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Type I IFN signaling in CD8− 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+ 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-γ–producing Th1 cells. Although type I IFN signaling modulated all subsets of splenic cDCs, CD8− cDCs were especially susceptible, exhibiting reduced phagocytic and Th1-promoting properties in response to type I IFNs. Additionally, rapid and systemic IFN-α 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− 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), Staphylococcus (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-γ production by Th1 cells, not by acting on the CD4+ 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+ T cells in mild and severe malaria models (19–21). In particular, not only do CD8− cDCs fail to cross-present antigens, but MHC class I–restricted and MHC class II–restricted (MHCI/II-restricted) ex vivo presentation of Plasmodium–expressed antigens by CD8− and CD8+ cDCs is also curtailed (16, 17). The factors responsible for systemic loss of cDC function during Plasmodium infection remain unclear.

CD8− cDCs have received less attention in recent years compared with CD8+ cDCs, which rapidly produce the Th1-inducing cytokine, IL-12, and cross-present exogenous antigen efficiently to CD8+ T cells (reviewed in refs. 22, 23). However, CD8− cDCs possess a greater intrinsic capacity for processing antigens onto MHCI molecules than CD8+ cDCs and, therefore, harbor significant potential for priming Th responses (24). It is puzzling, therefore, that CD8− cDCs underperformed substantially in terms of priming Th1 responses compared with CD8+ 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− 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 α-IFNAR1 or control IgG and Ifnar1−/− 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 α-IFNAR1, control IgG, or left untreated. PbA-infected Ifnar1−/− mice (n = 5) and uninfected WT and Ifnar1−/− mice (n = 5) were also tested. On day 4 p.i., flow cytometric analysis of IFN-γ production (without ex vivo stimulation) by splenic CD4+ TCRγδ+ cells was performed (2 independent experiments). (C) Time course analysis of parasitemia and IFN-α protein in whole spleen lysates and sera from PbA-infected WT mice (2–5 independent experiments). (D) Gating strategy for Ly6C+ monocytes and MHCII expression in blood. WT and Ifnar1−/− 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 TCRγδ+/CD220−/CD11b+/Ly6C+ single cells exhibiting low granularity (2 independent experiments). (E) WT and Ccr2−/− mice (n = 5–6) were treated with α-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+ Ly6C+ monocytes and (F) proportion and absolute numbers of CD4+ TCRγδ+ cells producing IFN-γ 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− 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− cDC-mediated generation of para-site-specific IFN-γ-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−/− 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 Antwerp-Kasapa (PbA) infection (12). To confirm that no developmental or immune homeostatic defects in Ifnar1−/− mice could account for this phenotype, we first studied WT PbA-infected mice treated with α-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 α-IFNAR1- and control IgG-treated mice by day 4 after infection (p.i.) (data not shown), consistent with our previous report in Ifnar1−/− mice (12). Furthermore, we observed substantial production of IFN-α 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-α, 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 cell responses (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 monocyte 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+ Ly6C+ monocytes in the blood, and their acquisition of cell surface MHCII (Figure 1D), increased strikingly in PbA-infected Ifnar1−/− 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−/− mice (data not shown) or WT mice treated with α-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−/− 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−/− mice treated with α-IFNAR1, compared with those in control-treated Ccr2−/− 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+ MHCIICδ 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 α-IFNAR1 treatment resulted in 50% less CD86 upregulation by cDCs (Figure 2C). Similar observations were made in Ccr2−/− mice, devoid of splenic monocyte recruitment, when treated with α-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...
Type I IFN signaling directly mediates systemic activation of splenic cDCs during PbA infection. (A) Analysis of cell surface CD86 expression by splenic CD8+ and CD8– cDCs (CD11c+MHCII+TCRβ–B220– 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+–/– mice (n = 5) and uninfected WT and Ifnar1+–/– 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+MHCII+TCRβ–B220– single cells from WT (CD45.1+)/Ifnar1+–/– (CD45.2+) 50:50 mixed BM chimeric mice (n = 6–7) 4 days p.i. with PbA (2 independent experiments). (E) C57BL/6 CD11c-Cre+/– Ifnar1fl/fl mice and Ifnar1fl/fl 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+ cDCs [CD8α+CD11c+MHCII+TCRβ–B220–]; CD8– cDCs [CD8α+CD11c+MHCII+TCRβ–B220–]; pDCs [PDCA-1+CD20+CD11c+]; B cells [CD19+B220+]; T cells [TCRβ+]; NK cells [NK1.1–]); as well as analysis of cell surface CD86 expression by CD8+ and CD8– cDCs (2 independent experiments). **P < 0.01; ***P < 0.001.
generated mixed BM chimeric mice with congenically marked Ifnar1+/+ and Ifnar1–/– splenic cDCs in equal proportions (Figure 2D). We then assessed CD86 expression by Ifnar1+/+ and Ifnar1–/– splenic cDC subsets in these animals at 4 days p.i. with PbA. Consistent with data from Ifnar1+/+ or α-IFNAR1–treated mice (Figure 2C), CD86 upregulation was approximately 50% reduced in CD8+ and CD8– Ifnar1–/– cDC subsets, compared with corresponding Ifnar1+/+ 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 Iggx-Cre<sup>−/−</sup> Ifnar<sup>+/−</sup> mice (CD11c<sup>−</sup>Cre Ifnar<sup>+/−</sup>) (CD11c<sup>−</sup>Cre Ifnar<sup>+/−</sup>) mice, in which IFNAR1 deficiency was confined to CD11c<sup>+</sup> cells (33, 34). We infected CD11c<sup>−</sup>Cre Ifnar<sup>+/−</sup> mice and Cre<sup>+−</sup> Ifnar<sup>+/−</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<sup>−</sup>Cre Ifnar<sup>+/−</sup> mice, compared with infected Ifnar<sup>+/−</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 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<sup>−</sup>Cre Ifnar<sup>+/−</sup> mice and Cre<sup>+−</sup> Ifnar<sup>+/−</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<sup>−</sup>Cre Ifnar<sup>+/−</sup> mice compared with that in Cre<sup>+−</sup> Ifnar<sup>+/−</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, P8TG, 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 Ifnar<sup>1−/−</sup> recipients, P8TG-infected mice (Figure 3C). Notably, however, expression of IFN-γ was again increased on a per cell basis in proliferated OTII cells from Ifnar<sup>1−/−</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<sup>−</sup>Cre Ifnar<sup>+/−</sup> mice compared with that in littermate Ifnar<sup>+/−</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 Ifnar<sup>1−/−</sup> and Ifnar<sup>+/−</sup> splenic cDCs, to investigate the effect of direct type I IFN signaling on phagocytosis (Figure 4A). Consistent with previous work (16), Ifnar<sup>1−/−</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 Ifnar<sup>1−/−</sup> CD8<sup>−</sup> cDCs remained sensitive to PbA-induced downregulation of phagocytosis, Ifnar<sup>1−/−</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–DT 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 Ifnar<sup>1−/−</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 Ifnar<sup>1−/−</sup> mice (data not shown), we then loaded WT and Ifnar<sup>1−/−</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, Ifnar<sup>1−/−</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 Ifnar<sup>1−/−</sup> infected mice (data not shown).

Given that OTII cells do not differentiate readily into T-bet+ Th1 cells (40), we next chose to stimulate ex vivo an alternative CD4<sup>−</sup> Th1 cell line, the Th1 clone 3D. Poly I:C–mediated Th1 suppression was partially prevented in CD11c<sup>−</sup>Cre Ifnar<sup>1−/−</sup> mice compared with that in littermate Ifnar<sup>1−/−</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
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-Eα-derived peptide (Eα52-68) when presented by MHCII I-Aβ molecules (41). When sorted TEa T cells were cultured with CD8+ cDCs from PbA-infected CD11c-Cre Ifnar1β/β mice or Ifnar1β/β 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
data and previous reports (16, 19). In contrast, TEa T cells significantly upregulated T-bet when stimulated with CD8– cDCs from infected Ifnar1fl/fl mice (Figure 5B). Most importantly, however, T-bet upregulation was strongest when stimulated with CD8– cDCs from infected CD11c-Cre Ifnar1fl/fl mice (Figure 5B).

Together, our ex vivo antigen presentation experiments indicate that CD8– 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– cDCs after type I IFN signaling was abrogated. An assessment of B7 family costimulatory molecule expression by CD8+ and CD8– cDCs from PbA-infected CD11c-Cre Ifnar1fl/fl mice and Ifnar1fl/fl littermate control mice (n = 5–6) infected 4 days previously with PbA (representative of 2 independent experiments). (C) FACS analysis of cell surface costimulatory molecule expression by splenic CD8+ and CD8– cDCs (CD11chiMHCIIhiTCRβ−B220− single cells) in CD11c-Cre Ifnar1fl/fl mice and Ifnar1fl/fl 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– cDCs from uninfected WT mice) in pooled (n = 6–10) CD8– cDCs sorted from spleens of CD11c-Cre Ifnar1fl/fl mice and Ifnar1fl/fl littermate controls. Data from 2 independent experiments presented. *P < 0.05; **P < 0.01.

Figure 5
Type I IFN signaling in cDCs impairs Th1 priming by CD8– cDCs ex vivo. (A) IFN-γ assessed in tissue culture supernatants after CD4+ OTII cells (3–6 replicate wells) were stimulated for 4 days with ex vivo OTII peptide–loaded, cell-sorted, splenic CD8+ and CD8– cDCs pooled from WT and Ifnar1−/− mice (n = 5–6) infected 4 days previously with PbA (data are mean ± SEM from a single experiment representative of 2). (B) T-bet expression by CD4+ TCR transgenic TEa T cells (4–5 replicate wells) stimulated for 5 days with ex vivo Eα52-68 peptide–loaded, cell-sorted, splenic CD8+ (400 ng/ml peptide) and CD8– cDCs (40 and 400 ng/ml peptide) pooled from CD11c-Cre Ifnar1fl/fl mice and Ifnar1fl/fl littermate control mice (n = 6–10) infected 4 days previously with PbA (representative of 2 independent experiments).
improved function of CD8– 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+Ifnar1fl/fl mice and Ifnar1fl/fl 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 α-IFNAR1 or Ctrl IgG (compared with PbA-infected WT mice). **\(P < 0.01\).
treated mice compared with that in control IgG-treated, CD8–
CD207+ cDC–depleted mice (Figure 6C). Together, our data are con-
sistent with type I IFN signaling to CD8– 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+ 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-α levels in PbA-
infected Ifnar1−/− mice (Figure 7A) and WT mice depleted of
phagocytic cells by clodronate liposome treatment (Figure 7B). In
both situations, serum IFN-α levels were greatly reduced, demon-
strating the existence of an IFNAR1-dependent signaling loop
requiring phagocytic cells. We also observed substantially lower
levels of serum IFN-α at day 4 p.i. in Cd11c-Cre Ifnar10/0 mice, com-
pared with those in infected Ifnar10/0 littermate controls (Figure
7C). Finally, given that CD8– 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-α/β mRNAs, not
only in pDCs, as expected (20), but also in CD8– cDCs (Figure 7D).
Taken together, our data suggest that during PbA infection, a feed-
forward loop comprised of IFN-α/β production by CD8– cDCs
and autocrine or paracrine type I IFN signaling in CD8– cDCs con-
tributes to the impairment of Th1 priming.

**Discussion**

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

Several groups have established that blood-stage *Plasmodium*
infection triggers profound systemic cDC activation and dys-
function 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– cDCs.

CD8– cDCs have received greater attention than CD8+ cDCs in many studies in recent years, particularly for their capacity
to efficiently cross-present exogenous antigen to CD8+ T cells
and produce IL-12 rapidly (reviewed in refs. 22, 23). Moreover,
recent studies highlight the unique ability of CD8+ cDCs to drive
immune-pathogenic CD8+ T cell responses during PbA infection
(39, 42). However, elegant in vivo studies, which targeted antigenic
proteins to splenic cDC subsets, demonstrated that CD8– cDCs
possess a greater intrinsic capacity for processing antigens onto
MHCIi than CD8+ 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 IC, CD8– cDCs substantially
underperformed compared with CD8+ cDCs (25, 26). Indeed, we
also observed that poly IC treatment reduces Th1 responses during
*Plasmodium* infection (Figure 3, D and E). Our data provide an
explanation for such suboptimal CD8– 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 develop-
ment. Our work supports a role for CD8– 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–
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– cDCs, regardless of whether they have taken up parasites or not. Ultimately, this process restricts IFN-γ production by T-bet+ Th1 cells.

Our data demonstrated that type I IFN signaling in CD8– 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– cDCs will have functional consequences for Th1 priming. The functional consequences of increased PDL2 levels on CD8– cDCs for Th1 priming also remain unstudied at present. However, given that PD1/PDL1 (but not PD1/PDL2) interactions potently suppress Th1 responses during experimental malaria (61, 62), and since PDL2 and PDL1 exhibit similar Kd values for PD1 (63), we hypothesize that PDL2 disrupts the suppressive interactions between PD1 on Th cells and PDL1 on CD8– cDCs. In addition to effects on costimulatory molecule expression, we observed a substantial decrease in Il10 mRNA expression in CD8– 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– 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 (rbc) might also contribute partially to an improved capacity for Th1 priming in CD8– cDCs. This aspect of CD8– cDC function remains to be addressed fully.

In considering suppression of CD8– 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– 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+ 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+ 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−/− mice (70, 71), langerin-DTR mice (38), Il6g-Cre−/−Ifnar1−/− mice (33), Ifnar1−/− mice (32), Ccr2−/− mice (72), Cx3cr1−/− mice (73), congenic (CD45.1) OTII mice (74), and Tεa x Rag1−/− 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 x 106 fresh syngeneic BM cells (from femurs of CD45.1+ WT and CD45.2+ mice, mixed at a 50:50 ratio) into lethally irradiated (11 Gy [137Cs source]) CD45.1+, CD45.1− 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.

*Pla* parasites were used after 1 in vivo passage in WT C57BL/6 mice. Transgenic *Pb* (clone Z31c11) constitutively expressing luciferase and GFP from the EF1-α promoter (*Pla*- luc) was used for *Pb* experiments, unless stated otherwise (35, 75). A transgenic *Pb* 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 106 rbc, 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 μl RPMI containing 5 μl/ml heparin sulphate. Diluted blood was stained simultaneously with Syto84 (5 μM; Life Technologies) to detect RNA/DNA and Hoechst33342 (10 μg/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 FACS Canto II Analyzer (BD Biosciences) and FlowJo software (Treestar). prbc were readily detected as being Hoechst33342– Syto84–, 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 days posttreatment 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-α protein and cytokines. Spleens were harvested into 2 ml 1× cold RPMI containing a general protease inhibitor cocktail (P2714, Sigma-Aldrich; used according to the manufacturer’s instructions) and passed through 100-μm 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°C until analyzed. IFN-α 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 1 (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), MCHC1 (M/14.15.2), CD4 (GK1.5), CD8α (53–6.7), IFN-γ (XM1.2), CD19 (6D5), CD45.1 (A20), CD45.2 (clone 104), Ly6C (HK1.4), CD11b (M1/70), CD40 (1C10), CD86 (GL1), OX40L (RM134L), ICOSL (B7–RPI), and PD-L2 (TY25) were purchased from Biolegend. Fluorescently conjugated mAbs against TCRβ chain (H57–597), NK1.1 (PK136), and PD-L1 (MH52) were purchased from BD Biosciences. Anti–T-bet mAb (eBio4B10) was purchased from ebioscience, and anti–granulocyte MHC (MHGB04) was purchased from Life Technologies. Cell surface and intracellular IFN-γ staining was performed as previously described (35, 36).

To sort purified cDCs, pooled spleens were treated as above with deoxyribonuclease I and collagenase type 4, and rbc were lysed as previously described (35, 36, 75), ensuring maximal recovery of splenic cDCs by treating spleens with deoxyribonuclease 1 (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), MCHC1 (M/14.15.2), CD4 (GK1.5), CD8α (53–6.7), IFN-γ (XM1.2), CD19 (6D5), CD45.1 (A20), CD45.2 (clone 104), Ly6C (HK1.4), CD11b (M1/70), CD40 (1C10), CD86 (GL1), OX40L (RM134L), ICOSL (B7–RPI), and PD-L2 (TY25) were purchased from Biolegend. Fluorescently conjugated mAbs against TCRβ chain (H57–597), NK1.1 (PK136), and PD-L1 (MH52) were purchased from BD Biosciences. Anti–T-bet mAb (eBio4B10) was purchased from ebioscience, and anti–granulocyte MHC (MHGB04) was purchased from Life Technologies. Cell surface and intracellular IFN-γ staining was performed as previously described (35, 36).

To sort purified 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+ 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–/7-aminoactinomycin D+, 7-aminoactinomycin D+/Annexin V−) CD4–/CD8– cDCs from naive OTII mice or cell-sorted CD4+ T cells from the spleens of naive TEs mice. The culturing medium used was RPMI medium containing 5% (v/v) fetal bovine serum, 2 mM l-glutamine, penicillin (100 U/ml)/streptomycin (100 μg/ml), sodium pyruvate (2 mM), β-mercaptoethanol (50 μM), and HEPES (200 μM). After 72 hours of culturing at 37°C/5% CO2, 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 Bioanalyser (BD Biosciences). Wells were then pulsed with 1 mCi [3H] thymidine for 18 hours, before measuring thymidine incorporation using a Betaplate Reader (Wallac). In addition, for TEs 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-α2, IFN-α4, IFN-α5, IFN-β1, IL-10, and HPR1 and TaqMan Gene Expression Master Mix (Applied Biosystems) on the Rotor Gene 6000 real-time rotary analyzer (Qagen). Relative quantification 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+ cDCs from Ifnar1KO mice 4 days p.i. HPR1 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. Normalized 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 ± 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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