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Type I interferons differentially modulate maternal host immunity to infection by *Listeria monocytogenes* and *Salmonella enterica* serovar Typhimurium during pregnancy

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Abstract

Problem: IFN- α receptor deficiency (IFNAR^{-/-}) enhances immunity to *Listeria monocytogenes* (LM) and *Salmonella enterica* serovar Typhimurium (ST) in the non-pregnant state by inhibiting pathogen-induced immune cell death. However, the roles of IFNAR signaling in modulating immunity to infection during pregnancy are not well-understood.

Method of Study: WT and IFNAR^{-/-} mice were infected systemically with LM or ST. Bacterial burden in spleen and individual placentas was enumerated at day 3 post-infection. Immune cell numbers and percentages were quantified in spleen and individual placentas, respectively, through flow cytometry. Cytokine expression in serum, spleen and individual placentas was measured through cytometric bead array.

Results: IFNAR^{-/-} mice exhibited decreased splenic monocyte numbers in non-pregnant and pregnant state, and an altered distribution of placental immune cell types in the non-infected state. IFNAR^{-/-} mice controlled LM infection more effectively than wild-type even during pregnancy, and this correlated with enhanced serum IL-12 expression, despite reduced splenic monocyte numbers relative to WT controls. In contrast, pregnant IFNAR^{-/-} mice unlike their non-pregnant

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Authorship

GA designed and performed the experiments, analyzed the data, wrote and edited the manuscript. KW designed and performed some experiments, and analyzed the data. SPM contributed to the discussion and ideas, and edited the manuscript. SS obtained the IFNAR^{-/-} mice, and edited the manuscript. LK conceptualized the study, designed the experiments, wrote and edited the manuscript, and contributed reagents.

Conflict of Interest Statement

The authors declare no conflict of interest.

counterparts exhibited increased susceptibility to ST infection which was associated with decreased serum IL-12 expression.

Conclusion: Type I IFN responses differentially impact host resistance to LM and ST infection during pregnancy through modulation of immune cell distribution and cytokine responses.

Keywords

Type I Interferon; *Listeria*; *Salmonella*; pregnancy; cytokines

Introduction

Type I interferons (IFNs) are a large family of cytokines that modulate innate and adaptive immune responses, including inflammatory cytokine expression, macrophage activation and T cell function.¹ The IFN- α and IFN- β subtypes are well-characterized for their roles in regulating host immunity to infections, and will be referred to as Type I IFNs in this study.² Type I IFNs signal through the ubiquitous transmembrane IFN- α receptor (IFNAR).³ They are produced by innate immune cell types, including macrophages and dendritic cells (DCs) upon pathogen detection by pattern recognition receptors and cytokine stimulation.^{3;4} Non-immune cells, including epithelial cells and fibroblasts, also promote activation of anti-microbial mechanisms through IFN- β production.³ Type I IFNs induce differential effects on host immunity to viral infections. IFNAR signaling typically exerts anti-viral functions through induction of IFN-stimulated genes (ISGs), which encode proteins that interfere with various stages of the viral life cycle, regulate host cell death and promote activation of innate and adaptive immune mechanisms.^{5;6} However, IFNAR signaling enhances susceptibility to chronic infection by lymphocytic choriomeningitis virus (LCMV) infection. This is associated with increased serum expression of anti-inflammatory interleukin-10 (IL-10) and programmed death-ligand 1, and decreased splenic numbers of cytokine-producing LCMV-specific CD4⁺ T cells.^{7;8} IFNAR signaling also induces differential host responses to bacterial infections. Type I IFNs enhance resistance to macrophage infection by *Legionella pneumophila* bacteria by promoting polarization of classically-activated M1 macrophage responses and production of anti-microbial nitric oxide.⁹ Protection mediated by type I IFNs from invasive lung infection by *Streptococcus pneumoniae* bacteria is associated with reduced lung epithelial permeability and enhanced alveolar epithelial type II cell survival.^{10;11} In contrast, IFNAR signaling compromises innate immunity to intracellular bacterial infection by *Listeria monocytogenes* (LM) and *Salmonella* Typhimurium by sensitizing immune cells to pathogen-induced death.^{12–14} Thus, the complex network of immune activation and regulatory roles mediated by type I IFNs differentially impact host immunity to infection.

Pregnancy features the complex interplay between inflammatory and immune-regulatory T-cell mechanisms to promote maternal tolerance to the semi-allogeneic fetus. As a consequence, this immune-altered condition can also modulate host susceptibility to infections. We previously showed that pregnancy does not compromise the inherent resistance of C57BL/6J wild-type (WT) mice to systemic LM infection.¹⁵ This is associated with robust systemic innate immune and antigen-specific CD8⁺ T-cell responses by pregnant mice similar to those of non-pregnant mice. However, pregnant mice exhibited progressive

bacterial growth in the placenta and enhanced fetal loss in the course of LM infection. Thus, preferential placental colonization by LM appeared to be differentially regulated by maternal systemic and local placental immunity in response to infection. In contrast, ST infection during pregnancy leads to profound systemic maternal and fetal disease.¹⁶⁻¹⁸ Pregnant mice exhibited reduced maternal splenic innate immune responses to infection, which was associated with decreased ratio of pro-inflammatory Th17 cells to anti-inflammatory regulatory T cells (Tregs) and enhanced IL-10 responses.^{16;18} In contrast, profound placental infection correlated with enhanced expression of pro-inflammatory cytokines, including interleukin-6 (IL-6), TNF and IL-18, leading to fetal loss.^{17;18} Thus, compartment-specific dichotomy between pro- and anti-inflammatory responses in pregnant mice impacted systemic and placental ST burden and disease progression.

The role of type I IFN responses in modulating innate immunity to intracellular bacterial infections during pregnancy, are not well-understood. In this study, we show that IFNAR deficiency modulates splenic and placental immune cell distribution in the non-infected state, and differentially modulates disease progression in response to LM and ST during pregnancy. Resistance to LM infection in non-pregnant IFNAR^{-/-} mice is retained during pregnancy and is associated with increased serum IL-12 expression relative to WT controls. In contrast, protection conferred by IFNAR deficiency against ST infection in the non-pregnant state is abrogated during pregnancy which correlated with reduced serum IL-12 expression. Taken together, our study shows the critical role for IL-12 in modulating host immunity to infection. Furthermore, the differential impact of type I IFNs on immunity to LM and ST during pregnancy are attributed to specific host-pathogen interactions.

Materials and Methods

Mice and Matings

C57BL/6J wild-type (WT) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). IFNAR^{-/-} mice bred on the WT background were obtained from Dr. Kaja Murali-Krishna (Emory University, Atlanta, GA, USA). Matings were performed by housing one male with one to two females for two nights. The presence of a copulatory plug after each day of mating was designated as day 0 of pregnancy. The rates of fetal resorptions, which are defined as uterine necrotic scars or as necrotic and/or hemorrhagic fetuses and placentas, were calculated using the formula $R/(R + V) \times 100$, where R is the number of resorbing fetuses and V is the number of viable fetuses per mouse. The study was performed in accordance with the guidelines of the Canadian Council on Animal Care and the protocols approved by National Research Council Canada in Ottawa, Canada.

Infections and Bacterial Enumeration

Listeria monocytogenes 10403S (LM) and *Salmonella enterica* serovar Typhimurium SL1344 (ST) were grown to mid-log phase at OD₆₀₀ = 1.0 and OD₆₀₀ = 0.8, respectively, under constant shaking in Bacto™ brain-heart infusion (BHI) medium (BD, Mississauga, ON, Canada) containing 50 µg/mL streptomycin (Sigma-Aldrich, Oakville, ON, Canada). Bacterial stocks were stored frozen in 20% glycerol at -80°C.

For mouse infections, frozen bacterial stocks were thawed and diluted in 0.9% sodium chloride (saline) solution. Age-matched non-pregnant and pregnant (day 11–12 of gestation) mice were injected intravenously (i.v.) via the lateral vein with 5×10^4 LM colony-forming units (CFUs), or 1×10^3 ST CFUs suspended in 200 μ l of saline solution. At day 3 post-infection, spleens and placentas (with intact deciduas) were homogenized in RPMI 1640 medium containing 8% fetal bovine serum (FBS). Serial 10-fold dilutions of tissue homogenates were plated onto BHI or Lysogeny broth agar plates (BD, Mississauga, ON, Canada) to enumerate LM and ST CFUs, respectively. Plates were incubated at 37°C overnight and bacterial colonies were enumerated. Data were presented as CFUs per spleen or placenta.

Tissues and sera from non-infected pregnant (day 14–15 of gestation) mice were used as negative controls in flow cytometric analysis of immune cell types and/or cytokine measurements in order to match the gestational stage of pregnant mice (infected during mid-pregnancy) at day 3 post-infection.

Flow Cytometric Analysis

Spleens and individual placentas (with intact deciduas) were homogenized and passed through a 100 μ m cell strainer to obtain a single-cell suspension. Total splenic cell numbers were determined by staining cells with the Acridine orange/propidium iodide (AO/PI) solution (Nexcelom Bioscience, Lawrence, MA, USA) and counting live cells using the Cellometer Auto 2000 (Nexcelom Bioscience, Lawrence, MA, USA). Placental homogenates were incubated in Hanks' Balanced Salt Solution (Gibco; Thermo Fisher Scientific, Burlington, ON, Canada) containing 0.2% Collagenase Type IV (Worthington Biochemical Corporation; Cedarlane, Burlington, ON, Canada). Immune cells were then isolated through Percoll® (GE Healthcare; Sigma-Aldrich, Oakville, ON, Canada) density gradient centrifugation. Briefly, placental homogenates were re-suspended in 5 mL 40% Percoll® and underlaid with 5 mL 70% Percoll®. Cells were then centrifuged at 2,000 rpm for 25 minutes without braking using the Heraeus Megafuge 40R (Thermo Fisher Scientific, Burlington, ON, Canada). Immune cells at the interphase between the 40%/70% gradient were isolated using plastic Pasteur pipettes.

Single-cell suspensions of spleens (1×10^6 cells) and individual placentas (whole tissue homogenates) were re-suspended in $1 \times$ phosphate-buffered saline containing 2% FBS (staining buffer) prior to incubation with unconjugated anti-CD16/CD32 (Fc γ RIII/II) antibody for 10 minutes. Cells were then stained with the following fluorochrome-conjugated antibodies for 30 minutes: CD45 (30-F11), CD11b (M1/70), Ly-6G (1A8), CD8a (53–6.7), CD45R/B220 (RA3–6B2) and CD11c (HL3) (BD Biosciences, Mississauga, ON, Canada); TCR- β (H57–597), CD4 (RM4–5) and CD49b (DX5) (eBioscience, Burlington, ON, Canada); F4/80 (BM8) and Ly-6C (HK1.4) (BioLegend; Cedarlane, Burlington, ON, Canada). Lastly, cells were washed and re-suspended in staining buffer prior to flow cytometry acquisition using the BD LSR Fortessa™. Flow cytometry data were analyzed using FlowJo® 7.6.5 (FlowJo, LLC, Ashland, OR, USA) software. Major immune cell types were identified according to cell surface marker expression, as described previously.¹⁹ Splenic and placental immune cell types were presented as absolute numbers and

percentages, respectively. The absolute numbers of splenic immune cell types were determined by acquiring 100,000 events of the CD45⁺ cell population and using the formula: (percentage of immune cell type gated on CD45⁺ cells × total splenocyte count) / 100. Placental immune cell types were quantified as the percentage of CD45⁺ cells upon acquisition of the whole tissue homogenate.

Cytokine Measurements

Serum was obtained from blood using Microtainer[®] serum separator tubes (BD, Mississauga, ON, Canada). Splenic and placental tissues were collected and homogenized in 1× lysis buffer (10 mM Tris-HCl pH 8.0, 150 mM sodium chloride, 1% NP-40 v/v, 10% glycerol v/v, 5 mM EDTA) containing cOmplete[™], Mini, EDTA-free Protease Inhibitor Cocktail (Roche; Sigma-Aldrich, Oakville, ON, Canada).²⁰ Total protein concentration per spleen or placenta was determined using the Pierce[™] BCA Protein Assay Kit (Thermo Scientific[™], Burlington, ON, Canada). Tissue homogenates were diluted to a final protein concentration of 500 µg/mL. Cytokine expression in splenic and placental tissues was presented as pg/mg of protein.

Cytokine expression levels in the serum and tissue homogenates were measured using the Cytometric Bead Array (CBA) Mouse Inflammation Kit (IL-6, IL-10, MCP-1, IFN-γ, TNF and IL-12p70) and individual Flex Sets (IL-1β and KC) (BD Biosciences, Mississauga, ON, Canada), as described previously.¹⁶ Samples were run using the BD LSRFortessa[™]. Data were analyzed using FACSArray[™] software (Soft Flow Hungary Ltd., Pécs, Hungary).

Statistical Analysis

All statistical analyses were performed using GraphPad[®] Prism 7 (GraphPad, La Jolla, CA, USA) software. Bacterial CFUs, immune cell numbers and cytokine expression levels were presented as mean ± SEM. Statistical significance of CFU, flow cytometry and cytokine expression data was determined through Mann-Whitney U test. Survival curves were analyzed using the Gehan-Breslow-Wilcoxon test. P values of 0.05 were considered statistically significant. *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001.

Results and Discussion

Type I IFNs modulate splenic and placental immune cell distributions and serum cytokine expression

We first determined the total splenic numbers in non-pregnant and pregnant mice WT and IFNAR mice by counting live cells using the Cellometer. In the non-infected state, pregnant WT and IFNAR^{-/-} mice exhibited elevated total splenic cell at day 14–15 of pregnancy relative to their non-pregnant counterparts (Fig. 1A). We then analyzed the distribution of immune cell subsets in the spleen and individual placentas through flow cytometry. The increased total splenic numbers in WT and IFNAR^{-/-} mice during pregnancy correlated with a general increase in splenic myeloid (macrophages and neutrophils) and lymphoid (B cells, T cells and NK cells) immune cell subsets during normal gestation (Fig. 1B, Supplemental Fig. 1A).²¹ It is proposed that the elevation of circulating granulocyte and monocyte numbers in the maternal host acts as a compensatory mechanism for pregnancy-related

changes in Th17 cells and Tregs.²² Furthermore, the spleen contains a reserve of monocytes that are rapidly recruited to inflammatory sites during infection.²³ We observed a decrease in splenic monocyte numbers in non-pregnant IFNAR^{-/-} mice compared to their WT counterparts, consistent with a previous study (Fig. 1B).²⁴ We also show that the reduced splenic monocyte numbers in IFNAR^{-/-} mice is retained during pregnancy. Thus, our results further highlight the role of type I IFNs in regulating monocyte distribution and that this mechanism is not impacted during pregnancy. Splenic T cell (CD4⁺ and CD8⁺) numbers were also decreased in pregnant IFNAR^{-/-} mice relative to pregnant WT controls, indicating a potential role for IFNAR signaling in modulating T cell distribution during pregnancy (Fig. 1B, Supplemental Fig. 1A). We also show for the first time an analysis of immune cell populations in the individual placenta. IFNAR^{-/-} mice exhibited reduced frequencies of monocytes, B cells and NK cells, and increased levels of macrophages and DC in the placenta relative to WT mice (Fig. 1C, Supplemental Fig. 1B). No significant differences were observed in placental neutrophil and T cell (CD4⁺ and CD8⁺) percentages between the two mouse strains. We then examined cytokine expression in the maternal systemic and placental compartments. Baseline levels of MCP-1 and KC were observed in the serum (Fig. 1D and Supplemental Fig. 1C). MCP-1 (CCL2) is a chemoattractant of monocytes, macrophages, NK cells and memory T cells.²⁵ Furthermore, KC promotes neutrophil recruitment and regulates neutrophil and T cell functionality during infection.²⁶ Non-pregnant and pregnant IFNAR^{-/-} mice exhibited decreased baseline levels of serum MCP-1 relative to their WT counterparts (Fig. 1D). This correlated with reduced monocyte numbers and percentages in spleens and individual placentas, respectively (Fig. 1B–D). Thus, modulation of monocyte distribution in the absence of IFNAR signaling is associated with downregulation of serum MCP-1 expression. Serum MCP-1 levels were also further reduced in pregnant IFNAR^{-/-} mice compared to non-pregnant controls (Fig. 1D). In contrast, we did not observe a statistically discernable reduction in serum MCP-1 expression levels between non-pregnant and pregnant WT mice. These results indicate that IFNAR signaling contributes to the regulation of baseline serum MCP-1 expression during pregnancy. Furthermore, baseline KC expression levels in the serum and spleen were similarly low in non-pregnant and pregnant IFNAR^{-/-} mice compared to WT controls (Supplemental Fig. 1C, 1D). A similar trend was observed in the placental expression of MCP-1 and KC between the two mouse strains (Supplemental Fig. 1E). Lastly, IL-6, IL-10, IFN- γ , TNF, IL-1 β and IL-12p70 expression levels in the serum, spleen and individual placentas were below the limits of detection (data not shown). Thus, IFNAR signaling modulates splenic and placental immune cell distributions and serum cytokine expression in the non-infected state.

IFNAR deficiency confers host protection from LM infection during pregnancy

The genetic resistance of C57BL/6J (WT) mice against LM is primarily attributed to the resistant allele at the *Hc* locus on chromosome 2.²⁷ We previously showed that this protection from infection is not adversely impacted within the systemic compartment of WT mice during pregnancy. WT mice infected with LM 10⁴ CFUs i.v. during mid-pregnancy (day 10–12 of gestation) exhibited significantly lower splenic bacterial numbers compared to non-pregnant controls at day 3 post-infection (day 13–15 of gestation).¹⁵ This was associated with potent systemic innate immune and antigen-specific CD8⁺ T-cell responses

similar to non-pregnant mice. However, placental bacterial numbers progressively increased in pregnant mice, correlating with fetal resorptions by days 3–5 post-infection (day 13–17 of gestation). These results indicate dichotomous control of LM infection in maternal systemic and placental compartments upon initiation of infection at mid-pregnancy. Furthermore, the protective role of IFNAR deficiency against systemic LM infection in non-pregnant mice is associated with reduced expression of IFN-inducible apoptosis-associated genes, decreased lymphocyte apoptosis and increased splenic numbers of TNF-producing macrophages.^{13;14;28} To determine whether the increased protection conferred by IFNAR deficiency against LM infection in the non-pregnant state is retained during pregnancy, we infected non-pregnant and pregnant (day 11–12 of gestation) WT and IFNAR^{-/-} mice with LM 5×10^4 CFUs i.v. At day 3 post-infection, IFNAR^{-/-} mice showed reduced splenic bacterial burden compared to WT mice in the non-pregnant state, as observed in previous studies (Fig. 2A).^{13;14} Pregnant WT mice also controlled splenic LM infection more efficiently compared to non-pregnant controls, as we observed previously.¹⁵ Next, pregnant IFNAR^{-/-} mice showed significantly reduced splenic bacterial burden relative to pregnant WT mice. A similar trend in bacterial numbers was also observed in individual placentas (Fig. 2B). Lastly, no significant differences were observed in fetal resorption rates among pregnant mice from both mouse strains (Fig. 2C). These results suggest that the protection conferred by IFNAR deficiency against LM infection is not adversely impacted by pregnancy. Furthermore, increased control of infection in non-pregnant IFNAR^{-/-} mice is associated with elevated splenic cell numbers relative to their WT counterparts (Supplemental Fig. 2A). Reduced splenic cell numbers in non-pregnant WT mice is consistent with accelerated macrophage and lymphocyte death in response to LM, as observed previously, leading to impaired innate immune cell responses.^{14;28} Furthermore, high LM burden enhances BM neutrophil exhaustion and reduces neutrophil infiltration into infected tissues.²⁹ In contrast, pregnant IFNAR^{-/-} mice exhibited reduced monocyte numbers, despite lower bacterial numbers in the spleen and individual placentas relative to WT controls (Fig. 2A, 2B, 2D). We surmise that pregnancy-associated increase in splenic immune cell numbers in the non-infected state may be sufficient to control bacterial growth in IFNAR^{-/-} mice upon infection, despite reduced monocyte numbers relative to WT mice (Fig. 1A, 1B, 2D). Reduced placental bacterial numbers were also associated with increased percentages of neutrophils and DCs, and decreased percentages of monocytes and NK cells in the placenta (Fig. 2B, 2E). Thus, our results suggest that deficiency in IFNAR signaling modulates splenic and placental immune cell distribution during LM infection. It is also possible that differential cytokine regulation during pregnancy is responsible for altered kinetics of infection in IFNAR mice relative to WT mice. Accordingly, we examined cytokine responses in the serum, spleen and individual placentas at day 3 post-infection. Increased control of LM infection in non-pregnant IFNAR^{-/-} mice relative to WT controls was associated with enhanced IL-12p70 expression in the serum at day 3 post-infection, consistent with previous studies (Fig. 2F).^{14;30} IL-12p70 is an immune-regulatory cytokine produced primarily by innate immune cells, including neutrophils, monocytes, macrophages and DCs.³¹ It plays critical roles in innate and adaptive immunity by promoting IFN- γ production upon NK cell activation and naïve CD4⁺ T cell differentiation into Th1 cells.³¹ IFN- α/β has been shown to inhibit IL-12 and IFN- γ expression during viral infection, further highlighting the immune-regulatory roles of IFNAR signaling in host immunity.³² Non-pregnant IFNAR^{-/-} mice also

exhibited reduced LM-induced expression of TNF, MCP-1, IFN- γ and KC in the serum, correlating with decreased bacterial burden in the spleen compared to their WT counterparts (Supplemental Fig. 2D, Fig. 2A, 2F). No significant differences were observed in IL-1 β and IL-10 expression in the serum among the non-pregnant groups (Supplemental Fig. 2D). During pregnancy, increased control of systemic LM infection in IFNAR^{-/-} mice was similarly associated with enhanced IL-12p70 expression compared to WT mice (Fig. 2A, 2F). However, serum expression levels of TNF, MCP-1, IFN- γ , IL-6 and KC were similar between pregnant WT and IFNAR^{-/-} groups (Fig. 2F, Supplemental Fig. 2D). IL-10 expression levels were similarly low in all non-pregnant and pregnant groups (Supplemental Fig. 2D). Interestingly, MCP-1 and TNF expression levels increased from the non-pregnant to pregnant state in IFNAR^{-/-} mice, indicating a potential role for type I IFN signaling in regulating bacterial-induced inflammation during pregnancy (Fig. 2F, Supplemental Fig. 2D). However, this enhanced inflammatory state was not detrimental to maternal and fetal protection from infection, suggesting a potential mechanism of homeostatic control during pregnancy. Analysis of splenic cytokine expression at day 3 post-infection showed reduced IL-6, IL-1 β , KC, MCP-1, TNF, IL-10 and IFN- γ expression in non-pregnant IFNAR^{-/-} mice relative to WT mice (Fig. 2G, Supplemental Fig. 2E). It is likely that the reduced splenic expression of these cytokines is attributed to decreased bacterial burden in pregnant IFNAR^{-/-} mice relative to their WT counterparts. Furthermore, deficiency in IFNAR signaling is associated with downregulation of MCP-1 production by BM-derived macrophages in response to LM infection.³³ Consistent with a previous study, we show that non-pregnant IFNAR^{-/-} mice exhibit enhanced control of LM infection despite reduced splenic MCP-1 expression relative to WT controls (Fig. 2A, 2G).³³ A similar trend in splenic bacterial burden and cytokine expression was observed in pregnant WT and IFNAR^{-/-} mice, except in IFN- γ expression where no significant differences were observed between the two groups (Fig. 2G). Splenic IL-12p70 expression was similarly low in all non-pregnant and pregnant groups (Supplemental Fig. 2E). Furthermore, non-pregnant and pregnant IFNAR^{-/-} mice showed reduced splenic IL-10 expression levels compared to their WT counterparts. In the placenta, KC, MCP-1, TNF, IL-6, IFN- γ and IL-12p70 expression were similar between the two mouse strains at day 3 post-infection (Supplemental Fig. 2F). This indicates that increased control of placental infection in IFNAR^{-/-} mice is not due to an altered placental inflammatory profile, but may be attributed to efficient initial control of infection in the systemic compartment. Thus, our results indicate compartment-specific regulation of cytokine responses in non-pregnant and pregnant hosts during systemic LM infection. Furthermore, enhanced serum IL-12 expression associated with IFNAR deficiency may be a critical indicator of host resistance to LM infection during pregnancy.

Pregnancy compromises host protection conferred by IFNAR deficiency against ST infection

Pregnancy enhances maternal and fetal disease progression in mice during ST infection. In the non-pregnant state, ST-resistant 129 $\times$ 1/SvJ mice infected with ST 10³ CFUs i.v. exhibit chronic disease for ~60 days prior to bacterial clearance.¹⁸ In contrast, pregnant 129 $\times$ 1/SvJ mice infected during mid-pregnancy (day 10–12 of gestation) show significantly decreased survival rates (~40%) relative to non-pregnant controls (100%) by day 7 post-infection. This reduced resistance against ST is attributed to impaired maternal splenic innate immune

responses, which are associated with increased expression of anti-inflammatory IL-10 and reduced ratios of pro-inflammatory Th17 cells to anti-inflammatory regulatory T cells (Tregs).^{16,18} In contrast, increased placental expression of pro-inflammatory IL-6, TNF and IL-18 correlate with profound bacterial colonization of the placenta.¹⁸ These results indicate that compartment-specific regulation of pro- and anti-inflammatory responses in pregnant mice modulates maternal systemic and placental disease progression. In the current study, we determined the role of IFNAR signaling in host immunity to ST infection during pregnancy by infecting non-pregnant and pregnant (day 11–12 of gestation) WT and IFNAR^{-/-} mice were infected with ST 10³ CFUs i.v. Non-pregnant IFNAR^{-/-} mice exhibit increased resistance to ST infection due to reduced bacterial-induced macrophage death.¹² As expected, non-pregnant IFNAR^{-/-} mice exhibited increased survival rates (median survival: 15 days) relative to their WT counterparts (median survival: 7 days). (Fig. 3A). However, the enhanced resistance of IFNAR^{-/-} mice against infection in the non-pregnant state was significantly reduced during pregnancy (median survival: 9 days). Furthermore, no significant differences in survival rates were observed between the two pregnant groups. Bacterial enumeration at day 3 post-infection showed reduced splenic bacterial numbers in non-pregnant IFNAR^{-/-} mice compared to WT controls (Fig. 3B). However, IFNAR^{-/-} mice showed a dramatic increase in splenic bacterial burden from the non-pregnant to pregnant state, to levels similar to those of pregnant infected WT mice. Splenic cell numbers from the non-pregnant to pregnant state were similar in both mouse strains during infection (Supplemental Fig. 3A). Furthermore, placental CFUs and fetal resorption rates were similarly high in pregnant WT and IFNAR^{-/-} mice upon infection (Fig. 3C, 3D). Thus, in contrast to LM infection, our results indicate that the protection conferred by IFNAR deficiency against ST infection is adversely impacted by pregnancy. Flow cytometric analysis of splenic immune cell populations at day 3 post-infection showed decreased monocyte percentages in non-pregnant and pregnant IFNAR^{-/-} mice relative to their WT counterparts (Fig. 3E). In a similar study, impaired bone marrow monocyte progenitor differentiation and reduced splenic accumulation of Ly6C⁺ monocyte-derived antigen presenting cells in response to lipopolysaccharide (LPS) was attributed to IFNAR deficiency.³⁴ LPS expressed by Gram-negative bacteria, such as *Salmonella*, induces immune activation upon recognition by host cells via Toll-like receptor 4 (TLR4).³⁵ These results indicate that defective monocyte recruitment and/or differentiation during ST infection may be associated with IFNAR deficiency, leading to altered splenic distribution of monocytes. Furthermore, no differences were observed in the splenic numbers of neutrophils, macrophages, DCs, NK cells, B cells and T cells (CD4⁺ and CD8⁺) in non-pregnant and pregnant IFNAR^{-/-} mice during infection relative to their WT counterparts (Supplemental Fig. 2B). We also observed a ~2-fold increase in neutrophil percentages in the placentas of IFNAR^{-/-} mice, which corresponded to an overall decrease in the percentages of other placental immune cell types relative to WT mice (Fig. 3F). Placental necrosis associated with neutrophil infiltration is a hallmark of immune pathology in ST infection during pregnancy.¹⁷ IFNAR deficiency has also been implicated in increased neutrophil migration but reduced monocyte infiltration into inflamed tissues during parasitic infection by *Leishmania amazonensis*.³⁶ Thus, our results suggest that type I IFNs play critical roles in modulating innate immune cell distribution in both splenic and placental compartments during infection. Analysis of serum cytokine expression at day 3 post-infection showed a reduction in IL-12p70 expression by pregnant

WT and IFNAR^{-/-} relative to their non-pregnant counterparts (Fig. 3G). This indicates that regulatory mechanisms associated with pregnancy may be exploited by ST, leading to impaired innate immune responses associated with decreased serum IL-12 expression. Splenic expression of IFN- γ , TNF and MCP-1 remained similar between non-pregnant WT and IFNAR^{-/-} mice (Fig 3H). In contrast, the expression levels of these cytokines were reduced in pregnant IFNAR^{-/-} mice relative to their WT counterparts. No significant differences were observed in the splenic expression levels of IL-6, KC, IL-1 β and IL-12p70 among all non-pregnant and pregnant groups (Fig. 3H, Supplemental Fig. 3E). Placental expression of KC and MCP-1 was reduced in IFNAR^{-/-} mice, which correlated with decreased monocyte percentages in the placenta relative to WT mice (Fig. 3I). Although KC is typically associated as a neutrophil chemoattractant, it has been shown to modulate monocyte recruitment using *in vivo* models of atherosclerosis and arteriogenesis.^{37;38} Therefore, it is possible that KC and MCP-1 expression contributed to the modulation of monocyte distribution in the placenta during ST infection. In contrast, placental expression levels of IFN- γ , TNF, IL-12p70 and IL-6 remained similar between the two mouse strains (Fig. 3I, Supplemental Fig. 3F). Thus, the protective role of IFNAR deficiency against LM and ST is largely dependent on pathogen-specific interactions with the host. Fetal-placental expression of type I IFNs during viral infection has been attributed major roles in the control of fetal viremia and the modulation of maternal survival.³⁹ In our study, reduced maternal systemic LM infection in pregnant IFNAR^{-/-} mice was associated with increased placental control of LM relative to their WT counterparts. In contrast, enhanced susceptibility of IFNAR^{-/-} mice to ST infection during pregnancy correlated with profound placental infection similar to WT mice. Our results indicate that bacterial burden in the placenta may also provide an additional source of infection, as observed during viral infection, thereby promoting disease progression.³⁹ However, the extent of the protection mediated by fetal-placental-derived type I IFNs against intracellular bacterial infections remains to be elucidated. Induction of IFNAR signaling during viral infection has been shown to decrease the threshold for pro-inflammatory cytokine expression in systemic and uterine compartments, and enhance susceptibility to secondary bacterial infection-driven pre-term births.⁴⁰ This results supports the prevailing “double-hit” hypothesis, wherein viral infection enhances the risk of invasive bacterial infection in the maternal host and adverse pregnancy outcomes.^{40–42} Furthermore, viral-induced inhibition of IFN- β expression enhances pro-inflammatory cytokine production and placental trophoblast cell sensitivity to LPS.⁴³ We have previously shown that TLR4 expression is associated with robust pro-inflammatory cytokine production in pregnant mice in response to ST infection, leading to increased maternal disease progression and fetal deaths.¹⁶ Taken together, these results highlight the complex signaling networks regulated by IFNAR and TLR-mediated inflammation in host immunity to infection during pregnancy. Achieving a balance between pro-inflammatory and anti-inflammatory responses in maternal systemic and fetal-placental compartments is critical in determining pregnancy outcomes.

Conclusion

Our study highlights the critical roles of IFNAR signaling in modulating immune cell distribution and cytokine expression, leading to differential responses to intracellular

infections during pregnancy. The increased resistance to LM infection and enhanced serum IL-12 response associated with IFNAR deficiency is not adversely impacted by pregnancy, despite the altered distribution of monocytes in maternal systemic and placental compartments. In contrast, the reduced resistance of pregnant hosts to ST infection is due to pregnancy-associated impairment of innate immune responses, including serum IL-12 expression, irrespective of IFNAR signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DC	dendritic cel
FBS	fetal bovine serum
IFNAR^{-/-}	IFN alpha receptor knockout mouse
i.v.	intravenous
LM	<i>Listeria monocytogenes</i> 10403S
NK	natural killer
ST	<i>Salmonella enterica</i> serovar Typhimurium SL1344
Treg	regulatory T cell
WT	C57BL/6J wild-type mouse

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Figure 1: IFNAR^{-/-} mice exhibit altered distributions of splenic and placental immune cell types relative to WT mice.

Spleen, individual placentas and serum were collected from non-pregnant and pregnant (day 14–15 of gestation) WT and IFNAR^{-/-} mice in the non-infected state. (A) Total splenic cell numbers were determined by counting live cells using the Cellometer. N = 6 mice per group. Immune cell populations in the spleen and individual placentas were identified through flow cytometry. (B) Splenic numbers of monocytes, macrophages, DCs, B cells, T cells and NK cells. N = 6 mice per group. (C) Placental percentages of monocytes, macrophages, DCs, B cells, T cells and NK cells. N = 51–52 individual placentas per pregnant group. (D) MCP-1 expression levels were determined through CBA. N = 4–5 mice per group. Data are presented as mean ± SEM. Statistical significance was analyzed by Mann Whitney U test. *: p = 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001. NPNI, non-pregnant non-infected; PNI, pregnant non-infected.

Figure 2: IFNAR^{-/-} mice retain increased resistance to LM infection from the non-pregnant to pregnant state.

Non-pregnant and pregnant (day 11–12 of gestation) WT and IFNAR^{-/-} mice were infected with LM 5 × 10⁴ CFUs i.v. Spleens, individual placentas and serum were collected at day 3 post-infection. (A and B) Bacterial burdens in spleens and individual placentas were enumerated. N = 6–8 mice per group; 47–63 individual placentas per pregnant group. (C) Fetal resorption rates were determined using the formula $R/(R + V) \times 100$, where R is the number of resorbing fetuses and V is the number of viable fetuses per mouse. N = 3–11 mice per group. Immune cell populations in the spleen and individual placentas were identified through were evaluated through flow cytometry. (D) Monocyte numbers in the spleen in non-pregnant and pregnant WT and IFNAR^{-/-} mice at day 3 post-infection. N = 4 mice per group. (E) Percentages of neutrophils, monocytes, DCs and NK cells in the placenta at day 3 post-infection. N = 24–27 individual placentas per pregnant group. Cytokine expression levels in the serum (F) and spleen (G) at day 3 post-infection. N = 4–5 mice per group. Data are presented as mean ± SEM. Statistical significance was analyzed by Mann Whitney U test. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$. NPI, non-pregnant infected; PNI, pregnant non-infected; PI, pregnant infected.

Figure 3: Pregnant IFNAR^{-/-} mice exhibit enhanced susceptibility to ST infection relative to non-pregnant controls.

Non-pregnant and pregnant (day 11–12 of gestation) WT and IFNAR^{-/-} mice were infected with ST 10³ CFUs i.v. (A) Mice were monitored every day following infection. Mouse deaths were recorded upon observing >20% loss in body weight and/or 2 or more severe signs of clinical illness, including piloerection, lack of grooming and morbidity. N = 7–8 mice per group. Spleen, individual placentas and serum were collected at day 3 post-infection. (B and C) Bacterial burden in spleen and individual placentas were enumerated. N = 7–13 mice per group; 35–70 individual placentas per pregnant group. (D) Fetal resorption rates were determined using the formula $R/(R + V) \times 100$, where R is the number of resorbing fetuses and V is the number of viable fetuses per mouse. N = 5–13 mice per group.

Immune cell populations in the spleen and individual placentas were identified through were evaluated through flow cytometry. (E) Monocyte numbers in the spleen in non-pregnant and pregnant WT and IFNAR^{-/-} mice at day 3 post-infection. N = 4–7 mice per group. (F) Percentages of neutrophils, monocytes, macrophages, DCs, B cells, T cells (CD4⁺ and CD8⁺) and NK cells in the placenta at day 3 post-infection. N = 38–40 individual placentas per pregnant group. Cytokine expression levels in the serum (G), spleen (H) and placenta (I) at day 3 post-infection. N = 5–9 mice per group; 10 individual placentas per pregnant group. Bacterial burden, resorption rates, cell numbers and cytokine expression levels are presented as mean ± SEM. Statistical significance was analyzed by Mann Whitney U test. *: p = 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001. NPI, non-pregnant infected; PNI, pregnant non-infected; PI, pregnant infected.