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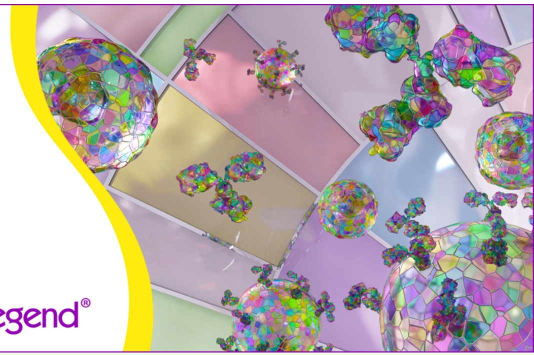

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Direct Stimulation of T Cells by Type I IFN Enhances the CD8$^+$ T Cell Response during Cross-Priming[1]

Agnes Le Bon,* Vanessa Durand,* Elisabeth Kamphuis,† Clare Thompson,* Silvia Bulfone-Paus,‡ Cornelia Rossmann,* Ulrich Kalinke,† and David F. Tough[2]*

Type I IFN (IFN-$\alpha\beta$), which is produced rapidly in response to infection, plays a key role in innate immunity and also acts as a stimulus for the adaptive immune response. We have investigated how IFN-$\alpha\beta$ induces cross-priming, comparing CD8$^+$ T cell responses generated against soluble protein Ags in the presence or absence of IFN-$\alpha\beta$. Injection of IFN-α was found to prolong the proliferation and expansion of Ag-specific CD8$^+$ T cells, which was associated with marked up-regulation of IL-2 and IL-15 receptors on Ag-specific cells and expression of IL-15 in the draining lymph node. Surprisingly, neither IL-2 nor IL-15 was required for IFN-α-induced cross-priming. Conversely, expression of the IFN-$\alpha\beta$R by T cells was shown to be necessary for effective stimulation of the response by IFN-α. The finding that T cells represent direct targets of IFN-$\alpha\beta$-mediated stimulation reveals an additional mechanism by which the innate response to infection promotes adaptive immunity. ***The Journal of Immunology,* 2006, 176: 4682–4689.**

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Cross-priming is a process by which functional CD8$^+$ T cell responses can be generated against Ags that are not expressed directly within APCs. Experimentally, cross-priming has been shown to occur following immunization with soluble or cell-associated Ags and after infection with viruses that are unable to infect APC (1–4). To elicit cross-priming, APC must target internalized Ags into a cross-presentation pathway, leading to the production of antigenic-peptide-MHC class I complexes (5). In addition, APC require an activation (or licensing) signal that endows them with the ability to generate a productive CD8 response; in the absence of licensing, APC stimulate an abortive response that culminates in tolerance rather than immunity (6–11). Among APC, dendritic cells (DC),[3] and the CD8α^+ DC subset in particular, appear to be chiefly responsible for the induction of cross-priming (10, 12–14).

Immune responses are regulated by signals associated with infection, including both direct (i.e., microbial components) and indirect (i.e., host inflammatory molecules) indicators of the presence of pathogens. One host-derived, infection-associated signal that has been reported to act as a stimulus for cross-priming is type I IFN (IFN-$\alpha\beta$) (15). IFN-$\alpha\beta$ is expressed rapidly by cells in response to infection and after exposure to various components of infectious agents (16–18). Although best characterized for its crucial role in innate defense to viral infection, IFN-$\alpha\beta$ also appears to be an important factor linking innate and adaptive immunity. IFN-$\alpha\beta$ has been shown to induce cross-priming when injected into mice together with Ag, and also when expressed by the host in response to virus infection (15). The ability of IFN-$\alpha\beta$ to stimulate cross-priming is in keeping with evidence that it can promote DC maturation (18). Moreover, DC appear to be a direct target of IFN-$\alpha\beta$ during cross-priming, since it was shown that a response could be induced in mice with selective expression of the IFN-$\alpha\beta$R on DC (15).

The mechanisms controlling the generation of productive CD8$^+$ T cell responses to cross-presented Ags remain incompletely understood. In general, CD8$^+$ T cell responses can be influenced by the availability of various cytokines, and IL-2 and IL-15 have been studied extensively in this respect. Administration of these cytokines has been shown to augment the CD8 response in the context of a range of immunization protocols (19–24). Furthermore, experiments in which the activity of IL-2 or IL-15 has been blocked have suggested that their endogenous production contributes to the generation of CD8 responses during infection with some viruses or in the induction of delayed-type hypersensitivity (25–29); DC were found to be the crucial source of IL-15 in the latter setting (29). Notably, however, analysis of CD8$^+$ T cell responses in some other models has failed to show a requirement for IL-2 or IL-15, indicating that distinct mechanisms can drive CD8 responses under different conditions of immunization/infection (30–32). At present, the role of IL-2 and IL-15 in cross-priming is unknown.

Induction of cross-priming by IFN-$\alpha\beta$ requires neither CD4$^+$ T cells nor CD40, and therefore does not involve Th-dependent licensing of DC (15). Instead, IFN-$\alpha\beta$ appears to act as a direct DC-licensing signal, because IFN-α can stimulate cross-priming when DC alone can respond to this cytokine. Interestingly, IFN-$\alpha\beta$ treatment causes DC to up-regulate expression of IL-15 and the α-chain of the IL-15R (33). This could suggest a possible mechanism by which IFN-$\alpha\beta$-exposed DC stimulate responding CD8$^+$ T cells, given that IL-15 is thought to be presented to target cells as an IL-15-IL-15Rα complex on the surface of IL-15-expressing cells (34–36). However, it is unknown whether DC represent the only direct targets of IFN-$\alpha\beta$ during the induction of cross-priming. In fact, although IFN-α was shown to stimulate cross-priming

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[3] Abbreviations used in this paper: DC, dendritic cell; BM, bone marrow; LN, lymph node; WT, wild type.

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in mice with selective expression of the IFN-$\alpha\beta$R on DC, the response in these mice was much lower (~10-fold) than in normal mice, suggesting that optimal stimulation requires IFN-$\alpha\beta$ activity on additional cell types.

There is evidence from in vitro studies indicating that IFN-$\alpha\beta$ can directly alter the behavior of T cells, although contrasting effects of IFN-$\alpha\beta$ have been observed depending on the conditions of the study. For example, whereas IFN-$\alpha\beta$ has long been recognized for its ability to strongly inhibit T cell proliferation in vitro (37, 38), preactivated T cells appear to be resistant to this antiproliferative activity (39) and pretreatment of T cells with IFN-$\alpha\beta$ has been reported to either inhibit (40) or enhance (41) subsequent proliferation upon stimulation through the TCR. In addition, IFN-$\alpha\beta$ was recently shown to enhance the proliferation and effector activity of $CD8^+$ T cells stimulated by artificial APC (consisting of microspheres coated with MHC-peptide complexes and costimulatory molecules) (42). Similarly, IFN-$\alpha\beta$ has been shown to both inhibit the apoptosis of activated T cells in vitro (43, 44), and to sensitize T cells to activation-induced cell death (45). Moreover, IFN-$\alpha\beta$ has been reported to have varied effects on T cell cytokine production and responsiveness, including up-regulation (46, 47) or down-regulation (48) of IL-2 and the IL-2R, up-regulation of the IL-18R (49), down-regulation of the IL-21R (50) and biphasic up- and down-regulation of IL-4R signaling (51). Overall, therefore, it is unclear from these in vitro observations what, if any, effect direct stimulation of T cells with IFN-$\alpha\beta$ has on the in vivo immune response.

In the present study, we have investigated the mechanisms by which IFN-$\alpha\beta$ stimulates cross-priming of $CD8^+$ T cell responses. Comparing responses in mice immunized by injection of a protein Ag alone or Ag + IFN-α, we find that IFN-α prolongs the expansion phase of $CD8^+$ T cells responding to cross-presented Ag, and that this is associated with increased T cell responsiveness to IL-2 and IL-15. However, neither IL-2 nor IL-15 was found to be required for IFN-α-induced cross-priming. By contrast, direct stimulation of T cells by IFN-α was shown to be essential for efficient induction of cross-priming.

Materials and Methods

Mice

C57BL/6 mice were purchased from Charles River Laboratories U.K. or from the specific pathogen-free unit at the Institute for Animal Health (Compton, U.K.). 129 SvEv (129), IFN-$\alpha\beta R^{-/-}$, OT-I, B6 CD45.1, H2-$Ab^{-/-}$, and $TCR\beta\delta^{-/-}$ mice were all purchased from the Institute for Animal Health. Bone marrow (BM) chimeras were produced by injecting 5×10^6 T cell-depleted BM cells i.v. into irradiated (900 cGy) recipients as indicated in the text. Chimeric mice were kept on antibiotic-containing drinking water for a month and were not used until at least 2 mo after BM transfer.

To generate mice with a conditional IFN-$\alpha\beta$R α-chain, IB10 embryonic stem cells were gene targeted such that exon 10 of the *IFNAR1* gene was loxP flanked. Upon Cre-mediated deletion of exon 10, a frameshift resulted in an open reading frame devoid of the transmembrane region and the cytoplasmic-signaling domain. Control experiments revealed complete IFN-$\alpha\beta$R inactivation upon exon 10 deletion (E. Kamphuis and U. Kalinke, manuscript in preparation). $IFNAR1^{flox/flox}$ mice generated from targeted embryonic stem cells and CD4-Cre mice (52) were backcrossed 10 times with C57BL/6 mice before both strains were intercrossed. Mice carrying the conditional *IFNAR1* locus homozygously and one *CD4-Cre* allele showed >95% efficient and T cell-specific IFNAR1 deletion as indicated by genetic and functional analysis (data not shown). Mice with a conditional *IFNAR1* gene were bred under specific pathogen-free conditions.

All animal experimentation was done with the approval of the U.K. Home Office and the Ethical Review Committee of the Institute for Animal Health.

Immunizations

One milligram of OVA (Sigma-Aldrich) was injected s.c. either in PBS alone or in PBS containing 10^5 U of IFN-α. Recombinant mouse IFN-α4 was produced by NSo mouse myeloma cells in serum-free medium as described (15). In IFN-α-treated mice, IFN-α was also injected 1 and 2 days after administration of Ag at the site of the primary injection, following a protocol previously shown to induce cross-priming (15).

Measurement of $CD8^+$ T cell response

Tetramer staining. PBMC isolated over a density gradient (Histopaque; Sigma-Aldrich) were incubated for 35 min at room temperature with allophycocyanin-conjugated K^b-SIINFEKL tetramers (Beckmann Coulter), washed, and stained with anti-CD8 FITC Ab (clone 53.6.7; BD Biosciences). Samples were analyzed by flow cytometry on a FACSCalibur (BD Biosciences) using CellQuest software (BD Biosciences).

In vivo CTL. Naive spleen cells were pulsed for 1 h with 1 nM SIINFEKL peptide at 37°C for 1 h. After extensive washing, cells were labeled with 0.1 μM CFSE ($CFSE^{low}$) (Molecular Probes). A control population of non-peptide-treated splenocytes was labeled with 1 μM CFSE ($CFSE^{high}$). $CFSE^{low}$ and $CFSE^{high}$ cells were mixed in a 1:1 ratio and 10^7 cells were injected i.v. into naive or immunized animals. Sixteen hours later, spleens were removed and single-cell suspensions were analyzed by flow cytometry to determine the ratio of $CFSE^{low}$ to $CFSE^{high}$ cells. The percentage of specific lysis was calculated as follows: percent-specific lysis = 100 − (100 × (% $CFSE^{low}$ immunized/% $CFSE^{high}$ immunized) − (% $CFSE^{low}$ control/% $CFSE^{high}$ control)).

Adoptive transfer of OT-I cells. $CD8^+$OT-I cells ($CD45.2^+$) were purified from lymph node (LN) cell suspensions by incubation with supernatant containing Abs specific for MHC class II (M5/114.15.2), B220 (RA6B2) and CD4 (GK1.5) followed by depletion of Ab-coated cells using sheep anti-mouse and sheep anti-rat Dynabeads (Dynal). The cells were routinely >98% $CD8^+$; 0.5×10^5 cells were transferred i.v. into $CD45.1^+$ congenic hosts.

BrdU labeling. LN cells were surface-stained with CD8 and CD45.2 and BrdU labeling assessed with anti-BrdU-FITC (BD Biosciences) as described elsewhere (53).

Duration of Ag presentation. Purified $CD8^+$ OT-I cells, labeled with 2.5 μM CFSE, were injected i.v. into B6 mice at different time points after recipients had been immunized by s.c. injection of OVA or OVA + IFN-α. The CFSE profile of the transferred cells was analyzed 3 days after transfer.

Analysis of cytokine receptor expression

FcRs on LN cells were blocked with anti-CD16/CD32 (clone 2.4G2), and cells were subsequently labeled with anti-CD8-allophycocyanin (clone 53.6.7) and anti-CD45.2-FITC (clone 104), plus either anti-CD25-PE (clone PC61), anti-CD122-PE (clone TMβ_1), or anti-CD132-PE (clone 4G3) (all Abs from BD Biosciences). To detect IL-15Rα, cells were incubated with biotinylated anti-IL-15Rα (R&D Systems) followed by anti-CD8-PE, anti-CD45.2-FITC and streptavidin-allophycocyanin (BD Biosciences).

Analysis of cytokine responsiveness

Five days after OT-I cell transfer and immunization, $CD8^+$ T cells were purified from draining LN and placed in wells of 96-well plates at 1×10^6/ml in the presence of various concentrations of recombinant mouse IL-2 or human IL-15 (R&D Systems). After 48 h of culture, cells were harvested, carefully counted, and labeled with anti-CD8 and anti-CD45.2 Abs. The samples were analyzed by FACS and the percent recovery calculated.

Semiquantitative IL-15 RT-PCR

Total RNA was extracted from draining LN cell suspensions from control or IFN-α-treated mice. To prepare cDNA, 5 μg of total RNA was incubated at 72°C for 10 min with 1 μg of oligo(dT)15 (Promega) in the presence of 40 U of RNasin (Promega). Reverse transcription was conducted using 20 U of avian myeloblastosis virus reverse transcriptase (Promega) for 90 min at 42°C in a final volume of 50 μl (50 mM Tris HCl, 50 mM KCL, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM DTT, 0.2 mM dNTPs (pH 8.3)). PCR was performed on 2 μl of each cDNA sample using 1.25 U of Thermoprime plus DNA polymerase (Advanced Biotechnologies) in a final volume of 50 μl containing 75 mM Tris, 20 mM ammonium persulfate, 0.1% Tween 20, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 200 nM sense primer, and 200 nM antisense primer (pH 8.8). The specific primer pairs used were as follows: IL-15, 5′-AAAACAGAGGCCAACTGGATAGAT GTA-3′ (sense) and 5′-CATGCAGTCAGGACGTGTTGATGAACAT-3′

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(antisense); β-actin, 5′-AGACGGGGTCACCCACACTGTGCCCATCTA (sense) and 5′-CTAGAAGCACTTGCGGTGCACGATGGAGGG-3′ (antisense). All primers were obtained from Sigma-Genosys. The samples were amplified for 35 cycles using the following conditions: 40 s denaturation at 94°C, 40 s annealing at 62°C, and 1-min extension at 72°C. Samples were further incubated at 72°C for 5 min. Amplified products were separated by agarose gel electrophoresis on a 1.2% Tris-acetate/EDTA gel and visualized using ethidium bromide staining and UV transillumination. The resulting gel image was then scanned and saved using Alpha Imager version 5.5 software. (Alpha Innotech). The relative density of each band was then determined using Quantity One 4.2.2 software (Bio-Rad).

In vivo blocking of IL-2

H2-Ab$^{-/-}$ mice were injected i.p. with 0.5 mg of purified anti-IL-2-neutralizing Ab (clone S4B6-1) or Chromopure rat IgG (Jackson ImmunoResearch Laboratories). The neutralizing activity of the anti-IL-2 Ab was confirmed by its ability to inhibit mouse IL-2-driven proliferation of an IL-2-dependent cell line (CTLL-2) in vitro. Ab treatment was given every other day, starting from the day of immunization with Ag, up to day 6.

Results

IFN-α extends the expansion phase of CD8$^+$ T cells responding to cross-presented Ag

As an approach to elucidate the mechanisms by which IFN-$\alpha\beta$ stimulates cross-priming, we sought to compare the properties of CD8$^+$ T cells that encounter Ag in the context of IFN-α injection with those of cells activated by injection of Ag alone. Previous studies of CD8$^+$ T cell responses elicited following cross-presentation of cell-associated Ags have shown that T cells undergo an "abortive" proliferation when triggered by Ag in the absence of DC-activating stimuli, which culminates in T cell tolerance (11, 54). Hence, it would be of interest to determine how CD8$^+$ T cells responding productively in the presence of IFN-α differ from cells that make an ultimately fruitless response.

We previously demonstrated that there was little if any systemic CD8$^+$ T cell response detectable in the spleen 8 days after s.c. immunization of mice with soluble OVA alone (15). Injection of OVA s.c. into normal B6 mice showed that this was also true when OVA-specific CD8$^+$ T cells were measured in the blood on day 8 using OVA 257–264 peptide (SIINFEKL)-H-2K^b tetramers (Fig. 1*A*), or when killing of SIINFEKL-pulsed target cells was assessed on day 9 using the in vivo CTL assay (Fig. 1*B*); this contrasted with the strong response observed after injection of OVA + IFN-α (Fig. 1, *A* and *B*). However, analysis of OVA-specific T cells at an earlier time point (day 5) in the draining LN revealed that an initial local response was elicited by injection of OVA alone. In fact, at day 5, similar numbers of OVA tetramer-staining cells were detected in the draining LN of mice injected with OVA or OVA + IFN-α (Fig. 1*C*). Subsequently, OVA-specific CD8$^+$ T cells declined in number in mice immunized with OVA alone, but continued to expand in mice given OVA + IFN-α.

The early response to OVA alone therefore allowed us to characterize CD8$^+$ T cells responding to Ag in the absence of IFN-α. Because the low number of peptide-specific cells present in normal mice makes analysis of these cells difficult, we used a TCR-transgenic T cell adoptive transfer model to increase Ag-specific cell frequencies. Purified CD8$^+$ T cells obtained from OT-I transgenic mice (CD45.2), which express a TCR specific for SIINFEKL-K^b (55), were injected into B6 CD45.1 mice, allowing detection of transgenic cells with CD45.2-specific Abs.

The premature decline in Ag-specific CD8 T cells after immunization with OVA alone suggests that there is accelerated death in this cell population compared with cells responding to OVA + IFN-α. However, reduced proliferation could also contribute to cell disappearance, tipping the dynamic balance between division and death toward a net cell loss. To determine whether proliferation rates differed for cells responding to OVA in the presence or absence of IFN-α, we gave recipients of OT-I cells a 4-h pulse of BrdU at different times after injection of OVA or OVA + IFN-α to label dividing cells. As shown in Fig. 2, OT-I cells were proliferating at equivalent rates 1 and 3 days after injection of OVA or OVA + IFN-α; close to 40% of cells incorporated BrdU into their DNA during the 4-h pulse on day 3, indicating extensive cell division. In contrast, a much lower percentage of OT-I cells was BrdU$^+$ in mice injected with OVA alone than in OVA + IFN-α-injected mice when pulsed at day 5 (6 vs 25%). These results indicated that an inability to sustain proliferation contributes to the abortive response of CD8 T cells recognizing cross-presented Ag in the absence of a licensing signal, and that IFN-α is able to prolong the proliferation phase of the response.

IFN-α does not prolong Ag presentation

The differential proliferation of responding CD8$^+$ T cells in OVA vs OVA + IFN-α-injected mice could be accounted for if there were a difference in the duration of Ag presentation under these two conditions. To address this possibility, we adoptively transferred CFSE-labeled OT-I cells into mice that had been injected at different times up to 5 days before with OVA or OVA + IFN-α. Division of OT-I cells, indicated by diminution of CFSE fluorescence at 3 days after adoptive transfer, was then used as an indicator of in vivo cross-presentation of the SIINFEKL peptide. Using this approach, we found that the proliferation of OT-I cells was very similar when transferred into recipients of OVA alone or OVA + IFN-α at all time points (Fig. 3), indicating that neither the magnitude nor the duration of peptide cross-presentation was substantially increased by injection of IFN-α. These data suggest that the abortive response of CD8 T cells in the absence of IFN-α is linked to a failure of these cells to receive secondary rather than primary (i.e., peptide-MHC) activation signals.

Injection of IFN-α enhances the responsiveness of Ag-specific CD8$^+$ T cells to IL-2 and IL-15

The inability of CD8$^+$ T cells primed with OVA alone to sustain expansion might reflect an inability of these cells to receive specific cytokine-mediated signals. Given the evidence that IL-2 and

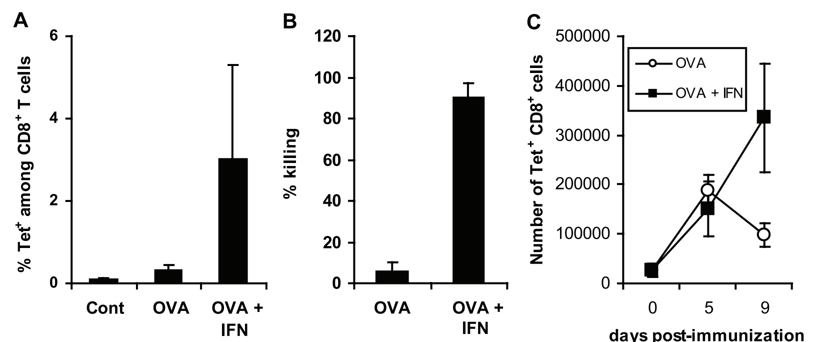

FIGURE 1. Stimulation of cross-priming by IFN-α. *A*, Percentage of CD8$^+$ T cells in the blood binding to SIINFEKL-K^b tetramers in unimmunized mice or in mice immunized 8 days before with OVA alone or OVA + IFN-α. *B*, Percent-specific killing of SIINFEKL-coated target cells in vivo, measured 9 days after immunization with OVA alone or OVA + IFN-α. *C*, Number of SIINFEKL-K^b tetramer-binding CD8$^+$ T cells in the draining LN at different times after immunization with OVA alone or OVA + IFN-α. Data show the mean ± SD for three mice per group, and are representative of results from at least two experiments in each case.

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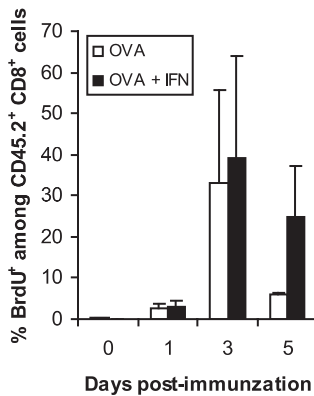

FIGURE 2. Prolonged proliferation of Ag-specific $CD8^+$ T cells when immunized mice are given IFN-α. $CD8^+$ T cells from OT-I mice (CD45.2) were adoptively transferred to B6 mice (CD45.1), and 1 day later recipients were immunized by injection of OVA or OVA + IFN-α. At the indicated time points, mice received an i.p. injection of 1 mg of BrdU. Mice were sacrificed 4 h later, and the percentage $BrdU^+$ cells among $CD45.2^+CD8^+$ T cells was determined. Data show the mean ± SD for three mice per group, and are representative of two independent experiments.

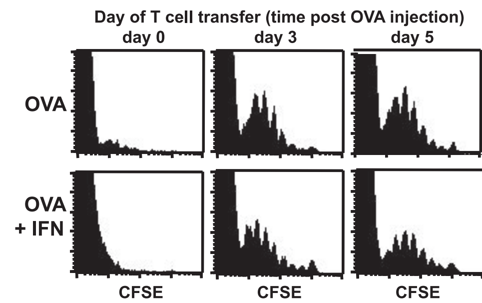

FIGURE 3. Similar cross-presentation of Ag with or without IFN-α injection. OVA was injected alone or with IFN-α into B6 mice. At the indicated time points after injection of Ag, mice received an i.v. injection of CFSE-labeled OT-I $CD8^+$ T cells. Three days after receiving OT-I cells, mice were sacrificed and CFSE labeling of cells in the draining LN was analyzed. Data are gated on total $CD8^+$ T cells and show representative staining profiles from one mouse of three in each group.

IL-15 contribute to the generation of $CD8^+$ T cell responses during certain viral infections (25–28), these cytokines seemed plausible candidates as downstream mediators of IFN-α during cross-priming. To evaluate potential differences in responsiveness to IL-2 and IL-15, the kinetics of expression of IL-2R and IL-15R subunits on the surface of OT-I cells in mice immunized with OVA or OVA + IFN-α was analyzed (Fig. 4*A*). Strikingly, much higher expression of IL- 2Rα (CD25) was observed on cells from OVA + IFN-α-injected mice, which reached maximal levels 3 days after immunization. The IL-15R α-chain was also substantially higher on cells responding in the presence of IFN-α, and similarly peaked on day 3. In addition, more subtle difference in IL-2/15Rβ (CD122) and common γ-chain (CD132) expression were apparent. CD122 was expressed at higher levels on cells from OVA + IFN-α-injected mice on days 5 and 8 postimmunization, while slightly greater CD132 expression by these cells was observed at day 1. Taken together, these results suggested that $CD8^+$ T cells primed in the context of IFN-α injection would be more responsive to IL-2 and IL-15 than cells activated by injection of Ag alone.

To address this possibility directly, $CD8^+$ T cells were purified from the draining LN of OT-I adoptive transfer recipients 5 days after immunization with OVA or OVA + IFN-α and placed in culture in medium alone or medium containing different concentrations of IL-2 or IL-15; naive OT-I $CD8^+$ T cells were also analyzed for comparison. The 5-day time point was chosen because it was after this time that responses diverged ± IFN-α (Fig. 1*C*). Responsiveness to cytokines was assessed by measuring the number of OT-I cells present after 2 days of in vitro culture.

The presence of IL-2 had no effect on the recovery of either naive OT-I cells or cells taken from mice immunized with OVA alone, both of which survived poorly in vitro (Fig. 4*B*). By contrast, IL-2 enhanced the recovery of OVA-specific cells obtained from OVA + IFN-α-injected mice in a dose-dependent manner. IL-2 clearly stimulated proliferation of these cells in vitro, as yields reached six times the number of cells initially plated (Fig. 4*B*). Cells from OVA + IFN-α-injected mice were also responsive to IL-15, which stimulated a dose-dependent increase in the yield of OT-I cells (Fig. 4*C*). However, IL-15 similarly enhanced the recovery of naive T cells and T cells obtained from mice injected with OVA alone; this is in keeping with previous findings that IL-15 can promote the survival of naive $CD8^+$ T cells and that the IL-15R α-chain may be dispensable for T cell responsiveness to IL-15 (35, 36, 56).

These results suggested that differential responsiveness of Ag-activated cells to IL-2 could contribute to the distinct behavior of $CD8^+$ T cells in OVA vs OVA + IFN-α-injected mice. In addition, although CD8 T cells in both types of mice responded to IL-15, IL-15 might play a role if the availability of this cytokine differed under the two conditions. This was worth considering since IFN-$\alpha\beta$ has been reported to up-regulate expression of IL-15 in DC and macrophages (33, 57). In keeping with this idea, s.c. injection of IFN-α into mice induced expression of IL-15 in the draining LN within 1 day (Fig. 4*D*). Therefore, higher concentrations of IL-15 are likely to be present at the site of T cell priming when mice are injected with OVA + IFN-α than with OVA alone.

IL-15 and IL-2 are not required for IFN-α-induced cross-priming

To assess the role of IL-15 and IL-2 in the induction of cross-priming by IFN-α, we determined how blocking the activity of these cytokines affected the generation of $CD8^+$ T cell responses after immunization. The contribution of IL-15 was investigated by comparing responses in wild-type (WT) B6 and IL-$15^{-/-}$ mice. These mice were injected with OVA or OVA + IFN-α, and in vivo CTL activity was measured 9 days later. As shown in Fig. 5*A*, IFN-α was able to induce OVA peptide-specific killing activity to a similar extent in both strains of mice, indicating that IL-15 was not required for IFN-α-induced cross-priming.

The importance of IL-2 in mediating the stimulatory effects of IFN-α was determined by treating OVA + IFN-α-injected mice with neutralizing anti-IL-2 Abs. Because IFN-α induces cross-priming by a CD4-independent mechanism (15), this experiment was conducted in CD4-deficient, H2-$Ab^{-/-}$ mice to eliminate any potential complications that could result from effects of neutralizing IL-2 on $CD4^+CD25^+$ regulatory T cells. Compared with mice treated with control Abs, no inhibition of OVA peptide-specific killing was evident when IL-2 was neutralized. Therefore, although immunization with OVA + IFN-α generates activated Ag-specific $CD8^+$ T cells that are responsive to IL-2 and IL-15, neither of these cytokines is required for IFN-α-induced cross-priming.

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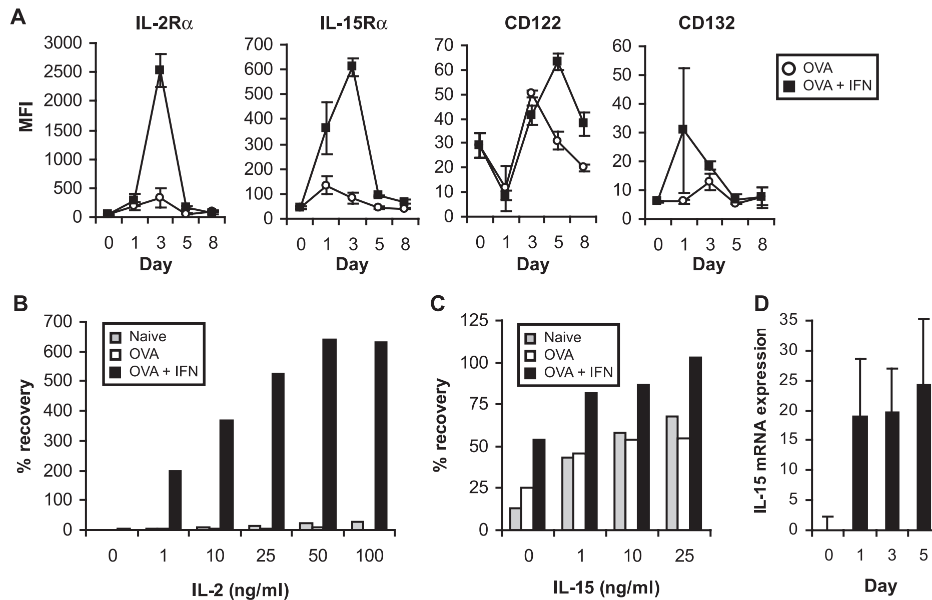

FIGURE 4. Increased responsiveness to IL-2 and IL-15 of $CD8^+$ T cells responding to Ag in the context of IFN-α injection. *A*, Recipients of OT-I cells were immunized by injection of OVA or OVA + IFN-α, and surface expression of IL-2 and IL-15 receptor subunits was measured on SIINFEKL-K^b tetramer-binding $CD8^+$ T cells at the indicated times. Data show mean ± SD for three mice per group. MFI, Mean fluorescence intensity. *B*, $CD8^+$ T cells were purified from the draining LN of recipients of OT-I cells that had been immunized 5 days before with OVA or OVA + IFN-α. These cells, as well as $CD8^+$ T cells from naive OT-I mice, were cultured in medium alone or medium containing the indicated concentration of IL-2 for 2 days. Data show the percent recovery of OT-I $CD8^+$ T cells at day 2 compared with the initial number plated. *C*, The effect of IL-15 on in vitro recovery of OT-I $CD8^+$ T cells was examined as described in *B* for IL-2. *D*, IL-15 mRNA expression was measured by RT-PCR analysis for total draining LN cells obtained from control mice or from mice at different times after injection of IFN-α. Data show the ratio of the intensities of bands corresponding to IL-15 vs β-actin, and are the mean ± SD for three mice per time point.

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Direct stimulation of T cells by IFN-α contributes to IFN-α-induced cross-priming

In our previous study, we showed that IFN-α was able to stimulate cross-priming of a $CD8^+$ T cell response when DC were the only cells directly responsive to IFN-αβ (15). However, the magnitude of the response observed under those conditions was ~10-fold lower than that in normal mice, detectable by IFN-γ ELISPOT but not by tetramer staining. One explanation for this was that DC are not the only direct target of IFN-α, and that optimal stimulation of the response requires IFN-α effects on other cells.

To determine whether T cells represent direct targets of IFN-α during the stimulation of cross-priming, we used two approaches to generate mice in which T cells were selectively deficient for expression of the IFN-αβR. In the first, we produced mixed bone marrow chimeras, reconstituting irradiated T cell-deficient TCRβ$\delta^{-/-}$ mice with a mixture of BM from TCRβ$\delta^{-/-}$ and IFN-αβR-deficient (IFN-αβ$R^{-/-}$) mice. In these chimeras (termed IFN-αβ$R^{-/-}$ T cell chimeras), all T cells were derived from IFN-αβ$R^{-/-}$ progenitors whereas other BM-derived cells were both IFN-αβ$R^{-/-}$ and IFN-αβ$R^{+/+}$. Control chimeras were made in a similar way, using a combination of TCRβ$\delta^{-/-}$ and 129 (WT) BM to reconstitute irradiated TCRβ$\delta^{-/-}$ mice. All cells in control chimeras were IFN-αβ$R^{+/+}$.

Both sets of chimeras were immunized by injection of OVA or OVA + IFN-α, and the response was assessed by measuring SIINFEKL-K^b-tetramer-binding cells in blood and in vivo killing of peptide-coated targets (8 and 9 days after immunization, respectively). As in normal mice, injection of OVA + IFN-α but not OVA alone effectively induced an OVA-specific $CD8^+$ T cell response in control chimeras (Fig. 6, *A* and *B*). In marked contrast, little if any response was detectable in IFN-αβ$R^{-/-}$ T cell chimeras immunized by injection of either OVA alone or OVA + IFN-α. These data indicated that direct stimulation of T cells by IFN-α contributes to the induction of cross-priming by this cytokine.

As a second approach to address this question, we used the Cre-loxP strategy (58) to selectively target IFN-αβR deficiency to T cells. Based on the loxP/Cre technology, mice were generated with a conditional *IFNAR1* gene (*IFNAR1$^{flox/flox}$*), which encodes one of the chains of the heterodimeric IFN-αβR, and these were

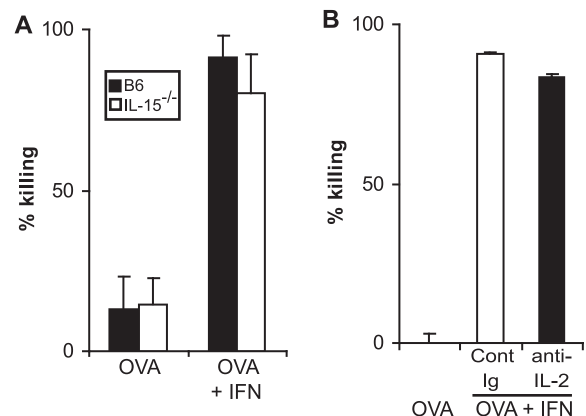

FIGURE 5. No requirement for IL-15 or IL-2 in IFN-α-induced cross-priming. *A*, B6 (■) or IL-$15^{-/-}$ mice (□) were immunized by injection of OVA or OVA + IFN-α, and the percent-specific killing of SIINFEKL-coated target cells in vivo was measured 9 days later. *B*, H2-$Ab^{-/-}$ mice were immunized by injection of OVA or OVA + IFN-α. OVA + IFN-α-injected mice were injected i.p. with 0.5 mg of anti-IL-2 or control Ab on days 0, +2, +4, and + 6 (relative to OVA injection). Cont, control. The percent-specific killing of SIINFEKL-coated target cells in vivo was measured 9 days after immunization. Data represent the mean ± SD for three mice per group.

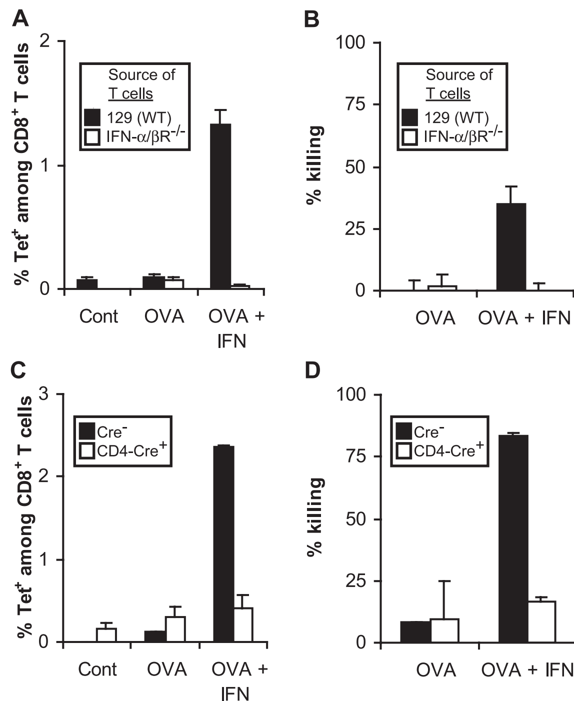

FIGURE 6. Direct stimulation of T cells by IFN-α contributes to IFN-α-induced cross-priming. *A* and *B*, Control chimeras (■) and IFN-$\alpha\beta$R$^{-/-}$ T cell chimeras (□) were immunized by injection of OVA or OVA + IFN-α. Cont, control. *C* and *D*, Cre^{-}IFNAR1$^{flox/flox}$ mice (■) and CD4-Cre^{+} IFNAR1$^{flox/flox}$ mice (□) were immunized by injection of OVA alone or OVA + IFN-α. The percentage of SIINFEKL-K^{b} tetramer-binding CD8^{+} T cells was measured 8 days after immunization (*A* and *C*), and the percent-specific killing of SIINFEKL-coated target cells in vivo was measured 9 days after immunization (*B* and *D*). Data represent the mean ± SD for three mice per group.

crossed to CD4-Cre transgenic mice (52). The regulatory sequences of the CD4 minigene direct expression of the Cre recombinase to double-positive thymocytes, resulting in deletion of the IFN-$\alpha\beta$R in all T cells (59). Normal numbers of T cells were generated in these mice, indicating that there is no requirement for IFN-$\alpha\beta$R signaling in T cell development; this is consistent with the normal lymphocyte production observed in IFN-$\alpha\beta$R$^{-/-}$ mice (data not shown).

CD4-Cre^{+}IFNAR1$^{flox/flox}$ mice and Cre-negative IFNAR1$^{flox/flox}$ mice were immunized by injection of OVA alone or OVA + IFN-α. As expected, injection of OVA + IFN-α into control (Cre^{-}IFNAR1$^{flox/flox}$) mice stimulated a strong OVA-specific CD8^{+} T cell response as measured by tetramers (Fig. 6*C*) or in vivo killing (Fig. 6*D*). Conversely, consistent with observations in IFN-$\alpha\beta$R$^{-/-}$ T cell chimeras, the ability of IFN-α to stimulate cross-priming was markedly reduced in CD4-Cre^{+}IFNAR1$^{flox/flox}$ mice. Therefore, these results provide strong evidence that T cells represent direct targets of IFN-α during the cross-priming of CD8^{+} T cell responses in vivo.

Discussion

Although it is well recognized that signals arising from the innate recognition of infection act to drive the generation of adaptive immune responses, much remains to be learned about the mechanisms involved. DC occupy a central role in the control of immune responses because of their ability to regulate the activation of naive T cells, and modulation of DC function by infection-associated stimuli represents an essential step in the link between innate and adaptive immunity. However, other cells that participate in immune responses are also capable of responding to direct and indirect markers of pathogen invasion. For example, both T and B cells express TLRs that allow them to detect various microbial components (60, 61). Therefore, it seems likely that sensing of infection by multiple immune cell types could influence the eventual development of immunity. The data presented here demonstrate that detection of IFN-$\alpha\beta$ by T cells acts as a stimulus for the CD8^{+} T cell response during cross-priming, and adds to previous evidence that IFN-$\alpha\beta$ acts as a licensing factor for DC (15). Thus, production of IFN-$\alpha\beta$ by the host serves as a signal for the induction of cross-priming by acting on multiple cell types.

Injection of IFN-α was shown to prolong the expansion phase of CD8^{+} T cells responding to cross-presented Ag. OVA peptide-specific CD8 T cells exhibited similar rates of cell division and expansion early (days 1–3) after immunization with OVA alone or OVA + IFN-α, but cells responding to Ag in the context of IFN-α injection proliferated faster at later time points. Whether this accounted fully for the observed differences in expansion is unknown; given that IFN-$\alpha\beta$ has been reported to reduce apoptosis of activated T cells in vitro (43, 44), a reduced death rate among responding T cells may also contribute (see below). The stimulatory effects of IFN-α on proliferation did not appear to be due to a prolongation of Ag presentation, because the kinetics of OVA peptide presentation in vivo was similar in OVA- and OVA + IFN-α-injected mice. These data indicate that although IFN-α licenses DC for cross-priming, it does not do so by promoting cross-presentation. Hence, although we have specifically studied cross-priming in this paper, it seems probable that direct stimulation of T cells by IFN-$\alpha\beta$ will also enhance CD8^{+} T cell responses against Ags processed through the endogenous MHC class I presentation pathway (i.e., direct priming); further studies will be required to assess this possibility.

The extension of the T cell expansion phase by injection of IFN-α was reminiscent of the reported effects of exogenous or host-produced IL-2 on the CD8^{+} T cell response (23, 28). In view of the fact that T cells responding to injection of OVA + IFN-α expressed very high levels of CD25, it therefore seemed likely that activation of the IL-2 pathway contributed to the adjuvant activity of IFN-β. Surprisingly, however, blocking the activity of IL-2 by injection of neutralizing Abs had no effect on the induction of cross-priming by IFN-α. Similarly, although IL-15R expression was increased on Ag-specific CD8^{+} T cells and IL-15 was expressed in the draining LN after IFN-α injection, normal enhancement of the response was observed in IL-15$^{-/-}$ mice. Thus, despite the responsiveness of Ag-specific T cells to IL-2 and IL-15, neither of these cytokines was required for IFN-α-induced cross-priming. The independence of the CD8 response from IL-2- and IL-15-mediated effects agrees with the results of some previous studies but conflicts with others. Notably, lymphocytic choriomeningitis virus infection has been reported to induce normal primary CD8^{+} T cell responses in the absence of either IL-2 or IL-15 (30, 32). This virus elicits the production of high concentrations of IFN-$\alpha\beta$ in vivo, and has been shown to stimulate cross-priming through an IFN-$\alpha\beta$-dependent mechanism (15). Therefore, induction of high levels of IFN-$\alpha\beta$ could be a key factor that allows for the CD8 response to occur independently of IL-2 and IL-15; whether this relates to direct effects of IFN-$\alpha\beta$ on T cells or other indirect mechanisms remains to be determined.

CD8^{+} rather than CD4^{+} T cells are likely to be the direct targets of IFN-α stimulation, because IFN-α-induced cross-priming occurs equally well in CD4^{+} T cell-deficient and WT mice (Fig. 5 and Ref. 15). Although effects on non-Ag-specific CD8^{+} T cells could in theory play a role (for example, by inducing secretion of cytokines), it seems probable that IFN-α mediates its stimulatory

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activity by directly triggering Ag-specific cells. One way in which it may do so is by inhibiting apoptosis. Thus, IFN-$\alpha\beta$ has been shown to enhance the survival of activated mouse and human T cells in vitro (43, 44), although the mechanisms by which it does so are unclear. For mouse T cells, rescue from cell death by IFN-$\alpha\beta$ does not appear to be associated with up-regulation of Bcl-2 or Bcl-x_L, antiapoptotic molecules typically associated with the promotion of cell survival by IL-2 family cytokines (43). We similarly found no difference in Bcl-2 expression by $CD8^+$ T cells responding to OVA vs OVA + IFN-α (data not shown). Conversely, human T cells rescued by IFN-$\alpha\beta$ have been shown to express high levels of Bcl-x_L (44). In addition, treatment of activated human T cells with IFN-α has been reported to induce expression of pim-1, a serine/threonine kinase that can accelerate lymphoid cell proliferation by enhancing cell survival (47); whether pim-1 is also induced by IFN-α in activated mouse T cells is unknown. Whatever the mechanism, protection of activated T cells from apoptosis could allow for more productive expansion of Ag-activated cells.

The results of this study show that stimulation of T cells by IFN-$\alpha\beta$ greatly enhances the cross-priming of $CD8^+$ T cell responses. Cross-priming during viral infections may aid in T cell priming by allowing noninfected DC to present viral Ags from internalized infected cells or viral particles. Because IFN-$\alpha\beta$ is commonly expressed after viral infection, IFN-$\alpha\beta$R signaling in T cells is likely to be relevant to the induction of cross-priming during infections as well as when IFN-α is used as an adjuvant. In this respect, it is worth noting that the IFN-α injection protocol used in this study leads to systemic IFN-$\alpha\beta$ concentrations that are similar to those observed after lymphocytic choriomeningitis infection (15). Interestingly, $CD8\alpha^+$ DC, which are responsible for initiating cross-priming, appear to be the most effective producers of IFN-$\alpha\beta$ among conventional DC (62). Moreover, uptake of infected cells by $CD8\alpha^+$ DC stimulates IFN-$\alpha\beta$ expression through a TLR3-dependent mechanism (63). Hence, in addition to cross-presented Ag and other costimulatory signals, $CD8\alpha^+$ DC might provide IFN-$\alpha\beta$ to $CD8^+$ T cells during the induction of cross-priming. This could lead to a high local concentration of IFN-$\alpha\beta$ at the site of interaction between the T cell and an IFN-$\alpha\beta$-expressing DC. Conversely, $CD8^+$ T cells could respond to systemic IFN-$\alpha\beta$ produced by other cells. In any case, our results indicate that IFN-$\alpha\beta$ links the innate response to infection with adaptive immunity not only through effects on DC but also by direct stimulation of T cells.

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Disclosures

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