Innate Immunity to Intraphagosomal Pathogens Is Mediated by Interferon Regulatory Factor 8 (IRF-8) That Stimulates the Expression of Macrophage-specific Nramp1 through Antagonizing Repression by c-Myc*

Michal Alter-Koltunoff‡, Sigal Goren§, Janna Nousbeck†, Carl G. Feng‡, Alan Sher‡, Keiko Ozato§, Aviva Azrieli‡, and Ben-Zion Levi‡‡

From the ‡Department of Biotechnology and Food Engineering, Technion-Israel Institute of Technology, Haifa 32000, Israel and the §Laboratory of Molecular Growth Regulation, NICHD, and the §§Immunobiology Section, NIAID, National Institutes of Health, Bethesda, Maryland 20892-8003

Macrophages are a central arm of innate immune defense against intracellular pathogens. They internalize microbes into phagosomes where the invaders are being killed by oxygen and nitrogen reactive species. Despite this battery of antimicrobial molecules, some are able to thrive within the phagosome thus termed intraphagosomal pathogens among which are Salmonella, Leishmania, and Mycobacteria. In mice, a single dominant gene termed Nramp1/Slc11a1 controls innate resistance to such pathogens. This gene is expressed exclusively in myeloid cells. Previously, we have shown that the restricted expression of Nramp1 is regulated by a myeloid cell-specific transcription factor termed IRF-8/ICSBP. It is demonstrated here that the induction of Nramp1 expression in activated macrophages is accompanied by a promoter shift from a repression state elicited by c-Myc to an activation state elicited by the induction of IRF-8 in activated macrophages. This transition from repression to activation is facilitated by a competitive protein-protein interaction with the transcription factor Miz-1. To show that IRF-8 is directly involved in the elimination of intraphagosomal pathogens through the regulation of Nramp1 gene expression, we bred wild type as well as IRF-8 and Nramp1 null mouse strains and examined macrophages derived from bone marrow and peritoneum. Our results clearly show that the absence of IRF-8 and Nramp1 leads to the same phenotype; defective killing of intraphagosomal pathogens. Importantly, Nramp1 is exclusively expressed in monocyte/macrophage cells (5), whereas its family member Nramp2 is ubiquitously expressed. The transcription factor termed Myc interacting zinc finger protein 1 (Miz-1) mediates the activation of Nramp1 that can be repressed by c-Myc (25). However, this interaction between Miz-1 and c-Myc does not explain the components of innate immunity and play a key role in host defense mechanisms against invading pathogens. These invading foreign bodies activate the macrophages, which in response engulf and subsequently entrap the pathogens in the phagosome/lysosome compartment. In this specialized compartment, the pathogens are subjected to massive attack by reactive oxygen and nitrogen intermediates (1). In addition, macrophages mediate innate resistance to host infection by several antigenically unrelated intraphagosomal pathogens including Salmonella, Leishmania, and Mycobacterium. This is controlled by a single dominant gene termed solute carrier family 11 member 1 (Slc11a1)² also known as natural resistance associated macrophage protein 1 (Nramp1), or Ity/Lsh/Bcg. Nramp1 is a proton/divalent cation antiporter with a unique role in innate resistance to intraphagosomal pathogens and autoimmune disease (for review, see Refs. 2–4). The exact function of Nramp1 as iron and divalent cation transporter is still controversial. It either functions to increase transphagosomal Fe²⁺ catalyzing the Haber-Weiss/Fenton reaction to generate the highly toxic hydroxyl radical essential for macrophage bactericidal activity. On the other hand, it is thought to deprive the intraphagosomal bacterium of Fe²⁺ and other divalent cations, which are critical for its survival in the phagosome (2). In mouse, Nramp1 has two alleles; one allele, Gly-169, restricts pathogen growth, whereas the second allele, Asp-169 (functionally null), is permissive for pathogen growth (5). Thus, a single gene product, Nramp1, is indispensable for innate immunity to intraphagosomal pathogens. Importantly, Nramp1 is exclusively expressed in monocyte/macrophage cells (5), whereas its family member Nramp2 is ubiquitously expressed. The transcription factor termed Myc interacting zinc finger protein 1 (Miz-1) mediates the activation of Nramp1 that can be repressed by c-Myc (25). However, this interaction between Miz-1 and c-Myc does not explain the
macrophage-restricted expression of Nramp1 and its inducibility by interferon-γ (IFN-γ) and LPS in comparison to its ubiquitously expressed homologue Nramp2.

Recently, we have demonstrated that Miz-1 interacts with another transcription factor termed interferon regulatory factor-8 (IRF-8) previously known as ICSBP. Furthermore, we have shown that these two factors in conjunction with a third partner, PU.1, synergistically activate Nramp1 (6). IRF-8 is expressed mainly in immune cells and its expression can be further induced by IFN-γ and Toll receptor ligands such as LPS or CpG (7). This factor is a key element for the differentiation of myeloid progenitor cells toward macrophages and for mature macrophage activity. Accordingly, IRF-8-/- mice exhibit clinical manifestation that resembles the human chronic myelogenous leukemia. Thus, IRF-8 drives bipotential myeloid progenitor cells toward mature macrophages while inhibiting the differentiation pathway toward granulocytes (8). In addition, it was shown that IRF-8 is an essential factor for proper function of mature macrophages. For example, IRF-8 null mice fail to mount Th1-mediated immune response due to lack of expression of IL-12 p40 subunit (9). IRF-8 belongs to a family of 9 cellular members sharing significant similarity at the DNA binding domain. Unlike other IRF members, IRF-8 is capable of binding to target DNA sequence only following association with other IRF or non-IRF transcription factors via the IFN association domain (10). IRF-8 associates with transcription factors like IRF-1 and IRF-2 as well as non-IRF members such as PU.1. The latter belongs to the Ets family of transcription factors and is indispensable for the development of lymphoid and myeloid cells (11). The interacting partner with IRF-8 dictates not only the DNA binding site but also the transcriptional activity, e.g. activation or repression. Through the formation of such heterocomplexes IRF-8 has an important role in the expression of macrophase genes such as the phagocosomal components, phagocyte oxidase complex (phox) (12), and iNOS (13), or the proinflammatory cytokines IL-12 (9), IL-23 (14), IL-18 (15), and IL-1β (16).

Previously, we have shown that IRF-8 can interact with Miz-1 only in immune cells (6). This interaction leads to synergistic activation of the Nramp1 promoter that is not observed in the non-immune cell line. This synergistic interaction is further enhanced with PU.1. These results clarified the molecular basis for the macrophage-restricted expression of Nramp1 and its inducibility by IFN-γ and LPS. In this article, we wished to further explore the Nramp1 promoter with respect to the identification of the exact binding sites for IRF-8 and PU.1. In addition, the competitive interplay between c-Myc and IRF-8 for the association with Miz-1 and the effect on the transcriptional activity of the Nramp1 promoter was investigated. Finally, a direct regulatory cascade linking IRF-8 and Nramp1 sequential expression to macrophages susceptibility/resistance to intraphagosomal pathogens such as Salmonella enterica serovar typhimurium and Mycobacterium bovis (bacille Calmette-Guerin (BCG)) is described.

**EXPERIMENTAL PROCEDURES**

**Animals**—The mouse strains, C57BL/6, 129/Sv (both were from Harlan Biotech, Israel), and C57BL/6 IRF-8-/- (17) were maintained in individually ventilated cages under pathogen-free conditions. The C57BL/6 IRF-8-/- mice were backcrossed with 129/Sv for 8 generations to replace the defective Nramp1 allele generating a new knock-out mouse strain 129/Sv IRF-8-/- . The presence of defective IRF-8 and functional Nramp1 were validated by PCR of genomic DNA from mouse tails. All animal work conformed to the guidelines of the animal care and use committee of the Technion.

**Cell Lines**—Murine bone marrow-derived RAW264.7 macrophage cell line was obtained from ATCC (Manassas, VA). CL2 macrophage cells, established from IRF-8 knock-out mice, were previously reported (9), R37 and R21 cells (constitutively expressing functional Nramp1 or antisense to Nramp1, respectively) were a kind gift from Dr. Barton (Southampton University, United Kingdom) (18). Cells were maintained in RPMI 1640, supplemented with 40 μM β-mercaptoethanol, 10% fetal calf serum, and antibiotics. 5 ng/ml M-CSF and CSF (R&D Systems, Minneapolis, MN) were added to the growth medium of CL2 cells and 50 μg/ml of gentamycin was added to the growth medium of R37 and R21 cells. Treatment with IFN-γ and LPS and killing assays were performed as described for peritoneal macrophages. NIH3T3 cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat inactivated fetal calf serum and antibiotics.

**Isolation of Bone Marrow and Peritoneal Macrophages**—Bone marrow cells were isolated from femurs of the various mouse strains and cultured for 7 days in Dulbecco’s modified Eagle’s medium supplemented with 30% CCL1 cell culture supernatant, 20% heat inactivated fetal calf serum, and antibiotics in nontissue culture plates. Peritoneal macrophages were harvested as described previously (6). 2.5 × 10⁷ cells were plated in tissue culture Petri dishes (100 mm) and kept at 37 °C and 6% CO₂ for 4 h and then non-adherent cells were removed by 2 washes with PBS.

**Intracellular Pathogens**—S. enterica serovar typhimurium 14028 (ATCC) stably expressing green fluorescent protein (GFP) was kindly obtained from Dr. Yaron (Technion, Israel). Following 18 h growth (LB medium without antibiotics) the bacterial culture was diluted 1:10 and A₆₀₀ was measured. Salmonella concentration was calculated as colony forming unit (CFU) per ml using 1 OD = 1.2 × 10⁹ CFU/ml. The cells were washed once with PBS and resuspended in mammalian culture medium. Macrophages were infected at multiplicity of infection (m.o.i.) of 15–30.

The Leishmania donovani stably expressing eGFP was a kind gift of Dr. Zilberstein (Technion, Israel). Following 18 h growth (LB medium without antibiotics) the bacterial culture was diluted 1:10 and A₆₀₀ was measured. Salmonella concentration was calculated as colony forming unit (CFU) per ml using 1 OD = 1.2 × 10⁹ CFU/ml. The cells were washed once with PBS, counted after fixation with paraformaldehyde, and adjusted to the required parasite m.o.i. of 10.

**Macrophage Killing Assay**—Killing assay with S. enterica serovar typhimurium was performed as described by Monak et al. (20) with minor modifications. Macrophages (either bone marrow derived or peritoneal) were washed twice with ice-cold PBS and the cells were gently scraped and plated at 3 × 10⁵ cells/well in 24-well plates in a medium without antibiotics.
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The cells were either not treated or treated for 18 h with 100 units/ml IFN-γ (CytoLab, Rehovot, Israel) and 50 ng/ml LPS (Sigma). Cells were infected in triplicates with S. enterica serovar typhimurium 14028 expressing at m.o.i. of 15–30. The 24-well plates were centrifuged at 250 × g for 5 min and placed in 5% CO₂ incubator at 37 °C for 40 min. This point in the experiment was determined as the zero point. At this stage gentamycin (50 μg/ml) was added to kill non-phagocytized bacteria and to assess intracellular growth. At the indicated time intervals post-infection, cells were washed 3 times with PBS, lysed by 200 μl of 1% deoxycholate, serially diluted, plated onto LB agar plates, and CFU (viable bacteria) were determined.

For killing assay with M. bovis (BCG), bone marrow-derived macrophages from various mouse strains were plated and activated with IFN-γ and LPS as described above. Macrophages were then infected with live M. bovis (BCG) at m.o.i. of 10 for 4 h, the adherent cells were washed twice, and fresh growth medium was added. Cells were harvested and lysed in 2 ml of sterile water, and 100-μl aliquots were used for preparing serial dilution of the bacteria. The diluted lysates were placed on oleic acid-albumin-dextrose-catalase-supplemented Middlebrook 7H11 Bacto-agar (Difco), and colonies were counted visually after 14 days.

Plasmids—Mammalian expression vectors encoding for Miz-1, IRF-8, IRF-1, and PU.1 were all described previously (6). The expression plasmid for c-Myc in pCDNA3.1 was a gift of Dr. Eilers (Marburg University, Germany). The reporter construct −1555 containing murine Nramp1 promoter in the reporter vector pGL3 was a gift from Dr. Barton (Southampton University). The various 5’ progressive Nramp1 promoter deletion constructs were generated by PCR and following sequence validation were cloned into pGL3 basic (Promega). Primer sequence is available upon request. Site-directed mutagenesis to the IFN-stimulated response element (ISRE, the binding site for IRF-8) and the PU.1 binding sites within the mouse Nramp1 promoter was performed using the QuikChange site-directed mutagenesis kit (Stratagene). Primer sequence is available upon request.

Cells Transfections and Reporter Gene Assays—Assays were performed exactly as described before (6) using the Dual Luciferase assay kit (Promega). Luciferase activities were determined and normalized for transfection efficiency. Fold of synergism was calculated as the ratio between the -fold of activation elicited by the two transfected factors divided by the sum of the -fold of activation for each factor alone. Each experiment was repeated at least 3 times.

Bifluorescence Complementation (BiFC)—The plasmid IRF-8-YCC was previously described (23). The plasmid Miz-1-YNC was generated by PCR amplification of the coding region of Miz-1 with primers adding EcoRI and HindIII at the 5’ and 3’ ends, respectively, and cloned into the corresponding restriction sites in the plasmid YNC (23). 3.5 × 10⁴ NIH3T3 cells/chamber were seeded in sterile 4-well slide chamber. 18 h later, cells were transfected by Lipofectamine (Invitrogen) with 0.25 μg of the YFP constructs and 0.5 μg of pHcRed1-C1 (Clontech) for 24 h. The later served as a control reporter plasmid to confirm that BiFC took place within a nucleus of a transfected cell. In addition, 0.75 μg of plasmid encoding for c-Myc was cotransfected as indicated. The cells were allowed to stand at 30 °C for 1 h, and YFP and red fluorescent protein signals were viewed under confocal microscopy.

Chromatin Immunoprecipitation (ChIP)—RAW264.7 cells were grown to a final concentration of 1 × 10⁶ with or without treatment with IFN-γ (100u/ml) for 12 h and ChIP assays were performed as described previously (24). Protein-DNA complexes were incubated for 6 h with 5 μg of anti-goat IgG or anti-goat IRF-8 antibodies (Santa Cruz Biotechnology), or acetyl-specific histone 3 antibody (Upstate Biotechnology). Immunoprecipitated DNA was analyzed for the presence of the murine Nramp1 promoter sequence by semiquantitative or quantitative PCR using the following primers: 5’-GGAGAGG-AACGAAGGTCAAAAC-3’ (sense), 5’-CTGGCAACTAGC-TCTCTCAAT-3′ (antisense); and 5’-GGGTATCCCAGGAGGA-CAGA-3′ (sense), 5’-CACACACTTGGCGGTTCGAA-3’ (antisense), respectively.

Retroviral Transduction—pMSCV-IREs-EGFP is a retroviral expression vector that has a bicistronic cassette that enables expression of IRES-driven EGFP, with no encoded gene in the first position. It was generated by digesting the bicistronic cassette from pIRE2-EGFP (Clontech) with Xhol and MfeI and cloned into pMSCV digested with Xhol and EcoRI. To generate the retroviral pMSCV-IRF8-IREs-EGFP expression vector, the cDNA for human IRF-8 was digested with Pmel and MfeI and cloned into the Hpal and EcoRI in the retroviral vector pMSCV-IREs-EGFP. Retroviruses were generated as described (25) by seeding 4 × 10⁶ 293FT cells in a 10-cm dish. The next day, cells were transfected using DNA-calcium phosphate precipitation containing 2.5 μg of plasmid pMD.G encoding vesicular stomatitis virus G protein, 7.5 μg of plasmid pMD.OGP encoding gag-pol, and 10 μg of the retroviral expression constructs. 48 h after transfection, the viral supernatant was collected, centrifuged at 800 × g, and used to infect bone marrow macrophages that were seeded at 7.5 × 10⁶ cells/well in a 12-well plate in a final volume of 1.5 ml. 24 h later, 0.5 ml of medium was remove and replaced with 0.5 ml of freshly prepared viruses and Polybrene was added to a final concentration of 8 μg/ml. The plates were spinoculated at 1200 × g for 90 min at 33 °C. After 24 h, 1 ml of the medium was replaced with fresh medium and 1 day later transfection efficiency was monitored under a fluorescent microscope and the transduced cells were selected with puromycin (3 μg/ml) for an additional 7 days and then used for Salmonella killing assay.

Statistical Analysis—Each experiment was repeated three times yielding similar results and statistical significance was determined by Student’s t test with a p < 0.05.

RESULTS

Deletion Analysis of the Nramp1 Promoter Revealed the Binding Sites for IRF-8 and PU.1—Our previous studies provided the molecular basis for the restricted expression of Nramp1 in activated macrophages. This is due to synergistic interaction between Miz-1, IRF-8, and PU.1. However, the exact binding sites for the myeloid-specific factors were not determined. Computer aided analysis identified several putative binding sites for IRF-8 (termed ISRE) and PU.1 along the −1555 bp of
the *Nramp1* promoter region. To test which of the putative binding sites serve as ISRE and PU.1 binding sites, reporter gene constructs harboring progressive 5′ promoter deletions were generated (see illustrations in Fig. 1A). RAW264.7 cells were cotransfected with these reporter constructs together with Miz-1, IRF-8, and PU.1 as indicated. 24 h later cells were harvested, and relative luciferase activities were determined and the -fold of synergism was calculated as described under “Experimental Procedures.” C, site-directed mutagenesis was performed to the ISRE (adjacent to position −96) and the PU.1 binding site (near position −139) on the −139 *Nramp1* reporter construct generating mutated reporter constructs termed −139ISREMut and −139PU.1mut, respectively (see Fig. 3A for detailed illustration). RAW264.7 cells were cotransfected with Miz-1, IRF-8, and PU.1 as described under panel A and indicated in the figure. Relative luciferase activities were determined and the -fold of synergism was calculated as described under “Experimental Procedures.” D, semiquantitative ChIP was performed in RAW264.7 cells before and following 12 h of activation with IFN-γ and LPS using the indicated antibodies. E, quantitative ChIP to determine IFR-8 binding activity to *Nramp1* promoter was performed as described under “Experimental Procedures.”

**Figure 1. Identification of IRF-8 and PU.1 binding sites on *Nramp1* promoter.** A, schematic illustration of the progressive 5′ *Nramp1* promoter deletions that were fused to the promoterless luciferase reporter construct pGL3-basic (Promega). Numbers indicate distance in bp from the transcription start site. The putative binding sites for PU.1, IRF-8 (ISRE), and Miz-1 (INR) are indicated. B, RAW264.7 cells were transfected with the different *Nramp1* promoter deletions constructs together with Miz-1, IRF-8, and PU.1 as indicated. 24 h later cells were harvested, and relative luciferase activities were determined and the -fold of synergism was calculated as described under “Experimental Procedures.” C, site-directed mutagenesis was performed to the ISRE (adjacent to position −96) and the PU.1 binding site (near position −139) on the −139 *Nramp1* reporter construct generating mutated reporter constructs termed −139ISREMut and −139PU.1mut, respectively (see Fig. 3A for detailed illustration). RAW264.7 cells were cotransfected with Miz-1, IRF-8, and PU.1 as described under panel A and indicated in the figure. Relative luciferase activities were determined and the -fold of synergism was calculated as described under “Experimental Procedures.” D, semiquantitative ChIP was performed in RAW264.7 cells before and following 12 h of activation with IFN-γ and LPS using the indicated antibodies. E, quantitative ChIP to determine IFR-8 binding activity to *Nramp1* promoter was performed as described under “Experimental Procedures.”

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moter. The binding of IRF-8 to Nramp1 promoter was evident by semi-quantitative PCR only under IFN-γ-induced conditions (Fig. 1D). The relative binding of IRF-8 to the Nramp1 promoter in the induced cells was increased by almost 5-fold (Fig. 1E). The specificity of the ChiP assay was evaluated using goat immunoglobulins (IgG, negative control) and acetyl-specific histone 3 antibody (positive control, data not shown). These results support the in vitro findings and provide in vivo evidence for the recruitment of IRF-8 to the Nramp1 promoter in the IFN-γ-activated macrophage cell line.

**c-Myc Negates IRF-8 and Miz-1 Transcriptional Synergy on Nramp1 Promoter**—As shown above, Nramp1 is induced in activated macrophages through synergistic association between IRF-8 and Miz-1. On the other hand, other reports demonstrated that c-Myc represses Nramp1 transcription by binding to Miz-1 and displacing the p300 co-activator, a paradigm for the repressive action of c-Myc (26–28). This suggested that Miz-1 interaction with either c-Myc or IRF-8 might affect Nramp1 enhancosome activity leading to repression or activation, respectively.

To test the effect of c-Myc on the synergistic activation of the Nramp1 promoter by IRF-8 and Miz-1, reporter gene assays were performed. The Nramp1 reporter construct (−1555) was cotransfected with expression vectors for IRF-8, Miz-1, and c-Myc. As previously shown (6), transfection of Miz-1 alone was sufficient to significantly activate the promoter (Fig. 2A, lanes 2), whereas IRF-8 and c-Myc alone exhibited no effect (Fig. 2A, lanes 3 and 4, respectively). As expected, cotransfection of Miz-1 and IRF-8 resulted in a significant activation of the promoter (−12-fold, Fig. 2A, lane 5). Interestingly, cotransfection of increasing amounts of c-Myc reporter construct inhibited Miz-1 and IRF-8 synergistic activation reaching the reporter level similar to that of Miz-1 and c-Myc alone (Fig. 2A, compare lanes 6–10 with lane 11, respectively). In analogy, the repression effect of c-Myc on Miz-1 (Fig. 2, lanes 11 and 2, respectively) could be partially alleviated by cotransfection of increasing amounts of IRF-8 expression vector (Fig. 2A, lanes 12–14). These results suggest that c-Myc and IRF-8 compete over the interaction with Miz-1. Whereas the first exert repression the latter negates this activity. Furthermore, it suggests that the protein-protein interaction between Miz-1 and c-Myc is stronger than that of Miz-1 and IRF-8 because IRF-8 could not completely reverse the repression effect of c-Myc.

BiFC assay was employed to visualize Miz-1, IRF-8, and c-Myc interactions in living cells (29). Expression vectors for IRF-8 fused to the C-terminal region of the YFP (IRF-8-YCC) and for Miz-1 fused to the N-terminal region of the YFP (Miz-1-YNC) were constructed. These plasmids were transiently transfected into NIH3T3 cells either together or alone. As seen in Fig. 2B, expression of both IRF-8-YCC and Miz-1-YNC resulted in nuclear fluorescence of the transfected cells (10% efficiency) demonstrating their interaction in living NIH3T3 cells. This supports our previous demonstrations of such interaction using mammalian two-hybrid and co-immunoprecipitation assays (6). Cotransfection of the expression vector encoding for c-Myc resulted in a sharp decrease of fluorescent nuclei, indicating that c-Myc interfered with IRF-8 and Miz-1 interaction (Fig. 2B). Transfection of expression vectors for IRF-8-YCC or Miz-1-YNC alone did not lead to any fluorescence (data not shown). Transfection efficiency was monitored by cotransfection of a red fluorescent protein expression vector (for details see “Experimental Procedures”). Together, these results suggest that interplay between c-Myc and IRF-8 expression following IFN-γ and LPS stimulation modulate the transcriptional activity of Nramp1 in macrophages.

**IFR-8 and PL1 Are Important Regulators of Nramp1 Activation during Infection with Intraphagosomal Pathogens**—We next wanted to study the significance of IRF-8 and PL1 on Nramp1 expression during infection with intraphagosomal pathogens. For that purpose, RAW264.7 cells were transfected with the Nramp1 reporter construct (−139) or with the same construct harboring point mutations in the ISRE or PL1 binding sites (see schematic illustration in Fig. 3A). Twenty h following transfection, cells were either not infected or infected with *S. enterica* serovar *typhimurium* (expressing GFP) at m.o.i. of 15 for 5 h. Infection efficiency was evaluated using fluorescent microscope, whereas the activation of the different Nramp1 promoters was measured by luciferase assay. As shown in Fig. 3B, the wild-type (WT) Nramp1 promoter is activated during *S. enterica* serovar *typhimurium* infection (nor-
null
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A

![Graph showing the decrease in CFU over time for WT and KO mice.](image)

and following activation with IFN-γ and LPS were infected with *S. enterica* serovar *typhimurium* and killing assay was performed as described under “Experimental Procedures.” Intracellular *S. enterica* serovar *typhimurium* viable counts were decreased by more than 10-fold 2 h postinfection and remained at this low level until the experiment was terminated (Fig. 5A, **solid line**). On the other hand, only a ~0.5 log decrease in the intracellular *S. enterica* serovar *typhimurium* viable counts was observed for peritoneal macrophages derived from *IRF-8*−/− mice (Fig. 5A, **dashed line**).

To evaluate the efficiency of phagocytosis and the functioning of the developing phagosome, the cells were infected with *S. typhimurium* expressing GFP and following 45 min of invasion the cells were stained with LysoTracker Red (Cambrex), which stains acidic compartments in living cells, and 4′,6-diamidino-2-phenylindole, which stains the nuclei. The cells were fixed and observed under fluorescent microscope. It is clear that both types of peritoneal macrophages equally phagocytized GFP expressing *S. enterica* serovar *typhimurium*, however, the Lyso-Tracker red staining of phagosomes of *IRF-8*−/− macrophages was significantly weaker, indicating that the phagosome is less acidified (Fig. 5B). This may be due to lack of *Nramp1* expression leading to a significant reduction in the acidity of the phagosome as reported previously (31–33). In these experiments the *IRF-8*−/− mouse strain (129/Sv *IRF-8*−/−) was wild type for *Nramp1* as will be elaborated hereafter.

Loss of Either *IRF-8* or *Nramp1* Results in an Aberrant Killing Response of Macrophage against *S. enterica* Serovar *typhimurium* and *M. bovis* (BCG)—Our studies thus far assign a pivotal role for *IRF-8* in *Nramp1* regulation and in innate resistance to intraphagosomal pathogens such as *S. enterica* serovar *typhimurium*. We next wanted to directly link the defective bactericidal response of *IRF-8*−/− macrophages with aberrant expression of *Nramp1*. The original *IRF-8* knock-out mouse strain was generated in C57BL/J6, which also have a nonfunctional *Nramp1* allele due to a single amino acid mutation (Asn183→Gln). Therefore, to genetically dissociate between the effect of *IRF-8* and *Nramp1* on the ability of macrophage to eliminate intraphagosomal pathogens, we had to generate a new mouse strain by cross-breeding for 8 generations the mouse strain 129/Sv harboring endogenous functional *Nramp1* and the C57BL/J6 mouse strain ablated for *IRF-8*. A comparative *S. enterica* serovar *typhimurium* killing study was performed with 4 different mouse strains. The two wild type mouse strains, C57BL/J6, which is practically knock-out for *Nramp1* and 129/Sv that harbors intact genes. In addition, 129/Sv *IRF-8*−/− (*Nramp1*+/+) and C57BL/J6 *IRF-8*−/−, which is also *Nramp1* defective and thus practically a double knock-out mouse strain. Peritoneal macrophages were collected from these four mouse strains and following 18 h of activation with IFN-γ and LPS, the cells were infected with *S. enterica* serovar *typhimurium*. As seen in Fig. 6A, the killing of *S. enterica* serovar *typhimurium* by the wild type 129/Sv mouse strain was the most effective. However, the killing ability of macrophages that are defective for *IRF-8*, *Nramp1*, or both was identical and markedly impaired compared with the wild type macrophages.

Similarly, bone marrow macrophages were collected from these 4 mouse strains and after 6 days of in vitro cultivation the cells were activated with IFN-γ and LPS for 18 h and then infected with *M. bovis* (BCG) as described under “Experimental Procedures.” Similar to the data observed in Fig. 6A, WT macrophages killed this intraphagosomal pathogen more effectively than macrophages that are defective for *IRF-8*, *Nramp1*, or both, which exhibited an almost identical killing pattern of *M. bovis* (BCG) (Fig. 6B). Linking mouse genetics with macrophages phenotypic susceptibility to intraphagosomal infection our results strongly suggests that *IRF-8* is a major regulator of *Nramp1*, enabling an efficient elimination of these pathogens at early stages of infection.

To directly show that *IRF-8* is an immediate regulator of *Nramp1 in vivo*, bone marrow macrophages were collected from the 129/Sv *IRF-8*−/− mouse strain and transduced with either empty pMSCV retroviral vector or with the same vector harboring *IRF-8*. After 7 days, the cells were activated with IFN-γ and LPS and subjected to killing assay as described under “Experimental Procedures.” It is clear from Fig. 6C that rescuing *IRF-8* expression led to a significant improvement in *S. enterica* serovar *typhimurium* killing profile. Taken together, our results demonstrate that ablation of either *IRF-8* or
These data suggest a direct genetic link between IRF-8 expression, Nramp1 regulation, and phagosome-mediated elimination of intraphagosomal pathogens such as S. enterica serovar typhimurium or M. bovis (BCG) at the early stage after infection.

**DISCUSSION**

Innate resistance to intraphagosomal pathogens is mediated by various genes enabling the elimination of the invader. Nramp1 was identified as one of the major constituents that confer mice resistance to such pathogens. In humans, aberrant expression of NRAMP1 is associated with susceptibility to infectious and autoimmune disease (34, 35).

Nramp1 is expressed exclusively in myeloid cells such as macrophages and dendritic cells (36–38). Our previous study demonstrated that synergistic activation of Nramp1 is mediated through association of Miz-1, a ubiquitously expressed factor, with the hematopoietic specific transcription factors IRF-8 and PU.1 and laid the molecular basis for the cell type restricted expression of Nramp1. PU.1 is constitutively expressed at relatively high levels in monocyte/macrophage cells and cannot account for the IFN-γ inducibility of Nramp1 (11). IRF-8, on the other hand, is induced by IFN-γ, and serves as the catalyst for the formation of the three party heterocomplex. The interaction between IRF-8 and PU.1 is a paradigm for macrophage-specific expression of many genes. Following protein-protein interaction, the heterocomplex is capable of binding to a DNA composite element of which half is the IRF binding site and half is the PU.1 binding site (also termed as ETS). Here we show that the binding sites for IRF-8 and PU.1 are separated by ~32 bp, however, protein-protein interaction is essential because mutation in the IRF association domain of IRF-8 eliminates the synergistic interaction with Miz-1 and the further enhancement with PU.1 (6). The region just upstream of the ISRE and PU.1 binding site is characterized by 27 × GT repeat in mice that is partially conserved in human. In humans, the segmented GT repeat is subjected to polymorphism that affects Nramp1 promoter activity (40, 41). This GT repeat can assemble in a condensed Z-DNA structure in vivo (40) that may lead to juxtaposition of the two binding sites and facilitate heterocomplex formation.

Previous studies suggested that the transcriptional enhancement elicited by Miz-1 is due to interaction with the co-activator p300. This is repressed by c-Myc, at least in part, through competition for binding with p300 (42). Here we show that this repression is also due to c-Myc interfering with Miz-1 and IRF-8 interaction. Miz-1 is a multidomain transcription factor and its interaction with c-Myc is via helix-loop-helix domain that lies between amino acids 683 and 715 (11, 27). The interaction with IRF-8 is mediated through a PEST domain that spans amino acids 190–273 (6). This interacts with previous studies demonstrating that IRF-8 association with other transcription factors is mediated through IRF association domain-PEST domain interaction. Therefore, it suggests that Miz-1 interacts with c-Myc and IRF-8 through different domains that are probably sequestered by the counterinteracting partner: c-Myc or IRF-8. We propose that in resting macrophages c-Myc levels are sufficient to repress Miz-1 activity by blocking possible

**Nramp1** or both lead to the same killing activity in macrophages. Additionally, rescuing IRF-8 expression improved the killing ability of IRF-8/−/− cells that have a WT Nramp1 allele.

**FIGURE 6.** IRF-8 and Nramp1 are essential for macrophage resistance to *Salmonella* and Mycobacteria. A, peritoneal macrophages were prepared from four mouse strains; 129/Sv (WT), 129/Sv IRF-8−/− (IRF-8 knock-out), C57BL/6 (which is practically Nramp1 knock-out), and C57BL/6 IRF-8−/− (which is practically double knock-out). 3 × 10^5 cells/well were seeded in a 24-well plate, activated with IFN-γ and LPS for 20 h, and then challenged with *S. enterica* serovar typhimurium at m.o.i. of 15. Following an invasion time of 45 min (determined as time 0), cells were lysed and intracellular *Salmonella* was determined as described in the legend to Fig. 4. B, bone marrow macrophages were extracted from the four mouse strains as described under panel A. After 6 days of maturation, cells were activated for 20 h with IFN-γ and LPS and then infected with *M. bovis* (BCG). Cells were lysed at various time points in 0.5 ml of sterile water and CFU was determined. C, bone marrow macrophages were extracted from 129/Sv IRF-8−/−. Twenty-four hours later the cells were transduced with retroviral vector encoding for IRF-8 fused at the carboxy terminus with eGFP, pMSCV-IRES-IRF-8-eGFP (solid line), or with the same vector expressing just eGFP (dashed line). Six days later the cells were challenged with *S. enterica* serovar typhimurium at m.o.i. of 15. Following an invasion time of 45 min (determined as time 0), cells were lysed at 2 and 4 h postinfection and intracellular *Salmonella* was determined as described in the legend to Fig. 4.
interaction with p300 and IRF-8. When the cells are activated by IFN-γ or pathogens, IRF-8 is induced and concomitant c-Myc expression is down-regulated as previously reported (43). Moreover, it has been recently shown that IRF-8 induction leads to c-Myc repression (44). Therefore, in activated macrophages, IRF-8 competes with c-Myc for the association with Miz-1. Upon IRF-8 interaction, PU.1 is also assembled leading to maximal activation of Nramp1 expression. This cell type-specific activation of Nramp1 by IRF-8 and PU.1 also occurs during infection with S. enterica serovar typhimurium and L. donovani as shown here. Taken together, this suggests that during macrophage activation the repression of Nramp1 due to Miz-1/c-Myc interaction is alleviated and c-Myc is being replaced by IRF-8/PU.1 binding that lead to the assembly of a cell type-specific enhanceosome. This signaling cascade is turned off due to the negative feedback loop that deactivate IFN-γ signaling and concomitantly lead to restoration of c-Myc expression and down-regulation of IRF-8 expression. Consequently, the enhanceosome architecture returns to the steady state status.

The importance of Nramp1 to the killing of intraphagosomal pathogens is well established both in vitro and in vivo (45, 46). In this article, we have established the role of IRF-8 as an important regulator of Nramp1. To do that, we have generated a new IRF-8−/− mouse strain with a genetic background containing a functional Nramp1 allele. Macrophages from all 4 possible combinations of WT and mutated genes for IRF-8 and Nramp1 were studied. Infection of these cells with S. enterica serovar typhimurium or M. bovis (BCG) clearly demonstrated that ablution of either IRF-8 or Nramp1 led to a similar deficiency in killing activity following pathogen invasion as also seen with macrophages retrieved from the double ablatted/mutated mouse strain. The fact that the killing efficiency of the IRF-8 null strain resembled that of the Nramp1 defective mouse strain and the double knock-out strain suggest that these two genes are phenotypically as well as genetically associated. Together with our Nramp1 promoter analysis we conclude that in vivo IRF-8 also controls the expression of Nramp1. To further establish this, we have complemented bone marrow-derived macrophages from IRF-8−/−/Nramp1−/− mice with retroviral vector allowing IRF-8 expression. This led to partial recovery of the killing ability that was statistically significant. This killing activity was better than the killing activity observed for the double knock-out macrophages complemented with just IRF-8 (data not shown). These results point to the pivotal role of IRF-8 as a transcriptional regulator of the expression of Nramp1 in vivo. This is in line with previous publications describing the sensitivity of IRF-8 null mice to intraphagosomal pathogens such as Leishmania and Toxoplasma (47, 48). IRF-8 is a key factor not only for monocyte/macrophage differentiation but also to the function of mature macrophages. As such, it is a major regulator of IL-12p40 and the sensitivity to the various pathogens was solely attributed to lack of Th1 response in the IRF-8 knock-out mouse strain due to lack of IL-12 expression. Later, it was demonstrated that IRF-8 is also a key regulator of phagosome essential genes (phagocytic oxidase (12), inducible nitric-oxide synthetase (13), inflammatory cytokine (16), and Toll receptors 4 and 9 (49, 50), which all are essential for macrophage-mediated killing of pathogens by affecting both innate and adaptive immune responses. Naturally, it is also involved indirectly being part of the signaling cascade of Toll receptors and IFNs (22, 51). To this list of genes regulated by IRF-8, which are essential for intraphagosomal pathogen elimination, we add Nramp1.

A recent publication (21) looking at BXH-2 mice harboring a point mutation in the IRF association domain of IRF-8 (R294C) demonstrated that these mice were also susceptible to S. enterica serovar typhimurium to a level comparable with that seen for mice lacking functional Nramp1 or TLR4. In addition, these mice were permissive to M. bovis (BCG), despite a resistant Nramp1G169 allele, and were unable to control splenic bacterial replication, which continued for up to 8 weeks postinfection. These impaired IRF-8 R294C mice also demonstrated increased replication of the Plasmodium chabaudi AS malarial parasite during the first burst of blood parasitemia, and recurring waves of high blood parasitemia late during infection (21). The conclusions were that IRF-8 is required for orchestrating early innate responses and also long-term immune protection against unrelated intracellular pathogens. In general, these results are in line with our results. However, the results described here are related only to the early stages of macrophage infection in vitro. Under these experimental conditions, immune components such as adaptive immunity are not studied.

To conclude, in this article we show that the diverse effects of IRF-8 on innate immunity are also funneled through macrophage-restricted regulation of Nramp1. The Nramp1 enhanceosome is subjected to a delicate balance between a repressor (c-Myc) during normal physiological condition and an activator (IRF-8) induced in response to infection with intraphagosomal pathogens. This interplay between repression and activation explain in molecular and genetic terms the ability of macrophages to eliminate such pathogens at early stages of infection.

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