

Disruption of type I interferon signaling causes sexually dimorphic dysregulation of anti-viral cytokines

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ABSTRACT

Type I interferons (IFNs) play a crucial role in the establishment of an antiviral state via signaling through their cognate type I IFN receptor (IFNAR). In this study, a replication-competent but highly attenuated strain of VSV (rVSVΔm51) carrying a deletion at position 51 of the matrix protein to remove suppression of anti-viral type I IFN responses was used to explore the effect of disrupted IFNAR signaling on inflammatory cytokine responses in mice. The kinetic responses of interleukin-6, tumor necrosis factor- α and interleukin-12 were evaluated in virus-infected male and female mice with or without concomitant antibody-mediated IFNAR-blockade. Unlike controls, both male and female IFNAR-blocked mice showed signs of sickness by 24-hours post-infection. Female IFNAR-blocked mice experienced greater morbidity as demonstrated by a significant decrease in body temperature. This was not the case for males. In addition, females with IFNAR-blockade mounted prolonged and exaggerated systemic inflammatory cytokine responses to rVSVΔm51. This was in stark contrast to controls with intact IFNAR signaling and males with IFNAR-blockade; they were able to down-regulate virus-induced inflammatory cytokine responses by 24-hours post-infection. Exaggerated cytokine responses in females with impaired IFNAR signaling was associated with more effective control of viremia than their male counterparts. However, the trade-off was greater immune-mediated morbidity. The results of this study demonstrated a role for IFNAR signaling in the down-regulation of antiviral cytokine responses, which was strongly influenced by sex. Our findings suggested that the potential to mount toxic cytokine responses to a virus with concomitant disruption of IFNAR signaling was heavily biased towards females.

1. Introduction

Interferons (IFNs) are a large family of pleiotropic cytokines that can be subdivided into three distinct groups and are produced by a variety of cells after viral infections. They play a dominant role in initiating host canonical antiviral defenses. Type I IFNs, including IFN- α and β , play a crucial role in the early establishment of an antiviral state by interacting with their cognate heterodimeric receptor, IFNAR, which is expressed on nucleated cells [1–9]. Cellular responses to type I IFNs are initiated by IFNAR-mediated signaling and include boosting of the late phase of antiviral immunity via a positive feedback loop [10]. Type I IFN-

mediated antiviral responses are also characterized by robust local induction of pro-inflammatory cytokines and chemokines that attract inflammatory cells of the innate immune system. The ability of type I IFNs to overcome viral infections relies on a finely tuned interplay of inflammatory and anti-inflammatory responses of both the innate and adaptive immune systems [11–17]. Severe viral infections are sometimes associated with a phenomenon known as a “cytokine storm” or “hypercytokinemia”, which is caused by an overly robust inflammatory response that can become toxic [18–36]. Coincidentally, these viruses that induce excessive, toxic cytokine responses are associated with mechanisms that facilitate potent suppression of type I IFN responses.

Abbreviations: IFNs, Interferons; IFNAR, IFN- α / β receptor; VSV, vesicular stomatitis virus.

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Antiviral immunity can be influenced by intrinsic host factors such as sex [37,38] and genetic background [3–5], the type of virus, the strategies used by viruses to evade immune responses, and the interplay between viruses and the host's immune system [3,8,33,39–47]. There is a sex-related disparity in antiviral responses, with a bias towards females experiencing a higher proportion of virus-associated morbidities and mortalities than males [37,38,48]. We sought to test the hypothesis that females would have more exaggerated cytokine responses to viral infection during IFNAR-blockade than males. To accomplish this, a replication-competent but highly attenuated recombinant strain of vesicular stomatitis virus (rVSVΔm51) was used [49,50]. This virus had the methionine at position 51 of the matrix protein deleted to remove

suppression of anti-viral type I IFN responses. This safe virus was developed to be administered intravenously (i.v.) at high doses to treat patients with cancers. Intravenous delivery of rVSVΔm51 into male and female mice was used in conjunction with i.v. administration of an IFNAR-blocking antibody to simulate potent inhibition of type I IFN responses during viremia. The results of this study indicated a role for IFNAR in the negative regulation of antiviral cytokine responses. Notably, this was influenced by sex, with a bias towards females.

2. Materials and methods

A summary of materials can be found in the following Key

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse IgG1 Isotype Control (clone HKSP84)	Leinco Technologies	Catalogue #I-117
Anti-Mouse Interferon Alpha/Beta Receptor-1 (clone MAR1-5A3)	Leinco Technologies	Catalogue #I-401
PE Rat Anti-Mouse Ly6G (clone 1A8)	BD Biosciences	Catalogue #551461
APC Anti-Mouse IL-6 (clone MP5-20F3)	BioLegend	Catalogue #504507
PE/Cyanine7 anti-mouse CD45 (clone 30-F11)	BioLegend	Catalogue #103113
Brilliant Violet 421™ anti-mouse/human CD11b (clone M1/70)	BioLegend	Catalogue #101235
Anti-Mouse CD16/CD32 (clone 93)	BioLegend	Catalogue #103020
Virus Strain		
Recombinant Indiana Strain of Vesicular Stomatitis Virus (rVSVΔm51)	Dr. Brian Lichty, McMaster University, Hamilton, Ontario, Canada	<i>Cancer Cell</i> , 2003 Oct;4(4):263-75. PMID: 14585354
Biological Samples		
Bovine Calf Serum	VWR	Catalogue #2100-500
Chemicals		
Heparin Sodium Salt from Porcine Intestinal Mucosa	Sigma-Aldrich	Catalogue #H3149
Ammonium Chloride (NH ₄ CL)	Fisher Scientific	Cat#A649500
Potassium Bicarbonate (KHCO ₃)	Fisher Scientific	Catalogue #P184-500
EDTA Disodium Salt (Na ₂ EDTA)	Fisher Scientific	Catalogue #BP120500
Trypan Blue Stain 0.4%	Gibco	Catalogue #15250
Phosphate Buffer Saline	Hyclone	Catalogue #SH0325601
Bovine Serum Albumin	Fisher Scientific	Catalogue #BP1600100
Zombie Aqua Fxable Viability Kit	BioLegend	Catalogue #423101/423102
Dulbecco's Modified Eagle Medium	Hyclone	Catalogue #SH3002201
Experimental Models: Organisms/Strains		
Female and Male Balb/c mice	Charles River Laboratories	strain code 028
Experimental Models: Cell Line		
Vero cells	America Type Culture Collection	#CCL-81
Critical Commercial Assays		
ProcartaPlex Mouse IL-12/IL-23p40 Simplex	ThermoFisher	Catalogue #EPX01A-26033-901
ProcartaPlex Mouse TNF alpha Simplex	ThermoFisher	Catalogue #EPX01A-20607-901
ProcartaPlex Mouse IL-6 Simplex	ThermoFisher	Catalogue #EPX01A-20603-901
Software and Algorithms		
Flowjo Software version 10	Becton Dickinson	https://www.flowjo.com/solutions/flowjo
GraphPad Prism version 8	TreeStar	https://www.graphpad.com/scientific-software/prism/
Other		
FACS Canto II Flow Cytometer	Becton Dickinson	
Heparinized Micro-Hematocrit Capillary Tubes	Fisher Scientific	Catalogue #22362566
Microtherma 2 Type "T" Thermometer	Braintree Scientific	Catalogue #TW2- 107
Traceable Excursion-Trac Datalogging Thermometer	ITM Instruments	

Resources Table:

2.1. Mice

Age-matched six-to-eight-week-old male and female Balb/c mice (Charles River Laboratories, USA; strain code #028) were used in all experiments. Mice were housed in an isolated pathogen-free environmentally-controlled facility at the University of Guelph. Mice were accommodated to the facility for one week prior to the initiation of experiments and were monitored regularly and provided with food and water *ad libitum*. Mouse studies adhered to the guidelines provided by the Canadian Council on Animal Care and were approved by the University of Guelph's Animal Care Committee (animal utilization protocol #3807).

2.2. Virus

The highly attenuated replication-competent rVSVΔm51 that was used in this study was kindly provided by Dr. Brian Lichty (McMaster University, Hamilton, Ontario, Canada) and has been previously described [49]. The virus was propagated in Vero cells (American Type Culture Collection; #CCL-81), concentrated by ultracentrifugation and purified by centrifugation on a sucrose density gradient followed by dialysis. Determination of viral titers was done using a standard plaque assay. Doses of 1×10^9 pfu of rVSVΔm51 in 200 μL of phosphate-buffered saline (PBS) were administered to mice via tail veins. The use of this virus was approved by the University of Guelph's Biosafety Committee (biohazard permit #A-367-04-19-05).

2.3. IFNAR-blockade

To block type I IFN receptors, mice received 1 mg of a murine IFNAR1-specific antibody (Leinco Technologies, USA; cat. #1-401) via tail veins 24- or two-hours before viral infection. Mice in control groups received intravenous injections of 1 mg of an IgG isotype control immunoglobulin (Leinco Technologies, USA; cat. #1-536).

2.4. Tissue processing and staining for flow cytometry

Blood samples were drawn from the *retro*-orbital sinus of mice using heparinized capillary tubes into 1.5 mL microtubes tubes containing 5 μL of heparin at a concentration of 3 μg/mL. The vasculature of the lungs was perfused with Hanks balanced salt solution to purge blood. Lungs were then minced, digested with collagenase IV and DNase (Thermo Fisher Scientific) for 30 min at 37 °C and then passed through a strainer with 70 μm pores to obtain a single-cell suspension. Erythrocytes were removed from blood and lung samples using a red blood cell lysing buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA). Leukocytes had their Fc receptors blocked by treatment with anti-CD16/CD32 (clone 93; BioLegend, USA; cat. #101320) in FACS buffer (PBS + 0.1% bovine serum albumin) for 15 min at 4 °C. Cells were then stained with anti-Ly6G, anti-CD45, and anti-CD11b (clones 1A8; A20; M1/70 all from BioLegend, San Diego, CA, USA) in FACS buffer for 20 min in the dark at 4 °C. After washing, the cells were stained with a fixable viability dye (Zombie Aqua; BioLegend; cat. #423102) as per the manufacturer's directions to facilitate exclusion of dead cells. The stained cells were then suspended in PBS containing 0.5% bovine serum albumin and immediately analyzed on a FACS Canto II flow cytometer with data acquired using FACSDiva 8.0.1 software (BD Biosciences, Mississauga, ON, Canada). Data were analyzed using FlowJo v.10.1 software (FlowJo LLC, Ashland, OR, USA). Exported data were graphed and statistically analyzed using GraphPad Prism version 8 software (GraphPad, San Diego, CA, USA).

For detection of intracellular cytokines, cells were left for four hours at 37 °C. Subsequently, brefeldin-A (BioLegend) was added for eight-

hours to inhibit protein export by the Golgi apparatus. After the incubation period, staining of surface markers proceeded as described above. After staining with a viability dye, the samples were fixed using fixation buffer (BioLegend) and permeabilized using intracellular staining perm-wash buffer (BioLegend) at 4 °C for 30 min and then stained with anti-IL-6 (clone MP5; BioLegend) at 4 °C for 20 min.

2.5. Assessment of body temperatures

A certified digital thermometer with a rectal probe (Microtherma 2 Type "T" Thermometer; Braintree Scientific, USA; cat. #TW2-107) was used to measure the body temperatures of mice. Proper calibration of the thermometer was confirmed by cross-referencing the room temperature with a second certified digital thermometer (Traceable Excursion-Trac Datalogging Thermometer, ITM Instruments, Canada).

2.6. Quantification of plasma-derived cytokines

Blood samples were acquired from the *retro*-orbital sinus of mice using heparinized capillary tubes. Blood was collected in 1.5 mL microtubes containing 5 μL of heparin at a concentration of 3 μg/mL. Blood was centrifuged at 4 °C for 10 min at 2,000g and the plasma was immediately collected, aliquoted into polypropylene tubes and archived at -80 °C until further analysis. The inflammatory cytokines interleukin (IL)-6, tumor necrosis factor (TNF)-α and IL-12(p40) were quantified in plasma using ProcartaPlex Mouse Simplex kits (Invitrogen eBioscience, USA; cat. #s EPX01A-20603-901, EPX01A-20607-901 and EPX01A-26033-901) according to the manufacturer's instructions and a Bio-Plex 200 system (Bio-Rad, Canada).

2.7. Determination of virus titers

Plasma samples were serially diluted in complete Dulbecco's Modified Eagle Medium (Hyclone) supplemented with 10% bovine calf serum (Hyclone, USA) and used for determination of 50% tissue culture-infected dose (TCID₅₀) and converting the values to plaque-forming units (pfu) by using the conversion factor 0.69, as previously described [51].

2.8. Depletion of neutrophils

Mice received 250 μg of anti-Ly6G (clone 1A8; BioXCell) in a volume of 200 μL injected intraperitoneally. Mice received the Ly6G-specific antibody 48 h before the administration of rVSVΔm51 with daily injections throughout the study.

2.9. Statistics

GraphPad Prism version 8 was used for all graphing and statistical analyses. Graphs show means and standard errors. If required, data were normalized by log transformation. Data were analyzed using one- or two-way analysis of variance with Sidak's multiple comparisons test when assessing one or two variables, respectively. Statistical significance was defined as $p \leq 0.05$.

3. Results

3.1. Mice developed a systemic inflammatory response to recombinant vesicular stomatitis virus (rVSVΔm51) that was characterized by an increase in the frequency of granulocytes in blood, which was potentiated by type I interferon receptor (IFNAR)-blockade

Neutrophils are the most prevalent granulocytes and are usually the first cells to traffic to sites of infection. As such, they can be used as a surrogate marker of acute inflammation. Male and female mice were infected intravenously with 1×10^9 pfu rVSVΔm51 to simulate viremia.

This was done with or without concomitant IFNAR-blockade. Blood-derived Ly6G⁺ granulocytes were then quantified by flow cytometry ten hours post-infection (Fig. 1). Regardless of sex, the frequency of circulating granulocytes in mice with intact IFNAR signaling was increased by approximately two-fold relative to uninfected controls. Interestingly, this frequency was significantly increased in both males and females by antibody-mediated blockade of IFNARs. This suggested that VSV-induced inflammatory responses were being modulated by type I interferon signaling.

3.2. Mice infected with rVSVΔm51 with concomitant IFNAR-blockade showed signs of sickness, with females having acute decreases in body temperatures

Due to its clinical development as an oncolytic virus, rVSVΔm51 has been shown to be safe when administered to mice [52]. However, since mice treated with an IFNAR blocking antibody had evidence of an exaggerated acute inflammatory response to this virus, they were monitored beyond ten hours to determine if any adverse events developed. Although all mice survived infection with rVSVΔm51, both male and female IFNAR-blocked mice showed multiple signs of morbidity at 24-hours post-infection (Table 1). This was in stark contrast to control mice with intact IFNAR signaling, which did not show any signs of sickness. In addition, female mice that had been infected during IFNAR-blockade had significant reductions in body temperatures (from 37.10 °C pre-infection to 32.05 °C and 32.90 °C at 10.5- and 24-hours post-infection, respectively) compared to males and IFNAR-intact females (Fig. 2). This suggested that female mice with compromised IFNAR signaling might be mounting more severe inflammatory

responses than their male counterparts that were not reflected in circulating neutrophils. This prompted an investigation of acute pro-inflammatory cytokine responses.

3.3. Inflammatory cytokine responses induced by rVSVΔm51 were exaggerated in females with disrupted IFNAR-mediated signaling

The cytokines IL-6, TNF-α and IL-12(p40) were selected as representative soluble markers of inflammation. At 10-hours post-infection with rVSVΔm51, female mice with blocked IFNARs showed an exaggerated production of all three of these cytokines in the blood compared to female controls and male IFNAR-blocked mice (Fig. 3). Specifically, females with IFNAR-blockade, females with intact IFNAR signaling and males with IFNAR blockade, respectively, had 6,337 versus 3,766 versus 2,674 pg/mL of IL-6, 1,784 versus 366 versus 442 pg/mL of TNF-α, and 880 versus 159 versus 201 pg/mL of IL-12(p40). This demonstrated a strong female bias towards more inflammatory cytokine responses against rVSVΔm51 if IFNAR signaling was compromised.

3.4. Female mice had an impaired ability to negatively regulate cytokine responses to recombinant vesicular stomatitis virus (rVSVΔm51) during type I interferon receptor (IFNAR)-blockade

Finding evidence of dysregulated cytokine responses 10-hours after infection with a virus in female mice that had concomitant IFNAR-blockade prompted a more detailed kinetic study over a period of 24-hours. Plasma samples were collected just prior to intravenous administration of rVSVΔm51 and at 2.5, 5, 10, and 26-hours post-infection. Groups included male and female mice with or without pre-treatment with an intravenously administered IFNAR-blocking antibody. Concentrations of IL-6, TNF-α and IL-12(p40) were determined (Fig. 4A). In all mice with intact IFNAR signaling, IL-6 and TNF-α responses peaked at 2.5-hours post-infection and had returned to baseline by 26-hours after infection. For IL-12(p40), the response peaked 5-hours after infection. Interestingly, the IL-6 response was blunted at 2.5-hours post-infection in males with IFNAR blockade, but this represented the only significant effect of interfering with IFNAR signaling in male mice. In stark contrast, females with IFNAR blockade experienced massively dysregulated cytokine responses that not only failed to return to baseline by 26-hours post-infection, they were still increasing in concentration at this time point for IL-6 and IL12(p40). Converting these kinetic cytokine response data to areas under the curves confirmed that females with IFNAR-blockade mounted exaggerated responses for all three cytokines compared to both groups of males and female controls with intact IFNAR signaling (Fig. 4B). These results provided evidence that females with disrupted IFNAR signaling were able to mount normal acute inflammatory cytokine responses to a virus but were subsequently unable to properly down-regulate these responses, especially for IL-6 and IL-12 (p40). This suggested a critical role for IFNAR signaling in the modulation of cytokine responses and unveiled a scenario in which females experienced a higher degree of inflammation.

3.5. IFNAR signaling was required for viral clearance

Type I interferon responses have been reported to be critical for clearance of viruses. To confirm this, rVSVΔm51 was quantified in plasma 24 h after infecting male and female IFNAR-intact versus IFNAR-blocked mice (Fig. 5). Mice with intact IFNAR signaling did not have virus titers above the limit of detection of the TCID₅₀ assay. As expected, mice that had been infected with concomitant IFNAR-blockade had difficulty controlling acute viremia. Notably, however, females with IFNAR-blockade had significantly lower viral titers than their male counterparts, which correlated with their more robust cytokine responses (Fig. 4). These findings confirmed the importance of type I interferon responses in the acute control of viral infections and suggested that exaggerated production of other virus-induced cytokines in

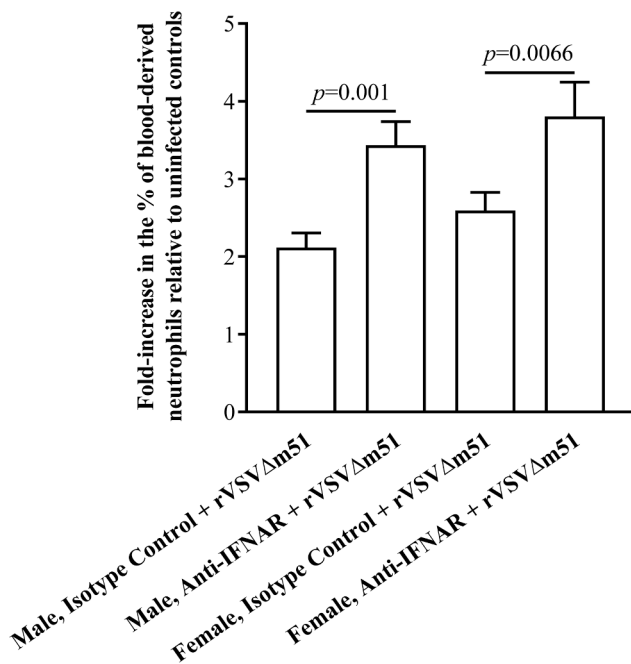


Fig. 1. Mice developed a systemic inflammatory response to recombinant vesicular stomatitis virus (rVSVΔm51) that was characterized by an increase in the frequency of granulocytes in blood, which was potentiated by type I interferon receptor (IFNAR)-blockade. Male and female Balb/c mice received intravenous injections of 1 mg of an isotype control immunoglobulin or a type I interferon receptor (IFNAR)-blocking antibody two hours before intravenous administration of 1×10^9 pfu of rVSVΔm51. Ten hours post-infection blood-derived Ly6G⁺ granulocytes were quantified by flow cytometry. Bars represent the mean fold-increase in the frequency of Ly6G⁺ cells relative to uninfected control mice. Standard errors are shown; $n = 4$ /group. Data are representative of three experimental replicates and were assessed using a one-way analysis of variance with Sidak's multiple comparisons test.

Table 1

Mice infected with a recombinant vesicular stomatitis virus (rVSVΔm51) with concomitant blockade of their type I interferon receptors showed signs of sickness. Male and female Balb/c mice received intravenous injections of 1 mg of an isotype control immunoglobulin or a type I interferon receptor (IFNAR)-blocking antibody two hours before intravenous administration of 1×10^9 pfu of rVSVΔm51. The mice were then monitored at 12- and 24-hours post-infection (hpi) to determine if clinical signs were present (+) or absent (-).

			Clinical Signs				
			Lethargic	Ruffled Fur	Isolated	Hunched	Laboured Breathing
Male	12 hpi	Isotype control	-	-	-	-	-
		Anti-IFNAR1	-	-	-	-	-
	24 hpi	Isotype control	-	-	-	-	-
		Anti-IFNAR1	+	+	+	+	+
Female	12 hpi	Isotype control	-	-	-	-	-
		Anti-IFNAR1	-	-	-	-	-
	24 hpi	Isotype control	-	-	-	-	-
		Anti-IFNAR1	+	+	+	+	+

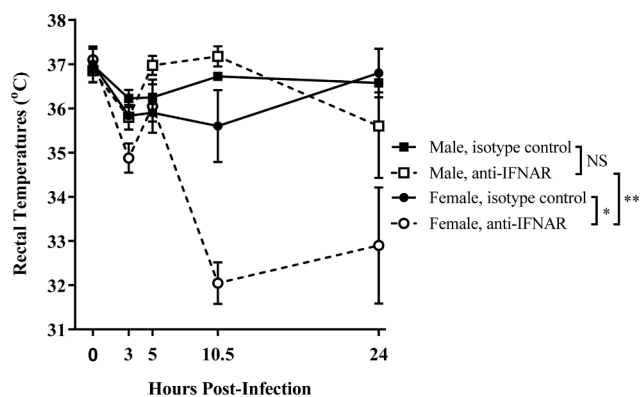


Fig. 2. Female mice with impaired type I interferon receptor (IFNAR) signaling had acute reductions of body temperatures after viral infection. Male and female Balb/c mice received intravenous injections of 1 mg of an isotype control immunoglobulin or a type I interferon receptor (IFNAR)-blocking antibody two hours before intravenous administration of 1×10^9 pfu of a recombinant vesicular stomatitis virus (rVSVΔm51). Rectal temperatures were assessed at multiple time points post-infection. Data are shown as means with standard errors; $n = 4$ /group. Data are representative of three experimental replicates and were assessed using a two-way analysis of variance with Sidak's multiple comparisons test. NS = not significant, $*p = 0.0372$, $**p = 0.0152$.

females (Fig. 4) could partially compensate for this, but at the expense of greater morbidity (Fig. 2).

3.6. Depletion of neutrophils in VSVΔm51-infected mice with IFNAR-blockade elevated pro-inflammatory cytokines

Since an increase in the frequency of granulocytes was demonstrated in the blood of mice that received recombinant rVSVΔm51, we sought to monitor infiltration of granulocytes into the organs. Oncolytic viruses can be detected in the lungs following intravenous administration [53] and accumulation of neutrophils becomes apparent in the lungs of mice within three hours of intravenous delivery of rVSVΔm51 [54]. Therefore, male and female mice were infected intravenously with 1×10^9 pfu of rVSVΔm51 to examine trafficking of neutrophils into the lungs. This was done with or without concomitant IFNAR-blockade. Ly6G⁺ CD11b⁺ neutrophils were then quantified by flow cytometry ten hours post-infection (Fig. 6A). Regardless of sex, the frequency of neutrophils in mice with intact IFNAR signaling increased by approximately four-fold relative to uninfected controls. Interestingly, this frequency was further increased in both males and females by antibody-mediated blockade of IFNARs (about ten-fold relative to uninfected controls). This suggested that VSV-induced inflammatory responses defined by the accumulation of neutrophils in tissues were being modulated by type I IFN signaling. We next sought to examine whether these infiltrated

neutrophils contributed to the cytokine response to VSV in mice with IFNAR-blockade. Intracellular cytokine staining revealed that neutrophils from both sexes produced a very tiny amount of IL-6 (Fig. 6B). These results prompted the evaluation of the role neutrophils in regulating cytokine responses. *In vivo* depletion of neutrophils prior to administration of rVSVΔm51 into female mice with IFNAR-blockade showed a further elevation of the pro-inflammatory cytokine IL-6 compared to controls (Fig. 6C). These findings suggest that while neutrophils actively participate in acute inflammation caused by rVSVΔm51, they play a regulatory role in cytokine responses to rVSVΔm51 by dampening IL-6 production in other cells when IFNAR signaling is impaired.

4. Discussion

Sex disparity in type I IFN production by cells of the immune system [37,55–61] in the context of viral infection [38], cancer [62], and immunological disorders [63–65] has been reported. Furthermore, sex disparity in other cytokine responses is a well-established phenomenon in a variety of contexts [66–69], including viral infections [38,48,70,71]. However, to the best of our knowledge, this study provides the first evidence of a direct, sex-biased link between IFNAR signaling and the regulation of an array of pro-inflammatory cytokine responses to a viral infection. Indeed, in this study, female mice had exaggerated concentrations of the inflammatory cytokines IL-6, TNF- α , and IL-12(p40) in plasma after infection with rVSVΔm51 when there was concomitant disruption of IFNAR signaling (Figs. 3 and 4). IL-6 and IL-12(p40) were the most dysregulated of the three cytokines in females, implying a particularly strong association with the sex of the host. Accordingly, a significant association has previously been reported between serum concentrations of IL-6, female sex, and autoimmune toxicity of anti-cytotoxic T-lymphocyte-associated protein-4-mediated checkpoint blockade therapy in patients with cancers [66]. While the present study provides additional evidence of sex-biased inflammatory responses, more importantly, it also shows a role for IFNAR signaling in the dimorphic regulation of antiviral cytokine responses, which might also be applicable to other contexts, including autoimmune diseases that feature differential IFN responses in females [63,64,72,73]. A synergistic interplay between estrogen and IL-6 has also been reported to be associated with the severity of experimental, drug-induced liver injury in female Balb/c mice. Specifically, estrogen-induced IL-6 was shown to promote sex-disparity in severity of drug-induced liver injury by reducing the expansion of splenic regulatory T cells in females [74].

While mechanisms underlying differential immune response between males and females are complex, the synergistic functions of the endocrine and immune systems, together with dimorphic gene expression and viral factors have been shown to be implicated in differential outcomes of viral infections between sexes [37,47,48,75]. Many hematopoietic and non-hematopoietic cells express receptors for sex

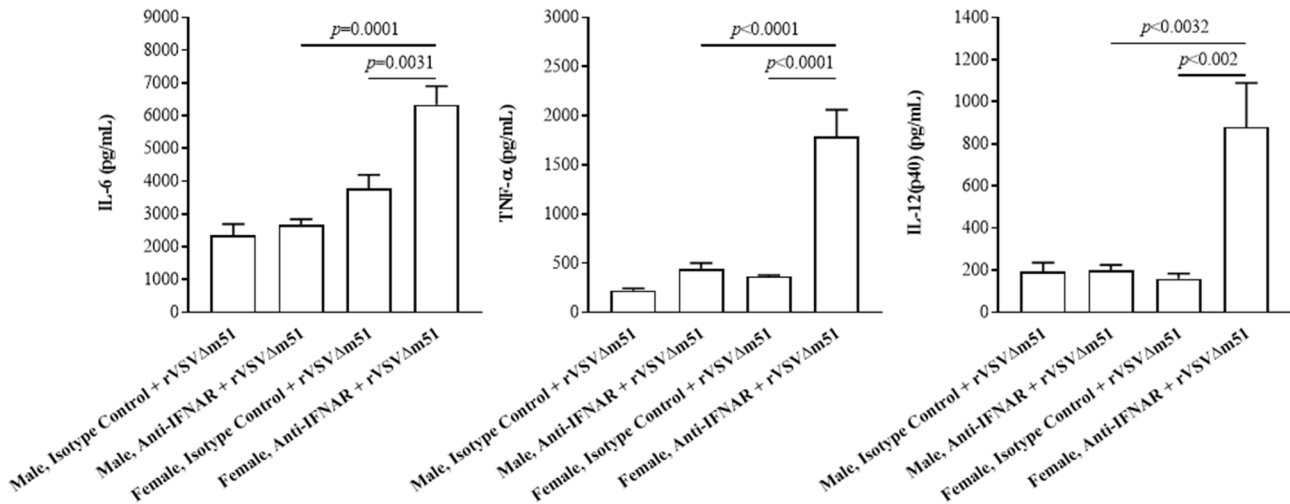


Fig. 3. Virus-induced inflammatory cytokine responses were exaggerated in females with disrupted type I interferon receptor-mediated signaling. Male and female Balb/c mice received intravenous injections of 1 mg of an isotype control immunoglobulin or a type I interferon receptor (IFNAR)-blocking antibody two hours before intravenous administration of 1×10^9 pfu of a recombinant vesicular stomatitis virus (rVSVΔm51). Plasma samples were collected ten hours post-infection to quantify the cytokines interleukin (IL)-6 (left panel), tumor necrosis factor (TNF)-α (middle panel) and IL-12(p40) (right panel) using a multiplex assay. Data are shown as means with standard errors; $n = 4$ /group. Data are representative of three experimental replicates and were assessed using a one-way analysis of variance with Sidak's multiple comparisons test.

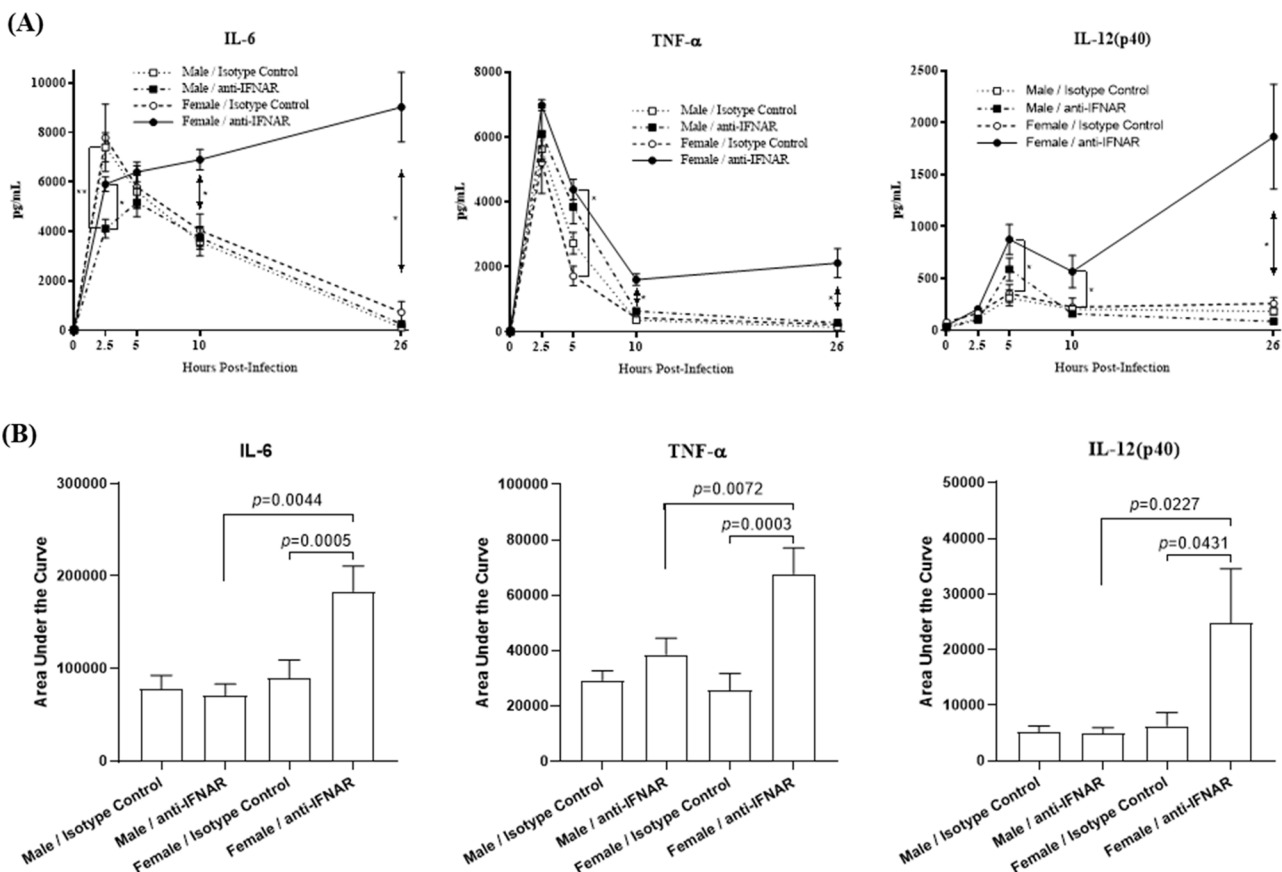


Fig. 4. Female mice had an impaired ability to negatively regulate cytokine responses to recombinant vesicular stomatitis virus (rVSVΔm51) during type I interferon receptor (IFNAR)-blockade. Male and female Balb/c mice received intravenous injections of 1 mg of an isotype control immunoglobulin or an IFNAR-blocking antibody two hours before intravenous administration of 1×10^9 pfu of rVSVΔm51. Plasma samples were collected 0, 2.5, 5, 10 and 26 h post-infection to quantify the cytokines interleukin (IL)-6 (left panels), tumor necrosis factor (TNF)-α (middle panels) and IL-12(p40) (right panels) using a multiplex assay. Data were graphed as (A) curves and (B) areas under the curves and shown as means with standard errors; data were pooled from two experiments; $n = 8$ /group. Data were assessed using (A) two-way analysis of variance with Tukey's multiple comparisons test, or (B) one-way analysis of variance with Sidak's multiple comparisons test.

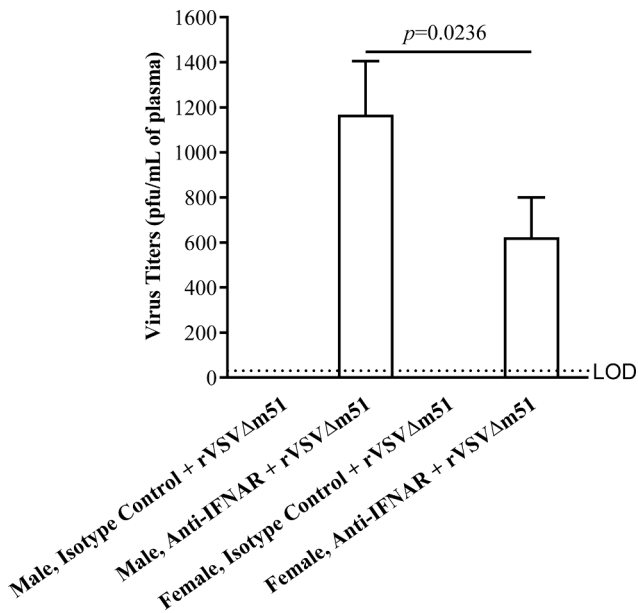


Fig. 5. Type I interferon receptor (IFNAR) signaling was required for viral clearance. Male and female Balb/c mice received intravenous injections of 1 mg of an isotype control immunoglobulin or an IFNAR-blocking antibody two hours before intravenous administration of 1×10^9 pfu of a recombinant vesicular stomatitis virus (rVSVΔm51). Plasma samples were collected 24-hours post-infection to quantify titers of circulating viruses using a standard assay to determine plaque-forming units (pfu). Data are shown as means with standard errors; $n = 4$ /group. Data are representative of three experimental replicates and were assessed using a one-way analysis of variance with Sidak's multiple comparisons test. Mice that had been infected without IFNAR-blockade had titers below the limit of detection (LOD) of the assay (*i.e.* approximately 10 pfu/mL).

steroid hormones [76–78] and immunity to viral infections can be impacted by variations in the levels of sex hormones. For example, female mice infected with influenza A virus mounted more robust inflammatory responses than their male counterparts, which made them more susceptible to infection-associated morbidities [36,38,79]. Furthermore, the presence of androgen or estrogen response elements

within the promoters of several genes implicated in innate immunity represent one of the potential mechanisms whereby hormones can introduce sex-related dimorphism in immune responses [80].

Another potential mechanism to introduce sex-related biases into immune responses is the differential encoding of immunoregulatory genes in sex chromosomes [37,63]. In contrast to a limited number of immunoregulatory genes encoded within the Y chromosome, the X chromosome encodes more than 1,100 genes, constituting 5% of the human genome, including some crucial regulatory genes that can introduce a sex bias in immune responses at both transcriptional and translational levels [37]. For example, Toll-like receptor (TLR) 7, is encoded on chromosome X and failure of second-X inactivation results in higher levels of expression of TLR7 in cells. This is associated with higher concentrations of cytokines and chemokines in females, than in males, in response to TLR7-mediated signaling [81]. TLR7-mediated type I IFN responses of plasmacytoid DCs of healthy females have also been shown to be influenced by the number of X chromosomes, as well as serum concentrations of sex hormones [55].

Physiological levels of male and female sex hormones have been shown to differentially affect inflammatory responses during viral infections. A hypothetical mechanism by which sex hormones potentially affect the outcomes of infections with influenza A virus and responses to influenza virus vaccines in both humans and mice was proposed by Klein *et al.* [38]. Specifically, infections in females, which cause a decrease in the levels of estradiol, are associated with upregulation of inflammatory cytokines and chemokines and excessive infiltration of leukocytes into inflamed sites, which could have severe pathological consequences in the lungs, and even potentiate death. Virally-suppressed testosterone in males, on the other hand, was not associated with potentiation of inflammatory responses inflammation-associated morbidities. Similar associations have been made for responses to influenza virus vaccines, with females experiencing more adverse events. Although the present study did not assess concentrations of sex hormones in rVSVΔm51-infected mice, it unveiled a novel association between sex and the severity of a viral infection. In the event of disrupted IFNAR signaling, male mice were as capable as control mice in down-regulating inflammatory cytokine responses at 24-hours post-infection, whereas females with impaired IFNAR signaling failed to control the cytokine responses (Figs. 3 and 4), which was associated with an acute decrease in body temperature as an adverse event (Fig. 2). Similar to hormone levels, this

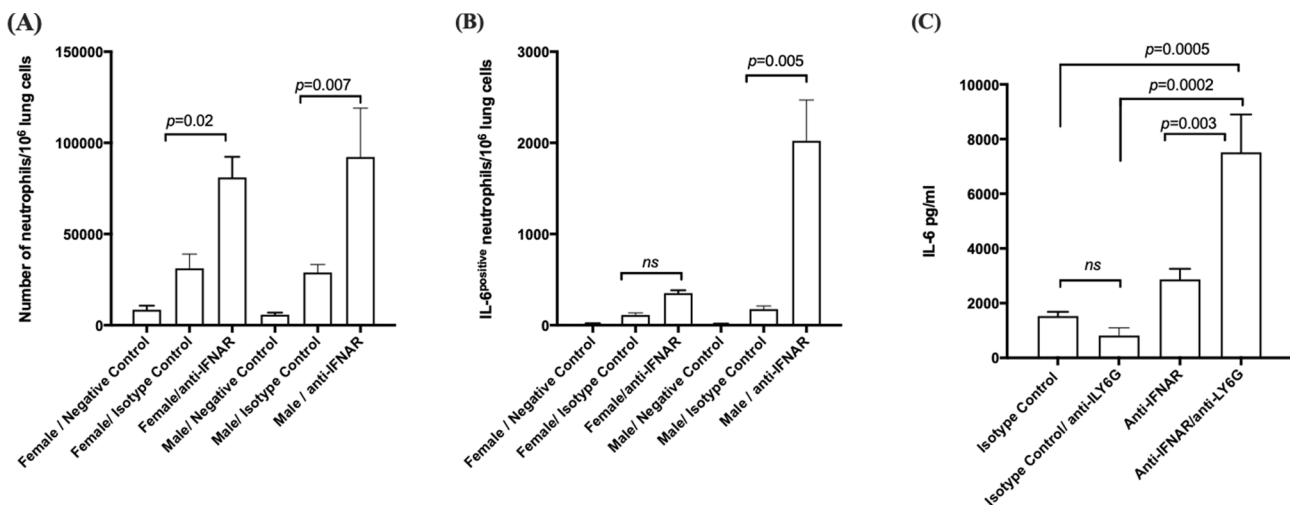


Fig. 6. Neutrophils accumulate in lungs and may play a regulatory role in cytokine responses to recombinant vesicular stomatitis virus (rVSVΔm51) during type I interferon receptor (IFNAR)-blockade. Male and female Balb/c mice received intravenous injections of 1 mg of an isotype control immunoglobulin or an IFNAR-blocking antibody two hours before intravenous administration of 1×10^9 pfu of rVSVΔm51. (A) Ten hours post-infection pulmonary neutrophils, defined as CD45⁺Ly6G^{high}CD11b^{high}, were quantified by flow cytometry. (B) Graphs are shown for the proportion of neutrophils that expressed interleukin (IL-6). (C) Plasma samples were collected at 10 h post-infection to determine the concentration of IL-6. All graphs show means and standard deviations ($n = 8$ /group). Data were assessed using one-way analysis of variance with Sidak's multiple comparisons test.

implicates a reduction in type I interferon signaling with a female bias towards increased inflammation and adverse events during a viral infection.

Both male and female mice failed to clear rVSV Δ m51 by 24-hours post-infection when there was a concomitant blockade of IFNAR signaling (Fig. 5). This agrees with other studies that have shown the indispensable role of IFNAR signaling in viral clearance [4,17,82]. Type I IFN responses have been reported to be particularly important in host protection against infections with VSV, as compared to infections with vaccinia virus or lymphocytic choriomeningitis virus that can be cleared by both type I and type II IFN responses [3,42,82]. Other viruses, like NDV, are also able to activate IFN-independent antiviral pathways in addition to IFN-mediated responses [41]. With that said, mice with IFNAR-blockade were able to eventually clear the virus, as evidenced by their long-term survival. The evidence at 24-hours post-infection showed that males with IFNAR blockade had greater viremia than females with IFNAR blockade (Fig. 5). This inversely correlated with cytokine responses (Figs. 3 and 4) the number of adverse events (Table 1 and Fig. 2). These results suggest that VSV-mediated viremia can be controlled by cytokine responses other than type I interferons, albeit less efficiently. Indeed, host antiviral defenses against VSV appear to be influenced by the type of cells that are infected. Studies of mouse embryonic fibroblasts and a human respiratory epithelial cell line indicated a role of type I IFN-independent mechanisms in providing protection against infection with VSV [41,43].

Neutrophils are an often-overlooked cell type in immunity against viral infection [83]. However, recent evidence indicates that these cells provide critical immune-regulation in diverse pathophysiological conditions, including viral infections and in the context of cancers [84]. We demonstrated increases in the frequencies of neutrophils in the blood and lungs following administration of rVSV Δ m51 in both males and females with concomitant antibody-mediated blockade of IFNARs. No significant sex differences were apparent (Figs. 1 and 6). This suggested that VSV-induced inflammatory responses that were being modulated by type I IFN signaling involved neutrophils, but the frequency of these cells could not explain the sex-mediated differences observed in the studies described here. Future studies are recommended to determine if there are functional differences between male and female neutrophils that could explain the sex-disparities.

Interleukin-6 stimulates the production of neutrophils by bone marrow progenitors and both human and mouse neutrophils have been shown to release IL-6 in the presence of IFN- γ [85]. Moreover, there is also evidence that type I IFNs potentiate the expression of IL-6 in human neutrophils [86]. We were not able to detect substantial amounts of IL-6 produced by neutrophils despite detecting high concentrations of IL-6 in the plasma of mice with or without IFNAR-blockade following treatment with rVSV Δ m51. Interestingly, *in vivo* depletion of neutrophils prior to administration of rVSV Δ m51 into mice with impaired IFNAR signaling led to elevation of the pro-inflammatory cytokine IL-6 compared to neutrophil-intact controls (Fig. 6C). These findings indicate that during the inflammation caused by rVSV Δ m51 and in a situation in which IFNAR signaling is impaired, neutrophils may actively participate in regulation of cytokine responses by inhibiting IL-6 produced by other effector cells. A growing body of evidence describes the existence of biochemically and physically distinct neutrophil subsets in healthy and pathological conditions [87]. Using flow cytometry, we have recently demonstrated that a subset of neutrophils produced IL-12 and gained surface receptors associated with antigen-presenting cell functions [54], while other groups characterized a subset of neutrophils that produced IL-10 and functioned as an immunomodulatory cell subset [88]. The current study suggests that a subset of neutrophils may play a regulatory role in cytokine responses to rVSV Δ m51 when IFNAR signaling is impaired. Further studies are needed to fully characterize neutrophil subsets, their tissue recruitment patterns, cytokine profiles and communication with other inflammatory cells, in infections and inflammatory disorders when IFNAR signaling is impaired.

5. Conclusions

This study modeled viremia using an attenuated virus to determine how blockade of type I IFN signaling impacted the immune response. This was based on the observation that pathogenic viruses that are associated with induction of pathogenic inflammatory responses often express proteins that can potentially interfere with type I IFN responses. The results presented here highlight a crucial role for IFNAR signaling in the negative regulation of antiviral cytokine responses. Indeed, blocking IFN responses during infection with rVSV Δ m51 resulted in a sexually dimorphic dysregulated cytokine response that delayed viral clearance. Importantly, viral clearance was less delayed in females with compromised IFN signaling, but this was at a cost of exaggerated pro-inflammatory cytokine responses that were associated with greater morbidity than their male counterparts. In contrast, male mice were able to down-modulate inflammatory responses to VSV during impaired IFNAR signaling. These findings have implications for infections with viruses that have mechanisms to potentially interfere with type I IFN signaling. Many viruses need to evade innate immune responses to survive in hosts, with type I IFN responses playing a dominant role in controlling their replication. The evidence presented here supports a direct link between IFNAR blockade and dysregulated cytokine responses. Although speculative, this could potentially be a mechanism of action contributing to cytokine storms that are induced by some pathogenic viruses. Of greater novelty, this study documented a scenario in which immune responses mounted by females caused greater morbidity, despite more efficiently clearing the virus that initiated the responses in the first place. Specifically, infection with a virus with concomitant blockade of type I IFN signaling could drive a female bias towards dysregulated cytokine responses. This concept warrants further investigation in the context of both infectious and non-infectious inflammation-mediated diseases that have a higher incidence in females.

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Author Contributions

All authors contributed to the conception and design of the study. MD and KEA contributed to the acquisition and analysis of data. All authors contributed to the interpretation of data. MD, KK and BWB contributed to drafting the article. All authors revised the article critically for important intellectual content. All authors provided final approval of the version to be submitted.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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