity by 51Cr-release assay. Day 7 polarized CD8+ T cells were incubated for 4hrs with 51Cr-labeled THP-1 cells (target) at the E:T ratios shown. CTL activity was assessed by quantification of 51Cr released into the supernatant by β emission. These experiments were performed with 5 different healthy donors with similar results.

Figure 2. **Regulation of human CD8+ T_{CM} development by IFN-α.** Day 7 cytokine polarized cells were stained with a panel of anti-human monoclonal antibodies, including CCR7, CD45RA, and CXCR3, to assess memory and effector phenotypes. (A) Analysis of surface markers CCR7 and CD45RA (top panel) and CCR7 and CXCR3 (bottom panel). The induction of CCR7^{hi}/CXCR3^{lo} cells by IFN-α is indicated by the orange gate. (B) Quantification of human effector and memory profile (top) and chemokine receptor profile (bottom) regulated by IL-12 and IFN-α. Black, (neutralized), magenta (IL-12), teal (IFN-α), orange (IL-12+IFN-α). These experiments were performed with 7 different healthy donors with similar results.
**Figure 3.** Human CD8+ T_{EM} and T_{CM} cells display distinct effector properties. Day 7 cytokine polarized cells were assessed for surface marker and cytokine expression. (A) Cells were gated on live CD8+ cells, and perforin and granzyme B levels were assessed. Cells were gated on either granzyme B single positive cells (magenta) or perforin-granzyme B double positive cells (orange) and examined for CCR7 and CXCR3 expression (lower panels). (B) Live CD8+ cells were gated through either CCR7^{hi}, CD127^{hi} cells (magenta) or CCR7 and CD127^{lo} cells (orange) and assessed for perforin and granzyme B expression (lower panels). (C) PMA and Ionomycin activated cells were gated on either CCR7^{lo}/CXCR3^{lo} (A, black), CCR7^{hi}/CXCR3^{lo} (B, magenta), CCR7^{hi}/CXCR3^{hi} (C, teal) or CCR7^{lo}/CXCR3^{hi} (D, orange) and examined for IFN-γ expression by intracellular staining (right panel).

**Figure 4.** CCR7 and CXCR3 expression demarcates distinct sub-populations of human CD8+ T cells with functional effector and memory properties. (A) Sorted CD8+ CD45RA+ cells were activated with IL-12 + IFN-α to day 7. Cells were then sorted into separate CCR7^{hi}/ CXCR3^{lo} or CXCR3^{hi}/CCR7^{lo} populations. (B) Sorted cells were rested overnight in the absence of IL-2 and subjected to a ^{51}Cr re-directed lysis assay with THP-1 target cells at the indicated E:T ratios. (C) Sorted cells were labeled with CFSE and left untreated (resting) or activated with 1.5μg/ml plate bound anti-human CD3 for 3 days (anti-CD3). On day 3, cells were assessed for proliferation by CFSE dilution. (D) Sorted cells were activated as described in panel C and examined at day 3 for expression of perforin and granzyme B by bi-variant dot plot analysis. (E) Sorted
cells were either left untreated (resting) or activated with 1.5μg/ml anti-CD3 for 3 days (anti-CD3). On day 3, CCR7hi/ CXCR3lo cells (left panel) and CXCR3hi/CCR7lo cells (right panel) were subjected to a re-directed lysis assay as described above. Each of these experiments was performed twice with two separate healthy donors with similar results.

**Figure 5. Reciprocal responsiveness to IL-12 and IFN-α/β in correlates to development of TEM and TCM cells.** (A) Day3, neutralized or IL-12+IFN-α activated cells were assessed for surface expression of the IL-12Rβ2 or IFNAR2 or (B) expression of CCR7, IL-12Rβ2 and IFNAR2. (C) Day5, PBSE labeled cells were assessed for IL-12Rβ2 and IFNAR2 expression as a function of division (top), and relative mean fluorescence intensity was quantified (bottom). (D) Day5, PBSE labeled cells were gated on division 0 (magenta), division 1-3 (teal), or division 4+ (orange), and chemokine receptor (middle panels) and cytokine receptor (right panels) expression was measured. (E-F) Day 5 cells were polarized with IL-12+IFN-α, reactivated with cytokines for 30 min. and assessed for intracellular STAT or phospho-STAT protein expression. (E) Total STAT, STAT4 (left panels) or phospho-STAT1, phospho-STAT4 (right panels) expression in live CD8+ gated cells. 2°+3° antibody alone (grey), unstimulated (Black), IFN-α- (teal) or IL-12-treated (orange). (F) Dot plot overlays of phospho-STAT1 (Top panel, right) or phospho-STAT4 (bottom panel, left) expression as a function of CFSE dilution. Unstimulated (black), IFN-α- (teal) or IL-12-treated (orange). Quantification of mean fluorescence intensity as a function of CFSE dilution (right panels); phospho-STAT1 (top), phospho-STAT4 (bottom).
Figure 6. **TCR signal strength regulates cytokine-dependent T_{CM} development.**

CD8$^+$ CD45RA$^+$ sorted cells were labeled with PBSE and polarized under either neutralizing or IL-12 + IFN-α conditions with 1μg/ml, 1.5μg/ml, 2.5μg/ml or 5μg/ml α-hCD3 and α-hCD28 for 5 days. (A) Assessment of division by PBSE dilution as a function of primary activation strength. The percentages of cells that are contained within each gate are indicated above the gate. (B) Cells were analyzed for expression of CXCR3 and CCR7 by bi-variant dot plot analysis.

Figure 7. **The development of human CD8$^+$ T_{EM} and T_{CM} cells is regulated by both cytokine signaling and strength of primary activation.** CD8$^+$ CD45RA$^+$ sorted cells were labeled with PBSE and polarized under neutralizing or IL-12 + IFN-α conditions with 1μg/ml, 2.5μg/ml or 5μg/ml anti-hCD3 and anti-hCD28 for 5 days. Cells were assessed for expression of IL-12Rβ2 (A) or IFNAR2 (B) as a function of division. Quantification of mean fluorescence intensity is displayed as a function of division for IL-12Rβ2 (A, right panels) or IFNAR2 (B, right panels) in neutralized (top panels) or IL-12+IFN-α (bottom panels) cells.
Figure 1
Figure 4
Figure 5
Figure 6
Figure 7

(A) IL-12Rβ2 and IFN-α levels with neutralization at different concentrations of 1μg/ml, 2.5μg/ml, and 5μg/ml. MFI (Mean Fluorescein Intensity) IL-12Rβ2 levels are shown for different divisions (0 to 4+).

(B) IFNAR2 and IL-12+IFN-α levels with neutralization at different concentrations of 1μg/ml, 2.5μg/ml, and 5μg/ml. MFI IFNAR2 levels are shown for different divisions (0 to 4+).
Reciprocal responsiveness to IL-12 and IFN-α specifies human CD8+ effector versus central memory T cell fates