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Research Article

Infectious disease

Many pathogens, including viruses, bacteria, and protozoan parasites, suppress cellular immune responses through activation of type I IFN signaling. Recent evidence suggests that immune suppression and susceptibility to the malaria parasite, *Plasmodium*, is mediated by type I IFN; however, it is unclear how type I IFN suppresses immunity to blood-stage *Plasmodium* parasites. During experimental severe malaria, CD4<sup>+</sup> Th cell responses are suppressed, and conventional DC (cDC) function is curtailed through unknown mechanisms. Here, we tested the hypothesis that type I IFN signaling directly impairs cDC function during *Plasmodium* infection in mice. Using cDC-specific IFNAR1-deficient mice, and mixed BM chimeras, we found that type I IFN signaling directly affects cDC function, limiting the ability of cDCs to prime IFN- $\gamma$ -producing Th1 cells. Although type I IFN signaling modulated all subsets of splenic cDCs, CD8<sup>−</sup> cDCs were especially susceptible, exhibiting reduced phagocytic and Th1-promoting properties in response to type I IFNs. Additionally, rapid and systemic IFN- $\alpha$  production in response to *Plasmodium* infection required type I IFN signaling in cDCs themselves, revealing their contribution to a feed-forward cytokine-signaling loop. Together, these data suggest abrogation of type I IFN signaling in CD8<sup>−</sup> splenic cDCs as an approach for enhancing Th1 responses against *Plasmodium* and other type I IFN-inducing pathogens.

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# Type I IFN signaling in CD8<sup>−</sup> DCs impairs Th1-dependent malaria immunity

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Many pathogens, including viruses, bacteria, and protozoan parasites, suppress cellular immune responses through activation of type I IFN signaling. Recent evidence suggests that immune suppression and susceptibility to the malaria parasite, *Plasmodium*, is mediated by type I IFN; however, it is unclear how type I IFN suppresses immunity to blood-stage *Plasmodium* parasites. During experimental severe malaria, CD4<sup>+</sup> Th cell responses are suppressed, and conventional DC (cDC) function is curtailed through unknown mechanisms. Here, we tested the hypothesis that type I IFN signaling directly impairs cDC function during *Plasmodium* infection in mice. Using cDC-specific IFNAR1-deficient mice, and mixed BM chimeras, we found that type I IFN signaling directly affects cDC function, limiting the ability of cDCs to prime IFN- $\gamma$ -producing Th1 cells. Although type I IFN signaling modulated all subsets of splenic cDCs, CD8<sup>−</sup> cDCs were especially susceptible, exhibiting reduced phagocytic and Th1-promoting properties in response to type I IFNs. Additionally, rapid and systemic IFN- $\alpha$  production in response to *Plasmodium* infection required type I IFN signaling in cDCs themselves, revealing their contribution to a feed-forward cytokine-signaling loop. Together, these data suggest abrogation of type I IFN signaling in CD8<sup>−</sup> splenic cDCs as an approach for enhancing Th1 responses against *Plasmodium* and other type I IFN-inducing pathogens.

## Introduction

Type I IFNs are crucial for protection against viruses but can facilitate survival of many pathogens, such as *Listeria* (1, 2), *Mycobacteria* (3–7), *Staphylococci* (8), and Lymphocytic choriomeningitis virus (9, 10), as well as protozoan parasites, such as *Leishmania* (11) and *Plasmodium* (12). Type I IFN-mediated susceptibility to bacterial infection involves immunomodulatory effects on macrophages, monocytes, and neutrophils via downregulation of cytokine receptor expression, induction of apoptosis, and modulation of recruitment to peripheral tissue sites (1–6, 8). In contrast, mechanisms of type I IFN-mediated immune suppression during protozoan parasitic infection remain less studied. A recent report highlighted the potential of *Plasmodium* DNA to trigger type I IFN responses in vitro (13). Moreover, type I IFN-related genes were transcribed in PBMCs from patients with malaria (13), and SNPs present in the gene encoding the type I IFN receptor chain, IFNAR1, have been linked to resistance to severe malaria (14, 15). Recently, we and others demonstrated that type I IFNs mediated susceptibility to *Plasmodium* using a murine model of severe malaria (12, 13). Through the use of BM chimeric mice, we also showed that type I IFNs suppressed IFN- $\gamma$  production by Th1 cells, not by acting on the

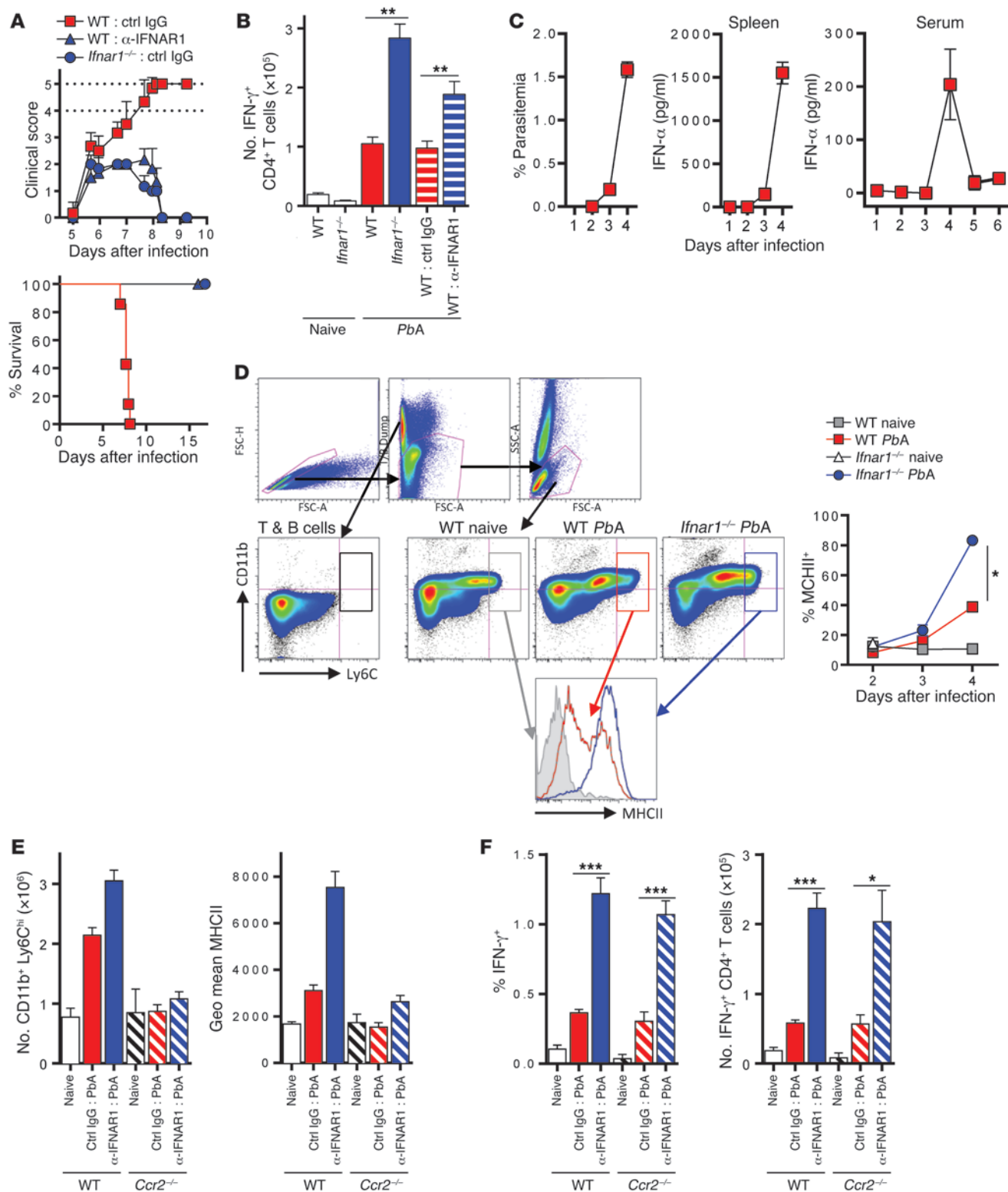
CD4<sup>+</sup> T cells themselves, but by signaling to an as yet unidentified hematopoietic cell population (12).

In models of severe malaria, splenic conventional DCs (cDCs) rapidly lose ex vivo antigen-presenting capacity and in vivo phagocytic capabilities as pathogen load increases (16–18). This is likely to negatively affect the generation and maintenance of Th cell responses, since cDCs are responsible for in vivo priming of CD4<sup>+</sup> T cells in mild and severe malaria models (19–21). In particular, not only do CD8<sup>+</sup> cDCs fail to cross-present antigens, but MHC class I-restricted and MHC class II-restricted (MHCII-restricted) ex vivo presentation of *Plasmodium*-expressed antigens by CD8<sup>+</sup> and CD8<sup>−</sup> cDCs is also curtailed (16, 17). The factors responsible for systemic loss of cDC function during *Plasmodium* infection remain unclear.

CD8<sup>−</sup> cDCs have received less attention in recent years compared with CD8<sup>+</sup> cDCs, which rapidly produce the Th1-inducing cytokine, IL-12, and cross-present exogenous antigen efficiently to CD8<sup>+</sup> T cells (reviewed in refs. 22, 23). However, CD8<sup>−</sup> cDCs possess a greater intrinsic capacity for processing antigens onto MHCII molecules than CD8<sup>+</sup> cDCs and, therefore, harbor significant potential for priming Th responses (24). It is puzzling, therefore, that CD8<sup>−</sup> cDCs underperformed substantially in terms of priming Th1 responses compared with CD8<sup>+</sup> cDCs, when specifically targeted in vivo in the presence of the potent type I IFN-inducing adjuvant, poly I:C (25, 26). The reasons for relatively poor CD8<sup>−</sup> cDC function in the presence of poly I:C adjuvant remain unclear.

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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## Figure 1

Type I IFN signaling limits Th1 responses during experimental severe malaria independently of suppressive effects on monocytes. (A) C57BL/6 WT mice ( $n = 6$ ) received  $\alpha$ -IFNAR1 or control IgG and *Ifnar1*<sup>-/-</sup> mice ( $n = 6$ ) received control IgG on days 0, 2, and 4 p.i. with *PbA*. Mice were assessed for clinical symptoms from day 5 p.i. and for survival (2 independent experiments). Dotted line at clinical score 4 indicates moribund threshold. (B) WT mice ( $n = 5$ ) were infected with *PbA* and treated with  $\alpha$ -IFNAR1, control IgG, or left untreated. *PbA*-infected *Ifnar1*<sup>-/-</sup> mice ( $n = 5$ ) and uninfected WT and *Ifnar1*<sup>-/-</sup> mice ( $n = 5$ ) were also tested. On day 4 p.i., flow cytometric analysis of IFN- $\gamma$  production (without ex vivo stimulation) by splenic CD4<sup>+</sup> TCR $\beta$ <sup>+</sup> cells was performed (2 independent experiments). (C) Time course analysis of parasitemia and IFN- $\alpha$  protein in whole spleen lysates and sera from *PbA*-infected WT mice (2–5 independent experiments). (D) Gating strategy for Ly6C<sup>hi</sup> monocytes and MHCII expression in blood. WT and *Ifnar1*<sup>-/-</sup> mice ( $n = 3$ –5) were infected with *PbA* and, at time points indicated, PBMCs were assessed by flow cytometry for cell surface expression of MHCII on TCR $\beta$ <sup>+</sup>/B220<sup>+</sup>/CD11b<sup>+</sup>/Ly6C<sup>hi</sup> single cells exhibiting low granularity (2 independent experiments). (E) WT and *Ccr2*<sup>-/-</sup> mice ( $n = 5$ –6) were treated with  $\alpha$ -IFNAR1 or control IgG and infected with *PbA*. On day 4 p.i., spleens were assessed for numbers of and, cell-surface MHCII expression by, CD11b<sup>+</sup> Ly6C<sup>hi</sup> monocytes and (F) proportion and absolute numbers of CD4<sup>+</sup> TCR $\beta$ <sup>+</sup> cells producing IFN- $\gamma$  without ex vivo restimulation (2 independent experiments). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

Given separate lines of evidence for type I IFN-mediated disease susceptibility (13), CD8<sup>-</sup> cDC dysfunction (16), and ineffective Th cell responses during severe *Plasmodium* infection (12, 27), we hypothesized that type I IFNs mediate systemic cDC dysfunction, which leads directly to ineffective Th1-mediated immunity. This hypothesis is supported by related data from other inflammatory disease models, including polymicrobial sepsis (28), and allogeneic reactivity after BM transplantation (29). In a murine cecal ligation puncture model of polymicrobial sepsis, type I IFNs impaired CTL responses via a direct effect on cDCs (28), but possible effects on Th cells were not investigated. In murine models of GVHD, type I IFN signaling in hematopoietic cells impaired Th cell responses (29), but the underlying cellular mechanisms were not fully characterized. Here, we used an established mouse model of severe malaria to test the above hypothesis and discovered that type I IFNs directly suppress CD8<sup>-</sup> cDC-mediated generation of parasite-specific IFN- $\gamma$ -producing Th1 cells.

## Results

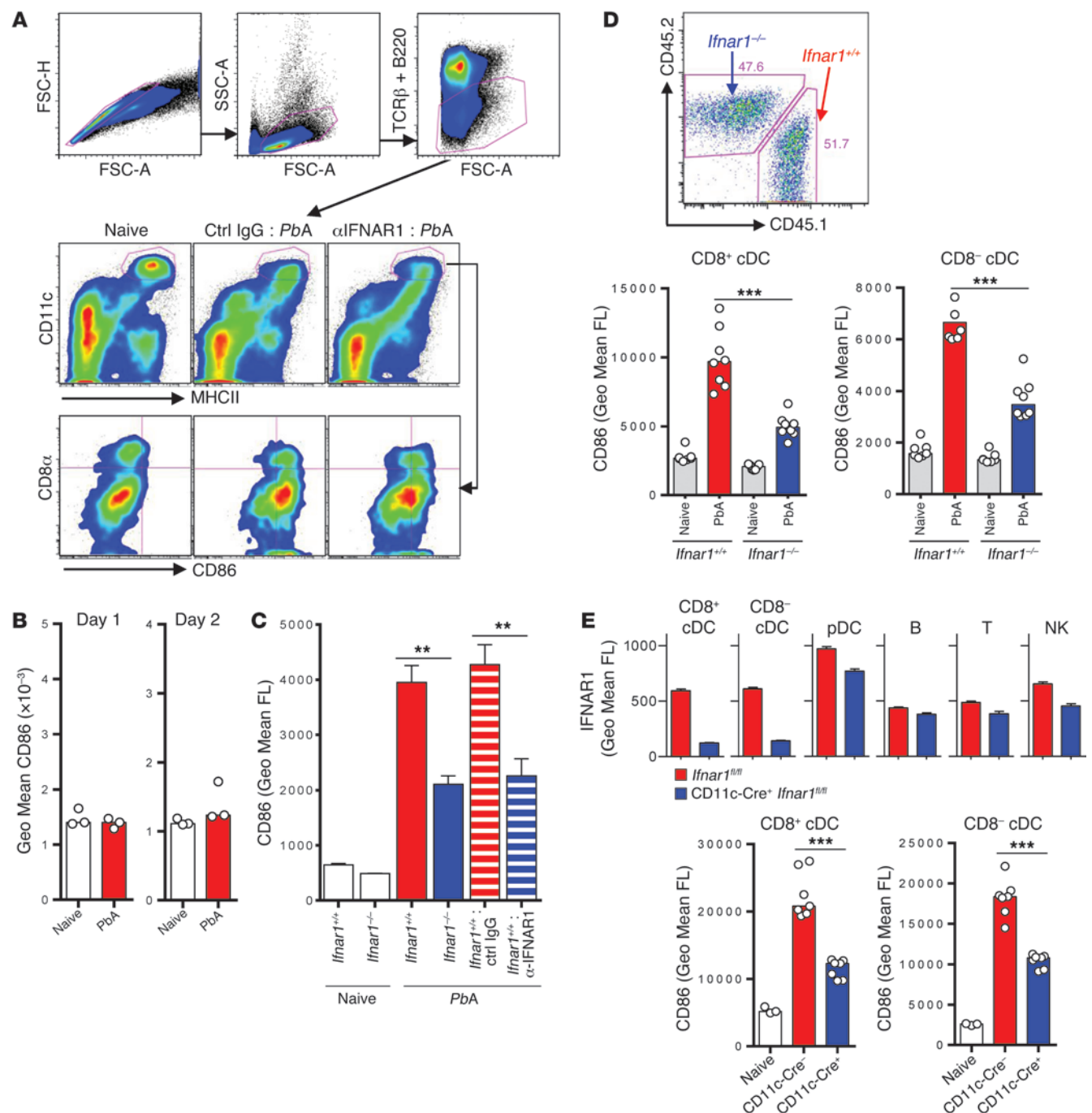
*Type I IFN signaling limits Th1 responses during experimental severe malaria independently of suppressive effects on monocytes.* We showed previously, using genetically deficient (*Ifnar1*<sup>-/-</sup>) mice, that type I IFN signaling in hematopoietic cells suppressed Th1 cell responses and mediated fatal disease symptoms during experimental severe malaria caused by *Plasmodium berghei* Antwerpen-Kasapa (*PbA*) infection (12). To confirm that no developmental or immune homeostatic defects in *Ifnar1*<sup>-/-</sup> mice could account for this phenotype, we first studied WT *PbA*-infected mice treated with  $\alpha$ -IFNAR1 blocking antibody. Consistent with genetic IFNAR1 deficiency (12), antibody-mediated blockade of type I IFN signaling completely prevented severe neurological symptoms and morbidity (Figure 1A) and substantially boosted Th1 responses (Figure 1B). Parasitemias remained equivalent between  $\alpha$ -IFNAR1- and control IgG-treated mice by day 4 after infection (p.i.) (data not shown), consistent with our previous report in *Ifnar1*<sup>-/-</sup> mice (12). Furthermore, we

observed substantial production of IFN- $\alpha$  protein in the spleens and sera during infection, the early kinetics of which mirrored increases in parasitemia (Figure 1C), but whose levels dropped in the sera after day 4 p.i. Together, these data confirm that type I IFN signaling, associated with a pathogen-induced spike of systemic IFN- $\alpha$ , suppressed Th1 responses and caused severe, fatal disease during *PbA* infection.

We next sought to determine cellular mechanisms by which type I IFN signaling suppressed Th1 responses. Given that hematopoietic type I IFN signaling suppressed antiparasitic Th1 cells indirectly (12), we hypothesized that the APCs responsible for Th1 priming would be directly impaired by type I IFN signaling. Previous reports identify cDC as the major APC responsible for priming Th1 responses to blood-stage *Plasmodium*, excluding roles for plasmacytoid DCs (pDCs) though not monocyte-derived DCs (moDCs) (20, 21). Therefore, we focused our study on cDCs and monocytes. Given an emerging paradigm that type I IFN modulates monocytic responses (4, 30, 31), we first considered the impact of type I IFN signaling on monocytes during *PbA* infection. We noted that the proportion of CD11b<sup>+</sup> Ly6C<sup>hi</sup> monocytes in the blood, and their acquisition of cell surface MHCII (Figure 1D), increased strikingly in *PbA*-infected *Ifnar1*<sup>-/-</sup> mice, compared with that in WT control-infected mice by day 4 p.i. These cells were also more abundant and expressed more cell surface MHCII in the spleens of *PbA*-infected *Ifnar1*<sup>-/-</sup> mice (data not shown) or WT mice treated with  $\alpha$ -IFNAR1 (Figure 1E), compared with control-treated, *PbA*-infected WT mice. This observation was unexpected given that type I IFN promotes, rather than suppresses, monocyte recruitment and activation (4, 30, 31) and suggested that increased monocyte activation could account for enhanced Th1 responses after type I IFN signaling blockade. However, although monocyte recruitment to the spleen and acquisition of cell surface MHCII was abrogated in *Ccr2*<sup>-/-</sup> mice (Figure 1E), Th1 responses were unaffected compared with those in WT mice, either in the presence or absence of type I IFN signaling (Figure 1F). Importantly, Th1 responses increased in *Ccr2*<sup>-/-</sup> mice treated with  $\alpha$ -IFNAR1, compared with those in control-treated *Ccr2*<sup>-/-</sup> mice (Figure 1F). These data show that, although type I IFN signaling suppressed monocyte responses during malaria, this process had no impact on the early development of Th1 cell responses during acute, severe *Plasmodium* infection.

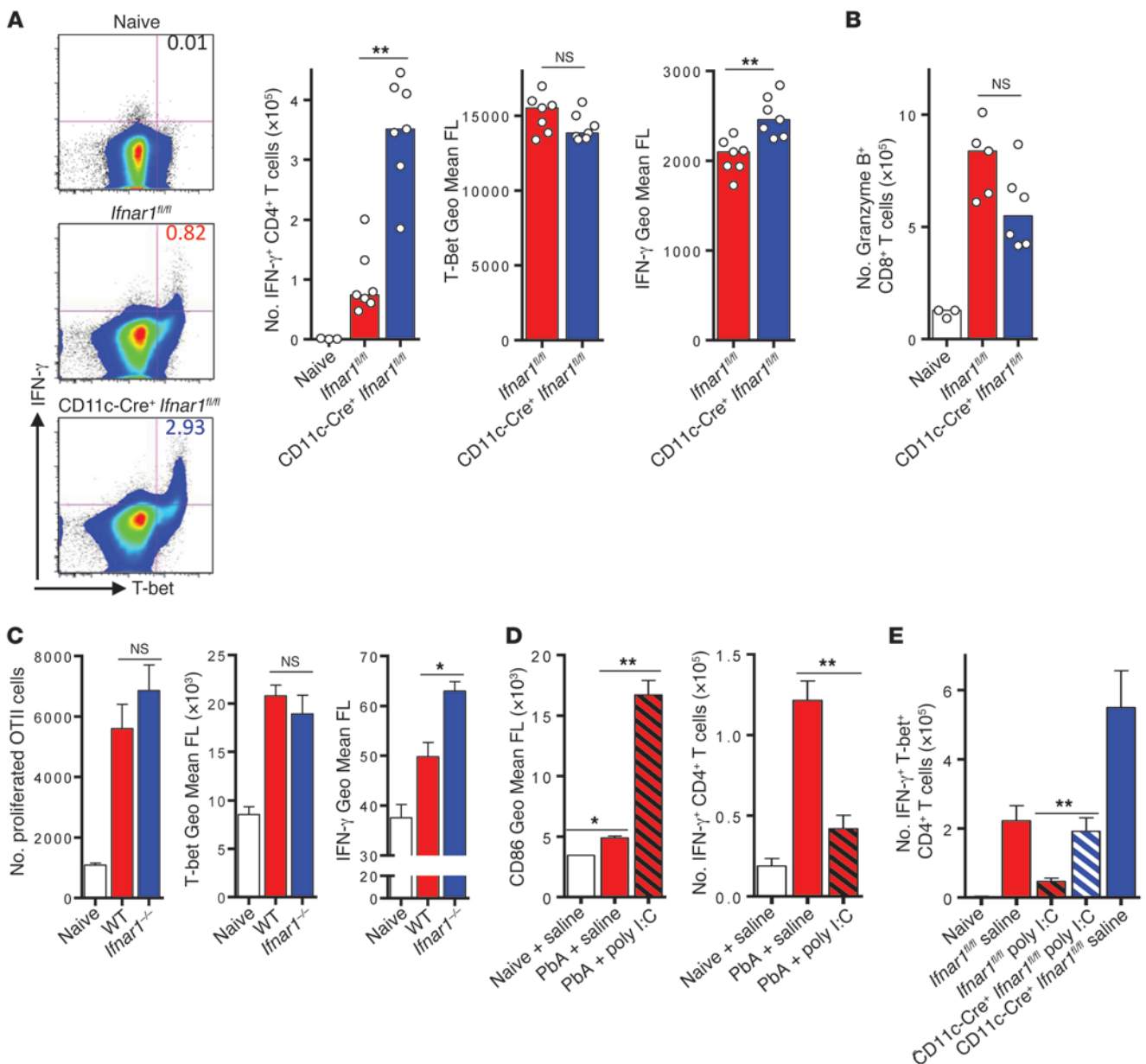
*Type I IFN signaling directly mediates systemic activation of splenic cDCs during PbA infection.* Since monocytes played no role in type I IFN-mediated suppression of Th1 responses (Figure 1, E and F), we hypothesized that splenic cDCs were the most likely APCs to be impaired by type I IFN signaling (20, 32). To test this, we first examined cell surface expression of the costimulatory molecule, CD86, on CD11c<sup>hi</sup> MHCII<sup>hi</sup> cDCs (Figure 2A), since it correlates well with their systemic activation and functional impairment during *PbA* infection (17). As expected, CD86 upregulation was observed, not during the first 2 days of infection (Figure 2B and ref. 17), but on day 4 p.i. (Figure 2C), when parasitemias had exceeded 1% (Figure 1C). Importantly, we noted that both IFNAR1 genetic deficiency and  $\alpha$ -IFNAR1 treatment resulted in 50% less CD86 upregulation by cDCs (Figure 2C). Similar observations were made in *Ccr2*<sup>-/-</sup> mice, devoid of splenic monocyte recruitment, when treated with  $\alpha$ -IFNAR1 or control IgG (data not shown). Together, these data indicate that type I IFN signaling contributed substantially to systemic cDC activation during *PbA* infection.

We next determined whether cDC systemic activation occurred via direct or indirect type I IFN signaling. To examine this, we first



**Figure 2**

Type I IFN signaling directly mediates systemic activation of splenic cDCs during PbA infection. (A) Analysis of cell surface CD86 expression by splenic CD8<sup>+</sup> and CD8<sup>-</sup> cDCs (CD11c<sup>hi</sup>MHCII<sup>hi</sup>TCRβ-B220<sup>-</sup> single cells) from WT mice ( $n = 5-6$ ) 4 days p.i. with PbA and after treatment with α-IFNAR1 or control IgG (2 independent experiments). (B) Analysis of cell surface CD86 expression by bulk splenic cDCs from WT mice ( $n = 3$ ) on days 1 and 2 p.i. with PbA (experiment performed once). (C) WT mice ( $n = 5$ ) were infected with PbA and treated with α-IFNAR1, control IgG, or left untreated. PbA-infected *Ifnar1*<sup>-/-</sup> mice ( $n = 5$ ) and uninfected WT and *Ifnar1*<sup>-/-</sup> mice ( $n = 5$ ) were also assessed. On day 4 p.i., flow cytometric analysis of cell surface CD86 expression by bulk splenic cDCs was performed (2 independent experiments). (D) Flow cytometric assessment of chimerism and cell surface CD86 expression in splenic CD11c<sup>hi</sup>MHCII<sup>hi</sup>TCRβ-B220<sup>-</sup> single cells from WT (CD45.1<sup>+</sup>)/*Ifnar1*<sup>-/-</sup> (CD45.2<sup>+</sup>) 50:50 mixed BM chimeric mice ( $n = 6-7$ ) 4 days p.i. with PbA (2 independent experiments). (E) C57BL/6 CD11c-Cre<sup>+</sup>/- *Ifnar1*<sup>fl/fl</sup> mice and *Ifnar1*<sup>fl/fl</sup> littermate control mice ( $n = 7-10$ ) were infected with PbA. 4 days p.i., flow cytometric analysis of cell surface IFNAR1 expression on indicated splenic cell types was performed (CD8<sup>+</sup> cDCs [CD8α<sup>+</sup>CD11c<sup>hi</sup>MHCII<sup>hi</sup>TCRβ-B220<sup>-</sup>]; CD8<sup>-</sup> cDCs [CD8α<sup>+</sup>CD11c<sup>hi</sup>MHCII<sup>hi</sup>TCRβ-B220<sup>-</sup>]; pDCs [PDCA-1<sup>+</sup>B220<sup>int</sup>CD11c<sup>int</sup>]; B cells [CD19<sup>+</sup>B220<sup>+</sup>]; T cells [TCRβ<sup>+</sup>NK1.1<sup>-</sup>]; NK cells [NK1.1<sup>+</sup>TCRβ<sup>-</sup>]) as well as analysis of cell surface CD86 expression by CD8<sup>+</sup> and CD8<sup>-</sup> cDCs (2 independent experiments). \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .



**Figure 3**

Type I IFN signaling in cDCs impairs Th1 priming in vivo. (A) FACS analysis of proportions, absolute numbers, and Geo Mean FL intensities for direct ex vivo IFN- $\gamma$  and T-bet expression by CD4<sup>+</sup> T cells (gated) from CD11c-Cre<sup>+</sup> *Ifnar1<sup>fl/fl</sup>* mice and *Ifnar1<sup>fl/fl</sup>* littermate control mice ( $n = 7-10$ ) infected with *PbA* for 4 days (2 independent experiments). (B) FACS analysis of granzyme B expression by splenic CD8<sup>+</sup> T cells from CD11c-Cre<sup>+</sup> *Ifnar1<sup>fl/fl</sup>* mice and *Ifnar1<sup>fl/fl</sup>* littermate control mice ( $n = 5$ ) infected with *PbA* for 4 days. (C) WT and *Ifnar1<sup>-/-</sup>* mice ( $n = 5-8$ ) received  $1 \times 10^5$  to  $2 \times 10^5$  CFSE-labeled CD4<sup>+</sup>CD45.1<sup>+</sup> OTII cells 1 day before infection with OTII peptide-expressing *PbA*. 4 days p.i., splenic OTII cells were assessed for CFSE loss and, subsequently, CFSE<sup>lo</sup> cells were assessed for direct ex vivo T-bet and IFN- $\gamma$  expression (2 independent experiments). (D) WT mice ( $n = 5$ ) were infected with *PbA* or left uninfected, and treated on day 2 p.i. with poly I:C or control saline. On day 3 p.i., bulk splenic cDCs were assessed by flow cytometry for cell surface CD86 expression, and on day 4 p.i., splenic CD4<sup>+</sup>TCR $\beta$ <sup>+</sup> single cells were assessed for intracellular IFN- $\gamma$  expression (without ex vivo stimulation) (performed once). (E) Number of splenic Th1 cells in CD11c-Cre<sup>+</sup> *Ifnar1<sup>fl/fl</sup>* mice and *Ifnar1<sup>fl/fl</sup>* littermate control mice ( $n = 5-6$ ) infected for 4 days with *PbA* and treated with poly I:C on day 2 p.i. \* $P < 0.05$ ; \*\* $P < 0.01$ .

generated mixed BM chimeric mice with congenically marked *Ifnar1<sup>+/+</sup>* and *Ifnar1<sup>-/-</sup>* splenic cDCs in equal proportions (Figure 2D). We then assessed CD86 expression by *Ifnar1<sup>+/+</sup>* and *Ifnar1<sup>-/-</sup>* splenic cDC subsets in these animals at 4 days p.i. with *PbA*. Consistent with data from *Ifnar1<sup>-/-</sup>* or  $\alpha$ -IFNAR1-treated mice (Figure

2C), CD86 upregulation was approximately 50% reduced in CD8<sup>+</sup> and CD8<sup>-</sup> *Ifnar1<sup>-/-</sup>* cDC subsets, compared with corresponding *Ifnar1<sup>+/+</sup>* cDC counterparts (Figure 2D), indicating that direct type I IFN signaling mediated systemic activation of both cDC subsets. To further establish direct type I IFN signaling as a mechanism for



systemic activation of cDCs, we used *Itgax-Cre<sup>+/-</sup> Ifnar1<sup>fl/fl</sup>* (*CD11c-Cre Ifnar1<sup>fl/fl</sup>*) mice, in which IFNAR1 deficiency was confined to CD11c<sup>hi</sup> cells (33, 34). We infected *CD11c-Cre Ifnar1<sup>fl/fl</sup>* mice and *Cre<sup>-</sup> Ifnar1<sup>fl/fl</sup>* littermate controls with *PbA*, and monitored IFNAR1 expression and CD86 upregulation on splenic cDCs at day 4 p.i. (Figure 2E). We first observed substantial reductions in IFNAR1 expression on CD8<sup>+</sup> and CD8<sup>-</sup> cDCs and minor reductions on other immune cells (Figure 2E). Then, consistent with our mixed BM chimeric data (Figure 2D), we once again observed approximately 50% less upregulation of CD86 by CD8<sup>+</sup> and CD8<sup>-</sup> cDCs in *PbA*-infected *CD11c-Cre Ifnar1<sup>fl/fl</sup>* mice, compared with infected *Ifnar1<sup>fl/fl</sup>* littermate controls (Figure 2E). Together, our data formally identify direct type I IFN signaling as a major cytokine-mediated mechanism for systemic activation of splenic cDCs during *PbA* infection.

*Type I IFN signaling in cDCs impairs their capacity to prime IFN-γ-producing Th1 cells in vivo.* To establish a causal link between type I IFN signaling in cDCs and Th1 suppression, we next compared Th1 responses in *PbA*-infected *CD11c-Cre Ifnar1<sup>fl/fl</sup>* mice and *Cre<sup>-</sup> Ifnar1<sup>fl/fl</sup>* littermate controls (Figure 3A). Strikingly, the proportion and absolute number of CD4<sup>+</sup> T cells expressing T-bet and IFN-γ was substantially increased in *CD11c-Cre Ifnar1<sup>fl/fl</sup>* mice compared with that in *Cre<sup>-</sup> Ifnar1<sup>fl/fl</sup>* littermate controls (Figure 3A). Furthermore, the amount of IFN-γ, not T-bet, produced on a per cell basis by Th1 cells was significantly enhanced in the absence of type I IFN signaling to cDCs (Figure 3A). Activation of polyclonal CD8<sup>+</sup> T cells, as assessed by granzyme B upregulation, was not significantly affected (Figure 3B). These data indicated that polyclonal Th1 responses were specifically boosted in magnitude and quality when cDCs were deprived of type I IFN signals. To confirm our findings in parasite-specific CD4<sup>+</sup> T cells, we next used an established transgenic system (16, 35–37), studying adoptively transferred, ovalbumin-specific, CFSE-labeled, OTII CD4<sup>+</sup> T cells after infection with a transgenic *PbA* strain, *PbTG*, which expresses the OTII peptide (Figure 3C). By day 4 p.i., the number of proliferated OTII cells, and their upregulation of T-bet, was equivalent in the spleens of WT and *Ifnar1<sup>-/-</sup>* recipient, *PbTG*-infected mice (Figure 3C). Notably, however, expression of IFN-γ was again increased on a per cell basis in proliferated OTII cells from *Ifnar1<sup>-/-</sup>* mice compared with WT recipient mice. Taken together, our data identify type I IFN signaling in cDCs as the causal mechanism suppressing IFN-γ production by parasite-specific Th1 cells during *PbA* infection.

To further establish a role for type I IFN signaling in suppressing Th1 responses, we next boosted type I IFN production in *PbA*-infected mice with a single dose of poly I:C and then studied splenic cDC and Th1 responses (Figure 3D). Poly I:C treatment triggered potent, systemic IFN-α production (data not shown) and was associated with substantially increased cDC activation the day after treatment and, most importantly, significantly impaired Th1 responses compared with those of saline-treated controls (Figure 3D). Poly I:C-mediated Th1 suppression was partially prevented in *CD11c-Cre Ifnar1<sup>fl/fl</sup>* mice compared with that in littermate *Ifnar1<sup>fl/fl</sup>* controls (Figure 3E), supporting the hypothesis that potent type I IFN signaling in cDCs impairs Th1 priming during *PbA* infection.

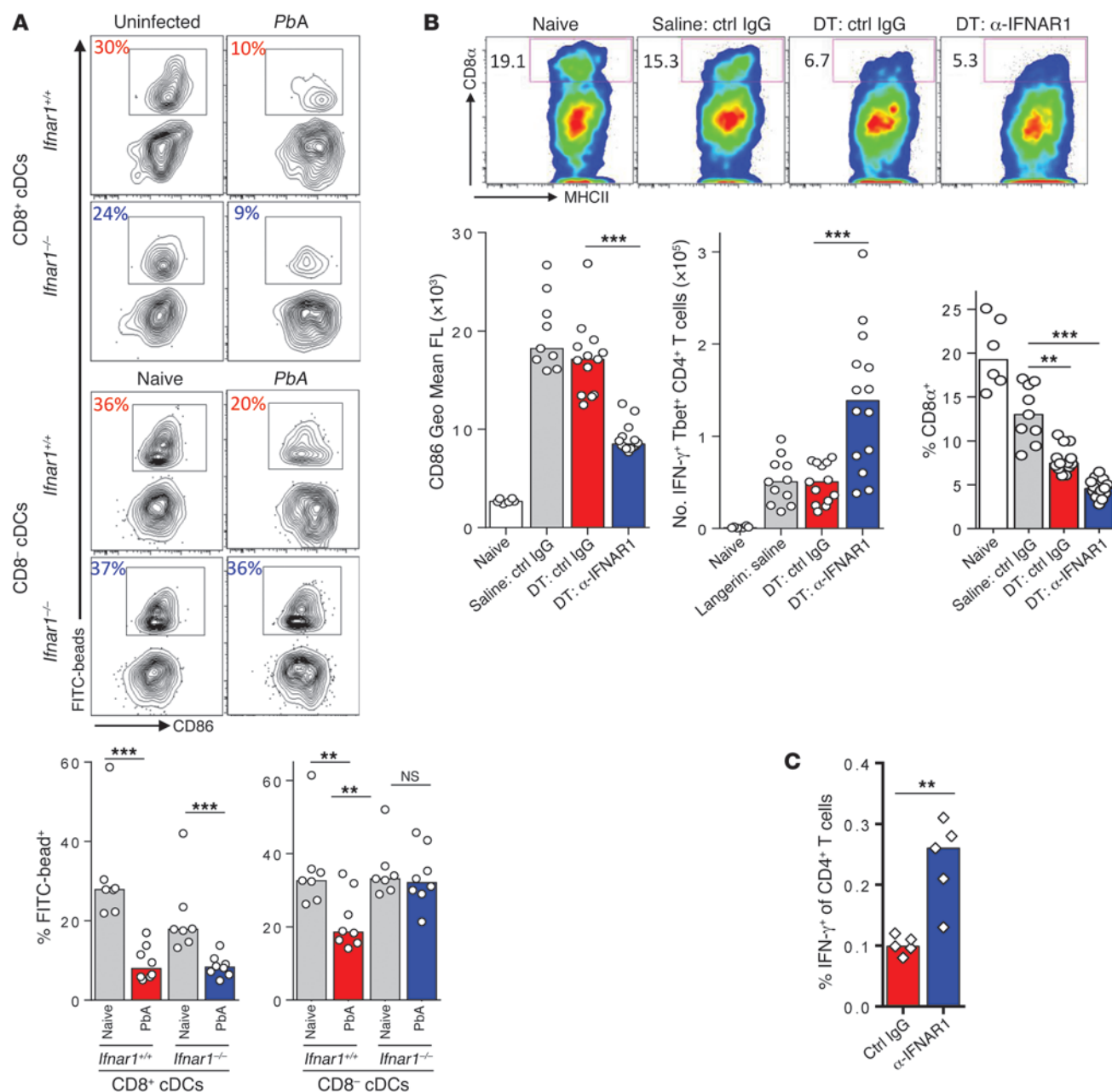
*Type I IFN signaling suppresses in vivo Th1 responses independently of CD8<sup>+</sup> CD207<sup>+</sup> splenic cDCs.* We next studied possible differential effects of type I IFN signaling on splenic cDC subsets in vivo. It was reported previously that the phagocytic capacity of cDC subsets was reduced dramatically during severe *Plasmodium* infection (16). Therefore, we used mixed BM chimeric mice, with equal

proportions of congenically marked *Ifnar1<sup>+/+</sup>* and *Ifnar1<sup>-/-</sup>* splenic cDCs, to investigate the effect of direct type I IFN signaling on phagocytosis (Figure 4A). Consistent with previous work (16), *Ifnar1<sup>+/+</sup>* CD8<sup>+</sup> and CD8<sup>-</sup> cDCs phagocytosed blood-borne, inert fluorescent beads efficiently prior to infection but poorly in mice after infection with *PbA* for 4 days. In stark contrast, while *Ifnar1<sup>-/-</sup>* CD8<sup>+</sup> cDCs remained sensitive to *PbA*-induced downregulation of phagocytosis, *Ifnar1<sup>-/-</sup>* CD8<sup>-</sup> cDCs retained their full phagocytic potential during infection (Figure 4A). These data provided clear in vivo evidence that direct type I IFN signals specifically impaired the function of splenic CD8<sup>-</sup> cDCs but not CD8<sup>+</sup> cDCs.

Therefore, we next hypothesized an important role for CD8<sup>-</sup> cDCs in type I IFN-regulated Th1 priming during *PbA* infection. To test this, we took advantage of the observation that many splenic CD8<sup>+</sup> cDCs express langerin (CD207), whereas CD8<sup>-</sup> cDCs do not (38). We were thus able to deplete splenic CD8<sup>+</sup> CD207<sup>+</sup> cDCs by diphtheria toxin (DT) treatment of langerin-DTR receptor (langerin-DTR) mice (with control mice receiving saline) (ref. 38 and Figure 4B). CD8<sup>+</sup> cDC-depleted and control langerin-DTR mice were infected with *PbA* and administered α-IFNAR1 or control IgG. We confirmed substantial, though not complete, depletion of CD8<sup>+</sup> cDCs within the cDC compartment in DT-treated langerin-DTR mice, compared with that in controls (Figure 4B), in accordance with other studies (38). This had no effect on upregulation of CD86 by CD8<sup>-</sup> cDCs or on the emerging Th1 response (Figure 4B). This is the first in vivo evidence that Th1 priming during blood-stage *Plasmodium* infection proceeds independently of CD8<sup>+</sup> CD207<sup>+</sup> cDCs. Importantly, blockade of type I IFN signals in mice depleted of CD8<sup>+</sup> CD207<sup>+</sup> cDCs triggered both a reduction in CD86 upregulation by remaining CD8<sup>-</sup> cDCs and a concomitant increase in Th1 responses (Figure 4B). To further test the independence of type I IFN-mediated Th1 suppression from CD8<sup>+</sup> cDCs, we administered α-IFNAR1 or control IgG to *PbA*-infected Clec9a-DTR mice that had been depleted of CD8<sup>+</sup> cDCs with DT (Figure 4C and ref. 39). Using this second model of CD8<sup>+</sup> cDC depletion, we again noted a significant increase in IFN-γ production by polyclonal CD4<sup>+</sup> T cells. Thus, taken together, our in vivo data indicated that CD8<sup>-</sup> cDCs primed Th1 cells more effectively after blockade of type I IFN signaling.

*Type I IFN signaling impairs Th1 priming by splenic CD8<sup>-</sup> cDCs.* To determine whether blockade of type I IFN signaling improved Th1 priming by CD8<sup>-</sup> cDCs, we first sort-purified splenic CD8<sup>-</sup> cDCs, along with CD8<sup>+</sup> cDCs for comparison, from *PbA*-infected WT and *Ifnar1<sup>-/-</sup>* mice (as well as from uninfected WT control mice). Given equivalent cell surface expression of MHCII by CD8<sup>-</sup> cDCs from infected WT and *Ifnar1<sup>-/-</sup>* mice (data not shown), we then loaded WT and *Ifnar1<sup>-/-</sup>* cDCs ex vivo with OTII peptide (recognized by ovalbumin-specific CD4<sup>+</sup> TCR transgenic T cells) and used these to drive de novo Th development in naive CD4<sup>+</sup> OTII T cells in vitro (Figure 5A). Consistent with previous reports, CD8<sup>+</sup> cDCs from all groups stimulated only modest IFN-γ production by OTII cells (16, 19), reflective of weak Th1 priming. In contrast, while WT CD8<sup>-</sup> cDCs drove appreciable IFN-γ expression by OTII cells, *Ifnar1<sup>-/-</sup>* CD8<sup>-</sup> cDCs exhibited the greatest capacity to do this, thus indicating a superior capacity to drive Th1 development (Figure 5A). In addition, we saw no difference in proliferation or Th2 or Th17 cytokine production by OTII cells stimulated with CD8<sup>-</sup> cDCs from either WT or *Ifnar1<sup>-/-</sup>* infected mice (data not shown).

Given that OTII cells do not differentiate readily into T-bet<sup>+</sup> Th1 cells (40), we next chose to stimulate ex vivo an alternative CD4<sup>+</sup>

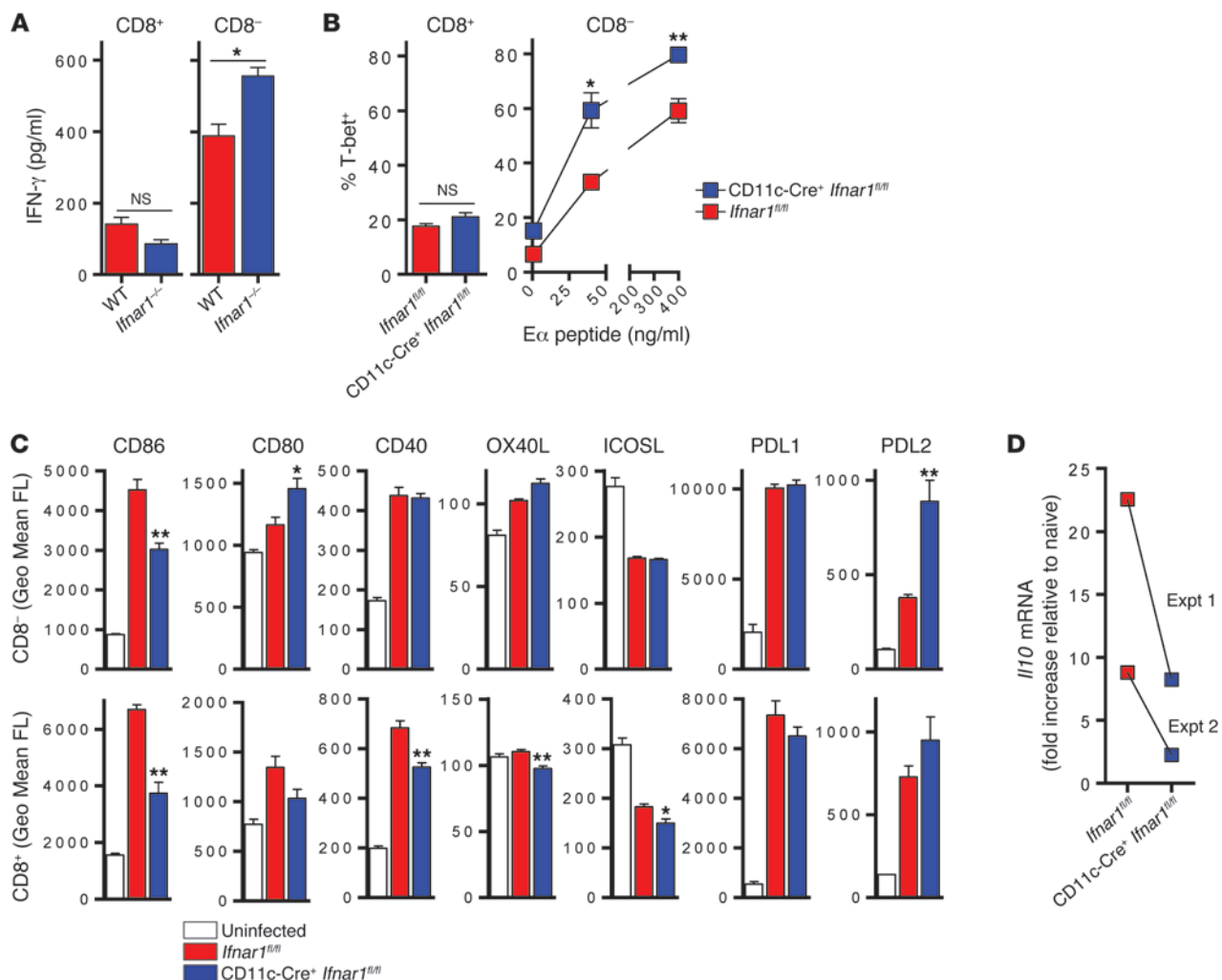


### Figure 4

Figure 1  
Type 1 IFN signaling suppresses in vivo Th1 responses independently of CD8<sup>+</sup> splenic cDCs. **(A)** 50:50 WT (CD45.1<sup>+</sup>)/*Ilfnar1*<sup>-/-</sup> (CD45.2<sup>+</sup>) mixed BM chimeric mice ( $n = 6-8$ ), either uninfected or infected 4 days previously with *PbA*, were injected i.v. with FITC<sup>+</sup> beads. 3 hours later, WT and *Ilfnar1*<sup>-/-</sup> splenic cDC (CD11c<sup>hi</sup>MHCII<sup>hi</sup>TCR $\beta$ -B220<sup>-</sup> single cells) subsets (CD8<sup>+</sup> and CD8<sup>-</sup>) were assessed by flow cytometry for uptake of FITC<sup>+</sup> beads. Percentages indicate proportion of each cDC population containing at least one bead (2 independent experiments). **(B)** C57BL/6 langerin-DTR mice were administered DT or control saline, prior to and during *PbA* infection; *PbA*-infected, DT-treated mice were also treated with  $\alpha$ -IFNAR1 or control IgG. (A group of mice was also left uninfected and nontreated.) 4 days p.i., flow cytometric analysis of the proportion of splenic CD11c<sup>hi</sup>MHCII<sup>hi</sup>TCR $\beta$ -B220<sup>-</sup> cDCs expressing CD8 $\alpha$  as well as cell surface CD86 expression by CD8<sup>-</sup> cDCs and the number of CD4<sup>+</sup> TCR $\beta$ <sup>+</sup> cells expressing T-bet and IFN- $\gamma$  (without ex vivo stimulation) were assessed (2 independent experiments). **(C)** The percentage of splenic CD4<sup>+</sup> T cells making IFN- $\gamma$  directly ex vivo from Clec9a-DTR (C57BL/6  $\times$  BALB/c F1 background) mice treated with DT, infected for 4 days with *PbA*, and treated with  $\alpha$ -IFNAR1 or control IgG (experiment performed once). \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

T cell from TEa TCR transgenic mice that upregulates T-bet more readily (40, 41). TEa cells express a T cell receptor specific for an I-E<sup>d</sup>-derived peptide (Ea52-68) when presented by MHCII I-A<sup>b</sup> molecules (41). When sorted TEa T cells were cultured with CD8<sup>+</sup>

cDCs from *PbA*-infected *CD11c-Cre Ifnar1<sup>fl/fl</sup>* mice or *Ifnar1<sup>fl/fl</sup>* littermates and loaded exogenously with Eα52-68 peptide (Figure 5B), they did not strongly upregulate intracellular T-bet protein expression, which is consistent with the above OTII

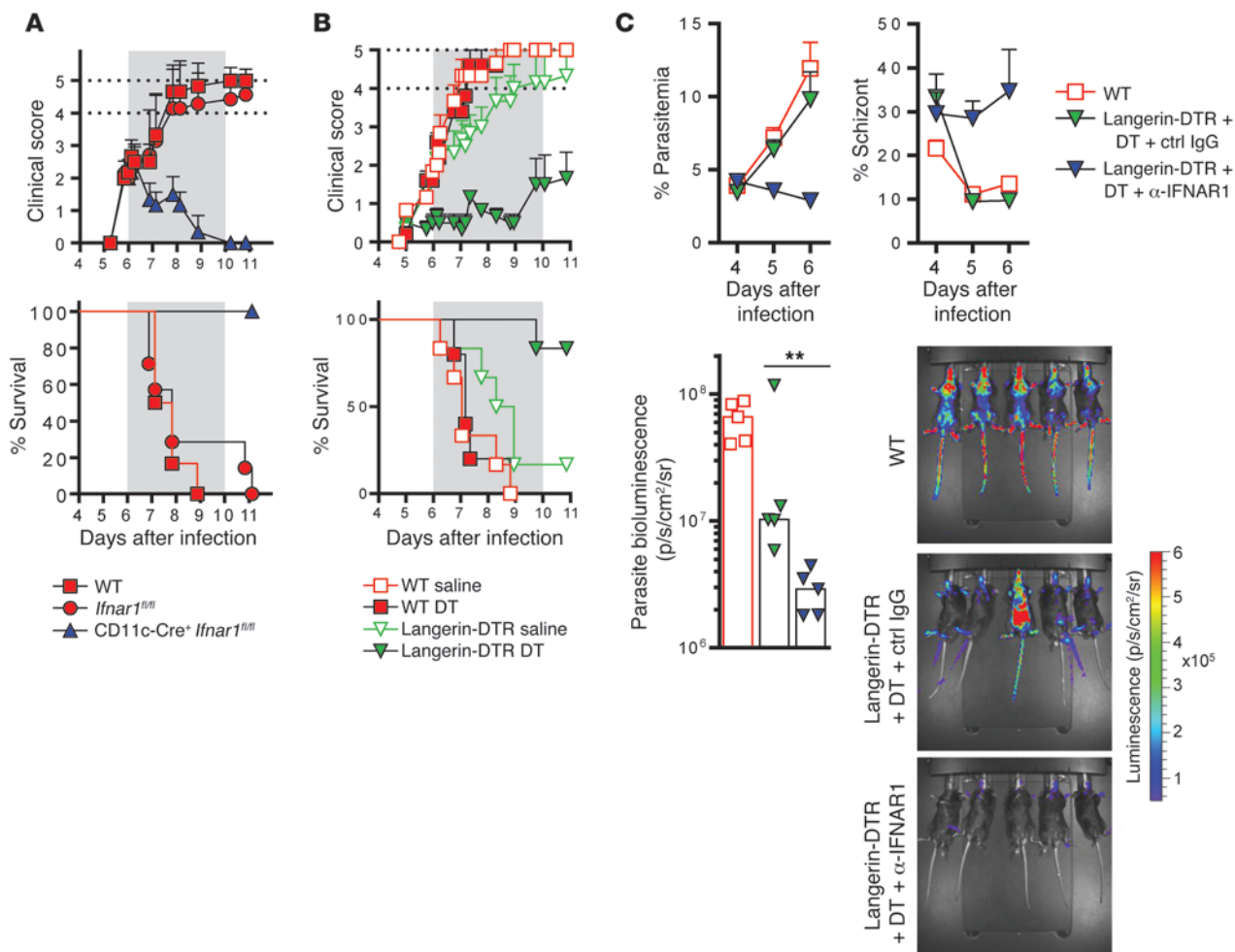
**Figure 5**

Type I IFN signaling in cDCs impairs Th1 priming by CD8<sup>-</sup> cDCs ex vivo. (A) IFN- $\gamma$  assessed in tissue culture supernatants after CD4<sup>+</sup> OTII cells (3–6 replicate wells) were stimulated for 4 days with ex vivo OTII peptide-loaded, cell-sorted, splenic CD8<sup>+</sup> and CD8<sup>-</sup> cDCs pooled from WT and *Ifnar1*<sup>-/-</sup> mice ( $n = 5$ –6) infected 4 days previously with PbA (data are mean  $\pm$  SEM from a single experiment representative of 2). (B) T-bet expression by CD4<sup>+</sup> TCR transgenic TEa T cells (4–5 replicate wells) stimulated for 5 days with ex vivo E $\alpha$ 52–68 peptide-loaded, cell-sorted, splenic CD8<sup>+</sup> (400 ng/ml peptide) and CD8<sup>-</sup> cDCs (40 and 400 ng/ml peptide) pooled from *CD11c-Cre<sup>+</sup> Ifnar1<sup>fl/fl</sup>* mice and *Ifnar1*<sup>fl/fl</sup> littermate control mice ( $n = 6$ –10) infected 4 days previously with PbA (representative of 2 independent experiments). (C) FACS analysis of cell surface costimulatory molecule expression by splenic CD8<sup>+</sup> and CD8<sup>-</sup> cDCs (*CD11c<sup>hi</sup>MHCII<sup>hi</sup>TCR $\beta$ -B220<sup>-</sup>* single cells) in *CD11c-Cre<sup>+</sup> Ifnar1<sup>fl/fl</sup>* mice and *Ifnar1*<sup>fl/fl</sup> littermate control mice ( $n = 5$ –6) infected 4 days p.i. with PbA. (D) Mean fold increase in *Il10* mRNA levels (compared with that in CD8<sup>-</sup> cDCs from uninfected WT mice) in pooled ( $n = 6$ –10) CD8<sup>-</sup> cDCs sorted from spleens of *CD11c-Cre<sup>+</sup> Ifnar1<sup>fl/fl</sup>* mice and *Ifnar1*<sup>fl/fl</sup> littermate controls. Data from 2 independent experiments presented. \* $P < 0.05$ ; \*\* $P < 0.01$ .

data and previous reports (16, 19). In contrast, TEa T cells significantly upregulated T-bet when stimulated with CD8<sup>-</sup> cDCs from infected *Ifnar1*<sup>fl/fl</sup> mice (Figure 5B). Most importantly, however, T-bet upregulation was strongest when stimulated with CD8<sup>-</sup> cDCs from infected *CD11c-Cre Ifnar1*<sup>fl/fl</sup> mice (Figure 5B). Together, our ex vivo antigen presentation experiments indicate that CD8<sup>-</sup> cDCs are specifically impaired from priming Th1 cells via type I IFN signaling to cDCs in vivo.

We next searched for possible mechanisms to account for the improved Th1-priming capacity of CD8<sup>-</sup> cDCs after type I IFN signaling to cDCs was abrogated. An assessment of B7 family costimulatory molecule expression by CD8<sup>+</sup> and CD8<sup>-</sup> cDCs from PbA-infected *CD11c-Cre Ifnar1*<sup>fl/fl</sup> mice and *Ifnar1*<sup>fl/fl</sup> littermates

revealed that, in addition to reduced CD86 upregulation in both cDC subsets, there were modest effects on many molecules, including CD80, CD40, OX40L, and ICOSL, which appeared mostly on CD8<sup>+</sup> cDCs (Figure 5C). Of particular interest, however, was a specific 2- to 3-fold increase in PDL2 expression on CD8<sup>-</sup> cDCs, but not CD8<sup>+</sup> cDCs, in the absence of cDC-specific type I IFN signaling (Figure 5C), an observation also seen on CD8<sup>-</sup> cDC-depleted, infected Clec9a-DTR mice treated with  $\alpha$ -IFNAR1 compared with control IgG (data not shown). Furthermore, we noted that upregulation of *Il10* mRNA by CD8<sup>-</sup> cDCs was reduced in *CD11c-Cre Ifnar1*<sup>fl/fl</sup> mice compared with that in controls (Figure 5D), while we found no evidence for increased *Il12p40* or *p35* mRNA levels (data not shown). Together these data indicate that

**Figure 6**

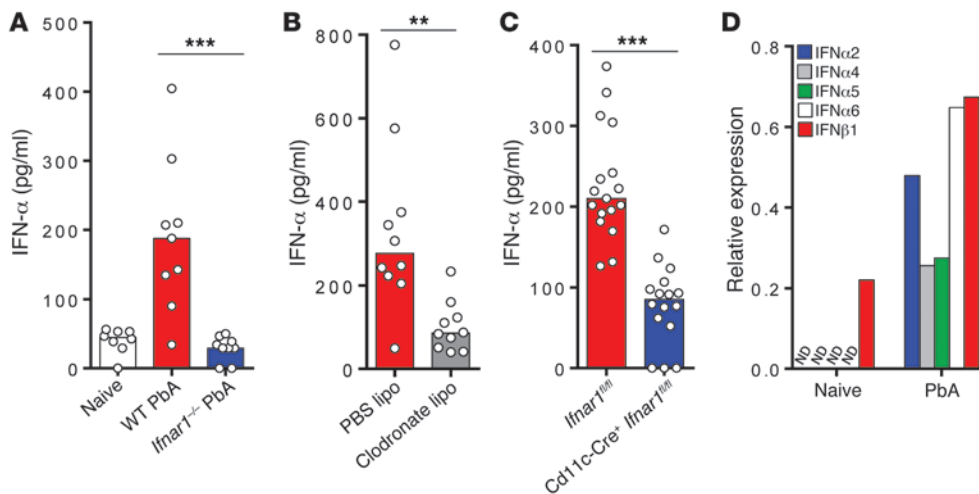
Type I IFN signaling in cDCs mediates disease susceptibility and impairs parasite control during *PbA* infection. (A) *CD11c-Cre<sup>+</sup> Ifnar1<sup>fl/fl</sup>* mice and *Ifnar1<sup>fl/fl</sup>* littermate controls ( $n = 8$ ) and WT mice were infected with *PbA* and scored for clinical symptoms and percent survival. Gray area indicates time frame for neurological symptoms in WT mice. Dotted line at clinical score 4 indicates moribund threshold. (B) Langerin-DTR and WT mice ( $n = 6$ ) were treated with DT or saline prior to and during infection with *PbA*. Mice were monitored for clinical scores and percent survival. Gray area indicates time frame for neurological symptoms in WT saline-treated group. Dotted line at clinical score 4 indicates moribund threshold. (C) Parasitemias and the proportion being mature-stage schizonts in peripheral blood, and whole body luciferase-expressing parasite-derived bioluminescence (day 6 p.i.) in langerin-DTR mice ( $n = 5$ ), given DT prior to and throughout infection with *PbA* and treated with  $\alpha$ -IFNAR1 or Ctrl IgG (compared with *PbA*-infected WT mice). \*\* $P < 0.01$ .

improved function of CD8<sup>+</sup> cDCs, as a result of type I IFN signaling blockade in cDCs, was associated with increased PDL2 expression and reduced *Il10* mRNA levels.

*Type I IFN signaling in cDCs mediates disease susceptibility and impairs parasite control during PbA infection.* We next determined the impact of preventing type I IFN signaling to cDCs on disease outcome during lethal *PbA* infection. First, we observed that *CD11c-Cre Ifnar1<sup>fl/fl</sup>* mice were completely protected from neurological symptoms and death, compared with *Ifnar1<sup>fl/fl</sup>* littermates or WT control mice (Figure 6A), thus indicating an absolute requirement for type I IFN signaling in cDCs for disease susceptibility in this model of severe malaria. To determine whether parasite control was improved by blocking type I IFN signaling in the absence of CD8<sup>+</sup> cDCs, we first confirmed a recent report that mice depleted of CD8<sup>+</sup> CD207<sup>+</sup> cDCs were protected from lethal neurological disease during *PbA*

infection (ref. 42 and Figure 6B). Consistent with this report, we found that langerin-DTR mice were resistant to disease when treated with DT but not saline. WT control mice given either DT or saline remained equally susceptible to lethal disease (Figure 6B).

We next determined whether type I IFN blockade could improve parasite control in disease-resistant, CD8<sup>+</sup> CD207<sup>+</sup> cDC-depleted mice (Figure 6C). Strikingly, we observed that DT-treated, langerin-DTR mice given  $\alpha$ -IFNAR1 exhibited much improved control of peripheral blood parasitemia compared with control IgG-treated counterparts (Figure 6C). Moreover, given our recent data that large proportions of mature-stage parasites in the bloodstream are indicative of a slow-growing parasite population (43), we also saw evidence of slowed parasite population growth as a result of  $\alpha$ -IFNAR1 treatment (Figure 6C). Finally, we observed nearly a 10-fold reduction in whole body parasite burdens in  $\alpha$ -IFNAR1-

**Figure 7**

Splenic IFN- $\alpha$  production depends on feed-forward, type I IFN signaling in cDCs. Assessment of serum IFN- $\alpha$  levels on day 4 p.i. in *PbA*-infected (A) WT and *Ifnar1*<sup>-/-</sup> mice (pooled from 2 independent experiments), (B) mice treated 1 day prior to infection with clodronate liposomes or control PBS liposomes (pooled from 2 independent experiments), and (C) *CD11c-Cre*<sup>+</sup> *Ifnar1*<sup>fl/fl</sup> mice and *Ifnar1*<sup>fl/fl</sup> littermate control mice (pooled from 2 independent experiments). (D) IFN- $\alpha$ / $\beta$  gene expression by qPCR in sorted splenic CD8<sup>-</sup> cDCs from pooled WT mice ( $n = 5$ ) 3 days p.i. with *PbA* or naive controls. Results indicate expression relative to sorted splenic pDCs from WT infected mice on day 3 p.i. ND, not detected. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

treated mice compared with that in control IgG-treated, CD8<sup>+</sup> CD207<sup>+</sup> cDC-depleted mice (Figure 6C). Together, our data are consistent with type I IFN signaling to CD8<sup>-</sup> cDCs impairing parasite control and thus contributing to lethal disease during *PbA* infection.

cDCs participate in a feed-forward loop that maximizes rapid type I IFN production. Our previous data suggested that pDCs are unlikely to be the sole source of suppressive type I IFN during *PbA* infection (20). In contrast, since cDCs prime *Plasmodium*-specific CD4<sup>+</sup> T cell responses, express type I IFN genes (20), and, as shown here, are themselves targets of type I IFN signaling, we hypothesized that cDCs operated in a cytokine-signaling loop, in which they not only responded to, but were also required for, the production of type I IFNs. To test this, we first measured serum IFN- $\alpha$  levels in *PbA*-infected *Ifnar1*<sup>-/-</sup> mice (Figure 7A) and WT mice depleted of phagocytic cells by clodronate liposome treatment (Figure 7B). In both situations, serum IFN- $\alpha$  levels were greatly reduced, demonstrating the existence of an IFNAR1-dependent signaling loop requiring phagocytic cells. We also observed substantially lower levels of serum IFN- $\alpha$  at day 4 p.i. in *CD11c-Cre Ifnar1*<sup>fl/fl</sup> mice, compared with those in infected *Ifnar1*<sup>fl/fl</sup> littermate controls (Figure 7C). Finally, given that CD8<sup>-</sup> cDCs were major targets of type I IFN-mediated suppression, we next determined whether these cells in particular might be a source of type I IFNs. By day 3 p.i., we noted marked upregulation of several IFN- $\alpha$ / $\beta$  mRNAs, not only in pDCs, as expected (20), but also in CD8<sup>-</sup> cDCs (Figure 7D). Taken together, our data suggest that during *PbA* infection, a feed-forward loop comprised of IFN- $\alpha$ / $\beta$  production by CD8<sup>-</sup> cDCs and autocrine or paracrine type I IFN signaling in CD8<sup>-</sup> cDCs contributes to the impairment of Th1 priming.

## Discussion

In this study, we show that type I IFN signaling directly modulates the function of CD8<sup>-</sup> cDCs during severe blood-stage *Plasmodium*

infection and, by doing so, limits their ability to drive IFN- $\gamma$  production by Th1 cells. Thus, we have provided a cellular mechanism to explain how CD4<sup>+</sup> T cell responses are impaired by type I IFNs during systemic disease. Moreover, while type I IFN-mediated susceptibility to nonviral pathogens has been shown to act via macrophages, monocytes, and neutrophils (1–3, 5, 6, 8, 11), this is the first report to identify a direct effect on cDCs. In this work, *Plasmodium*-induced type I IFN signaling suppressed both cDC function and monocytic responses, although monocytes played no role in Th1 priming over the short time frame of our acute model. We speculate that multiple type I IFN-dependent suppressive strategies could operate in other infection models, with pathogen susceptibility being variously dependent on cDC or monocyte suppression.

Several groups have established that blood-stage *Plasmodium* infection triggers profound systemic cDC activation and dysfunction using murine and human experimental systems (16–18, 44–47). However, the host-derived factors that contribute to this process have remained unclear, although one report suggested that systemic TNF mediates cDC paralysis (44). Nevertheless, our data clearly demonstrate a significant role for type I IFN signaling in functional impairment of CD8<sup>-</sup> cDCs.

CD8<sup>+</sup> cDCs have received greater attention than CD8<sup>-</sup> cDCs in many studies in recent years, particularly for their capacity to efficiently cross-present exogenous antigen to CD8<sup>+</sup> T cells and produce IL-12 rapidly (reviewed in refs. 22, 23). Moreover, recent studies highlight the unique ability of CD8<sup>+</sup> cDCs to drive immune-pathogenic CD8<sup>+</sup> T cell responses during *PbA* infection (39, 42). However, elegant *in vivo* studies, which targeted antigenic proteins to splenic cDC subsets, demonstrated that CD8<sup>-</sup> cDCs possess a greater intrinsic capacity for processing antigens onto MHCII than CD8<sup>+</sup> cDCs (24). However, in subsequent studies, in which Th1 development was assessed after similar *in vivo* targeting of cDC subsets in the presence of poly I:C, CD8<sup>-</sup> cDCs substantially underperformed compared with CD8<sup>+</sup> cDCs (25, 26). Indeed, we also observed that poly I:C treatment reduces Th1 responses during *Plasmodium* infection (Figure 3, D and E). Our data provide an explanation for such suboptimal CD8<sup>-</sup> cDC function: while these cells express the appropriate intracellular machinery to efficiently process onto MHCII, excessive type I IFN responses functionally paralyze these cells and impair their capacity to drive Th1 development. Our work supports a role for CD8<sup>-</sup> cDCs in driving Th cell differentiation *in vivo* (48–50) but, most importantly, shows that under conditions in which substantial type I IFN is produced, CD8<sup>-</sup> cDCs are prevented from priming strong Th1 responses.

In the model of acute, severe malaria used in this study, enhanced moDC responses did not appear to improve early Th1 develop-



ment. These data are entirely consistent with previous studies in which CCR2-expressing cells played little or no role during the first week of infection in controlling blood-stage *Plasmodium* infection (32, 51). Nevertheless, work using other malaria models and pathogens suggests that enhancing monocyte and moDC activity via type I IFN blockade could play a beneficial role in persistent and chronic *Plasmodium* infections, via direct killing of parasitized rbc, and maintenance of Th1 immunity (51–53).

During viral infection, type I IFNs are produced by pDCs in a TLR9-dependent manner (reviewed in ref. 54). However, while these cells are activated during *Plasmodium* infection, they do not play a major role in immune cell activation or disease outcome (20, 21). Data from nonviral infection models indicate that various macrophage populations in the spleen are major sources of type I IFNs (28, 55, 56). Our data are consistent with this notion and also propose that cDCs themselves could produce and respond to type I IFNs in an autocrine or paracrine manner (57). Thus, we propose a model in which appropriately positioned phagocytic cells in the spleen, such as macrophages and DCs located in the marginal zone, interact with parasitized rbc and produce type I IFNs. These cytokines then restrict PDL2 expression, increase *Il10* mRNA levels, and suppress phagocytosis in all splenic CD8<sup>+</sup> cDCs, regardless of whether they have taken up parasites or not. Ultimately, this process restricts IFN- $\gamma$  production by T-bet<sup>+</sup> Th1 cells.

Our data demonstrated that type I IFN signaling in CD8<sup>+</sup> cDCs induced a costimulatory phenotype that was suboptimal for Th1 priming and was associated with higher CD86/CD80 ratios and reduced PDL2 expression. The functional consequences of modulating CD86/CD80 expression levels remain unclear at present. However, given different binding affinities of CD80 and CD86 for CD28 and CTLA-4 (58–60), it is possible that changes in the relative expression of these molecules by CD8<sup>+</sup> cDCs will have functional consequences for Th1 priming. The functional consequences of increased PDL2 levels on CD8<sup>+</sup> cDCs for Th1 priming also remain unstudied at present. However, given that PD1/PDL1 (but not PD1/PDL2) interactions potentially suppress Th1 responses during experimental malaria (61, 62), and since PDL2 and PDL1 exhibit similar  $K_d$  values for PD1 (63), we hypothesize that PDL2 disrupts the suppressive interactions between PD1 on Th cells and PDL1 on CD8<sup>+</sup> cDCs. In addition to effects on costimulatory molecule expression, we observed a substantial decrease in *Il10* mRNA expression in CD8<sup>+</sup> cDCs deprived of type I IFN signals. Although we could not find evidence of increased *Il12p40* or *p35* mRNA expression in these cells (data not shown), it is possible that reduced cDC expression of the suppressive cytokine IL-10 might lead directly or indirectly to effects on Th1 cells. Finally, we also observed that type I IFN signaling in CD8<sup>+</sup> cDCs suppressed phagocytosis. It is therefore possible that, in addition to effects on costimulatory molecule expression and IL-10, a sustained ability to take up parasitized red blood cells (prbc) might also contribute partially to an improved capacity for Th1 priming in CD8<sup>+</sup> cDCs. This aspect of CD8<sup>+</sup> cDC function remains to be addressed fully.

In considering suppression of CD8<sup>+</sup> cDCs by type I IFN signaling more generally, it is likely that this process is beneficial in some instances for the prevention or amelioration of Th cell-mediated immune pathology, for example, in multiple sclerosis models (64, 65). Nevertheless, during the coevolution of *Plasmodium* and humans, it is possible that this mechanism permitted both the prevention of host immune pathology and the provision to the parasite of an opportunity to establish itself within the host. Given

a report that AT-rich DNA motifs in the *Plasmodium falciparum* genome trigger type I IFN production, it is tempting to hypothesize that *Plasmodium*, via its DNA, triggers type I IFN responses during the blood stage of infection to facilitate its own survival (13).

The functional impairment of CD8<sup>+</sup> cDCs by direct type I IFN signaling during blood-stage *Plasmodium* infection suggests that blockade of the IFNAR1/2 receptor complex on these cells could improve naturally acquired and vaccine-mediated protection against malaria. There is currently no licensed vaccine against *Plasmodium*, and the current best candidate was recently shown to provide only modest, short-lived, protection in phase III clinical trials in Africa (66). Given that humoral and cellular immunity to *Plasmodium* is likely to hinge upon effective CD4<sup>+</sup> T cell responses (67), these could be boosted by a vaccine strategy acting on the appropriate cDC population. Moreover, it is possible that people living in endemic regions, who experience repeated *Plasmodium* infections, may benefit from immune therapies aimed at transiently blocking suppressive type I IFN signals to specific cDC subsets. The benefit of such a highly targeted approach may be to improve protection against severe malaria without excessively compromising cytotoxic T cell-mediated immunity to viral pathogens. Finally, the question of whether viral vectors, which drive strong type I IFN responses, are indeed appropriate formulations for Th1-dependent vaccines should be considered carefully (68, 69). Further work is required to determine the affect of type I IFN signaling on vaccination and immune therapy against *Plasmodium* infection.

## Methods

**Mice, parasites, and infections.** C57BL/6 and congenic CD45.1<sup>+</sup> C57BL/6 mice (female, 6 to 10 weeks of age) were purchased from Australian Resource Centre (Canning Vale) and maintained under conventional conditions. C57BL/6 *Ifnar1*<sup>-/-</sup> mice (70, 71), langerin-DTR mice (38), *Itgax-Cre*<sup>+/-</sup> *Ifnar1*<sup>f/f</sup> mice (33), *Ifnar1*<sup>f/f</sup> mice (72), *Ccr2*<sup>-/-</sup> mice (73), congenic (CD45.1<sup>+</sup>) OTII mice (74), and TEa  $\times$  *Rag1*<sup>-/-</sup> mice (41) were all maintained in-house at QIMR Berghofer Medical Research Institute. Clec9a-DTR mice (39) were housed and used according to local animal ethics requirements at Nanyang Technological University.

BM chimeric mice were prepared by i.v. injecting  $5 \times 10^6$  fresh syngeneic BM cells (from femurs of CD45.1<sup>+</sup> WT and CD45.2<sup>+</sup> *Ifnar1*<sup>-/-</sup> mice, mixed at a 50:50 ratio) into lethally irradiated (11 Gy [<sup>137</sup>Cs source]) CD45.1<sup>+</sup> C57BL/6 recipient mice and treating them for 14 days with antibiotics in drinking water (neomycin sulphate, 1 g/l; Sigma-Aldrich). Engraftment in mixed BM chimeras was assessed after 8 to 12 weeks by flow cytometry. Chimeras were infected 12 weeks after BM transplantation.

*PbA* parasites were used after 1 in vivo passage in WT C57BL/6 mice. Transgenic *PbA* (clone 231c11) constitutively expressing luciferase and GFP from the EF1- $\alpha$  promoter (*PbA-luc*) was used for all *PbA* experiments, unless stated otherwise (35, 75). A transgenic *PbA* strain (*PbTG*) expressing model T cell epitopes, including the OTII epitope and control strains (*PbG*), was obtained from William R. Heath (University of Melbourne, Melbourne, Australia) and maintained and used as previously reported (37). Mice were infected i.v. with  $10^5$  prbc, and blood parasitemia was measured in Diff-Quick-stained (Lab Aids) thin blood smears obtained from tail bleeds. Alternatively, an adapted version of a previously established flow cytometric method (76–78) was used to measure parasitemia more rapidly. Briefly, a single drop of blood, from a tail bleed or cardiac puncture, was diluted and mixed in 200  $\mu$ l RPMI containing 5 U/ml heparin sulphate. Diluted blood was stained simultaneously with Syto84 (5  $\mu$ M; Life Technologies) to detect RNA/DNA and Hoechst33342 (10  $\mu$ g/ml; Sigma-Aldrich) to detect DNA for 30 minutes in the dark at room temper-



ature. Staining was quenched with 2 ml RPMI, and samples were immediately analyzed by flow cytometry using a BD FACSCanto II Analyzer (BD Biosciences) and FlowJo software (Treestar). prbc were readily detected as being Hoechst33342<sup>+</sup> Syto84<sup>+</sup>, with white blood cells excluded on the basis of size, granularity, and much higher Hoechst33342/Syto84 staining compared with prbc. Parasitemias were routinely measured at all day 4 p.i. analyses, with no statistically significant differences seen between *PbA*-infected WT C57BL/6 mice and those bearing any genetic modification.

**Preparation of splenic lysates and detection of IFN- $\alpha$  protein and cytokines.** Spleens were harvested into 2 ml ice-cold RPMI containing a general protease inhibitor cocktail (P2714, Sigma-Aldrich; used according to the manufacturer's instructions) and passed through 100- $\mu$ m cell strainers to create single cell suspensions. 0.5% v/v Triton X-100 (Sigma-Aldrich) was added for 15 minutes on ice to lyse cells. Lysates were centrifuged at 14,000 g for 5 minutes to pellet cellular debris. Lysate supernatants were immediately snap frozen and stored at  $-80^{\circ}\text{C}$  until analyzed. IFN- $\alpha$  was detected in splenic lysates or sera using an antibody-based, cytometric bead kit (FlowCytomix, eBioscience), according to the manufacturer's instructions, with bead populations analyzed on a FACSArray analyzer and results processed using FCAP array software (BD Biosciences). All other cytokines were measured using CBA Flex Sets (BD Biosciences) according to the manufacturer's instructions, with samples assessed as above.

**Flow cytometry and cell sorting.** Blood and spleen mononuclear cells were prepared as previously described (35, 36, 75), ensuring maximal recovery of splenic cDCs by treating spleens with deoxyribonuclease I (0.5 mg/ml; Worthington Biochemical) and collagenase type 4 (1 mg/ml; Worthington Biochemical) for 25 minutes at room temperature. Fluorescently conjugated mAbs against B220 (RA3-6B2), 120G8, CD11c-APC (N418), MHCII (M5/114.15.2), CD4 (GK1.5), CD8 $\alpha$  (53-6.7), IFN- $\gamma$  (XMG1.2), CD19 (6D5), CD45.1 (A20), CD45.2 (clone 104), Ly6C (HK1.4), CD11b (M1/70), CD40 (1C10), CD80 (16-10A1), CD86 (GL1), OX40L (RM134L), ICOSL (B7-RP1), and PDL2 (TY25) were purchased from Biolegend. Fluorescently conjugated mAbs against TCR $\beta$  chain (H57-597), NK1.1 (PK136), and PDL1 (MIH5) were purchased from BD Biosciences. Anti-T-bet mAb (eBio4B10) was purchased from eBioscience, and anti-granzyme B (MHGB04) was purchased from Life Technologies. Cell surface and intracellular IFN- $\gamma$  staining was performed as previously described (35, 36).

To sort purify cDCs, pooled spleens were treated as above with deoxyribonuclease I and collagenase type 4, and rbc were lysed as previously described (35, 36, 75). CD11c<sup>hi</sup> cells were enriched using CD11c microbeads according to the manufacturer's instructions (Miltenyi Biotec), and cDC subsets were identified during cell sorting as being (propidium iodide<sup>-</sup>/TCR $\beta$ /B220<sup>-</sup>/120G8<sup>-</sup>/CD11c<sup>hi</sup>) CD11b<sup>lo-mid</sup>, CD8 $\alpha$ <sup>+</sup> (purity >99%). Harvesting of cDCs from each group of mice within an experiment was staggered to minimize and normalize cDC isolation times for each group. Cells were recounted via Trypan Blue exclusion prior to plating.

**In vivo receptor blockade, cell depletion, and poly I:C treatment.** Anti-IFNAR1 blocking mAb (MAR1-5A3, Leinco Technologies Inc.) and its isotype control mAb were administered in 0.1 mg doses, via i.p. injection, on the day of infection and 2 days afterward. Cellular depletion in langerin-DTR and Clec9a-DTR mice was performed by i.p. injection of 10 ng/g DT (Sigma-Aldrich) in 200  $\mu$ l 0.9% saline (Baxter) per mouse the day prior to infection and on days 1 and 3 p.i. poly I:C (Sigma-Aldrich) was dissolved in RPMI and administered as a single 100  $\mu$ g dose i.p. on day 2 p.i.

**CD4<sup>+</sup> cell enrichment and adoptive transfer.** Spleens from CD45.1<sup>+</sup> OTII mice were aseptically removed, and mononuclear cells were prepared as described previously (35, 75, 79). CD4<sup>+</sup> cells were enriched using CD4 microbeads according to the manufacturer's instructions (Miltenyi Biotec) (CD4<sup>+</sup> cell purity was routinely 85%), labeled with CFSE (5  $\mu$ M; Life Technologies) in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free PBS for 15 minutes at room temperature in the

dark, and washed twice in 10 volumes Ca<sup>2+</sup>/Mg<sup>2+</sup>-free PBS. CFSE-labeled CD4<sup>+</sup> cells were resuspended in RPMI/2% normal mouse serum, and 200  $\mu$ l volumes were injected i.v. via a lateral tail vein using a 26-gauge needle.

**In vivo phagocytosis assay.** This protocol was performed essentially as previously described (16, 17).  $3.64 \times 10^{10}$  0.5  $\mu$ m Fluoresbrite Carboxylate Microspheres (Polysciences) were injected i.v. into mice, and, 3 hours later, spleens were harvested and processed as above.

**Ex vivo cDC priming of Th responses.**  $5 \times 10^4$  sort-purified cDCs (purity >99%) were plated in 96-well round-bottomed plates, with or without OTII peptide (40 ng/ml) or Ea52-68 peptide (40 or 400 ng/ml), at a 1:1 ratio with CD4<sup>+</sup>-enriched splenocytes from naive OTII mice or cell-sorted CD4<sup>+</sup> T cells from the spleens of naive TEa mice. The culturing medium used was RPMI medium containing 5% (v/v) fetal bovine serum, l-glutamine, penicillin (100 U/ml)/streptomycin (100  $\mu$ g/ml), sodium pyruvate (2 mM),  $\beta$ -mercaptoethanol (50  $\mu$ M), and HEPES (200  $\mu$ M). After 72 hours of culturing at  $37^{\circ}\text{C}/5\% \text{CO}_2$ , tissue culture supernatants were assessed for cytokines using cytometric bead arrays, according to the manufacturer's instructions (BD Biosciences), with samples acquired on a purpose-built FACSArray Bioanalyzer (BD Biosciences). Wells were then pulsed with 1 mCi [<sup>3</sup>H] thymidine for 18 hours, before measuring thymidine incorporation using a Betaplate Reader (Wallac). In addition, for TEa cells, intracellular T-bet staining was performed on individual replicate wells as above.

**Quantitative PCR.** RNA samples were quantitated using the Nanodrop ND-1000 (NanoDrop Technologies Inc.). RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed using TaqMan Gene Expression Probe Kits for genes encoding IFN- $\alpha$ 2, IFN- $\alpha$ 4, IFN- $\alpha$ 5, IFN- $\alpha$ 6, IFN- $\beta$ 1, IL-10, and HPRT and TaqMan Gene Expression Master Mix (Applied Biosystems) on the Rotor Gene 6000 real-time rotary analyzer (Qiagen). Relative quantitation of gene expression was performed using the relative standard curve method, as described by Applied Biosystems. Briefly, standard curves were prepared for all target and endogenous control genes using cDNA from sort-purified CD8<sup>-</sup> cDCs from *Ifnar1*<sup>fl/fl</sup> mice 4 days p.i. HPRT was used as the endogenous control. The amount of target gene or endogenous control in each sample was calculated from the appropriate standard curves. The target amount was then divided by the endogenous control amount to give the normalized target value. Normalizes target values were compared with a calibrator sample, as outlined in the figure legends, to yield relative expression levels.

**Statistics.** Comparisons between 2 groups were performed using nonparametric Mann-Whitney tests or Student's *t* tests for Gaussian distributions. *P* < 0.05 was considered significant. Kruskal-Wallis tests were performed to analyze 3 groups within an experiment. Graphs depict mean  $\pm$  SEM, except when individual mouse data points are depicted, in which case median values are shown. All statistical analyses were performed using GraphPad Prism 5 or 6 software.

**Study approval.** Animal procedures were approved and monitored by the QIMR Berghofer Medical Research Institute Animal Ethics Committee (approval no. A02-633M) in accordance with Australian National Health and Medical Research Council guidelines. Clec9a-DTR mice were housed and used according to local animal ethics requirements at Nanyang Technological University.

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