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Type I interferon signaling facilitates the development of IL-10-producing effector CD8+ T cells during influenza virus infection

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Abstract

Recent evidence has suggested that IL-10-producing effector CD8$^+$ T cells play an important role in regulating excessive inflammation during acute viral infections. However, the cellular and molecular cues regulating the development of IL-10-producing effector CD8$^+$ T cells are not completely defined. Here we show that type I interferons (IFNs) are required for the development of IL-10-producing effector CD8$^+$ T cells during influenza virus infection. We find that type I IFNs can enhance IL-27 production by lung antigen presenting cells, thereby facilitating IL-10-producing CD8$^+$ T cell development through a CD8$^+$ T cell non-autonomous way. Surprisingly, we also demonstrate that direct type I IFN signaling in CD8$^+$ T cells is required for the maximal generation of IL-10-producing CD8$^+$ T cells. Type I IFN signaling in CD8$^+$ T cells, in cooperation with IL-27 and IL-2 signaling, promotes and sustains the expression of IRF4 and Blimp-1, two transcription factors required for the production of IL-10 by effector CD8$^+$ T cells. Our data have revealed a critical role of the innate antiviral effector cytokines in regulating the production of a regulatory cytokine by effector CD8$^+$ T cells during respiratory virus infection. The potential implications of these findings for influenza virus infection are also discussed.

Introduction

CD8$^+$ T cells are the key to clear infectious virus, intracellular bacteria and transformed cells. Upon antigenic encountering in the secondary lymphoid organs, naïve CD8$^+$ T cells undergo stepwise stages of responses including activation, expansion and differentiation into effector CD8$^+$ T cells (i.e. cytotoxic T lymphocytes) (1, 2). Effector CD8$^+$ T cells acquire tissue tropic chemotactic receptors such as CCR5 and CXCR3 while simultaneously downregulate lymphoid tissue homing receptors such as CCR7 (1, 2), which allow them to

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infiltrate peripheral tissues to clear pathogen-infected or transformed cells. In the tissue, effector CD8$^+$ T cells use multiple mechanisms to combat invading enemies. Most prominently, effector CD8$^+$ T cells express key cytolytic molecules including granzymes, perforin and death receptor ligands and are able to clear virus infected cells or transformed cells through contact dependent mechanisms. In addition, effector CD8$^+$ T cells also express multiple anti-viral and anti-tumor pro-inflammatory cytokines, such as IFN-γ and TNF-α, to inhibit viral replication and kill tumor cells. Notably, these direct and indirect antiviral and tumor activities of effector CD8$^+$ T cells, if unchecked, also can cause severe inflammation and tissue destruction (1, 3–5).

Recently, we and others have demonstrated that effector CD8$^+$ T cells are able to produce the regulatory cytokine IL-10 in the lung and brain following acute viral infections (6–11). The production of IL-10 by effector CD8$^+$ T cells is important in counterbalancing exuberant inflammation as the blockade of IL-10 following infection has resulted in exaggerated inflammation and severe host diseases during influenza, respiratory syncytial virus (RSV) and coronavirus infections (7–12). Furthermore, we showed that IL-2 co-operates with innate cell-derived IL-27 to up-regulate IL-10 production by CD8$^+$ T cells specifically in the lung through the transcription factor Blimp-1-dependent mechanisms (6). However, the current understanding on the cellular and molecular mechanisms regulating the development of IL-10-producing effector CD8$^+$ T cells remain incompletely defined.

Type I IFNs are major anti-viral effector cytokines that have critical roles in shaping both innate and adaptive defense against viral infections. Initially discovered as the proinflammatory "signal 3" cytokines, type I IFNs were shown to promote CD8$^+$ T cell expansion and effector differentiation during bacterial and viral infections (13, 14). Moreover, type I IFN signaling in CD8$^+$ T cells can protect CD8$^+$ T cell from NK cell-mediated deletion during chronic virus infection (15, 16). Besides their role in enhancing anti-viral immune responses, type I IFNs signals also promotes IL-10 and PD-L1 production/expression and suppress effective CTL responses during chronic LCMV infection (17, 18), suggesting that type I IFN signaling possesses immune-regulatory functions. Similarly, recent evidence has demonstrated that type I IFNs are required for IL-10 production during influenza infection (19, 20). Arimori et al showed that IFNAR1-deficient mice exhibited diminished IL-10 levels in the lung, enhanced pro-inflammatory cytokine production and increased host mortality following influenza virus infection (20). However, how type I IFN signaling promotes IL-10 production during influenza infection is currently unknown. Furthermore, given that T cells, particularly effector CD8$^+$ T cells, are a major cellular source of IL-10 during influenza infection, it is important to determine whether and how type I IFN signaling affects IL-10 production of T cells.

In this report, we have examined the role of type I IFN signaling in the induction of IL-10-producing T cells during influenza infection. We found that type I IFN signaling is critically important in driving IL-10 production by effector CD8$^+$ T cells, but only plays modest role in promoting IL-10 production by CD4 T cells during influenza infection. We found that type I IFN signaling promotes IL-27 production by APCs to indirectly facilitate CD8$^+$ T cell IL-10 production in a CD8$^+$ T cell non-autonomous fashion. Interestingly, we found that direct type I IFN signaling in CD8$^+$ T cells is also critical for the maximal generation of

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IL-10-producing effector CD8+ T cells during influenza infection. Mechanistically, type I IFN signaling, in cooperation with IL-27 and IL-2 signaling, promotes and sustains the expression of Blimp-1 and IRF4, two transcription factors required for the production of IL-10 by effector CD8+ T cells. Our data have revealed the underlying molecular mechanisms by which innate antiviral effector cytokines regulates the production of a regulatory cytokine by effector CD8+ T cells during respiratory virus infection.

Materials and Methods

Mouse and infection

WT C57/BL6 mice were purchased from the Jackson Laboratory, Blimp-1 control (*Prdm1*$^{fl/fl}$), CD4-Cre transgenic, CD8-cre transgenic, IRF4 control (*Irf4*$^{fl/fl}$) and CD45.1 congenic mice were originally from the Jackson Laboratory and bred in house. T cell-specific Blimp-1 conditional knockout (cKO) mice were generated through breeding CD4-cre to *Prdm1*$^{fl/fl}$ mice. CD8 T cell-specific IRF4 cKO mice were generated through breeding CD8-cre to *Irf4*$^{fl/fl}$ mice. IFNAR1-deficient (*Ifnar1*$^{-/-}$) mice were originally from Dr. U. Deshmukh at the University of Virginia; Vert-X mice were originally from Dr. C. Karp from Cincinnati Children's Hospital; STAT2-deficient (*Stat2*$^{-/-}$) mice were originally from Dr. C. Schindler at the Columbia University. All mice were housed in a specific pathogen-free environment and all animal experiments were performed in accordance with protocols approved by the University of Virginia Animal Care and Use Committee (ACUC) or Indiana University Institutional Animal Care and Use Committee (IACUC). For influenza virus infection, mice were anesthetized first and then intranasally infected with Influenza A/PR8/34 strain (~150 pfu/mouse in serum-free IMDM media (Gibco) or PBS) as previously reported (8).

Quantitative RT-PCR

Lung single cell suspensions were prepared as previously described (6, 21). T cells were purified through positive selection via MACS-beads (Miltenyi Biotech) from pooled 2 – 3 lungs per group. mRNA from in vivo purified cells or in vitro cultured cells as indicated was isolated with RNeasy kit (Qiagen) or total RNA isolation kit (Sigma Aldrich) and treated with DNase I (Invitrogen). Random primers (Invitrogen) and Superscript II (Invitrogen) were used to synthesize first-strand cDNAs from equivalent amounts of RNA from each sample. RT-PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems). Data were generated with the comparative threshold cycle (Delta CT) method by normalizing to hypoxanthine phosphoribosyltransferase (HPRT). Sequences of primers used in the studies are available on request.

In vitro DC/T cell co-culture

BMDC were generated as described (22). CD8+ T cells were isolated from spleen and lymph nodes of indicated mice through MACS-beads (Miltenyi Biotech). BMDC and T cell co-culture was performed as previously described (6). Briefly, we mixed DC with CD8+ T cells at the ratio of 1 DC : 10 T cells in round-bottom 96 wells (5×10^4 T cells/well) in the presence of 0.1 μg/ml α-CD3. The conditions of the culture are indicated in the text. For RNA isolation and RT-PCR, T cells were harvested at day 3 of culture. For IFN-γ and IL-10

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staining, T cells were harvested at day 4 of the culture and restimulated with PMA (100 ng/ml, Sigma) or inomycin (1 μg/ml, Sigma) in the presence of Golgi-Stop (1 μl/ml, BD Biosciences) for 4 hours. Intracellular cytokine staining (ICS) was performed as we previously reported (6). To measure T cell cytokine production by ELISA, day 4 cultured T cells were restimulated with plate bound α-CD3 (1 μg/ml) for overnight. Supernatants were then measured by ELISA as previously described (6). The concentrations of the cytokines used in the culture are as following: recombinant human IL-2 (300 U/ml); mouse IL-27 (Biolegend), 10 ng/ml and IFN-αA (R&D) 250 U/ml respectively.

Antigenic stimulation of lung effector T cells

On day 6–7 of WT BMDC culture, BMDC were harvested and infected with influenza virus at approximated 100 M.O.I. for 6 h. Then BMDC were counted and mixed with total lung cells isolated from WT and transgenic mice as indicated in the text at a 1.5 to 1 ratio in the presence of Golgi-Stop (BD Biosciences, 1μl/ml) and hIL-2 (40 U/ml) for additional 6 h. The surface staining of cell surface markers, intracellular staining of cytokines was performed as described (8).

Generation of mixed bone marrow chimeras

To generate mixed bone marrow chimera, we lethally irradiated (1100 Rads) WT mice and then i.v. injected ~ 4 million 1:1 mixed BM cells from WT ($CD45.1^+$) or $CD45.2^+$ IFNAR1-deficient BM. After 12 week reconstitution, the chimeric mice were then infected with influenza A/PR8.

Broncho-alveolar lavage (BALF) cytokine determination

BALF was obtained by flushing the airway 3 – 4 times with a single use of 600 μl sterile PBS as previous described (8). Cells in BALF were spun down and supernatants were collected for multi-plex analysis (Millipore) according to the manufacturer manuals.

Cell sorting

Lung single cell suspensions were prepared from uninfected or infected mice (day 5 p.i.). Cells were stained with CD45, F4/80, CD11C, MHCII, CD11b and Ly6G. Lung eipthelia/stroma cells ($CD45^-$), Neutrophils ($CD11b^+$ $Ly6G^+$), DCs ($CD11c^{hi}$ $MHCII^{hi}$ $Ly6G^-$) and macrophages ($F4/80^+$ $MHCII^+$ $Ly6G^-$) were sorted based on their surface marker expression described above.

FACS analysis

All FACS Abs were purchased from Biolegend, BD Biosciences or eBioscience. Cells were acquired through FACS-Calibur, FACS-Canto or LSR II (BD Biosciences). Data were then analyzed by FlowJo software (Treestar).

Statistical analysis

Data are mean ± s.e.m. Paired or unpaired two-tailed Student's t-test was used. *P* values < 0.05 is considered significant.

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Online supplementary figures

Four supplementary figures can be found with this article online.

Results

IFNAR1 and STAT2 are required for the development of IL-10-producing effector CD8+ T cells during influenza infection

To determine the role of type I IFNs in the development of IL-10-producing effector CD8+ T cells, we infected WT and IFNAR1-deficient mice with influenza. At day 7 post infection (at the time when maximal IL-10 was made in vivo (6, 8), we stimulated lung T cells with influenza-infected WT bone marrow-derived dendritic cells (BMDCs) and measured IL-10 and IFN-γ production by T cells following stimulation as we previously reported (6, 8). We found that IFNAR1 deficiency modestly affected IFN-γ production by CD8+ T cells, but dramatically diminished IL-10 production by CD8+ T cells (Figure 1A). We then normalized IL-10+ CD8+ T cells to IFN-γ+ CD8+ T cells to determine the relative abundance of IL-10-producing cells in IFN-γ producing antigen specific CD8 T cells. We found that IFNAR1 deficiency significantly abrogated IL-10-producing CD8+ T cells within the antigen-specific CD8+ T cell population (Figure 1B). IFNAR1 deficiency also decreased the per cell production of IL-10 by effector CD8+ T cells as the mean fluorescence intensity (MFI) of IL-10 in IL-10+ cells diminished in the absence of IFNAR1 (Figure S1A). In addition, IFNAR1 deficiency diminished IL-10-producing CD4+ T cells (Figure 1C), although the relative abundance of IL-10-producing CD4+ T cells in antigen-specific IFN-γ+ CD4 T cells only modestly diminished in the absence of IFNAR1 (Figure 1D). Consistent with their diminished levels of IL-10-producing CD8+ and CD4+ T cells, IFNAR1-deficient mice exhibited diminished airway IL-10 levels and increased airway IFN-γ levels in vivo during influenza infection (Figure 1E and F), consistent with the recent findings that type I IFN signaling is required for IL-10 but not IFN-γ production in vivo during influenza infection (19, 20). Following type I IFNs engagement, IFNAR initiates downstream events to form a signaling complex consisting of STAT1/STAT2/IRF9, which mediate the major functions of type I IFNs (23, 24). We therefore examined whether STAT2 is required for the generation of IL-10-producing CD8+ T cells in vivo during influenza infection. We found that STAT2 deficiency significantly diminished IL-10-producing CD8+ T cells in the lung (Figure 1G, H). STAT2 deficiency also significantly decreased IL-10 MFI in IL-10+ CD8+ effector T cells (Figure S1B). Taken together, these data suggested that STAT2-dependent IFNAR1 signaling is required for the generation of IL-10-producing effector CD8+ T cells during influenza infection.

To further support this idea, we crossed IFNAR1-deficient mice to IL-10-IRES-eGFP reporter mice (Vert-X), as these mice faithfully report IL-10 expression by T and other cells without the need of in vitro restimulation (25). We infected IFNAR1-deficient Vert-X mice with influenza and then examined in vivo IL-10 production through the reporter eGFP expression on T cells at day 7 post infection. We found that, compared to those of IFNAR1-sufficient Vert-X mice, CD8+ T cells from IFNAR1-deficient mice exhibited diminished percentages of IL-10/eGFP+ cells (Figure 2A, B) and a lower per cell levels of IL-10/eGFP (Figure 2C), consistent with the idea that IFNAR1 signaling is required for the potent

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induction of IL-10-producing CD8+ T cells. We also examined IL-10/eGFP expression by influenza-specific PA224 tetramer+ CD8+ T cells and found that the deficiency of IFNAR1 resulted in decreased percentages of IL-10/eGFP+ cells within PA224+ CD8+ T cell population and diminished IL-10/eGFP MFI (Figure 2D – F). The deficiency of IFNAR1 did not significantly decrease IL-10/eGFP expression by CD4 T cells (Figure 2G, H). Taken together, these data suggested that IFNAR1 signaling is preferentially required for the development of IL-10 expressing CD8+ T cells.

Type I IFN signaling is required for the maximal production of IL-27

We recently have demonstrated that IL-27 is vital for the development of IL-10-producing effector CD8+ T cells during influenza infection. Type I IFNs have been shown to stimulate IL-27 production by antigen presenting cells (26). We found that antigen presenting cells (APCs, including macrophages, DCs and neutrophils) are the main sources of IL-27 expression (particularly p28 subunit) in the lung at day 5 post influenza infection, at the time when effector T cells begin to migrate to the lungs and upregulate IL-10 expression there (Figure 3A). Consistent with the previous findings, we found that type I IFNs were able to induce the expression of IL-27 in dendritic cells, macrophages and neutrophils (Figure S2), the main antigen presenting cell types in the infected lungs when CD8+ T cells start to produce IL-10 (27, 28). Therefore, we reasoned that type I IFNs induce IL-27 production, thereby supporting IL-10 production by CD8+ T cells. To address this hypothesis, we examined the expression of IL-27 subunits in the draining mediastinal lymph nodes (MLN) and the lungs (6, 8). We found that, compared to those of WT mice, Ebi3 and p28 expression in the lung of IFNAR1-deficient mice was significantly decreased (Figure 3B). Furthermore, airway IL-27 p28 protein levels were also diminished in IFNAR1-deficient mice following influenza infection (Figure 3C). Together, these data suggest that type I IFN signaling is required for the optimal production of the IL-10 inducing cytokine, IL-27, during influenza infection.

Cell-intrinsic IFNAR1 signaling is required for the maximal development of IL-10-producing effector CD8+ T cells

Type I IFNs are important “signal 3” cytokines that can directly drive CD8+ T cell differentiation (13, 14). We next want to examine whether direct type I IFN signaling in CD8+ T cells may influence IL-10 production beyond their cell non-autonomous effects in promoting IL-10 production by CD8+ T cells through the induction of IL-27 production. To do so, we lethally irradiated WT mice and then reconstituted the mice with 1:1 mixed CD45 mis-matched WT and IFNAR1-deficient bone marrow cells (Figure 4A). 12 weeks after reconstitution, we infected the chimeric mice with influenza and found that the T cell compartments in the lungs of the infected mice were successfully reconstituted with both WT or *Ifnar1*−/− cells (Figure S3A). We then checked IL-10 and IFN-γ production following in vitro restimulation of T cells. We found that IL-10 production by IFNAR1-deficient CD8+ T cells is significantly lower than that of WT CD8+ T cells even within the same hosts (Figure 4B, C). Furthermore, the IL-10 MFI is also significantly lower in IFNAR1-deficient CD8+ T cells than that of WT CD8+ T cells (Figure 4D). In contrast, IL-10 production by CD4+ T cells is comparable between WT and IFNAR1 deficient CD4+ T cells (Figure 4E and Figure S3B). Taken together, these data suggest that CD8+ T cell-autonomous type I

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IFN signaling is also essential for the maximal development of IL-10-producing effector $CD8^+$ T cells, but not IL-10-producing $CD4^+$ T cells.

Type I IFNs further promote IL-10 production by CD8+ T cells in the presence of IL-2 plus IL-27

To begin to examine the underlying cellular and molecular mechanisms by which type I IFN signaling promotes IL-10 production by $CD8^+$ T cells, we employed our previously reported $CD8^+$ T cell culture system (6). To this end, we cultured WT $CD8^+$ T cells in the absence or presence of IFN-α, IL-2 and IL-27 (IL-2/IL-27), or IFN-α plus IL-2/27 (IFN-α/IL-2/IL-27). We found that the inclusion of IFN-α alone in the culture minimally induced the development of IL-10-producing $CD8^+$ T cells in vitro (Figure 5A). However, IFN-α helped the development of IL-10-producing $CD8^+$ T cells in the presence of IL-2 and IL-27 (Figure 5A, B), which were reported to induce IL-10-producing effector $CD8^+$ T cells (6). IFN-α also promoted IL-10 mRNA expression in $CD8^+$ T cells in the presence of IL-2 and IL-27 (Figure 5C). In addition, although IFN-α alone minimally induced IL-10 secretion by effector $CD8^+$ T cells, it greatly enhanced IL-10 secretion by $CD8^+$ T cells in the presence of IL-2 plus IL-27 (Figure 5D). Taken together, these data suggested that type I IFNs co-operate with IL-2 and IL-27 to promote IL-10 production by $CD8^+$ T cells.

Blimp-1 and IRF4 are required for the development of IL-10-producing effector CD8⁺ T cells induced by type I IFNs plus IL-2 and IL-27

We next wished to determine the molecular mechanisms by which type I IFNs co-operate with IL-2 and IL-27 to promote the development of IL-10-producing $CD8^+$ T cells. We have shown previously that Blimp-1 is required for the development of IL-10-producing $CD8^+$ T cells in vitro and in vivo during influenza infection (6). We now observed that type I IFNs cooperated with IL-2 and IL-27 to induce the expression of Blimp-1 in $CD8^+$ T cells (Figure 6A, B). To determine whether Blimp-1 is required for the induction of IL-10-producing $CD8^+$ T cells in vitro, we cultured WT or Blimp-1-deficient $CD8^+$ T cells in the absence or presence of IFN-α, IL-2/IL-27 or IFN-α/IL-2/IL-27. We found that while IFN-α/IL-2/IL-27 induced abundant IL-10-producing $CD8^+$ T cells within WT $CD8^+$ T cells, these cytokine failed to stimulate IL-10 production by Blimp-1-deficient $CD8^+$ T cells (Figure 6C). Furthermore, Blimp-1 was also required for the secretion of IL-10 by $CD8^+$ T cells (Figure 6D). Thus, these data suggest that Blimp-1 is required for the production of IL-10 by $CD8^+$ T cells upon type I IFN signaling, consistent with the our previous findings that Blimp-1 is essential for the development of IL-10-producing $CD8^+$ T cells in vivo during influenza infection (6). We recently reported that IRF4 controls Blimp-1 expression in $CD8^+$ T cells (29). IRF4 was also shown to regulate IL-10 production by effector $CD4^+$ T cells and $Foxp3^+$ regulatory $CD4^+$ T cells (30, 31). We therefore examined whether type I IFNs signaling promoted IRF4 expression. We found that IFN-α was able to co-operate with IL-2/IL-27 to promote and sustain IRF4 expression in $CD8^+$ T cells (Figure 6E and Figure S4). Furthermore, we found that, following the treatment of IFN-α/IL-2/IL-27, IRF4 expression in $CD8^+$ T cells was required for the production of IL-10 by $CD8^+$ T cells (Figure 6F, G). To determine whether IRF4 is required for the induction of IL-10-producing $CD8^+$ T cells in vivo, we infected $CD8^+$ T cell-specific IRF4-deficient mice (IRF4 cKO) with influenza and examined IL-10 and IFN-γ production following in vitro restimulation. Consistent with our

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previous data (29), selective IRF4-deficiency in CD8$^+$ T cells diminished IFN-γ production by CD8$^+$ T cells, suggesting IRF4 is required for the development of anti-viral effector CD8$^+$ T cells (Figure 6H). When IL-10-producing CD8$^+$ T cells were normalized to IFN-γ producing effector CD8$^+$ T cell population, we found that IL-10 production was diminished in effector CD8$^+$ T cells in the absence of IRF4 (Figure 6I), suggesting that IRF4 is vital for the production of IL-10 by effector CD8$^+$ T cells in vivo.

Discussion

In this report, we have investigated the role of type I IFN signaling in regulating the development of IL-10-producing effector CD8$^+$ T cells during influenza infection. In accordance with previous observations (20), we found that IFNAR1-deficient mice exhibited diminished IL-10 production during influenza virus infection. We further showed that the effect of IFNAR1 deficiency on IL-10 production was more pronounced on effector CD8$^+$ T cells rather than effector CD4$^+$ T cells. We have also defined the downstream cellular and molecular mechanisms by which type I IFNs control the development of IL-10-producing effector CD8$^+$ T cells during influenza virus infection. We found that both indirect and direct effects of type I IFN signaling in CD8$^+$ T cells were required for the optimal induction of IL-10-producing CD8$^+$ T cells during influenza infection (Figure 6I).

Type I IFNs are major antiviral effector cytokines that play important roles in restricting the replication and dissemination of respiratory viruses in vitro and in vivo (24). Besides their direct anti-viral functions, type I IFNs are well known to regulate the development of innate and adaptive immunity during respiratory virus infections (32, 33). Early type I IFN signaling was important in the production of inflammatory cytokines during influenza infection (34, 35). Furthermore, type I IFN signaling plays a critical role in regulating the development anti-viral CD4$^+$ and CD8$^+$ cytotoxic T lymphocytes during influenza infection (21). Consistent with their role in promoting inflammation and immunity, overproduction of type I IFNs contributed significantly to the pathogenesis of influenza virus and mouse adapted coronavirus infection (36–38). On the other hand, our data here and results from Arimori et al have also suggested that type I IFNs could have regulatory role by restricting exuberant inflammatory responses through the induction of the anti-inflammatory cytokine IL-10 (20). Indeed, IFNAR1-deficient mice showed an exuberant host inflammatory response and IL-10 administration could ameliorate excessive lethal inflammation and associated mortality following influenza infection in IFNAR1-deficient mice (20). Such a regulatory role of type I IFN signaling in inflammation and immunity has been documented during chronic viral infections (17, 18). Thus, type I IFNs could display differing roles in regulating the development of inflammatory responses and immunity following viral infections, and their exact function in regulating inflammation and immunity would be dependent on the infectious organism as well as the timing and levels of the production of these pluripotent anti-viral cytokines.

In a similar way, IL-10 exerts its function in a timing-dependent fashion during influenza infection. Early production of IL-10 during influenza infection could hamper the activation of antiviral innate and adaptive immunity as evidenced by the enhanced resistance to influenza virus infection in IL-10-deficient mice (39–41). Conversely, the production of

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IL-10 at the time of effector T cell infiltration into the lung (i.e. day 5 and thereafter) could suppress the development of lethal pulmonary inflammation during influenza infection (8, 41). Similar beneficial effects of T-cell derived IL-10 have also been reported during respiratory syncytial virus and coronavirus infection (7, 9–11). Currently, the role of type I IFNs in regulating IL-10 production by human CD8$^+$ T cells has not been investigated. However, a recent clinical study has established that both type I IFNs and IL-10 were elevated in patients with severe influenza virus infection (42). These data suggested that type I IFNs might facilitate IL-10 production by effector T cells during human influenza infection. Whether IL-10 production is beneficial to the host by keeping inflammation under control or is detrimental to the host by suppressing anti-viral immunity in these patients warrants further investigation. However, understanding the timing and dose-dependent regulating of pulmonary inflammation and immunity by type I IFNs and IL-10 will be insightful for developing novel therapeutic strategies aiming to dampen excessive respiratory inflammation and diseases during severe influenza infection.

Type I IFNs have been shown to promote macrophage IL-10 production through the induction of the cytokine IL-27 (26). Similarly, we found that type I IFNs could stimulate IL-27 expression in major lung antigen presenting cells during influenza virus infection, including macrophages, dendritic cells and neutrophils. Furthermore, IL-27 levels were significantly diminished in the respiratory tract in the absence of type I IFN signaling. Thus, type I IFNs could promote IL-10 production by CD8$^+$ T cells through its indirect effects on regulating IL-27 levels in vivo. Surprisingly, through the mixed bone marrow chimera experiments, we have also established that direct type I IFN signaling is important in IL-10 production by CD8$^+$ T cells during influenza infection. Currently, the relative contribution of the indirect and direct effects of type I IFN signaling in CD8$^+$ T cells in promoting IL-10 production remain to be determined. Nevertheless, type I IFN signaling substantially enhances IL-10 by CD8$^+$ T cells in vitro in the presence of IL-2 and IL-27. At present, the mechanism(s) by which type I IFNs co-operate with IL-2 and IL-27 to promote IL-10 production is unknown. Type I IFN signaling has been shown to sustain the expression of high affinity IL-2 receptor on CD8$^+$ T cell surface (43). So it is possible that type I IFN signaling promotes IL-10 production by facilitating IL-2 and/or IL-27 signaling in CD8$^+$ T cells. Conversely, it is possible that IL-2 and IL-27 treatment could boost IFNAR1 expression and/or its downstream signaling to promote IL-10 expression.

Mechanistically, we found that type I IFN signaling promotes of IL-10 production through the concerted action of STAT2, IRF4 and Blimp-1. In a previous report we identified IRF4 expression as essential for the induction of Blimp-1 and its downstream anti-viral effector cytokine molecules in CD8$^+$ T cells (29). By normalizing IL-10-producing cells to IFN-γ producing cells, we showed that IRF4 is also critically important for IL-10 production by CD8$^+$ T cells. We speculate that IRF4 functions upstream of Blimp-1 to promote IL-10 expression in CD8$^+$ T cells. However it is also possible that IRF4 could directly bind to the IL-10 locus to promote IL-10 production in CD8$^+$ T cells since it was shown that the IL-10 locus contains IRF4 binding motifs (30). The strength of TCR signaling (i.e. high antigen dose and strong peptide affinity) was also shown to be critical for IRF4 expression in CD8$^+$ T cells. Here we have extended previous findings by demonstrating that IRF4 expression is also modulated by cytokine signaling (i.e. type I IFNs). Of note, high dose antigen

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stimulation has been shown to favor IL-10 production by CD4 T cells (44). We have shown previously that IL-10 production by $CD8^+$ T cells was highly restricted to the early time frame following virus specific T cell infiltration to the lungs during influenza infection (8), at the time when virus-derived antigens are plentiful in the infected lungs. Thus it is possible that both TCR-derived signaling and type I IFNs derived signaling are required for the optimal induction of IRF4 and downstream IL-10 production in vivo. These possibilities warrant further investigations.

In summary, our data have revealed potential regulatory roles of type I IFN signaling in inflammatory responses during acute viral infections, and may provide the groundwork for manipulating IL-10 production in effector $CD8^+$ T cells to control excessive host inflammation during acute respiratory virus infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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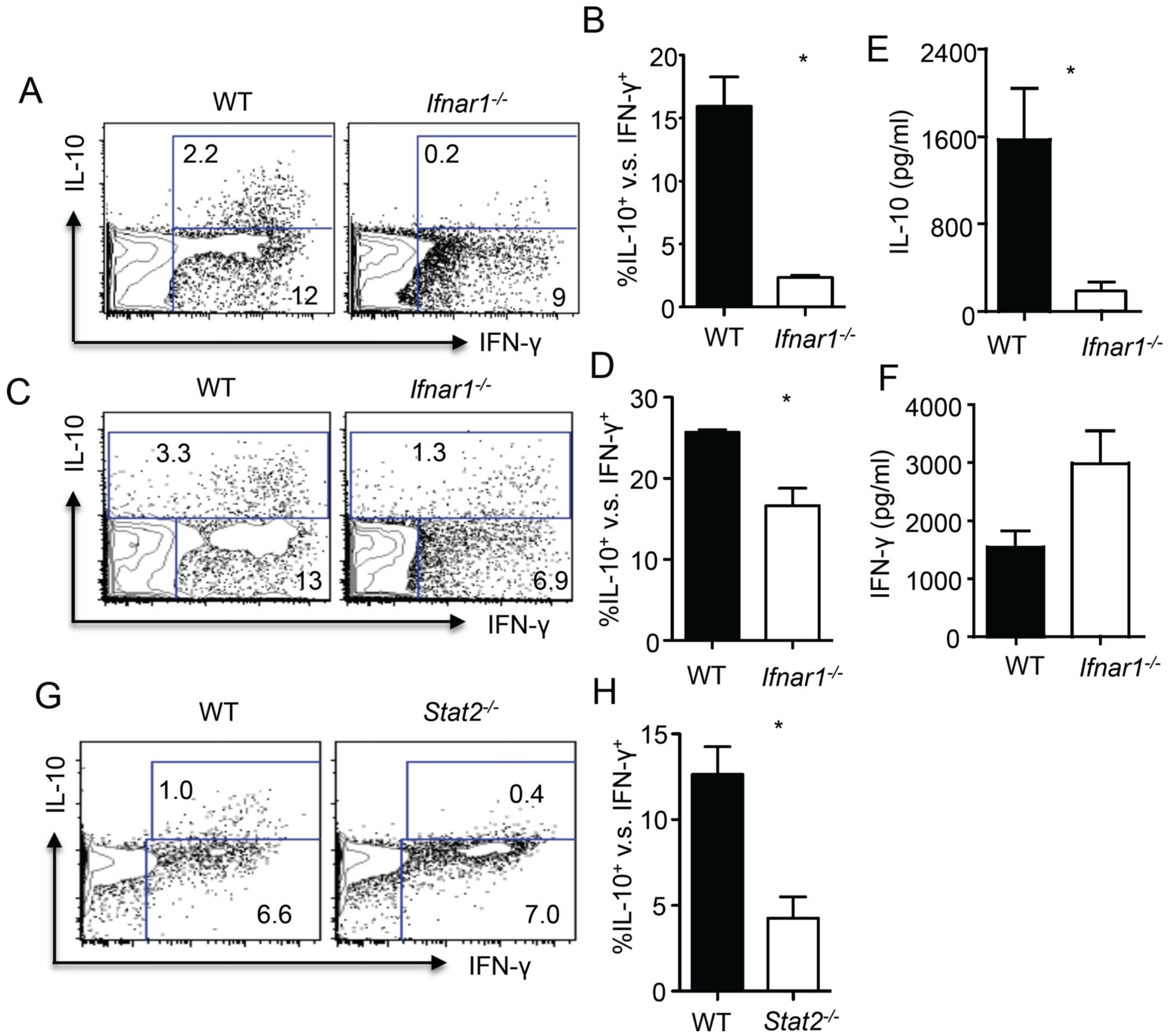

Figure 1. Type I IFN signaling is required for the optimal induction of IL-10-producing effector CD8+ T cells

(A–F) WT or *Ifnar1−/−* mice (n = 3) were infected with influenza. IL-10 production by lung T cells and IL-10 levels in the airway were determined at day 7 post infection.

(A). Production of IL-10 and IFN-γ by lung CD8+ T cells from WT or *Ifnar1−/−* mice following in vitro antigenic stimulation with influenza-infected WT BMDCs.

(B). Normalized percentages of IL-10+ cells in influenza-specific lung CD8+ T cells (IFN-γ+) from infected WT or *Ifnar1−/−* mice.

(C). Production of IL-10 and IFN-γ by lung CD4+ T cells following in vitro antigenic stimulation with influenza-infected WT BMDCs.

(D). Normalized percentages of IL-10+ cells in influenza-specific lung CD4+ T cells (IFN-γ+) from infected WT or *Ifnar1−/−* mice.

(E). IL-10 levels in the BALF from WT or *Ifnar1−/−* mice were determined through ELISA.

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(F). IFN-γ levels in the BALF from WT or *Ifnar1*$^{-/-}$ mice were determined through ELISA.
(G). Production of IL-10 and IFN-γ by lung CD8^{+} T cells from WT or *Stat2*$^{-/-}$ mice (n = 2 – 3) following in vitro antigenic stimulation with influenza-infected WT BMDCs.
(H). Normalized percentages of IL-10^{+} cells in influenza-specific lung CD8^{+} T cells (IFN-γ^{+}) from infected WT or *Stat2*$^{-/-}$ mice.
Data are representative of two to four separate experiments. Statistics were determined by unpaired two-tailed Student's t-test, $*P < 0.05$.

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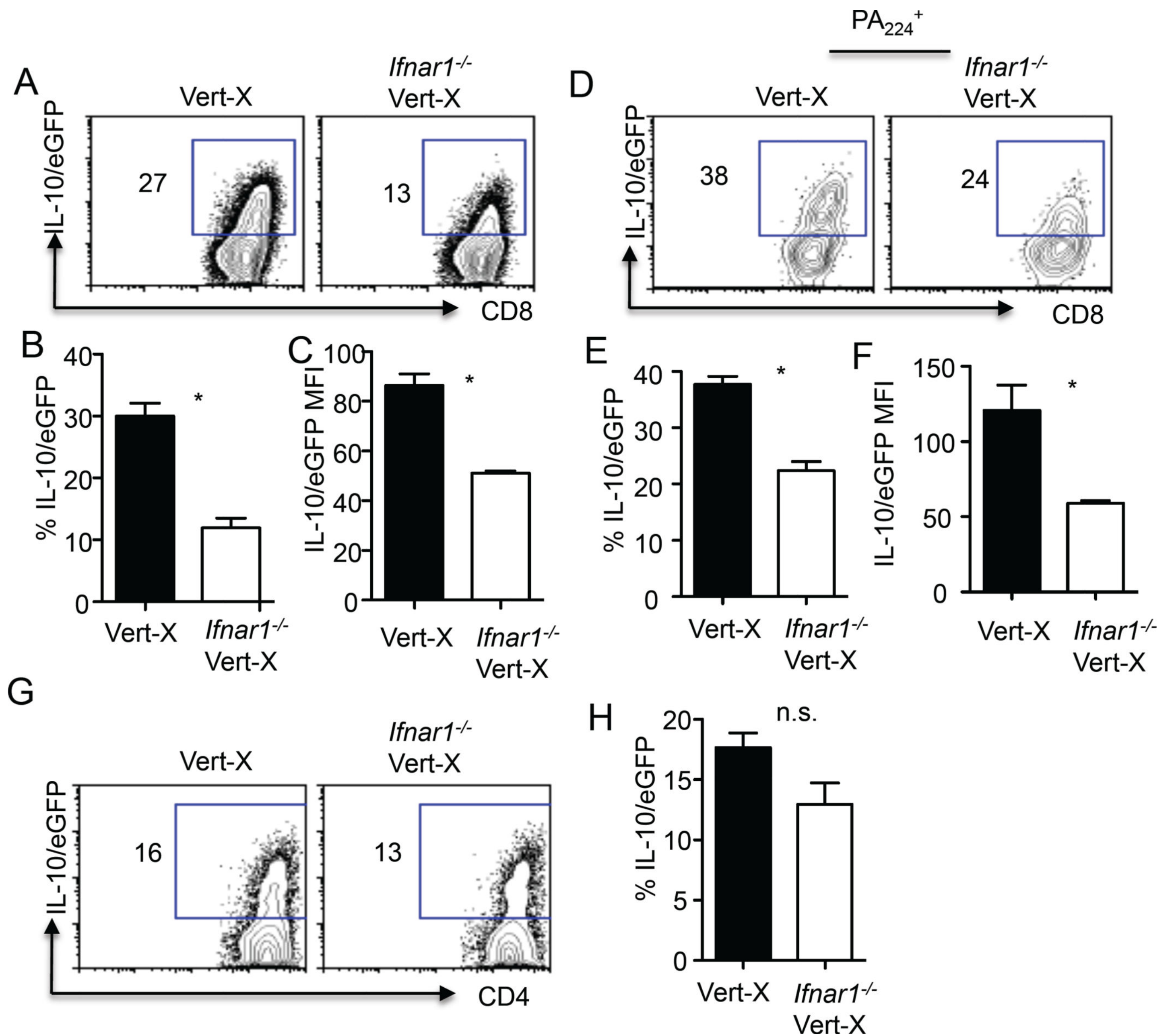

Figure 2. Type I IFN signaling is required for the optimal in vivo expression of IL-10 by CD8^{+} T cells

Vert-X or *Ifnar1$^{-/-}$* Vert-X mice (n = 3 – 4) were infected with influenza. IL-10 expression by T cells in vivo was measured through their eGFP expression by flow cytometry at day 7 p.i.

(A). Expression of IL-10-eGFP by total lung CD8^{+} T cells from infected Vert-X or *Ifnar1$^{-/-}$* Vert-X mice.

(B). Percentages IL-10-eGFP^{+} of cells in total lung CD8^{+} T cells from infected Vert-X or *Ifnar1$^{-/-}$* Vert-X mice.

(C). IL-10-eGFP expression levels (MFI) of the IL-10-eGFP^{+} of cells in total lung CD8^{+} T cells from infected Vert-X or *Ifnar1$^{-/-}$* Vert-X mice.

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(D). Expression of IL-10-eGFP by influenza-specific PA224$^+$ lung CD8$^+$ T cells from infected Vert-X or *Ifnar1*$^{-/-}$ Vert-X mice.

(E). Percentages IL-10-eGFP$^+$ of cells in influenza-specific PA224$^+$ lung CD8$^+$ T cells from infected Vert-X or *Ifnar1*$^{-/-}$ Vert-X mice.

(F). IL-10-eGFP expression levels (MFI) of the IL-10-eGFP$^+$ of cells in influenza-specific PA224$^+$ lung CD8$^+$ T cells from infected Vert-X or *Ifnar1*$^{-/-}$ Vert-X mice.

(G). Expression of IL-10-eGFP by total lung CD4$^+$ T cells from infected Vert-X or *Ifnar1*$^{-/-}$ Vert-X mice.

(H). Percentages IL-10-eGFP$^+$ of cells in total lung CD4$^+$ T cells from infected Vert-X or *Ifnar1*$^{-/-}$ Vert-X mice.

Data are representative of at least three separate experiments. Statistics were determined by unpaired two-tailed Student's t-test, $*P < 0.05$. n.s., non-significant.

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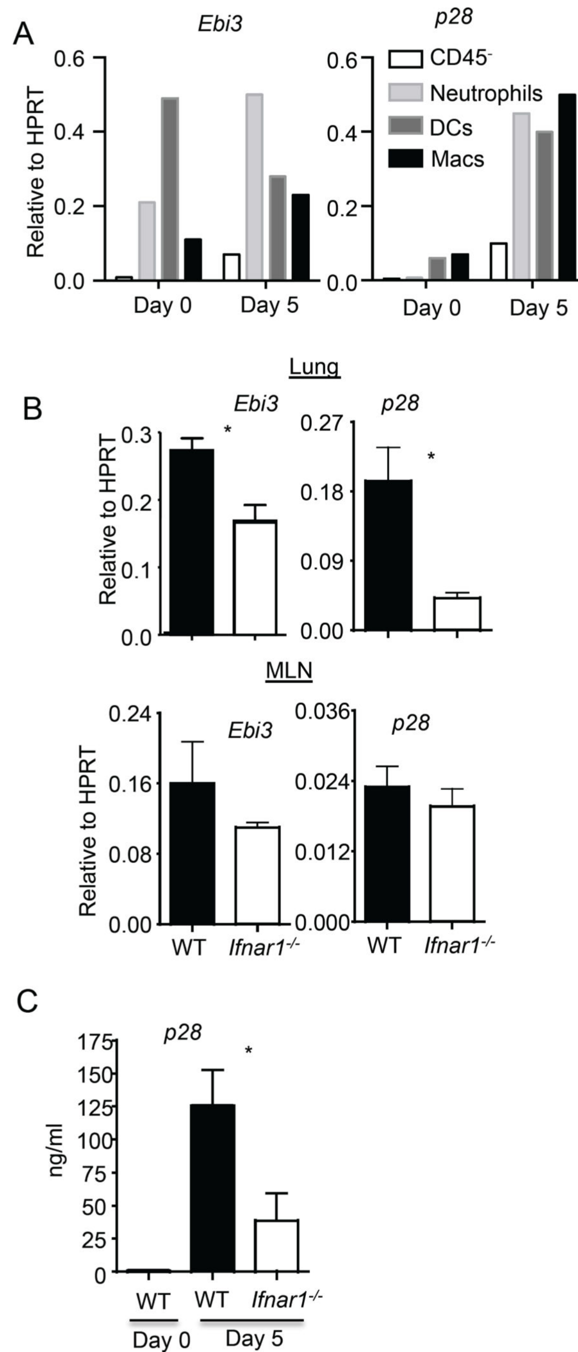

Figure 3. Type I IFN signaling is required for optimal IL-27 expression during influenza infection

WT mice were infected with influenza. IL-27 subunit (Ebi3 and p28) gene expression in different lung cells populations (A), mediastinal lymph nodes (MLN) and lungs (B) as well as p28 protein production in the airway (C) were determined at day 5 p.i.

(A). Ebi3 and p28 gene expression in various cell types sorted from the lungs of WT mice (pooled from 2 – 3 mice) at day 5 p.i.

(B) Ebi3 and p28 gene expression in the lung and draining MLN from WT or *Ifnar1*$^{-/-}$ mice (n=2 – 4) was determined through real-time RT-PCR.

(C). p28 protein levels in the BALF from WT or *Ifnar1*$^{-/-}$ mice (n = 2 – 4) were determined through ELISA.

Data are representative of two separate experiments. Statistics were determined by unpaired two-tailed Student's t-test, $*P < 0.05$.

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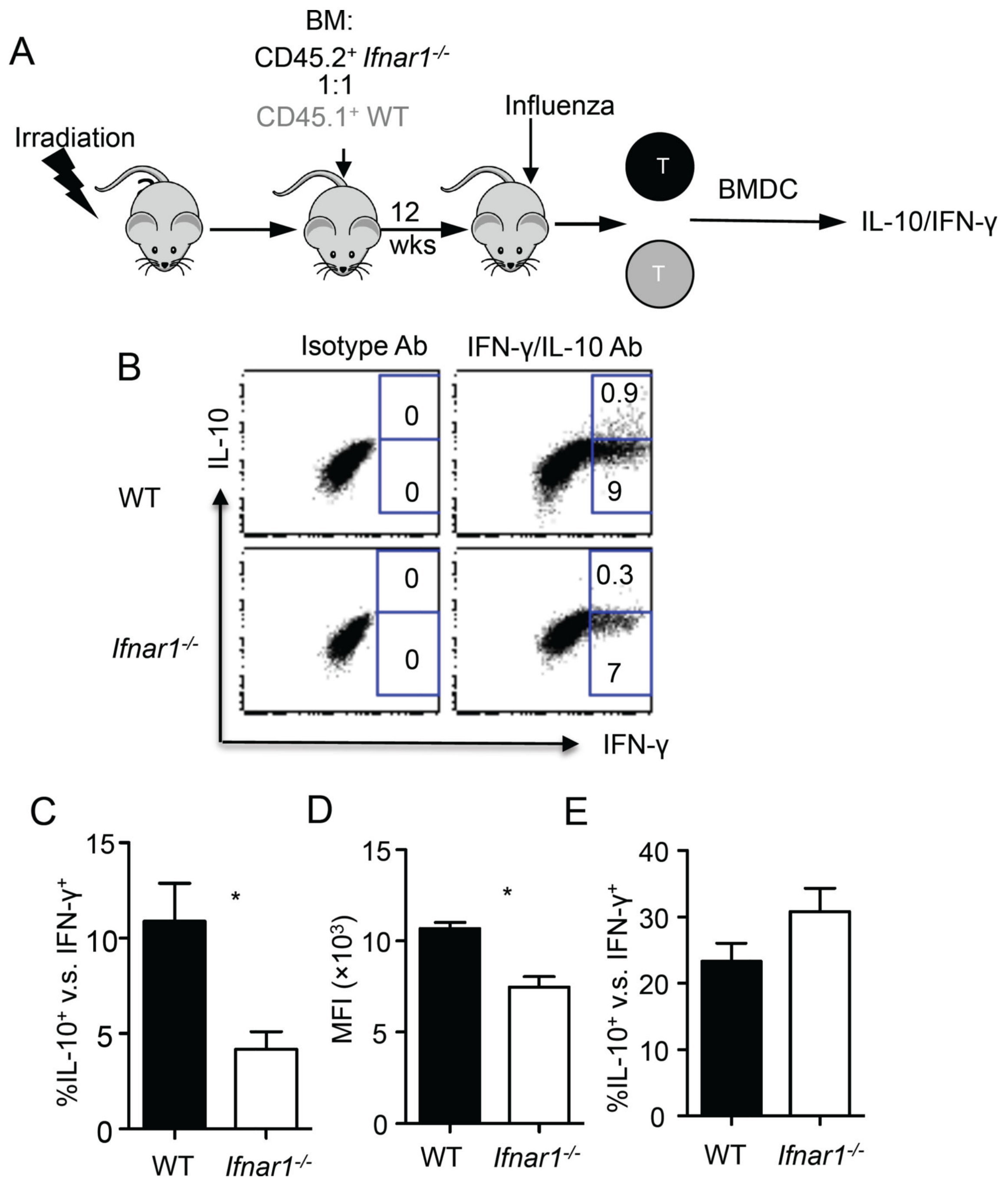

Figure 4. Direct type I IFN signaling in CD8+ T cells is required for optimal IL-10 production by effector CD8+ T cells

WT and *Ifnar1*−/− mixed bone marrow chimera mice (n= 5) were infected with influenza. IL-10 production by lung T cells was determined at day 7 post infection following stimulation with influenza-infected WT BMDCs.

(A). Schematics of the construction of BM chimeric mice.

(B). Production of IL-10 and IFN-γ by WT or *Ifnar1*−/− lung CD8+ T cells following in vitro antigenic stimulation with influenza-infected WT BMDCs.

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(C). Normalized percentages of IL-10^{+} cells in influenza-specific WT or *Ifnar1*$^{-/-}$ lung CD8^{+} T cells.

(D). IL-10 levels (MFI) produced by WT or *Ifnar1*$^{-/-}$ lung CD8^{+} T cells following in vitro antigenic stimulation with influenza-infected WT BMDCs.

(E). Normalized percentages of IL-10+ cells in influenza-specific WT or *Ifnar1*$^{-/-}$ lung CD4^{+} T cells (IFN-γ^{+}) from infected WT and *Ifnar1*$^{-/-}$ mice.

Data are representative of two separate experiments. Statistics were determined by paired two-tailed Student's t-test, $^{*}P < 0.05$.

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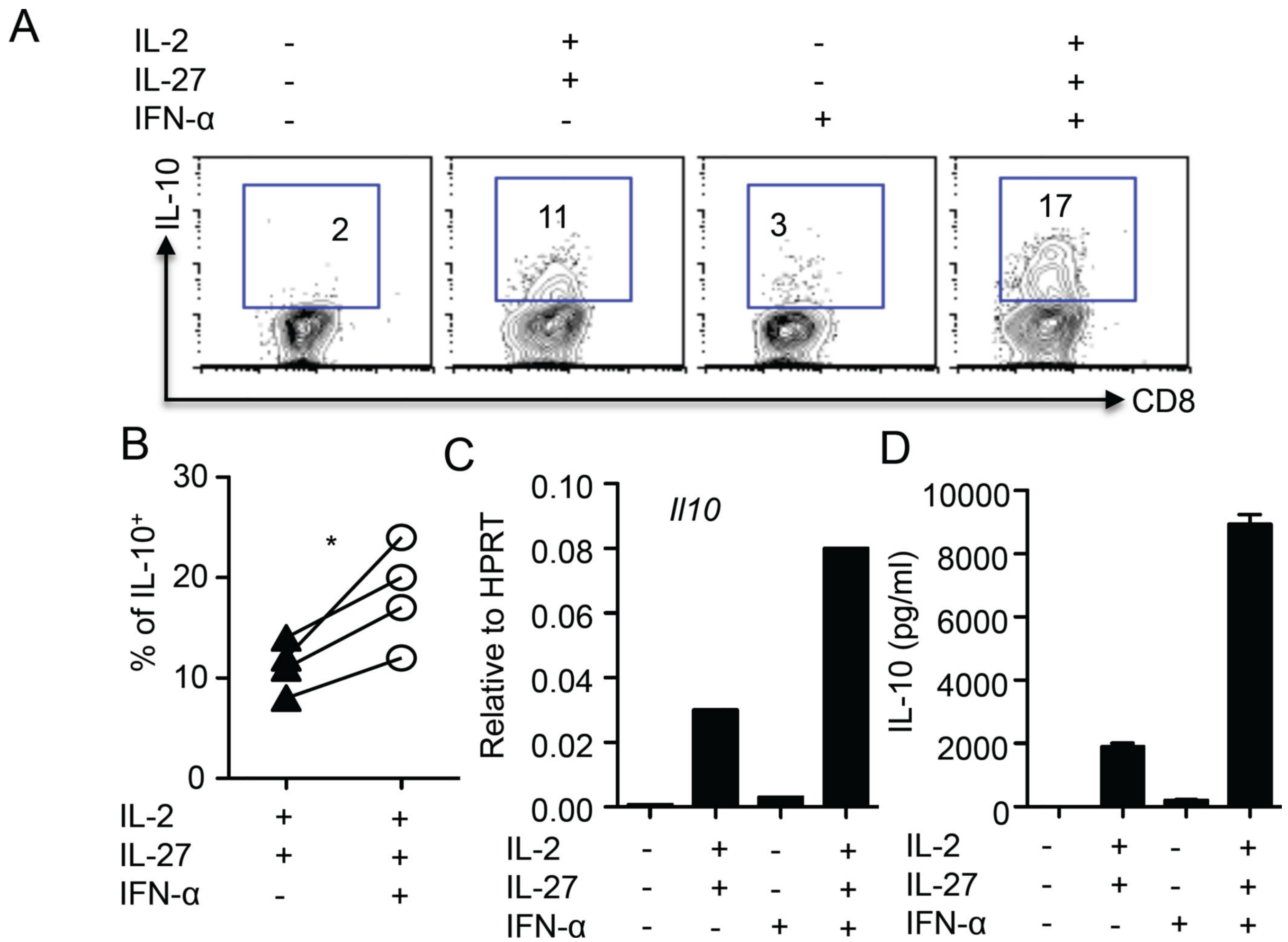

Figure 5. Type I IFNs further promote IL-10 production by CD8+ T cells in the presence of IL-2 plus IL-27

(A) Production of IL-10 by CD8$^+$ T cells following in vitro culture with indicated conditions (no cytokines, IFN-α, IL-2 plus IL-27 or IFN-α plus IL-2 and IL-27).

(B) Percentages of IL-10-producing CD8$^+$ T cells following in vitro culture with indicated conditions (IL-2 plus IL-27 or IFN-α plus IL-2 and IL-27) in total of 4 experiments.

(C) IL-10 gene expression in CD8$^+$ T cells following in vitro culture with indicated conditions (no cytokines, IFN-α, IL-2 plus IL-27 or IFN-α plus IL-2 and IL-27).

(D) IL-10 protein production in the supernatants following αCD3 restimulation of CD8$^+$ T cells cultured with indicated conditions (no cytokines, IFN-α, IL-2 plus IL-27 or IFN-α plus IL-2 and IL-27).

Data are representative of three to four separate experiments. Statistics were determined by paired two-tailed Student's t-test, *$P < 0.05$.

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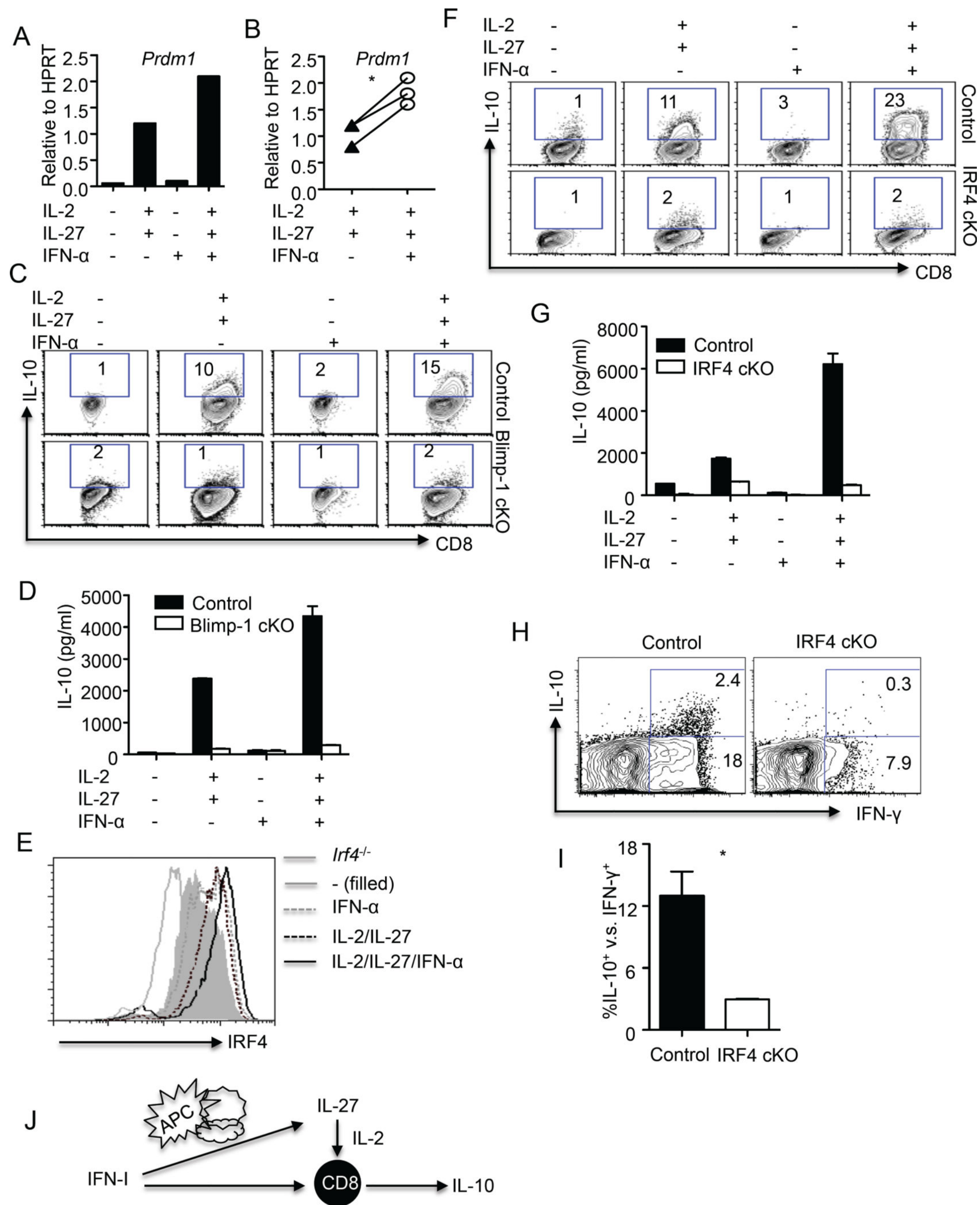

Figure 6. Blimp-1 and IRF4 are required for the development of IL-10-producing effector CD8$^+$ T cells induced by type I IFNs plus IL-2 and IL-27

(A) Prdm1 (Blimp-1 gene) expression in CD8$^+$ T cells following in vitro culture with indicated conditions (no cytokines, IFN-α, IL-2 plus IL-27 or IFN-α plus IL-2 and IL-27).
(B) Prdm1 expression in CD8$^+$ T cells following in vitro culture with indicated conditions (IL-2 plus IL-27 or IFN-α plus IL-2 and IL-27) in total of three experiments.
(C) Production of IL-10 by control or Blimp-1 cKO CD8$^+$ T cells following in vitro culture with indicated conditions (no cytokines, IFN-α, IL-2 plus IL-27 or IFN-α plus IL-2 and IL-27).

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(D) IL-10 protein production in the supernatants following restimulation of control or Blimp-1 cKO $CD8^+$ T cells cultured with indicated conditions (no cytokines, IFN-α, IL-2 plus IL-27 or IFN-α plus IL-2 and IL-27).

(E) IRF4 expression in $CD8^+$ T cells cultured with indicated conditions (no cytokines, IFN-α, IL-2 plus IL-27 or IFN-α plus IL-2 and IL-27).

(F) Production of IL-10 by control or IRF4 cKO $CD8^+$ T cells following in vitro culture with indicated conditions (no cytokines, IFN-α, IL-2 plus IL-27 or IFN-α plus IL-2 and IL-27).

(G) IL-10 protein production in the supernatants following restimulation of WT or IRF4 cKO $CD8^+$ T cells cultured with indicated conditions (no cytokines, IFN-α, IL-2 plus IL-27 or IFN-α plus IL-2 and IL-27).

(H) Production of IL-10 and IFN-γ by lung $CD8^+$ T cells from WT or IRF4 cKO mice (n= 2 – 3) following in vitro antigenic stimulation with influenza-infected WT BMDCs.

(I). Normalized percentages of IL-10^+ cells in influenza-specific lung $CD8^+$ T cells (IFN-γ^+) from infected control or IRF4 cKO mice.

(J). Model of type I IFN-dependent IL-10 production by $CD8^+$ T cells.

Data are representative of at least two separate experiments. Statistics were determined by paired (B) or unpaired (I) two-tailed Student’s t-test, $*P < 0.05$.