Type I Interferon Signaling Prevents IL-1β Driven Lethal Systemic Hyperinflammation during Invasive Bacterial Infection of Soft Tissue

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Summary

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Type I interferons (IFN-Is) are fundamental for antiviral immunity but their role in bacterial infections is contradictory and incompletely described. *Streptococcus pyogenes* activates IFN-I production in innate immune cells and IFN-I receptor 1 (Ifnar1)-deficient mice are highly susceptible to *S. pyogenes* infection. Here we report that IFN-I signaling protects the host against invasive *S. pyogenes* infection by restricting inflammation-driven damage in distant tissues. Lethality following infection in Ifnar1-deficient mice is caused by systemically exacerbated levels of the proinflammatory cytokine IL-1β. Critical cellular effectors of IFN-I in vivo are LysM+ and CD11c+ myeloid cells, which exhibit suppression of *Il1b* transcription upon Ifnar1 engagement. These cells are also the major source of IFN-β, which is significantly induced by *S. pyogenes* 23S rRNA in an Irf5-dependent manner. Our study establishes IL-1β and IFN-I levels as key homeostatic variables of protective, yet tuned, immune responses against severe invasive bacterial infection.

Introduction

Infectious diseases are a consequence of insufficient immune responses but deleterious effects of pathogens can also be caused by hyperstimulation and/or chronic activation of the immune system. Some pathogens can take advantage of both inadequate and exaggerated immune responses as exemplified by the highly versatile gram-positive human pathogen *Streptococcus pyogenes* (Group A Streptococcus) . The most common condition associated with *S. pyogenes* infection is streptococcal pharyngitis which is a largely self-limiting disease with substantial economic burden . In addition, *S. pyogenes* infections account for 650.000 annual cases of the life-threatening necrotizing fasciitis and streptococcal toxic shock syndrome with mortality exceeding 20% in spite of therapy . Human host factors determining susceptibility to *S. pyogenes* infections are not well characterized. Immunogenetics analyses suggest that a complex and precisely balanced immune response is required for successful protection. Mutations in the signaling adaptor Myd88 result in a dramatic susceptibility to pyogenic infections in children but not in adults indicating that a robust stimulation of the innate immune system is fundamental for defense against *S. pyogenes* under conditions where adaptive immunity is still underdeveloped. Several human leukocyte antigen class II haplotypes are

associated with strong protection against severe invasive *S. pyogenes* infections whereas others increase the risk of life-threatening disease . The high-risk haplotypes are often associated with an augmented cytokine storm. Experiments employing mice deficient in Myd88, Tnf, Tlr2, or Unc93b1, or mice depleted of macrophages or dendritic cells corroborate the fundamental role of the innate immune response . Adaptive IL-17-mediated immunity contributes to defense in the upper respiratory tract epithelium . However, cellular mechanisms ensuring protective yet balanced immune responses remain to be characterized.

Type I interferons (IFN-Is) are cytokines produced in response to viral, bacterial and fungal pathogens. The function of IFN-I in viral infections is well established and is always protective. IFN-I role in the context of bacterial infections is much less clear as both beneficial and detrimental effects have been reported, depending on the pathogen . Of particular relevance for bacterial infections appear to be immunosuppressive effects of IFN-Is. For example, immunosuppression by virus-induced IFN-Is compromises immunity against post-influenza bacterial pneumonia . Similarly, attenuation of immune responses has been linked with the detrimental role of IFN-I signaling in *Mycobacterium tuberculosis* infection models . A key question pertains to the protective mechanism of IFN-I in infections with important pathogens such as *S. pyogenes* or Group B Streptococcus .

It is well established that *S. pyogenes* activates IFN-I production in innate immune cells and that IFN-I receptor 1 (Ifnar1)-deficient mice are highly susceptible to subcutaneous *S. pyogenes* infection, a model of severe invasive cellulitis in humans . Our current study shows that IFN-I signaling plays a key role in homeostatic mechanisms required for an efficient yet not destructive immune reaction against invasive bacterial infection of soft tissue.

Results

IFN-I Induction by *S. pyogenes* in vivo Is Dependent on Irf5 and Occurs mostly in LysM+ and CD11c+ Myeloid Cells in Response to 23S rRNA

S. pyogenes was found by us and others to stimulate expression of IFN-β, the primary IFN-I, in murine bone marrow-derived macrophages (BMDMs) and GM-CSF-differentiated conventional dendritic cells (cDCs) . To identify cells producing IFN-I in vivo, we employed reporter mice

allowing Cre-Lox-regulated cell type-specific monitoring of the *Ifnb* promoter activity . Bioluminescence imaging of global reporter mice (*Ifnb*$^{+/\Delta\beta\text{-luc}}$) revealed increased *Ifnb* promoter activity at the site of infection and in inguinal lymph nodes 24 and 48 h after infection (Figure 1A and B). Reporter mice for the LysM+ (*Ifnb*$^{+/fl\ \beta\text{-luc}}$-LysMCre) or CD11c+ (*Ifnb*$^{+/fl\ \beta\text{-luc}}$-CD11cCre) lineages exhibited an overall lower luciferase signal prior to infection when compared to the uninfected global reporter mice, but the signal increased upon infection (Figure 1A and B). Importantly, the induction of luciferase by *S. pyogenes* at the site of infection was comparable in the three reporter mice (Figure 1B, right panel), indicating that LysM+ and CD11c+ myeloid cells are the major IFN-β producers in vivo. Since certain myeloid populations express both LysM and CD11c markers , the luciferase activity in *Ifnb*$^{+/fl\ \beta\text{-luc}}$-LysMCre and *Ifnb*$^{+/fl\ \beta\text{-luc}}$-CD11cCre mice might originate from the same or distinct cell populations. Neither basal nor infection-stimulated *Ifnb* promoter activity was detected in T cell-specific reporter mice (*Ifnb*$^{+/fl\ \beta\text{-luc}}$-CD4Cre) (Figure 1A and B). The relatively high luciferase signal in uninfected global reporter mice (Figure 1A and B) results from basal *Ifnb* gene expression which, for unknown reasons, occurs in various cells and tissues .

BMDMs employ the transcription factor Irf3 for IFN-β induction by *S. pyogenes*, while cDCs use Irf5 . To determine the roles of Irf3 and Irf5 in vivo we investigated the activity of the IFN-β promoter-driven luciferase reporter in mice lacking Irf3 (*Ifnb*$^{+/\ \Delta\beta\text{-luc}}$-*Irf3*$^{-/-}$) or Irf5 (*Ifnb*$^{+/\ \Delta\beta\text{-luc}}$-*Irf5*$^{-/-}$). The induction of the *Ifnb* promoter at the site of *S. pyogenes* infection in mice lacking Irf3 was comparable to *Ifnb*$^{+/\Delta\beta\text{-luc}}$ control reporter mice (Figure 1C and 1D). In contrast, mice lacking Irf5 exhibited severe impairment of *Ifnb* promoter induction (Figure 1C and 1D). These experiments showed that Irf5 is the major IFN-β activator in vivo, thereby resembling IFN-β production by cDCs in vitro. Irf5-dependent IFN-β induction in cDCs can be triggered by *S. pyogenes*-derived RNA . A short conserved sequence of the bacterial 23S rRNA, including that of *S. pyogenes*, was recently shown to induce pro-inflammatory cytokines (e.g. Tnf, Il-6) by triggering Tlr13 . To test whether *S. pyogenes* 23S rRNA is also capable of inducing IFN-β, we treated cDCs with total *S. pyogenes* RNA, purified *S. pyogenes* rRNA fractions or oligoribonucleotides comprising the Tlr13-stimulating 23S rRNA sequence (SA19) and its mutated version (mut-SA19). Both 23S rRNA and SA19 stimulated IFN-β production whereas 16S, 5S rRNAs and mut-SA19 did not (Figure 1E, left panel). Induction of IFN-β by 23S rRNA was entirely Irf5-dependent (Figure 1E), in agreement with the requirement of Irf5 for IFN-β induction by *S. pyogenes* in vivo (Figure 1C and 1D). Consistent with the reported broader role of Irf5 in Tlr signaling , Tnf induction was also

strongly dependent on Irf5 (Figure 1E). Finally, by using Tlr13-deficent cDCs we were able to show that *S. pyogenes* and *S. pyogenes* RNA induced IFN-β in a Tlr13-dependent way (Figure 1F).

In summary, our data reveal that myeloid cells expressing LysM and/or CD11c are the major source of IFN-β in *S. pyogenes*-infected mice. Our observation that IFN-β induction is largely Irf5-dependent in vivo implies that a CD11c+ DCs are the main IFN-β source. Furthermore, our data identify *S. pyogenes* 23S rRNA to be the IFN-β-inducing Tlr13 ligand in cDCs.

Protective IFN-I Signaling is Derived from CD11c+ and LysM+ Myeloid Cells and Is not Involved in Controlling the Bacterial Burden

To identify the effector cells of IFN-I signaling we infected mice lacking Ifnar1 in CD11c+ (*Ifnar1*$^{fl/fl}$-CD11cCre) or LysM+ (*Ifnar1*$^{fl/fl}$-LysMCre) cells and compared them with control (*Ifnar1*$^{fl/fl}$) as well as full *Ifnar1* knockout mice (*Ifnar1*$^{-/-}$) (Figure 2A). *Ifnar1* ablation in both CD11c+ and LysM+ cells resulted in significantly decreased survival, akin to the susceptibility of *Ifnar1*$^{-/-}$ mice (Figure 2A). In general, animals surviving longer than 5 days recovered regardless of the genotype. Thus, Ifnar1 signaling in CD11c+ and LysM+ cells is critically contributing to the protective effects of IFN-I in *S. pyogenes* infection.

To examine whether increased bacterial loads caused higher susceptibility of Ifnar1-deficient mice, we counted bacterial numbers at the site of primary infection (i.e. skin lesion), in the blood and in distant organs (spleen and liver) at 24 and 48 h post infection (p.i.), that is prior to occurrence of the first moribund animals. No differences in bacterial burden between WT and *Ifnar1*$^{-/-}$ mice were detected at any location or time point (Figure 2B and C). Therefore, the beneficial function of IFN-I must be exerted by mechanisms other than those involved in restricting bacterial burden.

IFN-I Signaling Prevents Hyperinflammation and Selectively Controls IL-1β Production

Given that the increased severity of infection in *Ifnar1*^{-/-} mice was not caused by higher bacterial burden, we assessed markers of liver and kidney injury. Serum levels of bilirubin, aspartate transaminase (AST) and glutamate dehydrogenase (GLDH) were significantly higher in *S. pyogenes*-infected *Ifnar1*^{-/-} when compared to control animals 48 h p.i., indicative of exacerbated hepatocellular injury (Figure 3A). Alanine transaminase (ALT) was also increased, albeit the difference was not significant (Figure 3A). Serum levels of creatinine and urea were also significantly elevated in *Ifnar1*^{-/-} mice suggesting a more severe impairment of renal function (Figure 3A).

To evaluate leukocyte infiltration and pathological changes in organ architecture, hematoxylin and eosin (H&E)-stained sections of the infected skin, liver, spleen and kidney were examined. Necrotic areas, which were frequently observed in all layers of the infected skin, were more often surrounded by areas of substantial leukocytic infiltrates in *Ifnar1*^{-/-} mice than in WT controls (Figure 3B). Analysis of spleen sections revealed massive infiltration of myeloid cells with expansion of the white pulp and retraction of the red pulp in *Ifnar1*^{-/-} mice (Figure 3C). The liver damage histopathology score in *Ifnar1*^{-/-} mice was significantly higher for hepatocellular vacuolation, but not for inflammatory infiltrates (Figure 3D). Kidney sections did not reveal any major pathological change except for rare tubular casts in both genotypes (Figure 3E). The lack of major morphological signs of kidney damage together with elevated blood levels of urea and creatinine in *Ifnar1*^{-/-} mice suggest a pre-renal kidney failure in the course of infection-triggered hypotension.

Exacerbated organ damage and leukocyte infiltration in *Ifnar1*^{-/-} mice demonstrated that the absence of IFN-I signaling results in hyperinflammation both at the site of infection as well as in distant tissues. To directly determine the inflammatory milieu we examined the amounts of the pro-inflammatory cytokines Tnf, IL-1α and IL-1β, the neutrophil chemoattractant Cxcl1 and the anti-inflammatory cytokine IL-10 in the infected skin, liver and spleen 48 h after the infection. The levels of IL-1β were considerably increased at all locations in *Ifnar1*^{-/-} animals when compared to WT controls (Figure 4A). In contrast, IL-1α was increased in the *Ifnar1*^{-/-} spleen but not at other sites, and Tnf and IL-10 levels were similar in all tissues in both genotypes (Figure 4B - D). Elevated IL-1α amounts in *Ifnar1*^{-/-} spleens might result from more excessive damage in this organ since damaged tissue is a rich source of this cytokine . Cxcl1 amounts were in general slightly higher in *Ifnar1*^{-/-} mice but the difference to WT controls was only significant in infected skin lesions (Figure 4E). Cytokine amounts were low in uninfected animals of each genotype

(Figure 4F). Interestingly, the basal IL-1β levels appeared higher in uninfected *Ifnar1*^{-/-} mice, suggesting that IFN-I signaling was attenuating IL-1β production also in untreated mice.

Collectively, these data demonstrate that the absence of IFN-I signaling in *S. pyogenes*-infected animals causes massive tissue damage and hyperinflammation associated with a profound and selective failure to control IL-1β production.

Increased Leukocyte Cellularity at the Site of Infection in *Ifnar1*^{-/-} Mice Results Largely from Neutrophil Infiltration

Tissue sections showed that IFN-I signaling attenuated leukocyte infiltration both at the site of infection and in distant organs. To examine the role of IFN-I signaling in recruiting immune cells, we employed an air pouch infection model which allows qualitative and quantitative analysis of infiltrating cells at the site of infection . Air pouch exudates of *Ifnar1*^{-/-} and WT mice were analyzed by flow cytometry and ELISA 24 h p.i. In agreement with previous studies , over 90% of infiltrating cells were Ly6G$^+$Ly6C$^+$ neutrophils (Figure 5A). These percentages were similar in both *Ifnar1*^{-/-} and WT mice (Figure 5A). However, the total numbers of all infiltrating cells as well as neutrophils were more than 2-fold higher in *Ifnar1*^{-/-} exudates (Figure 5A, lower right panel). IL-1β was markedly elevated in air pouches of *Ifnar1*^{-/-} animals (Figure 5B). IL-1α and Cxcl1 were also elevated in *Ifnar1*^{-/-} samples, albeit to a lesser extent than IL-1β, whereas Tnf and IL-10 levels were comparable in both genotypes (Figure 5B). Thus, the cytokine profiles in air pouches were similar to those in infected skin (Figure 4 and 5B).

To assess whether elevated IL-1β levels in *Ifnar1*^{-/-} exudates resulted from higher leukocytes numbers or from augmented IL-1β production we employed cell type-specific *Ifnar1* deletions. Air pouches generated in animals lacking Ifnar1 in neutrophils and macrophages (*Ifnar1*^{fl/fl}-LysMCre) or cDCs (*Ifnar1*^{fl/fl}-CD11cCre) contained comparable numbers of leukocytes as *Ifnar1*^{fl/fl} controls (Figure 5C, left panel). Hence, the increased leukocyte infiltration observed in *Ifnar1*^{-/-} mice (Figure 5A) was caused by the absence of IFN-I signaling in multiple lineages. However, IL-1β was increased in air pouches of *Ifnar1*^{fl/fl}-LysMCre but not *Ifnar1*^{fl/fl}-CD11cCre animals (Figure 5C, right panel) indicating that higher IL-1β levels resulted from uncontrolled production by

neutrophils and/or macrophages. Since neutrophils represent by far the largest population of recruited cells they probably contribute most significantly to the augmented IL-1β production at the infection site of *Ifnar1$^{-/-}$* mice.

IFN-I Signaling Limits IL-1β Production by Macrophages, Dendritic Cells and Neutrophils

To understand the mechanism of IL-1β inhibition by IFN-I we analyzed effects of exogenous and endogenous IFN-β on processing the IL-1β precursor (pro-IL-1β) in *S. pyogenes*-infected BMDMs. *S. pyogenes*-infected *Ifnar1$^{-/-}$* BMDMs secreted 2-fold more IL-1β than WT BMDMs (Figure 6A). Exogenous IFN-β suppressed IL-1β secretion by WT but not *Ifnar1$^{-/-}$* BMDMs (Figure 6A). Tnf levels were not affected by IFN-I signaling (Figure 6A). Western blot analysis of supernatants confirmed that IL-1β measured by ELISA was indeed the mature cytokine (Figure 6B). The analysis confirmed inhibition of IL-1β but not Tnf by IFN-I signaling (Figure 6B and S1). Pro-IL-1β was higher in cell lysates of infected *Ifnar1$^{-/-}$* BMDMs compared to WT cells, and exogenous IFN-β suppressed pro-IL-1β in WT but not *Ifnar1$^{-/-}$* BMDMs (Figure 6C and S2). Thus, pro-IL-1β was suppressed by IFN-I as was mature IL-1β release into cell supernatants. The levels of the IL-1β-processing enzyme caspase-1 (both procaspase-1 and the processed p20 form) were similar in infected WT and *Ifnar1$^{-/-}$* BMDMs although a slight decrease was detected upon treatment of WT BMDMs with IFN-β (Figure 6C). Together, these results indicate that IFN-I signaling is suppressing IL-1β largely prior to translation rather than at the posttranslational processing steps.

Il1b mRNA induced by *S. pyogenes* was also upregulated in *Ifnar1$^{-/-}$* BMDMs when compared to WT cells, and exogenous IFN-β suppressed *Il1b* mRNA in WT but not *Ifnar1$^{-/-}$* BMDMs (Figure 6D). Tnf mRNA induction was not affected by the genotype or IFN-β treatment (Figure 6D). *Il1b* mRNA levels are strongly influenced by 3' untranslated region (UTR)-mediated regulation of mRNA stability . However, mRNA decay assays did not show any effect of IFN-β on *Il1b* mRNA stability (Figure 6E), suggesting that IFN-I signaling regulated the transcription of *Il1b*. To measure changes in transcription we examined the levels of nascent transcripts (pre-mRNA). *S. pyogenes*-mediated *Il1b* pre-mRNA induction was higher in *Ifnar1$^{-/-}$* BMDMs than in WT cells and exogenous IFN-β suppressed *Il1b* mRNA in WT but not *Ifnar1$^{-/-}$* BMDMs (Figure 6F) confirming

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that IFN-I signaling inhibited *Il1b* gene transcription. *Il1a* induction was not affected by the lack of IFN-I signaling (Figure S3). IFN-I reduced *Il1b* gene transcription also in cDCs (Figure 6G). Similarly, IFN-β suppressed Il-1β but not Tnf production by peritoneal neutrophils (Figure 6H).

As *S. pyogenes* is a human-specific pathogen we asked whether IFN-I signaling suppressed IL-1β production in human cells. Primary human monocytes and macrophages were recently shown to produce IFN-β in response to bacterial RNA and infection, among others with *S. pyogenes*, in a TLR8-dependent way , implying that they are exposed to IFN-Is in the infected host. Similar to mouse cells, IFN-β suppressed IL-1β production and *IL1b* gene transcription but not TNF production in *S. pyogenes*-infected primary monocyte and macrophages (Figure 6I and J).

The inhibition of *Il1b* gene transcription by IFN-I signaling prompted us to examine the involvement of the IFN-I-activated transcription factors STAT1 and STAT2 by using knockout BMDMs. The suppressive effect of IFN-β on *Il1b* transcription and mRNA accumulation was abolished in STAT1$^{-/-}$ BMDMs but was partially retained in STAT2$^{-/-}$ cells (Figure 6L). Tnf induction was not regulated by STAT1 or STAT2 (Figure 6L, right panel). These data imply that both STAT1 homodimers and the ISGF3 complex (consisting of STAT1, STAT2 and IRF9) contribute to the inhibition of *Il1b* transcription by IFN-I.

In summary, these data demonstrate a specific suppression of *S. pyogenes*-induced IL-1β production by IFN-I signaling in macrophages, cDCs and neutrophils, thereby further supporting our notion that the key IFN-I effector cells in vivo are LysM+ and CD11c+ cells.

IFN-I Signaling Prevents Induction of IL-1β to Levels Exceeding the Protective Range

We wondered whether responses to physiological levels of IL-1β, i.e. levels reached under conditions of functional IFN-I signaling, contribute to immune protection against *S. pyogenes*. To this end, we first infected IL-1 receptor 1-deficient (*Il1r1$^{-/-}$*) mice with *S. pyogenes*. *Il1r1$^{-/-}$* mice were dramatically more susceptible to infection and displayed higher bacterial burden in distant

organs (Figure 7A and B). Thus, IL-1 receptor signaling is indispensable for clearance of bacteria and survival in our infection model.

IL-1R1 is a subunit of the IL-1 receptor which is activated by the closely related but functionally distinct cytokines IL-1α and IL-1β. As IL-1α levels were much less or not at all regulated by IFN-I signaling (Figure 4A and B), we reasoned that IL-1β was the cause of the hyperinflammation in *Ifnar1*$^{-/-}$ mice. Previous work has established that *S. pyogenes* induces IL-1β via the pore-forming toxin streptolysin O (SLO) by triggering the canonical NLRP3 inflammasome and caspase-1 . To address the role of IL-1β, we employed caspase-1 and caspase-11 double-deficient mice which lack the canonical and non-canonical inflammasome pathways (*Casp1*$^{-/-}$ *Casp11*$^{-/-}$) . These mice were significantly more susceptible to *S. pyogenes* infection when than WT controls indicating that IL-1β plays an indispensable role in activation of immune defense (Figure 7C).

These findings suggested that IFN-I signaling allows for sufficient production but prevents tissue-damaging accumulation of IL-1β . To support this hypothesis we treated *Ifnar1*$^{-/-}$ mice with Ac-YVAD-CHO which inhibits caspase 1 and caspase 4 . Initially, we observed that Ac-YVAD-CHO was able to attenuate IL-1β synthesis in *S. pyogenes*-infected WT mice (Figure 7D). The inhibitor significantly reduced IL-1β production in the organs but it was only marginally effective in the infected skin (Figure 7D). Remarkably, treatment of *Ifnar1*$^{-/-}$ mice with Ac-YVAD-CHO resulted in full protection against *S. pyogenes* infection showing that unrestricted IL-1β production caused by the absence IFN-I signaling was indeed deleterious (Figure 7E). In further support of this, *Ifnar1*$^{-/-}$ mice treated with soluble IL-1R antagonist IL-1RA (anakinra) exhibited improved resistance against *S. pyogenes* infection (Figure 7F).

These findings, in conjunction with the detrimental hyperinflammation and overproduction of IL-1β observed in *S. pyogenes*-infected *Ifnar1*$^{-/-}$ mice, demonstrate that IL-1β signaling must operate within a narrow range as both excessive and defective IL-1β responses lead to lethal disease. IFN-I signaling helps maintain the level of IL-1β activity within the desired range.

Discussion

In this study we examined the role of IFN-I signaling as well as the mechanisms of IFN-I induction in the context of invasive soft tissue infection with pyogenic bacteria. Our findings reveal that IFN-I signaling is an essential component of a feedback system, which prevents lethal IL-1β-mediated hyperinflammation during an otherwise manageable systemic bacterial infection. This discovery is unexpected since the immunomodulatory functions of IFN-Is have thus far been associated with deleterious effects on immunity against pathogenic bacteria such as *M. tuberculosis* and *Francisella tularensis* . By contrast, our study shows that the immunosuppressive function of IFN-I signaling is advantageous during a bacterial infection.

Both *S. pyogenes* DNA and RNA were previously shown to induce IFN-β in murine macrophages and cDCs, respectively . DNA, which triggered the TBK1/STING/IRF3 pathway, was a comparably poorer IFN-β inducer than RNA which signaled via Myd88 and Irf5, but not Irf3 . Our current study demonstrates that in the context of the whole organism, the Irf5-dependent pathway is a non-redundant source of *S. pyogenes*-elicited IFN-β whereas the Irf3 axis is dispensable. Irf5-depedent IFN-β production is triggered by *S. pyogenes* 23S rRNA in a Tlr13 dependent way. The functional Tlr13 equivalent in humans is TLR8, which was reported to trigger IFN-β production in response single stranded bacterial RNA, including that of *S. pyogenes* . The precise signature of RNA recognized by TLR8 and the role of TLR8 in human infections remain incompletely understood**.**

We propose that cells positive for both LysM and CD11c markers are the key IFN-β-producing cells in *S. pyogenes*-infected mice. First, the global IFN-β reporter mice induce the reporter to an extent similar to that of LysM+ and CD11c+ reporter mice, implying that no major IFN-β-producing cell population was missed when using such mice. Second, the activation of IFN-β in reporter mice is almost entirely dependent on Irf5 which is in agreement with the reported requirement for Irf5 in cDCs in vitro . LysM+ macrophages can be excluded as a significant IFN-β source since the macrophage-specific Irf3-driven IFN-β activation is not important in vivo. Neutrophils can be excluded since they reportedly lack the ability to activate IFN-β expression upon bacterial infection . Although we did not directly investigate the plasmacytoid DC population, these cells are not likely to add significantly to IFN-β levels as they are CD11c low and were reported to lack IFN-β gene activation upon streptococcal infection . This reasoning

suggests that the IFN-β-expressing cells are Tip-DCs (Tnf- and iNOS-producing DCs), which express both LysM and CD11c and were reported as a major source of IFN-β in response to *Listeria monocytogenes* infection .

We observe two major effects of IFN-I signaling: restriction of IL-1β production by infiltrating leukocytes and reduction of the numbers of infiltrating leukocytes. Ifnar1-deificent neutrophils are probably the main source of the excessive IL-1β at the site of infection because they constitute more than 90% of infiltrating cells. On the other hand, the increased leukocyte infiltration in the absence of IFN-I signaling is a process caused by multiple cell types since the lack of Ifnar1 in LysM+ neutrophils and macrophages or CD11c+ DCs alone is not sufficient to sustain the enhanced infiltration.

Inhibition of IL-1β production by the IFN-I pathway occurs in our system at the level of *Il1b* transcription. As this inhibition requires STAT1 and is partially dependent on STAT2, the effect can be a consequence of binding of STAT1 dimers and/or ISGF3 to the *Il1b* promoter, or a result of STAT1- or ISGF3-driven expression of a transcriptional repressor. In agreement, IFN-γ-stimulated STAT1 can suppress *Il1b* expression by reducing NF-κB occupancy at the *Il1b* promoter . We do not observe a significant contribution of other mechanisms previously implicated in IFN-I-mediated IL-1β suppression, i.e. inhibition of IL-1β translation and NLRP3 activity . These studies employed different modes of IL-1β induction (e.g. LPS, alum), which could result in other mechanisms of inhibition by IFN-I. However, Guarda and colleagues found that IFN-β reduced cellular pro-IL-1β pools in Salmonella-infected BMDMs by undefined mechanisms . These data together with our findings suggest that transcription inhibition is the key mechanism of IL-1β suppression by IFN-I signaling in responses to bacterial infection.

Previous studies demonstrated that IL-1β or signaling via the Il-1 receptor is required for host protection against bacterial pathogens including *Streptococcus pneumoniae*, *Legionella pneumophila*, *Klebsiella pneumoniae* or *Mycobacterium tuberculosis* . Our study adds *S. pyogenes* to this list of pathogens. This finding was unexpected since a deletion of the NLRP3 inflammasome does not alter resistance against *S. pyogenes* in an intraperitoneal challenge model . However, consistent with our observations, IL-1β helps prevent lethal bacteremia after intravenous infection with the same pathogen . These inconsistent findings might result from differences intrinsic to the *S. pyogenes* infection models used. Notably, we employed

subcutaneous infection which is a valid model of invasive cellulitis in humans . Alternatively, *S. pyogenes* might activate IL-1β synthesis in certain cells independently of NLRP3 or inflammasomes in general, as recently reported for sterile bone inflammation . Indeed, the incomplete blockage of IL-1β production in *S. pyogenes*-infected mice treated with the caspase inhibitor Ac-YVAD-CHO, as observed in our study, might result from inflammasome-independent IL-1β synthesis. However, a low tissue penetrance of the inhibitor could also explain the remaining IL-1β amounts.

The lethal hyperinflammation in *Ifnar1*^{-/-} mice was rescued completely by Ac-YVAD-CHO but only partially by anakinra. Anakinra exhibits a rapid pharmacokinetics which might diminish its beneficial effects. Alternatively, since caspase inhibition also prevents pyroptosis, the effects of Ac-YVAD-CHO might be more pleiotropic. Anakinra, and to some extent also Ac-YVAD-CHO impair both IL-1α and IL-1β signaling. However, only IL-1β is systemically upregulated in infected *Ifnar1*^{-/-} mice indicating that it is the key cytokine causing the destructive hyperinflammation. Nonetheless, future studies should clarify the roles of both IL-1 subtypes.

Our study highlights the importance of infection-induced homeostatic mechanisms for successful host defense. This report demonstrates a fundamental role of IFN-I signaling in preventing lethal IL-1β-driven inflammation during bacterial infection. Moreover, we present clear evidence for the requirement to precisely regulate the minimal and maximal IL-1β levels in defense against *S. pyogenes* infection. Our work introduces the cytokines IL-1β and IFN-Is as adjustable parameters determining the outcome of an invasive bacterial infection. This knowledge together with the characterization of the underlying cellular network and identification of 23S rRNA as IFN-I inducer might be of relevance for developing new immunomodulation-based approaches for treating severe soft tissue infections cause by *S. pyogenes*.

Author Contributions

Conceptualization, V.C. and P.K.; Methodology, A.P., O.G. and S.L.; Investigation, V.C., A.P., F.E., M.J., U.D., S.L. and P.K.; Resources, A.K., C.K., U.K., B.S., M.M., D.S. and S.L.; Writing – Original Draft, V.C., M.J. and P.K.; Funding Acquisition, B.S., M.M. and P.K.

Acknowledgements

Experimental procedures

Experimental Model of *S. pyogenes* Infection

Subcutaneous infection of mice with *S. pyogenes* was carried out as described . Briefly, bacteria were cultured overnight, then diluted 1:24 and grown for 4 hours at 37°C until mid-logarithmic phase. Bacteria were harvested (6500 rpm for 8 min), washed twice in sterile PBS and then resuspended at a concentration of 2×10^8 CFU per 50 µl as controlled by plating serial 10-fold dilutions on blood agar plates. Mice were anesthetized by injection of a mixture of 10 mg/ml ketamine and 1 mg/ml xylazine (Graeub) in NaCl, the fur at one flank was partially shaved, and the inoculum (50 µl) was injected subcutaneously (s.c.). Infected animals were monitored for their health status every 4 to 8 h, and were euthanized when reaching behavioral and pathophysiological humane endpoints. Survival was monitored for 6 days. For experiments other than survival, animals were euthanized at indicated time points. Survival of mice was analyzed by the Logrank Test in GraphPad Prism 5 (GraphPad software, San Diego, USA). Survival curves comparison between two groups was performed using t-test. P values ≤0.05 were considered as significant.

Generation of Skin Air Pouches

Air pouch infections were carried out as described . Briefly, air pouches were raised on the dorsum of anesthetized mice by subcutaneous injection of 900 µl of air together with 100 µl of 1 x 10^6 CFU of bacterial suspension. After 24 hours, mice were sacrificed, air was aspirated from the pouches and exudates were collected by injection and subsequent aspiration of 1 ml PBS.

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Samples were centrifuged at 1200 rpm for 5 minutes, and supernatants were used immediately or kept at –20 C for the evaluation of cytokines production. Infiltrating cells were counted using a hemocytometer, and cell viability was determined by trypan blue staining. Analysis of recruited cells was carried out by flow cytometry: cells were incubated with anti-Fc receptor (CD16/32), and stained using antibodies to Ly6C, Ly6G, CD11c, F4/80 (BD Biosciences, eBioscience). Data were acquired using a Fortessa fluorescence-activated cell sorting (FACS) (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Inhibition of IL-1ß in vivo

To inhibit IL-1ß processing in vivo, mice received the caspase 1- and 4-specific inhibitor Ac-YVAD-CHO (Enzo Life Sciences) at a concentration of 5 mg/kg of body weight, or PBS, intraperitoneally 2 h before the infection with *S. pyogenes.* To inhibit IL-1 signaling in vivo, recombinant human IL-1RA (anakinra, Kineret®; kindly provided by Swedish Orphan Biovitrum) was diluted in PBS and i.p.-injected to the mice at 6, 24, 48, 72 and 96 hours post infection at a concentration of 50 mg/Kg body weight.

Detection of IFN-ß Promoter Activation in vivo

For in vivo imaging, mice were injected i.v. with 150 mg/kg of D-luciferin (Perkin Elmer) in PBS immediately prior to scanning, anesthetized using isofluran (Baxter) and monitored using an IVIS 200 imaging system (CaliperLS). Photon flux was quantified using the Living Image 4.4 software (CaliperLS).

Ethics Statement

Animal experiments, carried out according the Austrian law for animal experiments (BGBl. I Nr. 114/2012) were approved and authorized through the permissions BMWF-66.006/0024-II/3b/2012 and BMWF-66.006/0006-II/3b/2013 issued by the Austrian Ministry of Science to PK.

Statistical Analysis

Statistical analyses were performed on Prism (GraphPad) using Student's t-test. Not significant (ns) $p \geq 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

References

Abram, C.L., Roberge, G.L., Hu, Y., and Lowell, C.A. (2014). Comparative analysis of the efficiency and specificity of myeloid-Cre deleting strains using ROSA-EYFP reporter mice. J Immunol Methods *408*, 89-100.

Barry, K.C., Fontana, M.F., Portman, J.L., Dugan, A.S., and Vance, R.E. (2013). IL-1α Signaling Initiates the Inflammatory Response to Virulent Legionella pneumophila In Vivo. The Journal of Immunology *190*, 6329-6339.

Bergstrom, B., Aune, M.H., Awuh, J.A., Kojen, J.F., Blix, K.J., Ryan, L., Flo, T.H., Mollnes, T.E., Espevik, T., and Stenvik, J. (2015). TLR8 Senses Staphylococcus aureus RNA in Human Primary Monocytes and Macrophages and Induces IFN-beta Production via a TAK1-IKKbeta-IRF5 Signaling Pathway. J Immunol *195*, 1100-1111.

Carapetis, J.R., Steer, A.C., Mulholland, E.K., and Weber, M. (2005). The global burden of group A streptococcal diseases. The Lancet infectious diseases *5*, 685-694.

Chen, G.Y., and Nunez, G. (2010). Sterile inflammation: sensing and reacting to damage. Nature reviews *10*, 826-837.

Cole, J.N., Barnett, T.C., Nizet, V., and Walker, M.J. (2011). Molecular insight into invasive group A streptococcal disease. Nat Rev Microbiol *9*, 724-736.

Dileepan, T., Linehan, J.L., Moon, J.J., Pepper, M., Jenkins, M.K., and Cleary, P.P. (2011). Robust antigen specific th17 T cell response to group A Streptococcus is dependent on IL-6 and intranasal route of infection. PLoS pathogens *7*, e1002252.

Eigenbrod, T., Bode, K.A., and Dalpke, A.H. (2013). Early inhibition of IL-1beta expression by IFN-gamma is mediated by impaired binding of NF-kappaB to the IL-1beta promoter but is independent of nitric oxide. J Immunol *190*, 6533-6541.

Eigenbrod, T., Pelka, K., Latz, E., Kreikemeyer, B., and Dalpke, A.H. (2015). TLR8 Senses Bacterial RNA in Human Monocytes and Plays a Nonredundant Role for Recognition of Streptococcus pyogenes. J Immunol *195*, 1092-1099.

Fieber, C., Janos, M., Koestler, T., Gratz, N., Li, X.D., Castiglia, V., Aberle, M., Sauert, M., Wegner, M., Alexopoulou, L.*, et al.* (2015). Innate Immune Response to Streptococcus pyogenes Depends on the Combined Activation of TLR13 and TLR2. PLoS ONE *10*, e0119727.

Fieber, C., and Kovarik, P. (2014). Responses of innate immune cells to group A Streptococcus. Frontiers in cellular and infection microbiology *4*, 140.

Goldmann, O., Rohde, M., Chhatwal, G.S., and Medina, E. (2004). Role of macrophages in host resistance to group A streptococci. Infect Immun *72*, 2956-2963.

Granowitz, E.V., Vannier, E., Poutsiaka, D.D., and Dinarello, C.A. (1992). Effect of interleukin-1 (IL-1) blockade on cytokine synthesis: II. IL-1 receptor antagonist inhibits lipopolysaccharide-induced cytokine synthesis by human monocytes. Blood *79*, 2364-2369.

Gratz, N., Hartweger, H., Matt, U., Kratochvill, F., Janos, M., Sigel, S., Drobits, B., Li, X.D., Knapp, S., and Kovarik, P. (2011). Type I interferon production induced by Streptococcus pyogenes-derived nucleic acids is required for host protection. PLoS pathogens *7*, e1001345.

Gratz, N., Siller, M., Schaljo, B., Pirzada, Z.A., Gattermeier, I., Vojtek, I., Kirschning, C.J., Wagner, H., Akira, S., Charpentier, E., and Kovarik, P. (2008). Group A Streptococcus Activates Type I Interferon Production and MyD88-dependent Signaling without Involvement of TLR2, TLR4, and TLR9. J Biol Chem *283*, 19879-19887.

Guarda, G., Braun, M., Staehli, F., Tardivel, A., Mattmann, C., Forster, I., Farlik, M., Decker, T., Du Pasquier, R.A., Romero, P., and Tschopp, J. (2011). Type I interferon inhibits interleukin-1 production and inflammasome activation. Immunity *34*, 213-223.

Harder, J., Franchi, L., Munoz-Planillo, R., Park, J.H., Reimer, T., and Nunez, G. (2009). Activation of the Nlrp3 Inflammasome by Streptococcus pyogenes Requires Streptolysin O and NF-{kappa}B Activation but Proceeds Independently of TLR Signaling and P2X7 Receptor. J Immunol.

Henry, T., Kirimanjeswara, G.S., Ruby, T., Jones, J.W., Peng, K., Perret, M., Ho, L., Sauer, J.D., Iwakura, Y., Metzger, D.W., and Monack, D.M. (2010). Type I IFN signaling constrains IL-17A/F secretion by gammadelta T cells during bacterial infections. J Immunol *184*, 3755-3767.

Hernandez-Cuellar, E., Tsuchiya, K., Hara, H., Fang, R., Sakai, S., Kawamura, I., Akira, S., and Mitsuyama, M. (2012). Cutting edge: nitric oxide inhibits the NLRP3 inflammasome. J Immunol *189*, 5113-5117.

Hoeksema, M.A., Scicluna, B.P., Boshuizen, M.C., van der Velden, S., Neele, A.E., Van den Bossche, J., Matlung, H.L., van den Berg, T.K., Goossens, P., and de Winther, M.P. (2015). IFN-gamma Priming of Macrophages Represses a Part of the Inflammatory Program and Attenuates Neutrophil Recruitment. J Immunol *194*, 3909-3916.

Kayagaki, N., Warming, S., Lamkanfi, M., Vande Walle, L., Louie, S., Dong, J., Newton, K., Qu, Y., Liu, J., Heldens, S.*, et al.* (2011). Non-canonical inflammasome activation targets caspase-11. Nature *479*, 117-121.

Kotb, M., Norrby-Teglund, A., McGeer, A., El-Sherbini, H., Dorak, M.T., Khurshid, A., Green, K., Peeples, J., Wade, J., Thomson, G.*, et al.* (2002). An immunogenetic and molecular basis for differences in outcomes of invasive group A streptococcal infections. Nature medicine *8*, 1398-1404.

Kratochvill, F., Machacek, C., Vogl, C., Ebner, F., Sedlyarov, V., Gruber, A.R., Hartweger, H., Vielnascher, R., Karaghiosoff, M., Rulicke, T.*, et al.* (2011). Tristetraprolin-driven regulatory

circuit controls quality and timing of mRNA decay in inflammation. Molecular systems biology *7*, 560.

Kruger, A., Oldenburg, M., Chebrolu, C., Beisser, D., Kolter, J., Sigmund, A.M., Steinmann, J., Schafer, S., Hochrein, H., Rahmann, S*., et al.* (2015). Human TLR8 senses UR/URR motifs in bacterial and mitochondrial RNA. EMBO reports.

Li, P., Allen, H., Banerjee, S., Franklin, S., Herzog, L., Johnston, C., McDowell, J., Paskind, M., Rodman, L., Salfeld, J., and et al. (1995). Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. Cell *80*, 401-411.

Li, X.D., and Chen, Z.J. (2012). Sequence specific detection of bacterial 23S ribosomal RNA by TLR13. eLife *1*, e00102.

Lienenklaus, S., Cornitescu, M., Zietara, N., Lyszkiewicz, M., Gekara, N., Jablonska, J., Edenhofer, F., Rajewsky, K., Bruder, D., Hafner, M*., et al.* (2009). Novel reporter mouse reveals constitutive and inflammatory expression of IFN-beta in vivo. J Immunol *183*, 3229-3236.

Lin, A.E., Beasley, F.C., Keller, N., Hollands, A., Urbano, R., Troemel, E.R., Hoffman, H.M., and Nizet, V. (2015). A Group A Streptococcus ADP-Ribosyltransferase Toxin Stimulates a Protective Interleukin 1beta-Dependent Macrophage Immune Response. MBio *6*.

Loof, T.G., Goldmann, O., Gessner, A., Herwald, H., and Medina, E. (2010). Aberrant inflammatory response to Streptococcus pyogenes in mice lacking myeloid differentiation factor 88. The American journal of pathology *176*, 754-763.

Loof, T.G., Rohde, M., Chhatwal, G.S., Jung, S., and Medina, E. (2007). The contribution of dendritic cells to host defenses against Streptococcus pyogenes. The Journal of infectious diseases *196*, 1794-1803.

Lukens, J.R., Gross, J.M., Calabrese, C., Iwakura, Y., Lamkanfi, M., Vogel, P., and Kanneganti, T.D. (2014). Critical role for inflammasome-independent IL-1beta production in osteomyelitis. Proc Natl Acad Sci U S A *111*, 1066-1071.

Mancuso, G., Gambuzza, M., Midiri, A., Biondo, C., Papasergi, S., Akira, S., Teti, G., and Beninati, C. (2009). Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells. Nat Immunol *10*, 587-594.

Mayer-Barber, K.D., Andrade, B.B., Barber, D.L., Hieny, S., Feng, C.G., Caspar, P., Oland, S., Gordon, S., and Sher, A. (2011). Innate and adaptive interferons suppress IL-1alpha and IL-1beta production by distinct pulmonary myeloid subsets during Mycobacterium tuberculosis infection. Immunity *35*, 1023-1034.

Medina, E. (2010). Murine model of cutaneous infection with Streptococcus pyogenes. Methods Mol Biol *602*, 395-403.

Mishalian, I., Ordan, M., Peled, A., Maly, A., Eichenbaum, M.B., Ravins, M., Aychek, T., Jung, S., and Hanski, E. (2011). Recruited macrophages control dissemination of group A Streptococcus from infected soft tissues. J Immunol *187*, 6022-6031.

Nakamura, S., Davis, K.M., and Weiser, J.N. (2011). Synergistic stimulation of type I interferons during influenza virus coinfection promotes Streptococcus pneumoniae colonization in mice. J Clin Invest *121*, 3657-3665.

Novikov, A., Cardone, M., Thompson, R., Shenderov, K., Kirschman, K.D., Mayer-Barber, K.D., Myers, T.G., Rabin, R.L., Trinchieri, G., Sher, A., and Feng, C.G. (2011). Mycobacterium tuberculosis triggers host type I IFN signaling to regulate IL-1beta production in human macrophages. J Immunol *187*, 2540-2547.

Oldenburg, M., Kruger, A., Ferstl, R., Kaufmann, A., Nees, G., Sigmund, A., Bathke, B., Lauterbach, H., Suter, M., Dreher, S*., et al.* (2012). TLR13 recognizes bacterial 23S rRNA devoid of erythromycin resistance-forming modification. Science *337*, 1111-1115.

Radwan, M., Stiefvater, R., Grunert, T., Sharif, O., Miller, I., Marchetti-Deschmann, M., Allmaier, G., Gemeiner, M., Knapp, S., Kovarik, P*., et al.* (2010). Tyrosine kinase 2 controls IL-1ss production at the translational level. J Immunol *185*, 3544-3553.

Rijneveld, A.W., Florquin, S., Branger, J., Speelman, P., Van Deventer, S.J., and van der Poll, T. (2001). TNF-alpha compensates for the impaired host defense of IL-1 type I receptor-deficient mice during pneumococcal pneumonia. J Immunol *167*, 5240-5246.

Sato, M., Suemori, H., Hata, N., Asagiri, M., Ogasawara, K., Nakao, K., Nakaya, T., Katsuki, M., Noguchi, S., Tanaka, N., and Taniguchi, T. (2000). Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. Immunity *13*, 539-548.

Serbina, N.V., Salazar-Mather, T.P., Biron, C.A., Kuziel, W.A., and Pamer, E.G. (2003). TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. Immunity *19*, 59-70.

Shahangian, A., Chow, E.K., Tian, X., Kang, J.R., Ghaffari, A., Liu, S.Y., Belperio, J.A., Cheng, G., and Deng, J.C. (2009). Type I IFNs mediate development of postinfluenza bacterial pneumonia in mice. J Clin Invest *119*, 1910-1920.

Solodova, E., Jablonska, J., Weiss, S., and Lienenklaus, S. (2011). Production of IFN-beta during Listeria monocytogenes infection is restricted to monocyte/macrophage lineage. PLoS ONE *6*, e18543.

Stifter, S.A., and Feng, C.G. (2015). Interfering with immunity: detrimental role of type I IFNs during infection. J Immunol *194*, 2455-2465.

Takaoka, A., Yanai, H., Kondo, S., Duncan, G., Negishi, H., Mizutani, T., Kano, S., Honda, K., Ohba, Y., Mak, T.W., and Taniguchi, T. (2005). Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. Nature *434*, 243-249.

Trinchieri, G. (2010). Type I interferon: friend or foe? J Exp Med *207*, 2053-2063.

Tsatsaronis, J.A., Walker, M.J., and Sanderson-Smith, M.L. (2014). Host responses to group a streptococcus: cell death and inflammation. PLoS pathogens *10*, e1004266.

von Bernuth, H., Picard, C., Jin, Z., Pankla, R., Xiao, H., Ku, C.L., Chrabieh, M., Mustapha, I.B., Ghandil, P., Camcioglu, Y., *et al.* (2008). Pyogenic bacterial infections in humans with MyD88 deficiency. Science *321*, 691-696.

Wellington, C.L., Ellerby, L.M., Hackam, A.S., Margolis, R.L., Trifiro, M.A., Singaraja, R., McCutcheon, K., Salvesen, G.S., Propp, S.S., Bromm, M., *et al.* (1998). Caspase cleavage of gene products associated with triplet expansion disorders generates truncated fragments containing the polyglutamine tract. J Biol Chem *273*, 9158-9167.

Wessels, M.R. (2011). Clinical practice. Streptococcal pharyngitis. N Engl J Med *364*, 648-655.

FIGURE LEGENDS

Figure 1. IFN-I Induction by *S. pyogenes* in vivo Is Dependent on Irf5 and Occurs in LysM+ and CD11c+ Myeloid Cells in Response to 23S rRNA

(**A** and **B**) *Ifnb*$^{+/\Delta\text{ß-luc}}$, *Ifnb*$^{+/fl \text{ ß-luc}}$-LysMCre, *Ifnb*$^{+/fl \text{ ß-luc}}$-CD11cCre and *Ifnb*$^{+/fl \text{ ß-luc}}$-CD4Cre were s.c. infected with *S. pyogenes* (*S.p.*) (1×10^6 CFU). *Ifnb* reporter luciferase activity was monitored before (0 h) and 24 and 48 h post infection (p.i.). *Ifnb* induction at the infection site and inguinal lymph nodes (A). Luciferase activity at the infection site at indicated time points shown as absolute values (B, left panel) or normalized to the levels before infection (B, right panel).

(**C**) *Ifnb*$^{+/\Delta\text{ß-luc}}$-*Irf3*$^{-/-}$, *Ifnß*$^{+/\Delta\text{ß-luc}}$-*Irf5*$^{-/-}$ were infected and analyzed as in (A), and luciferase activity 24, 48 and 72 h p.i. normalized to the basal levels before infection was determined (**D**).

(**E** and **F**) cDCs derived from WT, *Irf5*$^{-/-}$ or *Tlr13*$^{-/-}$ mice were transfected with *S. p.* total RNA, rRNA (23S, 16S, 5S), SA19 oligoribonucleotide, mutated SA19 (SA19-mut) or mammalian RNA, or stimulated with LPS. Supernatants were collected 6 h p.i. and IFN-ß (E and F) and Tnf (E) were determined. Error bars: SDs (n=3).

Statistical analysis: Student's t-test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

Figure 2. IFN-I targets LysM+ and CD11c+ cells in vivo without influencing the control of bacterial burden

(**A**) *Ifnar1*^{fl/fl}, *Ifnar1*^{fl/fl}-CD11cCre, *Ifnar1*^{fl/fl}-LysMCre, *Ifnar1*^{-/-} and WT mice were infected s.c. (2×10⁸ CFU of *S. p.*), and animal survival was monitored over 6 days. Data show Kaplan-Meier survival curves for WT, *Ifnar1*^{fl/fl}, *Ifnar1*^{fl/fl}-CD11cCre (n=8 per genotype), and *Ifnar1*^{fl/fl}, *Ifnar1*^{fl/fl}-LysMCre (2 pooled experiments, n=16 per genotype). Student's t-test, * $p < 0.05$; ** $p < 0.01$

(**B**) WT and *Ifnar1*^{-/-} mice were infected as in (A) and bacterial burdens in the blood, spleens, livers, and infected lesions were determined 24 h and 48 h p.i. Bacterial load is shown as CFU/ml of blood and CFU/g of analyzed organ per infected animal. Plots: mean ± SEM of CFUs.

Statistical analysis: Student's t-test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

Figure 3. Deficiency in IFN-I signaling results in increased inflammation and organ damage upon *S. pyogenes* infection

(**A**) WT and *Ifnar1*^{-/-} mice were infected s.c. (2×10⁸ CFU of *S. p.*). Blood values of bilirubin, AST, ALT, GLDH, urea and creatinine 48 p.i. are shown; mean ± SEM (n=8).

(**B**) H&E staining of infected skin sections from WT and *Ifnar1*^{-/-} mice, and quantification of leukocytic infiltrates (right panel) are shown. Skin lesions show areas of coagulative and lytic necrosis (N) extending from the dermis (D) into the panniculus carnosus (PC) in both genotypes. Infiltration of leukocytes (arrows in insets of left and middle panels) at the border of the necrotic area is significantly increased in *Ifnar1*^{-/-} (right panel).

(**C**) H&E staining of spleen sections of infected WT and *Ifnar1*^{-/-} mice, and quantification of myeloid infiltration and red pulp (RP) replacement by white pulp (WP) (right panel) are shown. RP and WP borders are marked by lines. Infiltrates of myeloid cells are more numerous in *Ifnar1*^{-/-} spleens. The spleen of *Ifnar1*^{-/-} mice exhibits a pronounced replacement of RP by WP.

(**D**) H&E staining of liver sections of infected WT (left panel) and *Ifnar1*^{-/-} (middle panel) mice, and quantification of hepatocellular vacuolation and leukocytic infiltrates (graphs, right panel) are shown. Larger and more numerous aggregates of infiltrating leukocytes (arrows in insets) are observed in the liver of *Ifnar1*^{-/-} than WT mice. CV, centrolobular vein.

(**E**) Tubular casts in kidneys of infected mice are rare and they occur at a comparable frequency in WT and *Ifnar1*^{-/-} animals. Pathology scores in (B - E) were determined by blinded scoring. Bars represent mean ± SEM.

Statistical analysis: Student's t-test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 4. Systemic levels of IL-1ß but not other cytokines are increased in *S. pyogenes*–infected *Ifnar1*^{-/-} mice

(**A** - **E**) WT and *Ifnar1*^{-/-} mice (10 mice per genotype) were infected s.c. (2×10^8 CFU of *S. p.*) for 48 h and IL-1β (A), IL-1α (B), Tnf (C), IL-10 (D) and Cxcl1 (E) levels in the skin, liver and spleen homogenates were determined. Data show one of three independent experiments. Bars represent mean ± SEM (n=10 per genotype). Student's t-test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, ns, not significant.

(**F**) Basal cytokine levels in the skin, liver and spleen of uninfected WT and *Ifnar1*^{-/-} mice (2 mice per genotype).

Figure 5: Air pouch infection model reveals augmented neutrophil recruitment in the absence of IFN-I signaling

(**A** - **D**) *S. p.* (2x10^6 CFU) and air were injected s.c. in the back of WT, *Ifnar1*^{−/−}, *Ifnar1*^{fl/fl}, *Ifnar1*^{fl/fl}-CD11cCre and *Ifnar1*^{fl/fl}-LysMCre. Analyses were carried out 24 h p.i.

(**A**) Neutrophils (Ly6G+ Ly6C+) represent the majority of cells infiltrating air pouches and they are increased in *Ifnar1*^{−/−} mice. Representative FACS contour plots for one animal per genotype (upper panels) and plots displaying the analysis of 6 animals per genotype (lower left panel) are shown. Values are means ± SEM (n=10) of two pooled experiments performed independently.

(**B**) Cytokines in air pouch exudates from *Ifnar1*^{-/-} and WT mice; values are means ± SEM (n=11) of two pooled experiments performed independently.

(**C**) Live cells and Ly6G+ Ly6C+ neutrophils in air pouches of *Ifnar1*^{fl/fl}, *Ifnar1*^{fl/fl}-CD11cCre and *Ifnar1*^{fl/fl}-LysMCre mice were analyzed as in (A).

(**D**) IL-1ß levels in air pouches of *Ifnar1*^{fl/fl}, *Ifnar1*^{fl/fl}-CD11cCre and *Ifnar1*^{fl/fl}-LysMCre mice; values are means ± SEM (n=10-11) of two pooled experiments performed independently.

Statistical analysis: Student's t-test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

Figure 6. IFN-I signaling inhibits *Il1b* gene transcription in myeloid cells

(**A** – **F**) BMDMs from WT and *Ifnar1*^{−/−} mice were infected for 4 h (and 6 h where explicated) with *S. p.* (MOI = 100) in the presence (added simultaneously) or absence of IFN-ß, or left uninfected. IL-1ß and Tnf levels in the supernatants are shown (A). Processed IL-1ß (p17) was detected in supernatants by Western blot (B) (see also Figure S1). Unprocessed IL-1ß (pro-IL-1ß), processed IL-1ß (p17), procaspase-1 and cleaved caspase-1 p20 subunit were detected by Western blot in whole cell extracts (C) (see also Figure S2). *Il1b* and *Tnf* mRNA levels were determined by qRT-PCR (D). *Il1b* mRNA stability was examined by qRT-PCR analysis of RNA isolated from cells 0, 30, 60 and 90 min after transcription blockage using actinomycin D (5

µg/ml) (E). *Il1b* gene transcription rate was determined using qRT-PCR analysis of *Il1b* pre-mRNA (F) (see also Figure S3).

(**G**) cDCs were treated for 6 h as in (A), and the levels of *Il1b* pre-mRNA and *Tnf* mRNA were determined by qRT-PCR.

(**H**) Casein-elicited peritoneal neutrophils were treated for 6 h as in (A) and IL-1ß and Tnf were determined by ELISA.

(**I, J**) Primary human monocytes and macrophages were infected for 4 h (MOI 10) or transfected with 1 µg of *S. p*. RNA. Where specified, IFN-ß was added at the time of infection. IL-1ß and Tnf were determined by ELISA (I). *Il1b* pre-mRNA levels were determined by qRT-PCR (J).

(**L**) BMDMs from WT, *Stat1$^{-/-}$* or *Stat2$^{-/-}$* mice were infected as in (A). *Il1b* pre-mRNA and *Tnf* mRNA were determined by qRT-PCR.

Error bars: mean with SD (n>3). Student's t-test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

Figure 7. IFN-I-mediated control of IL-1ß levels is required for the protective function of IL-1ß signaling during *S. pyogenes* infection

(**A**, **B**) IL-1 signaling is required for defense against *S. pyogenes* infection. Kaplan-Meier survival curves of *Il1r1$^{-/-}$* and WT mice (n=8 per genotype) (A). Bacterial loads (CFU/g of organ) in *Il1r1$^{-/-}$* (n=4) and WT (n=5) mice 24 h p.i (s.c., $2×10^8$ CFU) (B). Plots indicate the mean ± SEM of bacterial counts.

(**C**) Mice deficient in IL-1ß processing (*Casp1$^{-/-}$ Casp11$^{-/-}$* mice, n=9) are more susceptible to *S. p.* infection ($2×10^8$ CFU, s.c.) than WT mice (n=7).

(**D**) Caspase inhibitor Ac-YVAD-CHO decreases the concentrations of IL-1ß in the liver and spleen of *S. p.*–infected mice. WT mice (n=6) were intraperitoneally (i.p.) injected with PBS or Ac-YVAD-CHO 2 h before infection. IL-1ß levels were assessed 48 h p.i.

(**E, F**) Ac-YVAD-CHO and anakinra (rIL1-RA) treatment enhance the resistance of *Ifnar1$^{-/-}$* mice against *S. p.* infection. Kaplan-Meier survival curves of *Ifnar1$^{-/-}$* mice injected i.p. with PBS (n=6) or Ac-YVAD-CHO (n=6) 2 h prior to infection ($2×10^8$ CFU) (E). Kaplan-Meier survival curve of *Ifnar1$^{-/-}$* mice treated with 50 mg of anakinra per kg body weight or PBS at 6, 24, 48, 72 and 96 h p.i. ($2×10^8$ CFU) (two pooled experiments, n=17).

Statistical analysis: Student's t-test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.