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Expression of IRF8 in gastric epithelial cells confers protective innate immunity against *Helicobacter pylori* infection

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Abstract

Interferon regulatory factor 8 (IRF8) is expressed in many types of blood cells and plays critical roles in cellular differentiation and function. However, the role of IRF8 in nonhematopoietic systems remains poorly understood. In this study, we provide evidence that IRF8 is a transcriptional modulator of the gastric mucosa necessary for limiting *H. pylori* colonization. *H. pylori* infection significantly upregulated expression of IRF8, which in turn promoted IFN-γ expression by gastric epithelial cells (GECs). Mice deficient of IRF8 exhibited increased *H. pylori* colonization and aborted induction of mucosal IFN-γ. Genome-wide analyses of IFN-γ-treated GECs by ChIP-seq and RNA-seq led to the identification of IRF8 target genes, with many belonging to the IFN regulated gene family that was previously observed in immune cells. Our results identify the IRF8-IFN-γ circuit as a novel gastric innate immune mechanism in host defense against infection of *H. pylori*.

Introduction

IRF8 is a transcription factor of the interferon regulatory factor (IRF) family with a wide range of functions in immune cell development, activation, immunomodulation and function. Under steady state conditions, based on an IRF8 reporter, IRF8 is expressed in

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developing lymphoid and myeloid cells but not in mature neutrophils, T cells or megakaryocytes (1, 2). A knockout of the mouse gene encoding IRF8 (*Irf8*$^{-/-}$ mice) results in a variety of developmental abnormalities of dendritic cells (DC), B cells, Langerhans cells and monocytes (Ref in (3)). IRF8 exerts its regulatory functions by binding to specific DNA sequences. Since IRF8 itself has weak DNA binding activity, heterodimerization with a partner molecule is essential to assert its transcriptional regulatory activity (4). Depending on the partner molecule, IRF8 either activates or represses expression of its target genes. Genome-wide analyses of IRF8 target genes in germinal center B cells, myeloid cells and brain during an inflammatory response against certain pathogens revealed that IRF8 regulates a relatively conserved set of genes involved in antigen presentation, interferon responses, DNA repair, RNA expression and protein processing (5–7).

While IRF8 expression and function were once thought to be restricted to the hematopoietic system, more recent evidence indicates a much broader tissue distribution. Studies in the nervous system (8), heart (9) and other muscle cells (10) have identified previously unrecognized functions of IRF8. For example, expression of IRF8 in neurons confers protection against ischaemic-reperfusion-induced brain damage (8). Expression of IRF8 in cardiomyocytes prevents development of cardiac hypertrophy by inhibiting calcineurin signaling (9). These data underscore the importance of cell context-dependent expression and functions of IRF8. However, it remains unclear how IRF8 is regulated in these non-hematopoietic cells and what gene programs IRF8 controls.

Here we report that IRF8 is expressed in gastric epithelial cells (GECs), another non-hematopoietic cell type. Expression levels of IRF8 are enhanced following infection with *H. pylori*. Mice deficient in IRF8 exhibit higher bacteria loads than wild type controls. We also show that IRF8 is induced by IFN-γ and that IRF8, in turn, promotes IFN-γ production forming a positive regulative loop to amplify inflammation. Furthermore, using ChIP-seq and RNA-seq analyses, we identified IRF8 target genes in GECs, providing a detailed understanding of how IRF8 functions in gastric mucosal innate immunity.

Materials and methods

Mice and infection

B6 (WT), *Irf8*$^{-/-}$, *Irf8*$^{f/f}$ and IRF8-EGFP reporter mice were previously described (2, 11, 12). Vil-Cre mice (Stock# 4586) were purchased from the Jackson Laboratory and bred with *Irf8*$^{f/f}$ to generate *Irf8*$^{f/f}$Vil-Cre mice. All mice were maintained in a specific pathogen-free facility at the National Institutes of Health (Bethesda, MD) according to guidelines proved by NIAID (ASP LIG-16) and NIDDK (NIDDK-K052-NIHMD-13) Animal Care and Use Committees. Infection of mice with the *H. pylori* SS1 strain (kindly provided by Drs. A. Lee and J. O'Rourke, University of New South Wales, Sydney, Australia) was performed as described before (13). Briefly, mice were inoculated with an oral challenge dose of 10^7 CFU at day 1, 3 and 5. At 4 and 8 weeks following infection, stomach tissues were processed for histology and tissue culture for enumeration of *H. pylori*. For infection of cells *in vitro*, mouse GEC were prepared by enzyme digestion as previously described (14). After culture for 5–8 days, subconfluent cells were incubated with *H. pylori* with a MOI of 1:100 for 2 days.

Immunohistolochemistry (IHC) and flow cytometry

Paraffin sections of stomach tissues were stained with a polyclonal anti-IRF8 Ab (Santa Cruz Biotechnology) and/or DAPI using standard procedures and imaged using a Nikon ECLIPSE TE2000-U confocal microscope. For intracellular staining, GECs were fixed and permeabilized using a Fix & Perm Kit (Life Technology) and stained with APC-conjugated anti-cytokeratin Ab (pan-reactive, EXBIO Praha). Cells were then analyzed by a FACS LSRII flow cytometer (BD Biosciences) and FlowJo software.

Microarray, qPCR, immunoblotting, RNA-seq and ChIP-seq

Microarray analysis was done using RNA extracts from stomach tissues of infected and control mice and Affymetrix chips. Data were analyzed by the NIDDK Bioinformatics facility. Quantitative real time-PCR (qPCR) was performed as described previously (15). The GSM06 GECs (a gift of Dr. Yoshiaki Tabuchi at University of Toyama, Japan) were stimulated with 10 ng/ml of IFN-γ for different times before analysis. Primers are listed in Supplemental Table I. Western blot and ChIP analyses were performed as previously described (15). ChIP-seq and RNA-seq analyses were performed as described previously (16). The GEO accession number is GSE67476 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=yrwtacaspzoxlqp&acc=GSE67476).

Statistics analysis

The unpaired two-tailed Student *t* test or Mann Whitney *U* test were used to compare differences of two groups. $P<0.05$ was considered statistically significant.

Results and Discussion

Expression of IRF8 is increased in stomach tissues following infection with *H. pylori*

Microarray-based gene expression profiling of gastric tissues from *H. pylori* infected and mock-infected mice revealed a 2-fold increase of *Irf8* transcripts in infected stomach tissues (Fig. 1A). This was confirmed by qPCR (Fig. 1B). Infection of a mouse GEC cell line, GSM06, with *H. pylori* also resulted in a time-dependent increase in *Irf8* expression (Fig. 1C). Immunohistochemical analyses revealed that normal stomach tissues expressed low levels of IRF8 as evidenced by barely positive staining of IRF8 in nucleated glandular cells (Fig.1D). However, infection with *H. pylori* resulted in a robust increase of IRF8 in GEC (Fig. 1D). By using an IRF8-EGFP reporter mouse in which an IRF8-EGFP fusion protein is expressed under the control of natural regulatory elements of the *Irf8* gene (2), we detected a dramatic upregulation of IRF8-EGFP expression in infected gastric glandular cells (Fig. 1E).

In an in vitro assay, cultured primary GECs of IRF8-EGFP and WT mice uniformly expressed cytokeratin, a marker for gastric epithelial identity (Fig. 1F, top panels). Following coculture with *H. pylori*, GECs of IRF8-EGFP reporter mice but not WT mice upregulated expression of IRF8-EGFP (Fig. 1F, bottom panels), consistent with the data from IHC analyses. As a positive control, IFN-γ, a known potent stimulus for IRF8 expression, significantly up-regulated expression of IRF8-EGFP. The results demonstrated that IRF8 was readily induced in isolated GEC indicating that expression induced by exposure to H. *pylori* shown in the IHC analyses was not dependent on other gastric cell

types. From this, we conclude that infection with *H. pylori* significantly increased expression of IRF8 in GEC.

IRF8 deficiency impairs gastric innate immunity against infection with *H. pylori*

To determine whether IRF8 contributes to gastric inflammatory responses against infection with *H. pylori*, we infected *Irf8*$^{-/-}$ and WT mice with the *H. pylori* SS1 strain and assessed changes in pathology and bacterial loads in stomach tissues at later time points. At 4 and 8 weeks post infection, the extent of stomach tissue damage was indistinguishable between *Irf8*$^{-/-}$ and WT controls (data not shown), but bacterial loads in *Irf8*$^{-/-}$ mice were significantly greater at both time points than in controls (Fig. 2A).

Because *Irf8*$^{-/-}$ mice are defective in many aspects of innate and adaptive immunity including development and function of DCs, B cells, Th17 cells and monocytes (Ref in (3, 17)), the decreased bacterial clearance in *Irf8*$^{-/-}$ mice could be due to a collective dysfunction of these immune effector cells. To gain more insights into GEC-intrinsic effects of IRF8 resulting from *H. pylori* infection, we first generated chimeric mice by reconstituting lethally irradiated *Irf8*$^{-/-}$ mice with hematopoietic stem cells (HSCs) of WT mice. Eight weeks after reconstitution, the mice were infected with *H. pylori* and examined 2 months later. The *Irf8*$^{-/-}$ mice reconstituted with WT HSCs still exhibited higher bacterial loads than WT control mice reconstituted with WT HSCs (Fig. 2B). Second, we generated IRF8 conditional deletion mice using Vil-Cre mediated deletion of the IRF8 gene in villin-expressing gastric tissues. The bacterial loads in *Irf8*$^{f/f}$Vil-Cre mice were significantly higher than *Irf8*$^{f/f}$ control mice at 4 and 8 weeks post infection, respectively (Fig. 2C). These data strongly support a GEC-intrinsic effect of IRF8 in limiting *H. pylori* colonization. Next, we investigated *H. pylori* induced IFN-γ production in GECs *in vitro*. Previous studies demonstrated that infection with *H. pylori* induced expression of IFN-γ in gastric tissues (24). Consistent with this result, the expression of IFN-γ transcripts was significantly increased in gastric tissues following *H. pylori* infection (Fig. 3A). Infection of the GSM06 GEC cell line and WT primary GECs with *H. pylori* also induced expression of IFN-γ (Fig. 3B). However, infection failed to induce IFN-γ expression in *Irf8*$^{-/-}$ GECs (Fig. 3B). Importantly, IFN-γ transcripts were negligible or absent in gastric tissues of *Irf8*$^{-/-}$ mice infected with *H. pylori* for 4 and 8 wks (Fig. 3C). Taken together, these data suggest that expression of IFN-γ in *H. pylori*-infected GECs is IRF8-dependent.

IFN-γ contributes to *H. pylori*-induced inflammation and regulates expression of IRF8

As noted previously, IFN-γ is a potent inducer of IRF8 expression in immune cells (18). Interestingly, IFN-γ also induced IRF8 expression in GSM06 cells in a time-dependent manner (Fig. 3D). Similarly, stimulation of primary GECs of IRF8-EGFP mice with IFN-γ also increased IRF8-EGFP expression (Fig. 1F, bottom panel). It is worth noting that GECs express IFN-γ receptors constitutively as determined by qPCR analyses of transcript levels for the IFN-γ receptors, *Ifngr1 and Ifngr2* (data not shown). Taken together, these data suggested that IFN-γ drives IRF8 expression, which, in turn, promotes IFN-γ production. This positive feedback loop is expected to amplify IFN-γ-mediated immunity in the early phase of infection, which could change the course of later adaptive immunity to *H. pylori* by priming CD4^{+} T cells for Th1 differentiation (Ref in (19)).

IRF8 controls expression of a large number of genes in gastric epithelial cells

Previous high-throughput genomic analyses of tissue-specific IRF8 target genes in stomachs were based on analyses of whole tissues, obviating the opportunity to discern the contributions of gastric cells as compared to infiltrated inflammatory immune cells that often express high levels of IRF8 (2). To overcome this obstacle, we performed genome-wide analysis of IRF8 target genes by employing ChIP-seq and RNA-seq using the gastric epithelial cell line, GSM06, stimulated with IFN-γ, a factor that was more efficient than *H. pylori* in inducing IRF8 protein expression (Fig. 1F), enabling us to generate high quality ChIP materials for analysis. To demonstrate that this approach is relevant to the biology of a true infection, we validated some of the target genes identified following IFN-γ treatment using cultured mouse primary GECs infected with *H. pylori*.

We identified 666 IRF8-specific ChIP-seq peaks in DNA from cells stimulated with IFN-γ. The distribution of IRF8 binding sites with 20% proximal to the transcription start site, 32% intronic and 35% intergenic (Fig. 4A) was similar to that recently described for IRF8-induced mouse monocyte differentiation (20). *de novo* motif analysis revealed that IRF8-bound genomic sequences were greatly enriched (71%) for the sequence, GAAANNGAAA, which matches the core consensus interferon-stimulated response element (ISRE) sequence (Fig. 4B). Of the 666 IRF8 ChIP-seq peaks, 314 bound within genes annotated by RefSeq (Fig. 4C).

RNA-seq analyses of gene expression by the IFN-γ-stimulated and control cells identified 1037 IFN-γ regulated genes including 605 that were up-regulated and 432 that were down-regulated (Fig. 4D). The over-lap of IRF8-bound genes identified by ChIP-seq with IFN-γ stimulated genes identified by RNA-seq comprised 77 genes including 73 that were up-regulated and 4 that were down-regulated (Fig. 4C; Supplemental Table II). Ingenuity functional analysis revealed that 48% of these genes are involved in endocrine system disorders, gastrointestinal diseases, and immunological Diseases, and 41% of these genes belong to pathways of antimicrobial response, inflammatory response, and endocrine system disorders (Supplemental Table II). Interestingly, the most increased gene, *Ifit3*, but not the most decreased gene *Ifi202b*, in infected gastric tissues (Fig. 1A) was found to be a direct target of IRF8 (Supplemental Table II). Ifit3 has recently been identified as a novel antiviral protein (21, 22) and could be an important innate factor against microbial infection.

Previous gene microarray profiling analysis in the lungs of *M. tuberculosis* infected mice and ChIP-seq analysis in the brain of malaria infected mice have led to identification of a common core of 53 IRF8-bound genes that were upregulated in both conditions (5). Given that different tissues express significantly different gene profiles and that IRF8 targets can vary with tissue, this core list could be an underestimate. Nevertheless, we compared this "common core" list with our 77-gene list and identified 17 genes that overlapped in all three tissues. These genes ontologically belong to innate immunity (*Ifit3, Nlrc5, Oasl2, Trim21*), adaptive immunity, antigen processing and presentation (*Cd274, H2-T22, Psmb8, Tap2*), GTP signaling (*Gbp2, Gbp3, Igtp, Irgm1, Irgm2*), and ubiquitination (*Rnf19b, Usp18*). The small number of overlap also indicates that IRF8-controlled gene programs are mostly cell context-dependent.

To determine whether IRF8 is required for expression of these genes, we analyzed expression of *Usp18, Upp1, Nlrc5, Ifit3, Ifit1,* and *Ifi35* in primary GECs of WT and *Irf8*$^{-/-}$ mice. The cells were treated with IFN-γ or infected with *H. pylori* for 2 days with gene expression levels analyzed by qPCR. As shown in Fig. 4E, transcript levels of all 6 genes were significantly higher in primary GECs of WT mice than IRF8-deficient controls following stimulation with IFN-γ (Fig. 4E, $p<0.001$). As expected, deficiency of IRF8 abrogated *H. pylori*-induced upregulation of these genes (Fig. 4E). In addition, the degree of altered gene expression in IFN-γ-treated GECs was significantly greater than for *H. pylori*-infected GECs, suggesting that IFN-γ is a prominent stimulator for elicitation of robust GEC gene expression programs.

Previous studies support a Th1-biased adaptive immune response during *H. pylori*-induced gastric infection (19). Our study extends this view by providing new evidence that infected GECs exhibit an IRF8-IFN-γ positive regulatory circuit that would facilitate Th1 cell differentiation. Because GECs produce IFN-γ (Fig. 3 and (14)) and IFN-γ expression in GECs is completely dependent on IRF8 (Fig. 3), these data suggest that IRF8 could be the major regulator for IFN-γ production in infected gastric tissues. Importantly, the IRF8-IFN-γ regulatory circuit may amplify local inflammatory responses to promote bacterial clearance. Thus, it is possible that downregulation of IRF8 in GECs may facilitate chronic infection and enhance the likelihood of *H. pylori*-induced malignant transformation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This paper is dedicated to the memory of Dr. William G. Coleman, Jr. who initiated and supervised this collaborative study with HCM. We thank Alfonso Macias for maintaining the mouse colony.

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Abbreviations used in this article

IRF8	interferon regulatory factor 8
qPCR	quantitative real time PCR
DC	dendritic cell

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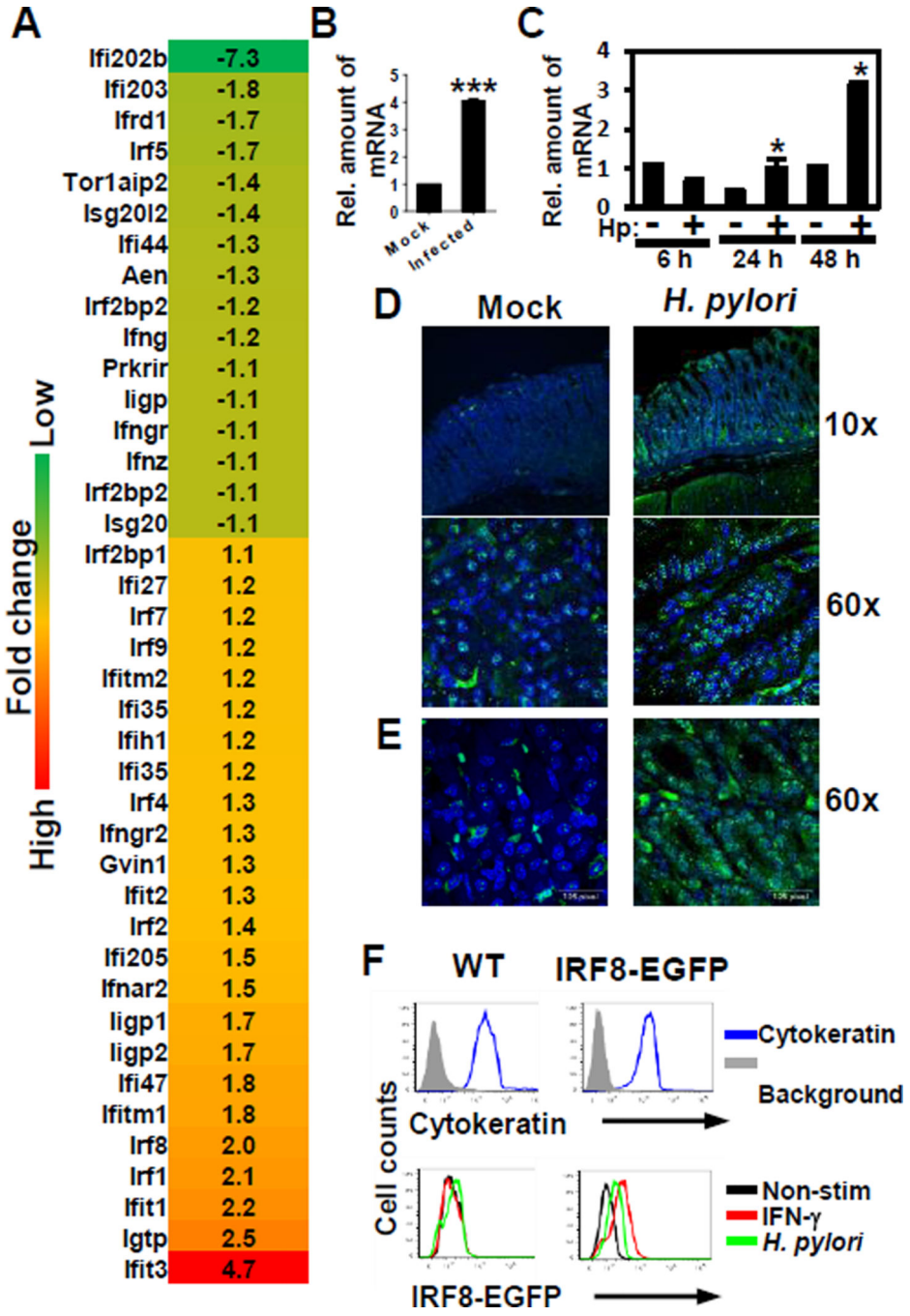

Fig. 1.

Expression of IRF8 in stomach. (A) Microarray analysis of IRF8 expression in stomach tissues of mice (n=3) infected with *H. pylori* for 2 months. Shown are fold changes in expression of genes belonging to the interferon family and the IRF family. (B) qPCR analysis of IRF8 transcripts in stomach tissues of mice (n=3) infected with *H. pylori*. (C) qPCR analysis of IRF8 expression in GSM06 cells infected with *H. pylori* for different times. Cont, non-infected controls. Hp, *H. pylori*-infected. Error bars represent triplicate assays *p<0.05. Data are from one of three experiments. (D and E) Fluorescence

histological analysis of IRF8 expression in stomach tissues of B6 (D) and IRF8-EGFP reporter (E) mice infected with *H. pylori* for 8 weeks. Stomach tissue sections were immunostained using indirect immunofluorescence with Abs against IRF8 (D) or direct imaging for EGFP fluorescence (E). Sections were also counterstained with DAPI (blue) to aid in the identification of nucleated cells and the localization of IRF8 to the nuclei. Data are representative of six mice per group. (F) IRF8-EGFP expression in primary GECs. Cultured GEC of WT and IRF8-EGFP mice were co-cultured with *H. pylori* or IFN-γ for 2 days and analyzed by flow cytometry. Top panel is GECs stained intracellularly with anti-cytokeratin Abs to show gastric epithelial cell identity. Background is determined by unstained samples. Data are representative of two independent experiments with similar results.

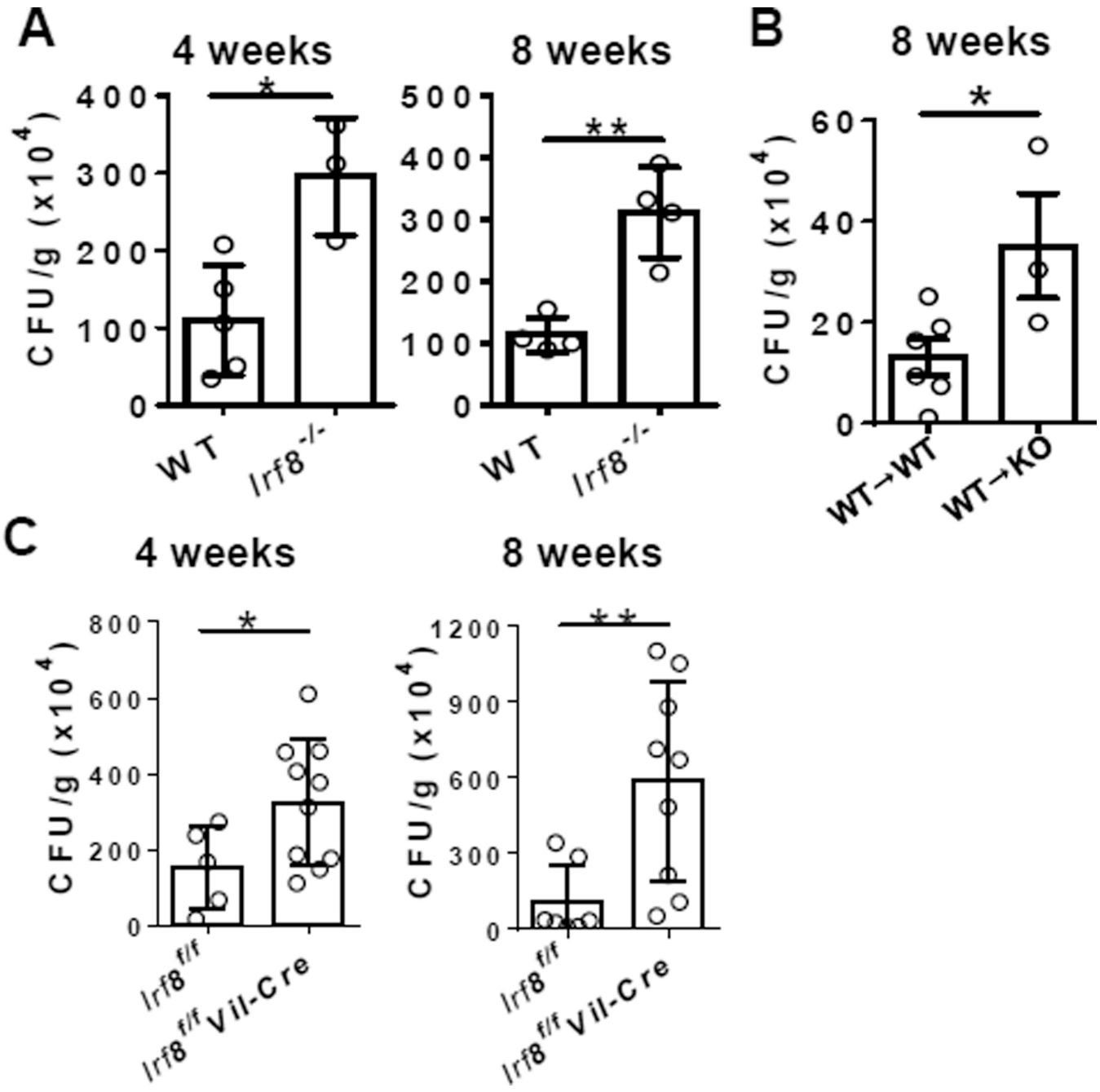

Fig. 2.

IRF8 deficiency impaired bacterial clearance in the stomach. (A) *Irf8*$^{-/-}$ and WT mice were infected with *H. pylori* for 4 and 8 weeks and analyzed for bacterial load. **p*<0.05; ***p*<0.01. Each symbol represents a mouse. (B) Lethally irradiated WT and IRF8−/− mice reconstituted with WT HSCs for 2 months were infected with *H. pylori* for 8 weeks and analyzed for bacterial load as in (A). Each symbol represents a mouse. **p*<0.05. Data are pooled from two experiments. (C) *Irf8*$^{f/f}$Vil-Cre mice were infected and analyzed with *H. pylori* as in (A). Each symbol represents a mouse. **p*<0.05; ***p*<0.01.

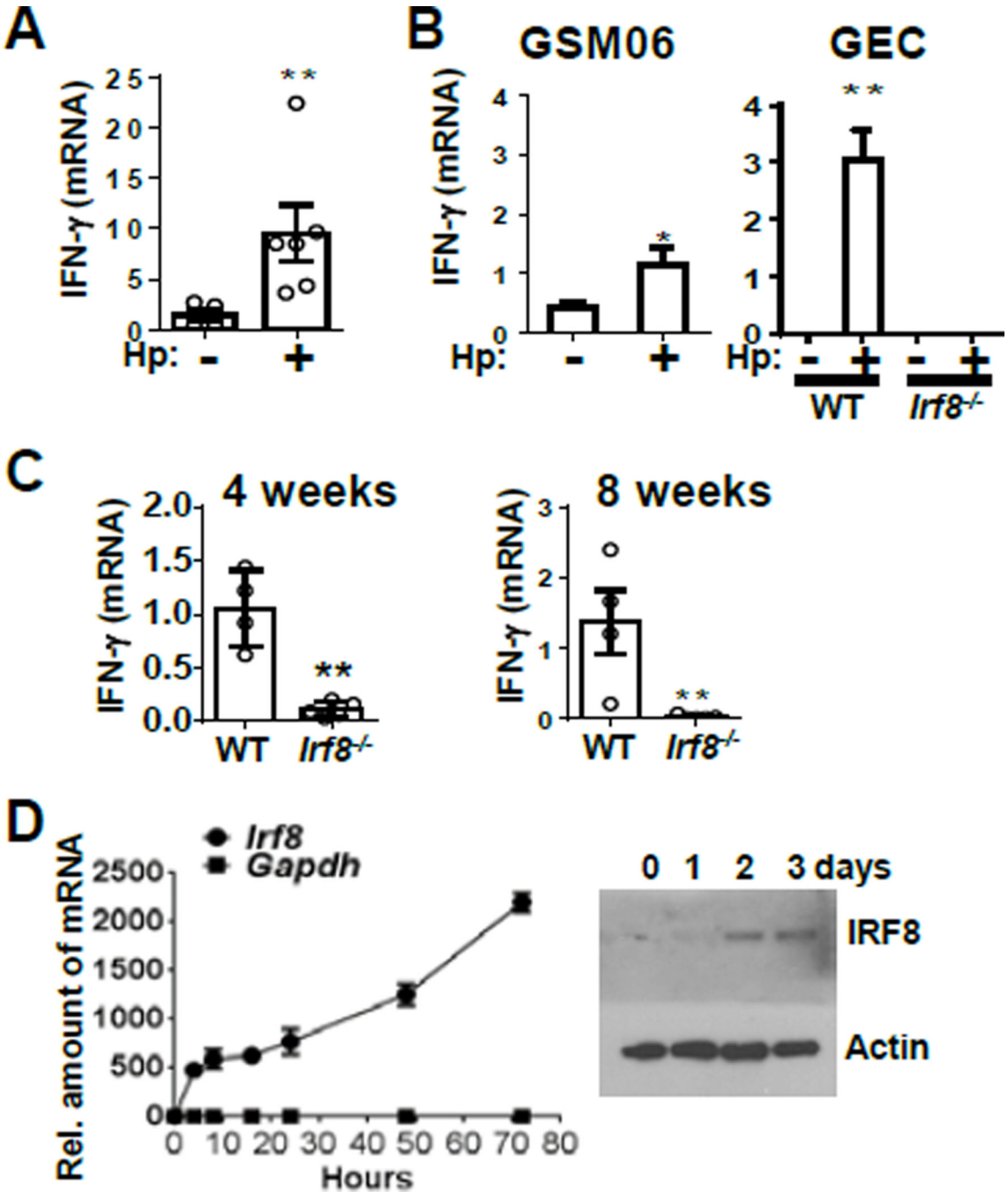

Fig. 3.

IFN-γ and IRF8 expression in stomach tissues. (A) Stomach tissues of WT mice infected with *H. pylori* for 8 weeks were analyzed by qPCR for IFN-γ expression. Each symbol represents a mouse. ***p*<0.01. (B) IFN-γ expression in GSM06 cells and primary GECs of WT and *Irf8*$^{-/-}$ mice infected with *H. pylori* for 24 h. Error bars are for triplicate assays. Data are representative of two independent experiments. (C) IFN-γ expression in stomach tissues of *Irf8*$^{-/-}$ and WT mice infected with *H. pylori* for 4 and 8 weeks. Each symbol represents a mouse. ***p*<0.01. (D) GSM06 cells were stimulated with IFN-γ for 24 h. IRF8

expression was analyzed by qPCR (left panel) and immunoblotting (right panel). Data represents three independent experiments.

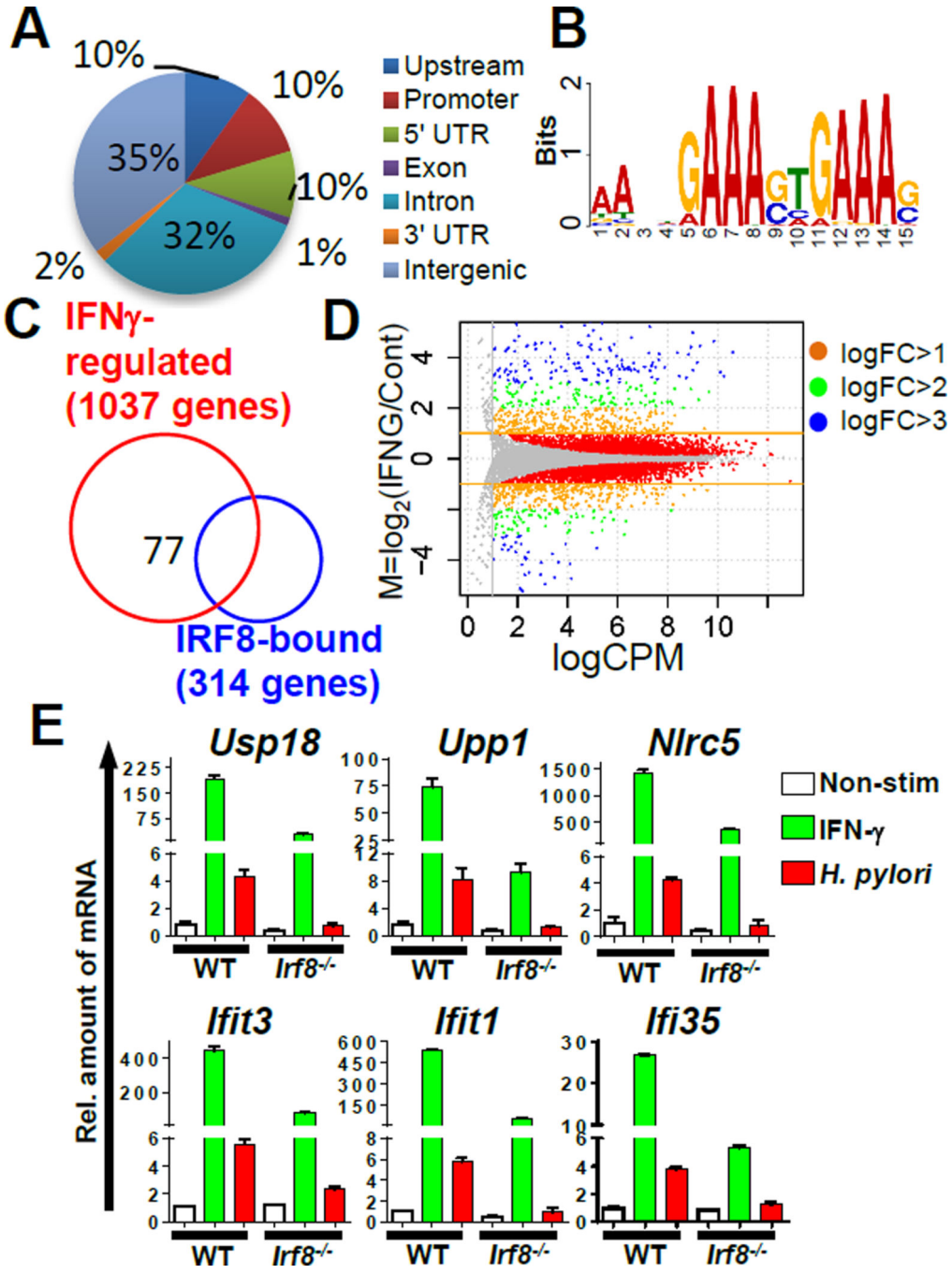

Fig. 4.

Genome-wide analysis of IRF8 targets in GECs. (A) Genomic binding distribution of IRF8 binding induced by IFN-γ (upstream: [−15kb, −5kb], promoter: [−5kb, TSS]). (B) De novo motif analysis indicated that 474 out of 666 binding sites contained the IRF core motif GAAANNGAAA. (C) 77 genes are bound by IRF8 and also differentially expressed following stimulation with IFN-γ. (D) RNA-Seq analysis shows that IFN-γ regulated a number of genes, including 605 that were up-regulated and 432 that were down-regulated (FDR < 0.05, FC > 2). (E) IRF8-bound genes including *USP18*, *Upp1*, *Nlrc5*, *Ifit1*, *Ifit3* and

Ifi35 are stimulated by IFN-γ and *H. pylori* in an IRF8-dependent fashion. The primary GECs of WT and *Irf8*$^{-/-}$ stomach tissues were cocultured with *H. pylori* or IFN-γ for 2 days. Data represent two independent experiments.