Cutting Edge

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Cutting Edge: NOX2 NADPH Oxidase Controls Infection by an Intracellular Bacterial Pathogen through Limiting the Type 1 IFN Response

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The NOX2 NADPH oxidase (NOX2) produces reactive oxygen species to kill phagosome-confined bacteria. However, we previously showed that *Listeria monocytogenes* is able to avoid the NOX2 activity in phagosomes and escape to the cytosol. Thus, despite the established role of NOX2 limiting *L. monocytogenes* infection in mice, the underlying mechanisms of this antibacterial activity remain unclear. In this article, we report that NOX2 controls systemic *L. monocytogenes* spread through modulation of the type I IFN response, which is known to be exploited by *L. monocytogenes* during infection. NOX2 deficiency results in increased expression of IFN-stimulated genes in response to type I IFN and leads to 1) promotion of cell-to-cell spread by *L. monocytogenes*, 2) defective leukocyte recruitment to infection foci, and 3) production of anti-inflammatory effectors IL-10 and thioredoxin 1. Our findings report a novel antimicrobial role for NOX2 through modulation of type I IFN responses to control bacterial dissemination. *The Journal of Immunology*, **2021, 206: 323–328.**

The NOX2 NADPH oxidase (NOX2) plays a key role in modulating immune responses to infection (1, 2). This multimeric enzyme is composed of both membrane-bound ($gp91^{phox}$/$p22^{phox}$) and cytosolic subunits ($p40^{phox}$/$p47^{phox}$/$p67^{phox}$/Rac) (3), which once assembled mediates the generation of reactive oxygen species (ROS) in the lumen of phagosomes to directly kill engulfed microorganisms (4). NOX2 also regulates other cellular processes relevant to host defense, including cytoskeleton dynamics, autophagy, and cytokine/chemokine signaling (5).

Loss of NOX2 activity in humans causes chronic granulomatous disease (CGD), characterized by severe susceptibility to common pathogens such as *Staphylococcus aureus* and *Aspergillus sp* (6). CGD is also characterized by an impaired control of inflammation. Indeed, the granulomas observed in CGD are typically sterile but fail to resolve (7). Partial loss of NOX2 function, involving reduced (but not ablated) ROS production or mistargeting of the NOX2 enzyme complex, has also been linked to chronic inflammatory diseases, including arthritis, lupus, and inflammatory bowel disease (8–10). However, the mechanisms through which NOX2 regulates both immunity and inflammatory responses remain unclear.

Recent studies have linked NOX2 to the regulation of type I IFN responses, where patients with CGD and NOX2-deficient mice exhibit an upregulated type I IFN signature (11). This regulation of type I IFN responses by NOX2 was examined in the context of viral infection (12). It was reported that RNA and DNA viruses promote NOX2-derived ROS production, leading to oxidation of a critical cysteine residue in TLR7, thereby abrogating antiviral signaling. Pharmacologic inhibition of NOX2 promoted type I IFN production in mice and suppressed viral infection. These studies identify NOX2 as a potential therapeutic target for treating viral infections. However, the impact of enhanced type I IFN responses on bacterial infections in situations of deficiency or inhibition of NOX2 remains unclear. This is especially relevant because some intracellular bacterial pathogens, such as *Listeria monocytogenes*,

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Abbreviations used in this article: BMDM, bone marrow–derived macrophage; CGD, chronic granulomatous disease; IFITM3, IFN-induced transmembrane protein 3; ISG, IFN-stimulated gene; *Isg15*, IFN-stimulated gene 15; NOX2, NOX2 NADPH oxidase; p.i., postinfection; ROS, reactive oxygen species; TRX1, thioredoxin 1; WT, wild-type.

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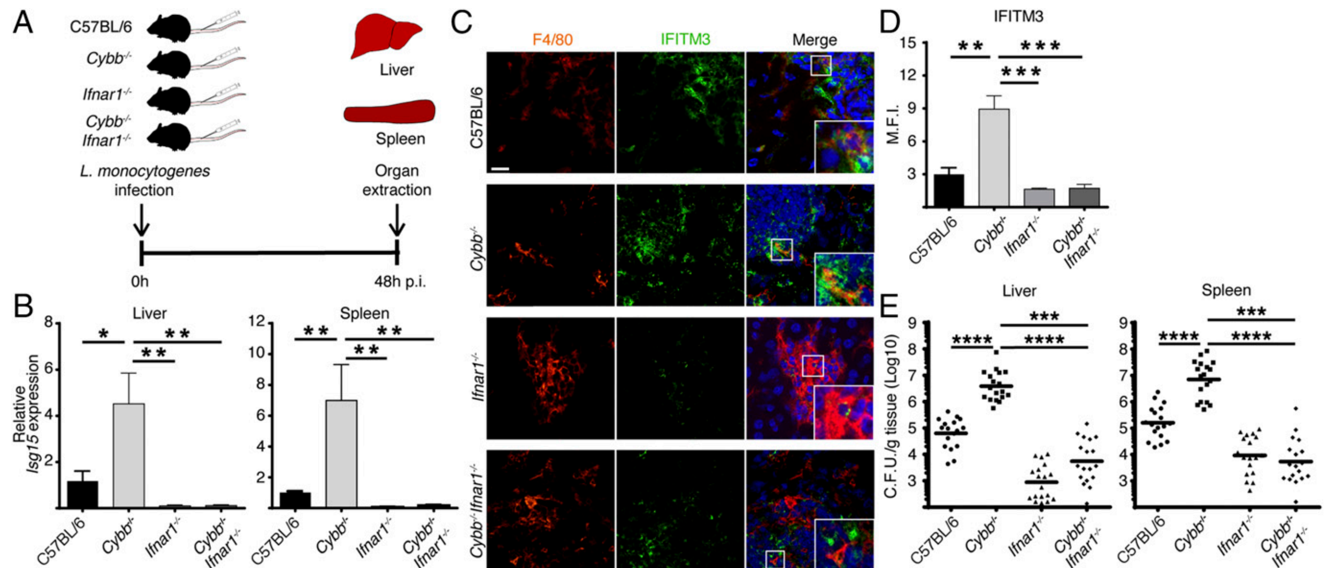

FIGURE 1. NOX2 deficiency leads to enhanced type I IFN responses during *L. monocytogenes* infection that promote bacterial growth. (**A**) Experimental design for *L. monocytogenes* infection. Livers and spleens were obtained 48 h p.i. and homogenized for mRNA extraction or assessing bacterial load. (**B**) The mRNA of *Isg15* in the liver and spleen are shown, data represent mean ± SD for three independent experiments with a total of six mice each per group for each experiment. (**C**) Liver sections were stained with anti-F4/80, anti-IFITM3, and DAPI. Representative confocal slices of 6 μm are shown. Scale bar, 70 μm. Insets are higher magnifications of the boxed areas. (**D**) Mean fluorescence intensity of IFITM3 was quantified across 10 total sections per animal shown in (**C**), which represent mean ± SD of three independent experiments with a total of six mice per group for each experiment. (**E**) Bacterial load (CFU per gram of tissue) in livers and spleens 48 h p.i. Data shown represent mean ± SD of three independent experiments with a total of six mice each. The p value was calculated using one-way ANOVA with Tukey post hoc test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

are known to exploit the type I IFN response as part of their pathogenic strategy (13).

L. monocytogenes is a facultative intracellular pathogen that can cause listeriosis, a severe systemic disease involving dissemination to many organs of its host (14). After uptake by host cells, these bacteria can escape from phagosomes using the pore-forming toxin listeriolysin O (LLO) and two phospholipase C enzymes (15). Upon entry to the cytosol, *L. monocytogenes* expresses actin assembly-inducing protein (ActA), a cell surface protein, to initiate actin-based motility for cell-to-cell spread (16). NOX2 was shown to control systemic *L. monocytogenes* infection in mice (17). However, we reported that LLO disrupts NOX2-mediated ROS production in phagosomes and that NOX2 does not affect intracellular growth of wild-type (WT) *L. monocytogenes*, suggesting that NOX2-derived ROS restricts *L. monocytogenes* infection by a mechanism other than direct killing in phagosomes (18).

The type I IFN response is exploited by *L. monocytogenes* (13, 19), affecting many aspects of the immune response against this intracellular bacterial pathogen (20). Previously, we showed that type I IFN promotes cell-to-cell spread of *L. monocytogenes* by enabling actin-based motility (21). In this article, we tested the hypothesis that NOX2 controls *L. monocytogenes* infection by limiting the type I IFN response.

Materials and Methods

Animals

Cybb$^{-/-}$, *Ifnar1*$^{-/-}$, and *Cybb*$^{-/-}$/*Ifnar1*$^{-/-}$ mice (on a C57BL/6 background) were previously characterized and bred in house at the Hospital for Sick Children Animal Care Facility. C57BL/6 mice, originally from The Jackson Laboratory, were also bred in house and used as controls. All experiments described in this study were carried out in accordance with the Guide for the Humane Use and Care of Laboratory Animals and were approved by the Hospital for Sick Children's Animal Care Committee.

Abs and reagents

Primary Abs are as follows: rabbit anti–*L. monocytogenes* (223021; BD), rat anti-F4/80 (ab16911; Abcam), rabbit anti-Thioredoxin1 (ab26320; Abcam), rabbit anti–p-STAT1 (9167; Cell Signaling), rabbit anti–IFN-induced transmembrane protein 3 (IFITM3) (PA5-11274; Thermo Fisher Scientific), rabbit anti-SOCS3 (2923; Cell Signaling), and anti–β-Actin (A5441; Sigma-Aldrich). Secondary Abs are as follows: goat anti-rabbit 488 (A11070; Molecular Probes), goat anti-rat 568 (ab175476; Abcam), and DAPI (no. D1306; Invitrogen).

Bacterial strains

L. monocytogenes 10403S (WT) and Δ*actA* (DP-L3078) were from D. Portnoy, University of California at Berkeley.

Bone marrow–derived macrophage generation

Bone marrow–derived macrophages (BMDM) were generated from dissected femurs and tibias as described (21) and used for experiments after 7–9 d.

Immunofluorescence

Immunostaining was conducted as described (21). Samples were imaged using a spinning disc confocal Leica DM16000B inverted microscope, Hamamatsu ORCA Flash 4 sCMOS camera, and Volocity 6.54 software, using a 10× objective. Images were imported to Image J software for analysis and assembled in Adobe Illustrator for labeling.

Infection focus assay

BMDM were seeded onto coverslips in 24-well tissue culture plates at 8×10^5 cells per well to generate a monolayer. After 18 h, the monolayer was infected with WT *L. monocytogenes* at a multiplicity of infection of 0.01 in RPMI 1640. After 1 h postinfection (p.i.), cells were washed three times with PBS, and RPMI 1640 containing 10% FBS and 50 μg/ml gentamicin (Wisent 311–420-CL) was added to the cultures. At 18 h p.i., cells were fixed with 2.5% PFA for 30 min at 37°C and prepared for fluorescence microscopy.

Mouse infections and tissue preparation

Mice were infected with 5×10^4 WT *L. monocytogenes* in 200 μl of PBS via i.v. injection in the lateral tail vein. Mice were euthanized by CO_2 inhalation 48 h p.i. Liver sections were embedded in OCT compound (Tissue-Tek; Sakura) and snap frozen at −80°C. Transverse sections of 5 μm were taken on a cryostat (CM1850; Leica) and mounted onto Superfrost Plus slides.

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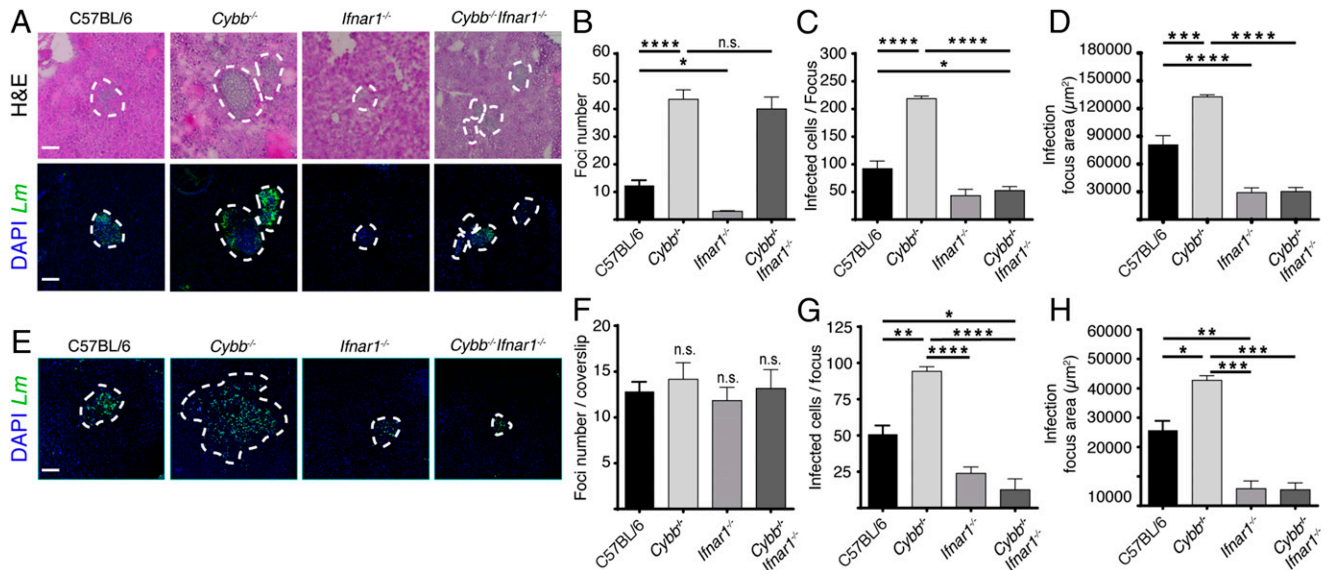

FIGURE 2. NOX2 deficiency promotes *L. monocytogenes* tissue dissemination and cell-to-cell spread in a type I IFN–dependent manner. Indicated mice strains were infected with 5×10^4 *L. monocytogenes* by i.v. tail injection. Livers were obtained 48 h p.i. and then embedded, mounted, and sectioned. (**A**) Consecutive liver sections were stained with H&E (upper panel) or DAPI and anti–*L. monocytogenes* Ab (bottom panels). Representative slices of 6 μm are shown. Dashed lines represent outer limits of infection foci. Scale bar, 70 μm. Ten total sections per organ were assessed to determine (**B**) foci number, (**C**) number of infected cells per focus, and (**D**) the infection focus area. Data panels represent mean ± SD for three independent experiments with a total of six mice per group for each experiment. The *p* values were calculated using a one-way ANOVA with Tukey post hoc test. $*p < 0.05$, $***p < 0.001$, $****p < 0.0001$. (**E–G**) BMDM were generated from the indicated mice strains, and an infection focus assay was performed. Confluent monolayers were infected with a multiplicity of infection of 0.01 of *L. monocytogenes* for 18 h and then fixed. Cells were stained with DAPI and anti–*L. monocytogenes* Ab and analyzed by fluorescence microscopy. (E) Representative images are shown, dashed lines indicate outer limits of infection foci. Scale bar, 70 μm. Volocity Software was used to quantify (F) the foci number, (G) the number of infected cells per focus, and (**H**) the infection focus area. Data represent mean ± SD for three independent experiments. The *p* values were calculated using one-way ANOVA with Tukey post hoc test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

Serial sections were collected and stained with H&E prior to immunofluorescence imaging to ensure images were taken at the foci center.

Intracellular growth of L. monocytogenes *in BMDM*

BMDM were plated at 5×10^5 cells per well in 24-well tissue culture plates, 24 h prior to infection. Intracellular growth of *L. monocytogenes* was conducted as described (21).

RNA isolation and quantitative PCR

RNA was isolated using the RNeasy kit (74104; QIAGEN), and cDNA was synthesized using iScript Reverse Transcription Supermix for reverse transcription. Ten nanograms of cDNA per reaction was used for quantitative PCR using SsoFast EvaGreen Supermix (1708840; Bio-Rad) with Hprt as housekeeping (22).

ELISA

IFN-β levels were measured in culture supernatants by ELISA (Ref 4200-1; PBL Assay Science). Bio-Tek System (Gen 5 2.0 Software) was used to read plates at 450 nm.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism v.6.0 g. The average ± SD is shown in figures, and *p* values were calculated as described in figure legends. A $p < 0.05$ was considered statistically significant.

Results and Discussion

NOX2 deficiency leads to enhanced type I IFN responses during L. monocytogenes *infection*

We examined *L. monocytogenes* infection of mice using a systemic model of disease (Fig. 1A). At 48 h p.i., livers and spleens were processed to assess markers of the type I IFN response. Using quantitative PCR, we observed that IFN-stimulated gene 15 (*Isg15*) mRNA was significantly upregulated in the liver and spleen of NOX2-deficient ($Cybb^{-/-}$) animals compared with WT controls (Fig. 1B). Accordingly, increased expression of IFITM3 was also observed in the livers of $Cybb^{-/-}$ mice (Fig. 1C, 1D). Differences in *Isg15* mRNA or IFITM3 protein were not observed in mice lacking the type I IFNR Ifnar1 ($Ifnar1^{-/-}$) or in a double-knockout mouse lacking both NOX2 and the type I IFN receptor ($Cybb^{-/-}/Ifnar1^{-/-}$). We conclude that NOX2 deficiency leads to enhanced type I IFN responses during *L. monocytogenes* infection.

NOX2 deficiency promotes systemic L. monocytogenes *infection in a type I IFN–dependent manner*

Next, we examined the impact of enhanced type I IFN responses on *L. monocytogenes* infection. After 48 h p.i., livers and spleens were harvested to assess bacterial load in these tissues (Fig. 1E). In NOX2-deficient mice, we observed an increased bacterial load, consistent with prior studies (17). In contrast, $Ifnar1^{-/-}$ mice displayed a reduced *L. monocytogenes* load, as expected (23). Remarkably, the double-knockout mice showed a significantly reduced bacterial load compared with NOX2-deficient mice, indicating a protective effect of *Ifnar1* deficiency to *L. monocytogenes* infection even in absence of the antimicrobial NOX2. These findings suggest that in vivo, NOX2 deficiency promotes systemic *L. monocytogenes* growth in a type I IFN-dependent manner.

NOX2 deficiency promotes L. monocytogenes *tissue dissemination in a type I IFN–dependent manner*

To examine bacterial growth during systemic infection, we processed tissues for H&E staining (Fig. 2A, upper panels) and immunofluorescence microscopy analysis (Fig. 2A, bottom panels). We found that $Cybb^{-/-}$ mice displayed more infection foci with respect to control livers (Fig. 2B). The number of infected cells per focus (Fig. 2C) and the size of

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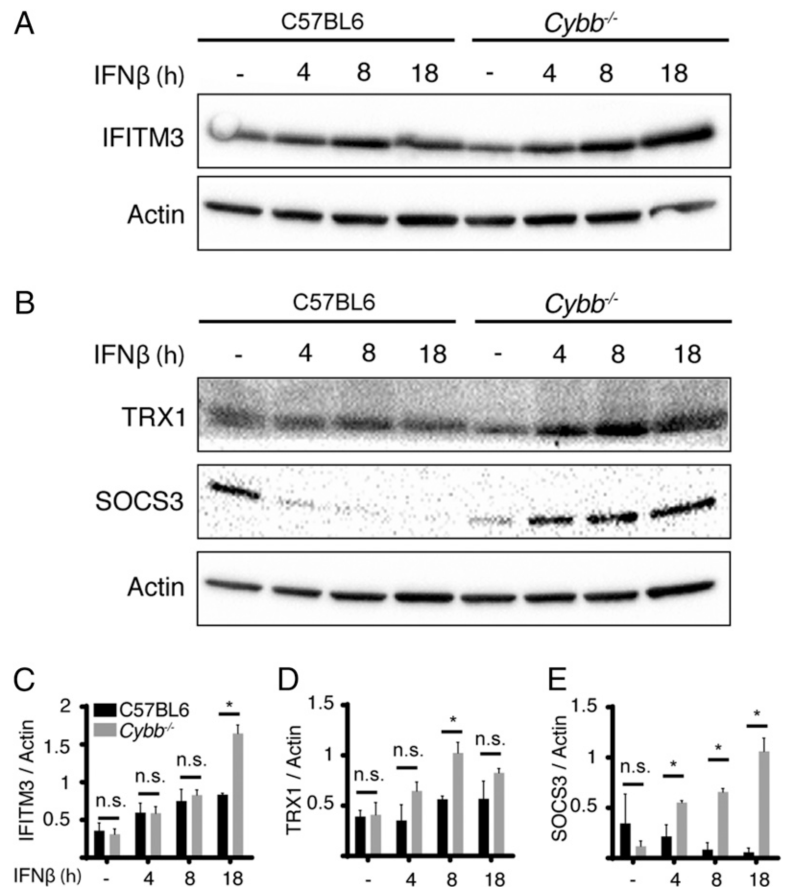

FIGURE 3. NOX2 deficiency leads to enhanced ISG expression in response to type I IFN. BMDM were generated from C57BL/6 and *Cybb*$^{-/-}$ mice, and lysates from unstimulated and stimulated with IFN-β (12,000 IU) were collected. Cell lysates were processed by Western blot to measure expression of ISG proteins IFITM3 (**A**), TRX1, and SOCS3 (**B**) using β-actin as a loading control. TRX1 and SOCS3 were blotted as strips from the same membrane and have the same actin control. Panels show a representative experiment. (**C**–**E**) Densitometric analysis was performed using ImageJ software. Panels represent mean ± SD for three independent experiments. The p values were calculated using a Student t test. Not significant (n.s.) $p > 0.05$, *$p < 0.05$.

each infection focus (Fig. 2D) in *Cybb*$^{-/-}$ mice was also increased, consistent with prior studies (17). In contrast, *Ifnar1*$^{-/-}$ mice showed a reduced infected cell number as well as a reduced infection foci size, as expected (21). The increased number of infection foci observed in *Cybb*$^{-/-}$ mice was also observed in double-knockout animals (Fig. 2A, 2B), suggesting that NOX2 deficiency has type I IFN–independent effects on the establishment of infection foci in tissues. The nature of the NOX2-dependent factors that limit establishment of infection foci in tissues during systemic disease will be an important subject for future studies. In contrast, double-knockout tissues displayed a reduction in the size of infection foci (Fig. 2C), and the number of infected cells per focus compared with *Cybb*$^{-/-}$ mice (Fig. 2D). These findings indicate that NOX2 deficiency impacts dissemination of *L. monocytogenes* within tissues in a type I IFN–dependent manner.

NOX2 deficiency promotes L. monocytogenes *cell-to-cell spread in macrophages in a type I IFN–dependent manner*

Direct cell-to-cell spread of *L. monocytogenes* in tissues is thought to be a major factor impacting the size of infection foci observed in tissues and overall bacterial growth (24). Indeed, *L. monocytogenes* mutants lacking *actA* are severely attenuated for virulence in mice (25). Previously, we showed that type I IFN promotes cell-to-cell spread by *L. monocytogenes* (21). Therefore, we examined cell-to-cell spread of *L. monocytogenes* in BMDM from our knockout mice. We performed an infection focus assay, whereby BMDM were infected with a low multiplicity of infection of *L. monocytogenes*, and bacterial spread from cell-to-cell was examined after 18 h (Fig. 2E). We observed that in *Cybb*$^{-/-}$ BMDM, *L. monocytogenes* spread was significantly increased, as measured by the number of infected cells per focus (Fig. 2F) and infection focus area (Fig. 2G). *L. monocytogenes* spread in *Ifnar1*$^{-/-}$ BMDM was significantly reduced, as expected (21). However, the double-knockout BMDM displayed a significantly reduced *L. monocytogenes* spread with respect to *Cybb*$^{-/-}$ BMDM (Fig. 2F, 2G). Importantly, intracellular growth of *L. monocytogenes* in BMDM was not affected by loss of *Cybb* and/or *Ifnar1* (Supplemental Fig. 1). It is noteworthy that we assessed intracellular growth of a Δ*actA* mutant of *L. monocytogenes* to avoid the confounding effect of cell-to-cell spread (Fig. 2E). We conclude that NOX2 deficiency promotes *L. monocytogenes* cell-to-cell spread in BMDM in a type I IFN–dependent manner.

NOX2 deficiency impairs leukocyte recruitment to L. monocytogenes *infection foci*

We considered other mechanisms by which NOX2 deficiency could impact *L. monocytogenes* infection. First, we looked at leukocyte migration to infection sites because NOX2 is required for expression of CCR1 and CCR2 (26). Using the marker F4/80, we observed a reduced number of recruited leukocytes to infection foci in *Cybb*$^{-/-}$ mice (Supplemental Fig. 2A, 2B) (27). In contrast, leukocyte recruitment was enhanced in *Ifnar1*$^{-/-}$ mice, as expected (28). Leukocyte recruitment was restored to levels comparable to WT in the double-knockout animals but notably did not recapitulate the phenotype of *Ifnar1*$^{-/-}$ mice. We conclude that loss of type I IFN signaling can overcome the leukocyte recruitment defect seen in NOX2-deficient mice.

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NOX2 deficiency upregulates anti-inflammatory effectors in a type I IFN-dependent manner

We also examined the impact of NOX2 deficiency on anti-inflammatory mediator production during *L. monocytogenes* infection. First, we examined IL-10, which is known to modulate *L. monocytogenes* infection (29). It is noteworthy that IL-10 is upregulated in CGD (30) and autoimmune diseases, such as systemic lupus erythematous (31). We observed significantly increased *IL-10* mRNA in the livers of $Cybb^{-/-}$ mice (Supplemental Fig. 3A). IL-10 has been implicated in the anti-inflammatory response mediated by type I IFN (32). Consistent with this, we observed decreased *IL-10* mRNA in $Ifnar1^{-/-}$ mice. However, the double-knockout mice showed significantly reduced *IL-10* mRNA with respect to $Cybb^{-/-}$ mice. We also examined thioredoxin 1 (TRX1), an oxidative stress-limiting protein associated with the anti-inflammatory signaling linked to IL-10 (33). Similar expression profiles were observed for both mRNA expression (Supplemental Fig. 3B) and protein expression of TRX1 (Supplemental Fig. 3C, 3D). Thus, NOX2 deficiency promotes the production of anti-inflammatory effectors in a type I IFN–dependent manner.

NOX2 deficiency leads to enhanced IFN-stimulated gene expression in response to type I IFN

We examined the impact of NOX2 on type I IFN responses in vitro using BMDM. Secretion of IFN-β in response to *L. monocytogenes* infection was not affected in cells from $Cybb^{-/-}$ mice compared with WT control cells (Supplemental Fig. 4A). Similarly, the response to type I IFN treatment (in the absence of infection) was unaffected, as measured by phosphorylation of STAT1 (Supplemental Fig. 4B). However, we observed enhanced expression of several IFN-stimulated genes (ISGs) (TRX1, IFITM3, and SOCS3) in NOX2-deficient BMDM (Fig. 3). Thus, our findings indicate that NOX2 acts downstream of STAT1 to limit ISG expression in response to type I IFN.

NOX2 plays an important role in immunity by delivering ROS to phagosome-confined bacteria (1). However, the immunomodulatory role of NOX2 in mediating resistance to intracellular bacterial pathogens is unclear. In this article, we show that the previously observed increase in susceptibility of NOX2-deficient ($Cybb^{-/-}$) mice to *L. monocytogenes* infection (17) is due to an augmented type I IFN response in these animals. Dissemination of bacteria within the tissues of $Cybb^{-/-}$ animals is enhanced, with both increased numbers and size of infection foci. Although intracellular growth of *L. monocytogenes* in the cytosol of macrophages is known to be unaffected by NOX2 deficiency (18), we find that cell-to-cell spread by *L. monocytogenes* is enhanced in NOX2-deficient macrophages in a type I IFN–dependent manner. In summary, our findings reveal a novel antimicrobial role for NOX2 through immunomodulation that suppresses bacterial dissemination.

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Disclosures

The authors have no financial conflicts of interest.

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