The Journal of Immunology

IFN-γ Priming of Macrophages Represses a Part of the Inflammatory Program and Attenuates Neutrophil Recruitment

Marten A. Hoeksema, Brendon P. Scicluna, Marieke C. S. Boshuizen, Saskia van der Velden, Annette E. Neele, Jan Van den Bossche, Hanke L. Matlung, Timo K. van den Berg, Pieter Goossens and Menno P. J. de Winther

J Immunol 2015; 194:3909-3916; Prepublished online 6 March 2015;
doi: 10.4049/jimmunol.1402077
http://www.jimmunol.org/content/194/8/3909

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

IFN-γ Priming of Macrophages Represses a Part of the Inflammatory Program and Attenuates Neutrophil Recruitment

Marten A. Hoeksema,* Brendon P. Scicluna,[†,‡] Marieke C. S. Boshuizen,* Saskia van der Velden,* Annette E. Neele,* Jan Van den Bossche,* Hanke L. Matlung,[§] Timo K. van den Berg,[§] Pieter Goossens,[¶] and Menno P. J. de Winther*

Macrophages form a heterogeneous population of immune cells, which is critical for both the initiation and resolution of inflammation. They can be skewed to a proinflammatory subtype by the Th1 cytokine IFN-γ and further activated with TLR triggers, such as LPS. In this work, we investigated the effects of IFN-γ priming on LPS-induced gene expression in primary mouse macrophages. Surprisingly, we found that IFN-γ priming represses a subset of LPS-induced genes, particularly genes involved in cellular movement and leukocyte recruitment. We found STAT1-binding motifs enriched in the promoters of these repressed genes. Furthermore, in the absence of STAT1, affected genes are derepressed. We also observed epigenetic remodeling by IFN-γ priming on enhancer or promoter sites of repressed genes, which resulted in less NF-κB p65 recruitment to these sites without effects on global NF-κB activation. Finally, the epigenetic and transcriptional changes induced by IFN-γ priming reduce neutrophil recruitment in vitro and in vivo. Our data show that IFN-γ priming changes the inflammatory repertoire of macrophages, leading to a change in neutrophil recruitment to inflammatory sites. ***The Journal of Immunology*****, 2015, 194: 3909–3916.**

Macrophages demonstrate considerable plasticity that allows them to respond efficiently to environmental signals and adapt their phenotype in response to cytokines and microbial signals. In the classical paradigm, different polarized macrophage subsets are referred to as classically activated (M1) and alternatively activated (M2) macrophages in reflection of the Th1 and Th2 nomenclature in T cells. M1 macrophages mainly secrete proinflammatory cytokines, whereas M2 macrophages are known for their anti-inflammatory and wound-healing characteristics. The initial concept for macrophage polarization described different phases, starting with macrophage differentiation, followed by priming with pro- or anti-inflammatory cytokines and subsequently activation during an infection (1, 2).

The Th1 cytokine IFN-γ is known to skew macrophages to a proinflammatory phenotype. IFN-γ is a cytokine produced by NK cells, T cells, and APCs (3). Upon binding to the IFN-γ receptor, it primarily induces STAT1 homodimerization and subsequent translocation to the nucleus (4). In macrophages, one of the most important effects of IFN-γ is the activation of microbicidal effector functions through the induction of NADPH oxidase and inducible NO synthase, which are both effective tools for killing bacteria, viruses, parasites, and fungi (5). Furthermore, IFN-γ increases MHCII expression, which is essential for efficient Ag presentation (6).

Macrophage activation by cytokines, such as TNF, or TLR ligands, such as LPS, initiates various proinflammatory signaling pathways. These include the MyD88-independent or -dependent activation of NF-κB, a major transcriptional regulator of mainly proinflammatory and prosurvival genes (6, 7).

Thus, STAT1 and NF-κB are both critical transcription factors in inducing a proinflammatory repertoire in macrophages. The general concept is that IFN-γ signaling augments proinflammatory NF-κB signaling (4, 8). For instance, in combination with a TLR trigger, IFN-γ can activate an initiation complex with STAT1 and NF-κB, leading to increased *Nos2* transcription compared with TLR activation alone (9). Furthermore, by inducing IFN regulatory factor 1 expression, IFN-γ increases the expression of a specific subset of LPS-induced genes (10).

In this study, we investigated how IFN-γ priming affects the LPS-induced gene program by genome-wide expression analysis. Surprisingly, we found that IFN-γ, besides augmenting a set of inflammatory genes, also represses a specific subset of LPS-inducible genes. We show that this repression is dependent on STAT1 and results in reduced recruitment of NF-κB p65 to promoters of repressed genes. Furthermore, we show that this subset of genes is epigenetically silenced, suggesting that IFN-γ–stimulated macrophages deactivate

*Experimental Vascular Biology Laboratory, Department of Medical Biochemistry, Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, the Netherlands; [†]Center for Experimental Molecular Medicine, Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, the Netherlands; [‡]Center for Infection and Immunity, Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, the Netherlands; [§]Sanquin Research and Landsteiner Laboratory, Department of Blood Cell Research, Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, the Netherlands; and [¶]Centre d'Immunologie de Marseille-Luminy, Aix-Marseille Université, UM2, 13288 Marseille, France

Received for publication August 13, 2014. Accepted for publication February 2, 2015.

This work was supported by the Netherlands Heart Foundation (2010B022), the Netherlands Organization for Scientific Research (TOP91208001), the Netherlands CardioVascular Research Initiative (CVON2011-19), and an Academic Medical Center fellowship to M.P.J.d.W.

The microarray data presented in this article have been submitted to the National Center for Biotechnology's Gene Expression Omnibus (http://www/ncbi.nlm.nih.gov/geo/) under accession number GSE60290.

Address correspondence and reprint requests to Prof. Menno P.J. de Winther, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105AZ Amsterdam, the Netherlands. E-mail address: m.dewinther@amc.uva.nl

The online version of this article contains supplemental material.

Abbreviations used in this article: ChIP, chromatin immunoprecipitation; qPCR, quantitative PCR.

a specific transcriptional program, and that this silencing has consequences for neutrophil recruitment.

Materials and Methods

Macrophage culture

All wild-type, STAT1$^{-/-}$ (11), and IFN-β$^{-/-}$ mice were female and on a C57BL/6 background. Femurs and tibia were flushed with ice-cold PBS. Bone marrow cells were cultured in RPMI 1640, 25 mM HEPES, 2 mM L-glutamine, 10% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml) (all Life Technologies) supplemented with 15% L929-conditioned medium for 8 d. After 8 d, bone marrow cells were differentiated to macrophages; ∼80% of cells were F4/80 and CD11b double positive (Supplemental Fig. 1A). Cells were primed with 10 U/ml IFN-γ (Hycult Biotech) for 18 h and subsequently activated with 10 ng/ml LPS (Sigma-Aldrich) for 6 h. Media were frozen and stored at −20°C for ELISA and NO assay.

RNA and protein isolation

Cells were lysed with 400 μL lysis buffer of the High Pure RNA isolation kit (Roche, Basel, Switzerland), which was used for RNA isolation, according to the manufacturer's protocol. For protein isolation, cells were lysed with a SDS lysis buffer containing Complete protease inhibitor mixture (Roche) and PhosSTOP phosphatase inhibitor mixture (Roche) for 1.5 h at 4°C and then harvested.

Microarray

For microarray, yield and purity (260:280 nm) were determined by Nanodrop (Thermo Fischer). The integrity (RNA integrity number > 8.0) of the total RNA was determined by using the RNA Nano Chip Kit on the Bioanalyzer 2100 and the 2100 Expert software (Agilent). The Illumina TotalPrep-96 RNA Amplification Kit was used to generate biotin-labeled (biotin-16-UTP) amplified cRNA starting from 200 ng total RNA. A total of 750 ng biotinylated cRNA was hybridized onto the Illumina MouseRef-8v2 Expression BeadChip. The samples were scanned using the Illumina iScan array scanner. Preparation of cRNA, chip hybridization, washing, staining, and scanning were carried out at ServiceXS (Leiden, the Netherlands). The raw scan data were read using the *beadarray* package (version 2.4.2) (12), available through Bioconductor (13), using the R statistical package (version 2.13.2; R Foundation for Statistical Computing, Vienna, Austria). Estimated background was subtracted from the foreground for each bead. For replicate beads, outliers >3 median absolute deviations from the median were removed, and the average signal was calculated for the remaining intensities. BASH analysis was performed to detect and remove spatial artifacts (14). For each probe, a detection score was calculated by comparing its average signal with the summarized values for the negative control probes. Resulting data were preprocessed by the neqc function, which performs a normal-exponential convolution model analysis that estimates nonnegative signal, quantile normalization, and log2 transformation (15). Quality control was performed both on bead level and on bead summary data. The arrayQualityMetrics package v2.6.0 (16) was used for further quality assessment of the normalized bead summary data. Probes were reannotated using the package illuminaMousev2.db from Bioconductor (17). All nonnormalized and normalized data are available at the Gene Expression Omnibus of National Center for Biotechnology Information (GEO, http://www.ncbi.nlm.nih.gov/geo/) with accession number GSE60290.

Microarray data analysis and bioinformatics

Differential probe intensities were identified using the R software package *limma* version 3.10.3 (18), as previously described (19). Briefly, empirical Bayesian methods were used to generate *p* values obtained from moderated *t* statistics or *f* statistics. These *p* values are then adjusted for multiple comparisons with Benjamini and Hochberg's method to control the false discovery rate. Probes were collapsed to gene symbols for data presentation and interpretation. Bioinformatic analysis for biofunctional and canonical signaling pathways was performed by means of the Ingenuity Pathway Analysis software (www.ingenuity.com), as previously described (20). All parameters were kept as default. Significant pathway associations were delineated at Benjamini and Hochberg–adjusted $p < 0.05$.

Single-site analysis in oPOSSUM (21) was used to determine over-represented transcription factor binding sites. Motif analysis was performed with HOMER (22) using default settings.

Quantitative PCR

RNA concentrations were determined using the Nanodrop (Thermo Scientific); cDNA was synthesized from total RNA with iScript (Bio-Rad). Quantitative PCR (qPCR) was performed with 4 ng cDNA using Sybr Green Fast (Applied Biosytems) on a ViiA7 PCR machine (Applied Biosystems). Gene expression was normalized to the mean of ARBP and GAPDH expression. Primer sequences are available on request.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed, as described elsewhere (23). For AcH3 ChIP, 12×10^6 cells were used. For p65 ChIP, 40×10^6 cells were used and DSG (Sigma-Aldrich) was used to cross-link protein interactions for 30 min at a concentration of 2 mM before formaldehyde fixation. Relative enrichments are presented as percentage input. ChIP-qPCR was performed using Sybr Green Fast (Applied Biosytems) on a ViiA7 PCR machine (Applied Biosystems). Primer sequences are available on request.

ChIP sequencing

A previously published ChIP experiment (24), GSE38379, was normalized to a total of 10^7 uniquely mapped tags using HOMER (22) and visualized by preparing custom tracks for the University of California, Santa Cruz genome browser.

ELISA

Ab pair kits were used for ELISA to measure IL-1β, IL-6, IL-12p40, and TNF (all: Life Technologies) and Cxcl1 (R&D Systems) production. Flat-bottom 96-well plates (NUNC) were coated with capture Ab overnight. Then plates were blocked with 0.5% BSA (Sigma-Aldrich) in PBS for 2 h. After 1 h of incubation with cytokine standards and samples, the plate was incubated with detection Ab dilution. Streptavidin HRP was used to detect bound Abs, and tetramethylbenzidine (Thermo Scientific) was used as a substrate for the color reaction. This reaction was stopped with 1.8 M H_2SO_4, and absorbance was measured on a microplate reader at a wavelength of 450 nm.

NO production

For the quantification of NO production, 50 μl each sample was pipetted in a flat-bottom 96-well plate. A standard curve from 200 μM to 3.125 μM $NaNO_2$ was diluted in cell medium. Then 50 μl Griess (2.5% H_3PO_4, 1% sulfanylamide, and 0.1% naphtylene diamine in milliQ) reagent was added to the cell medium, and absorbance was measured at 550 nm on a microplate reader.

NF-κB TransAM

For nuclear extraction, cells were scraped in PBS/5 mM EDTA (Life Technologies Invitrogen), spun down, and resuspended in a buffer containing 10 mM HEPES (Life Technologies Invitrogen), 1.5 mM MgCl (Sigma-Aldrich), 0.5 mM DTT (Sigma-Aldrich), and phosphatase and protease inhibitors (Roche). The outer membrane of the cell was disrupted with the detergent Nonidet P40 (Sigma-Aldrich). After 10 min of incubation, the nuclei were spun down at 4000 rpm for 10 min, and the supernatant was discarded. The nuclear pellet was then dissolved in a SDS lysis buffer and incubated on ice for 30 min. The extract was spun down at 6000 rpm for 15 min, and the supernatant was transferred to a new tube and snap frozen in −80°C. Bicinchoninic acid was used to determine protein content, and 10 μg protein was used for the p65 NF-κB TransAM. NF-κB activity was measured with the p65 NF-κB TransAM (Active Motif), and the absorbance was measured at 450 nm with a reference wavelength of 655 nm.

Western blot

Protein concentrations of samples were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific). Equal amounts of protein samples were loaded with 2-ME (Promega) containing loading buffer on a SDS polyacrylamide gel and blotted on a polyvinylidene fluoride membrane (Amersham). The blots were blocked in PBS with 0.05% Tween-20 and 5% nonfat dry milk (Sigma-Aldrich). Next, blots were incubated with primary Abs for p65 (Millipore), P-p65 Ser^{536} and GAPDH (both Cell Signaling Technology) in PBS with 0.05% Tween-20, and 5% BSA (Sigma-Aldrich). Goat anti-rabbit HRP (DAKO) in PBS with 0.05% Tween and 1% nonfat dry milk (Sigma-Aldrich) was used as secondary Ab. The signals were captured with a chemiluminescent imager using the ECL substrate kit (Thermo Scientific).

Neutrophil recruitment

Neutrophils were isolated from female mouse bone marrow of C57BL/6 mice with a MACS neutrophil isolation kit (Miltenyi Biotec). After counting, neutrophils were labeled with calcein-AM (Life Technologies),

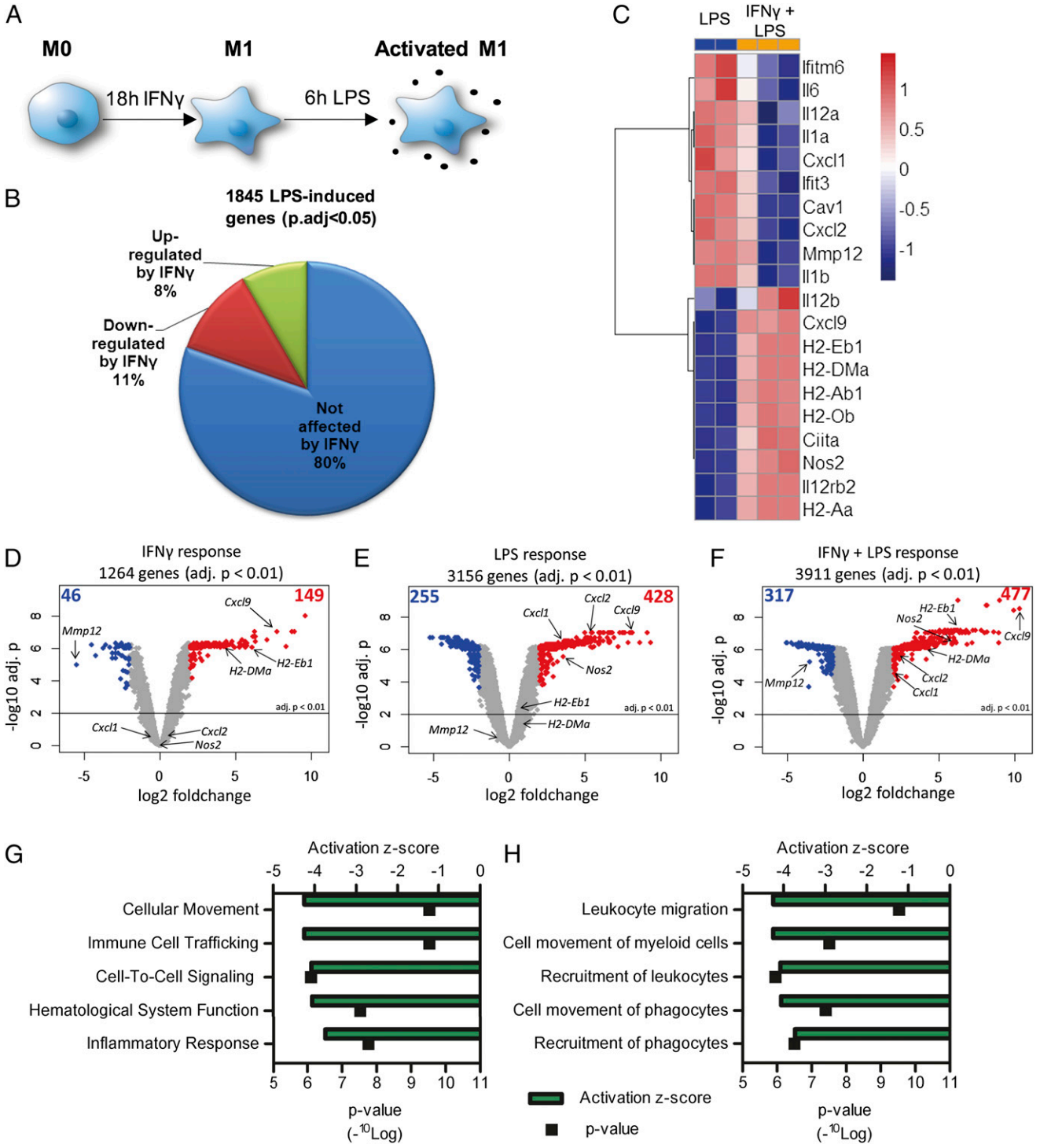

FIGURE 1. IFN-γ priming represses part of the LPS-induced inflammatory program. Macrophages were differentiated from mouse bone marrow, primed overnight with 0 or 10 U/ml IFN-γ, and subsequently activated with 10 ng/ml LPS for 6 h. (**A**) Schematic overview of the different stimulations used in the described experiments. (**B**) Figure representing the proportions of LPS-induced genes that are upregulated or downregulated following IFN-γ priming. (**C**) Heatmap with selected genes showing the effect of IFN-γ priming on LPS-induced gene expression. (**D–F**) Volcano plots indicating the effects of the IFN-γ and LPS stimulations on selected affected individual genes. (**G**) Pathways that were downregulated by IFN-γ priming, sorted on *z*-score, as analyzed with Ingenuity Pathways Analysis. (**H**) Subgroup of cell movement canonical pathways affected by IFN-γ priming.

and chemotaxis in response to macrophage supernatants (5× diluted) was measured in a Transwell chemotactic assay, as described previously (25).

In vivo neutrophil recruitment to the peritoneum

Female C57BL/6 mice were injected i.p. with PBS or IFN-γ (diluted in PBS, 5000 U/mouse, 18 h prior to the LPS injection). After 18 h, they were injected i.p. with LPS (diluted in PBS, 10 mg/kg) or PBS only as control. Six hours later, the mice were sacrificed, the peritoneum was flushed with 5 ml PBS, and blood and spleen were harvested. Peritoneal cells were spun down, and peritoneal fluid was kept for ELISA analysis. After lysis of the erythrocytes (for spleen and blood samples) and Fc block, the peritoneal cells, blood cells, and splenic cells were analyzed by FACS to determine neutrophil counts. We selected neutrophils as cells positive for CD45 (allophycocyanin-Cy7, 30-F11; BioLegend), CD11b (FITC, M1/70; eBioscience), and Ly6G (PE, 1A8; BD Pharmingen). For the peritoneal

cells, CountBright absolute counting beads (Life Technologies) were added to determine absolute cell counts.

Statistical analysis

Data are expressed as means ± SE and were analyzed by unpaired Student *t* test or two-way ANOVA for grouped analysis. Data were analyzed using GraphPad Prism 5. The *p* values <0.05 were considered statistically significant.

Results

IFN-γ priming results in repression of genes involved in leukocyte recruitment

To investigate the effect of IFN-γ priming on LPS-induced gene expression in macrophages, we stimulated bone marrow–derived macrophages with IFN-γ for 18 h and subsequently activated them with LPS for 6 h (Fig. 1A). To check for successful priming, we measured the expression of two cytokines that are typically expressed in proinflammatory macrophages, IL-12 and TNF. Indeed, expression of both cytokines (Supplemental Fig. 1C, 1D), as well as NO production (Supplemental Fig. 1E), which was measured after 24 h of LPS stimulation, was enhanced in IFN-γ–primed macrophages. In a microarray analysis of the samples, we found 1845 genes significantly upregulated upon activation with LPS (adjusted *p* value <0.05). Interestingly, prior priming with IFN-γ enhanced the expression of only 8% of these LPS-induced genes, whereas it repressed 11% of the LPS-induced genes (Fig. 1B). In particular, a subset of proinflammatory cytokines and chemokines showed a decreased gene expression as a result of IFN-γ priming (Fig. 1C). We also investigated the effects of LPS on IFN-γ–induced gene expression; in this study, we found that LPS enhanced the expression of most IFN-γ–induced genes (Supplemental Fig. 1B), for example, *Cxcl9* (see Fig. 1D–F, in which the regulation of genes by IFN-γ and LPS stimuli is visualized in volcano plots). Biofunctional pathway analysis using Ingenuity Pathway Analysis revealed that especially cellular movement pathways (Fig. 1G) were downregulated. Within this group of cellular movement, more specifically pathways regulating migration of leukocytes, myeloid cells, and phagocytes (Fig. 1H) were negatively affected by IFN-γ priming. Among the top 20 associating canonical signaling pathways, cholesterol synthesis, NF-κB signaling, TLR/pattern recognition receptor signaling, and chemokine signaling were prominent (Supplemental Fig. 2). Downregulated genes included cytokines *Il6* and *Il1b* and the chemokine *Cxcl1*. We performed qPCR to validate the gene expression changes of these genes (Fig. 2A–C) and performed ELISA to determine cytokine/chemokine production. Indeed, also on protein level, we found a clear reduction in LPS-induced IL-6, IL-1β, and CXCL1 production (Fig. 2D–F). To determine whether IFN-γ only prevented the upregulation of these LPS-induced genes or whether it really suppresses LPS-induced gene expression, we performed a timing experiment in which we also added IFN-γ at later time points. We found that also coincubation of IFN-γ with LPS or treatment of cells with IFN-γ after the LPS stimulus decreased expression of these genes (Supplemental Fig. 1F–H).

STAT1 is involved in the repression of LPS-induced genes by IFN-γ priming

To investigate which transcription factors are involved in repression of LPS-induced genes by IFN-γ priming, we investigated the enrichment of binding motifs in repressed genes using oPOSSUM (21). We selected genes that were repressed by at least 50% and analyzed the promoter regions (up to 2000 bp upstream of the transcriptional start site) for enriched motifs. Considering a *z*-score >10 and adjusted *p* value <0.01, analysis revealed that the STAT1-binding motif was significantly enriched in those repressed genes (Fig. 3A). Also using HOMER (22), we performed motif

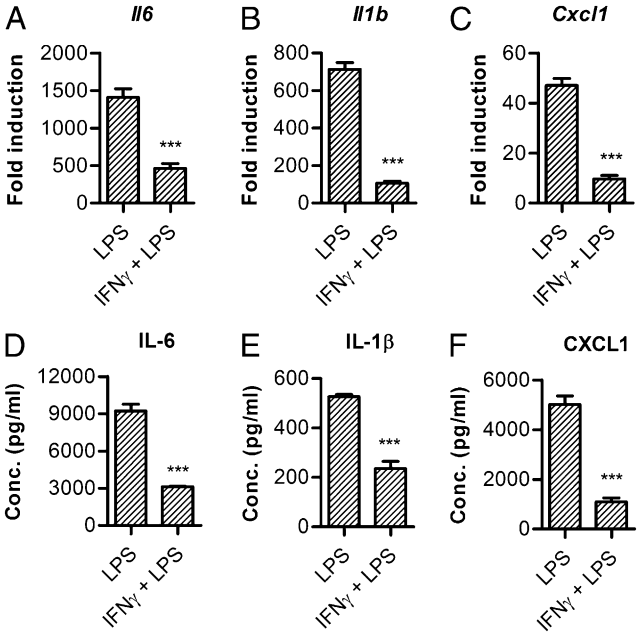

FIGURE 2. Gene and protein expression of IFN-γ priming downregulated genes. *Il6* (**A**), *Il1b* (**B**), and *Cxcl1* (**C**) gene expression was measured and corrected for the expression of household genes *CycloA* and *ARBP*. (**D**) IL-6 secretion by macrophages was measured after 6 h of LPS. (**E**) IL-1β secretion was measured after 6 h of LPS, in the presence of ATP for the last hour. (**F**) CXCL1 secretion was measured after 6 h of LPS treatment. One of at least three representative experiments with triplicates was shown. Unpaired *t* test was performed for statistical analysis. Error bars indicate SEM. ***$p < 0.001$ compared with nonprimed macrophages.

analysis on the same group of repressed genes (>50% repression). In this study as well, we found that the STAT1 motif was the most significant motif overrepresented in this subset of genes (Fig. 3B). These findings suggest a dominant role for STAT1 transcription factor activity in repressing a LPS-induced transcriptional program.

To further investigate the role of STAT1 in the inhibition of genes after IFN-γ priming, we repeated the priming and activation in STAT1-deficient macrophages. We found that IFN-γ does not repress the expression of *Il6*, *Il1b*, and *Cxcl1* in the absence of STAT1 (Fig. 3C–E, Supplemental Fig. 3A, 3B). Moreover, *Il1b* and *Cxcl1* expression was even derepressed in LPS-activated STAT1$^{-/-}$ macrophages (Fig. 3D, 3E). These data support the hypothesis that STAT1 mediates the repression of a subset of LPS-induced genes post–IFN-γ priming. As previous literature suggests that CXCL1 inhibition in postinfluenza bacterial pneumonia was due to repression by IFN-β (26), we also assessed the effect of IFN-γ priming in LPS-activated IFN-β$^{-/-}$ macrophages. In this study, CXCL1 production was still inhibited by IFN-γ priming (Supplemental Fig. 3C), indicating that the priming effects are not dependent on IFN-β. IFN-β$^{-/-}$ macrophages do produce more CXCL1 in response to LPS, confirming that autocrine IFN-β signaling does inhibit CXCL1 production, as described previously (26), in the absence of IFN-γ priming. Also, the effect of IFN-γ priming on IL-6 production was independent of IFN-β, as IFN-γ priming still results in repression of IL-6 production (Supplemental Fig. 3D).

IFN-γ priming does not inhibit NF-κB activity but attenuates its recruitment to the promoters of inhibited genes

As NF-κB is the master regulator of inflammatory responses in macrophages, we assessed global NF-κB activation by two different methods, as follows: phosphorylation of NF-κB p65 at serine 536 (Fig. 4A) and by use of a NF-κB TransAM assay to

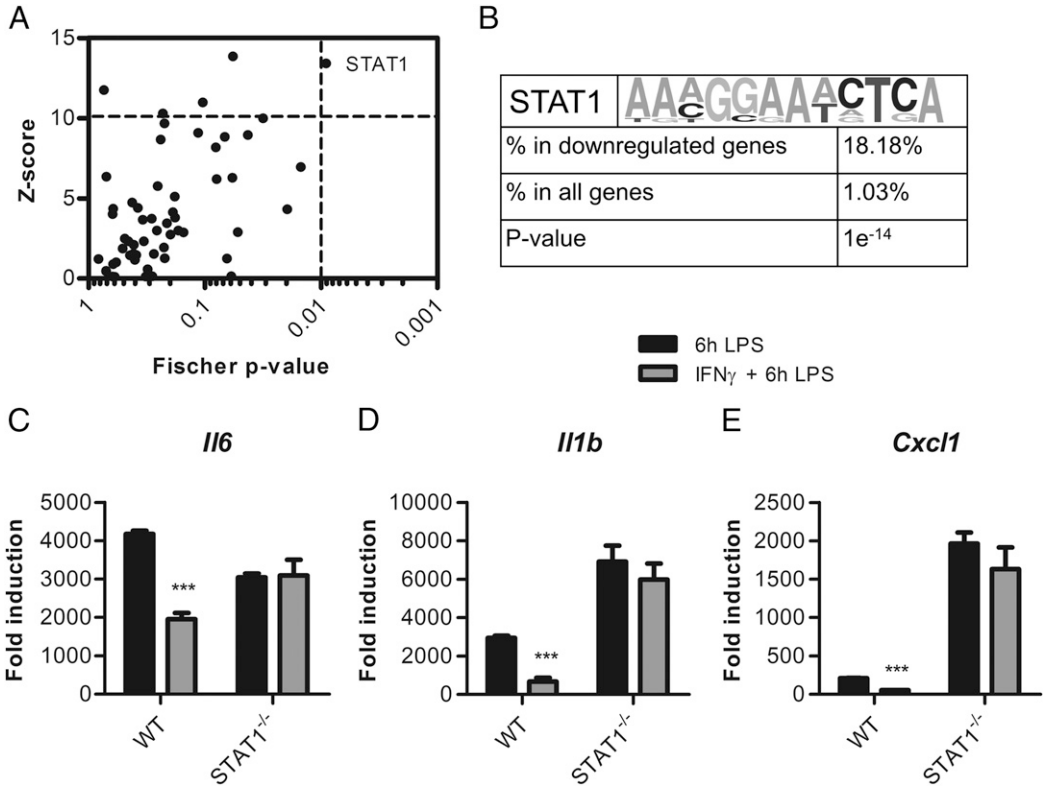

FIGURE 3. Repression by IFN-γ depends on STAT1. (**A**) Single-site analysis with oPOSSUM reveals that STAT1-binding motifs are overrepresented in the IFN-γ priming-repressed (>50%) gene subset. (**B**) Motif analysis with HOMER demonstrates that STAT1 is the most overrepresented motif in the IFN-γ priming-repressed (>50%) gene subset. (**C–E**) Macrophages were cultured from bone marrow from wild-type (WT) and STAT1$^{-/-}$ mice and stimulated, as previously described. *Il6* (C), *Il1b* (D), and *Cxcl1* (E) gene expression was measured and corrected for household gene expression. One of at least three representative experiments with triplicates was shown. Two-way ANOVA with Bonferroni correction was performed for statistical analysis. Error bars indicate SEM. ***$p < 0.001$ compared with nonprimed WT macrophages.

assess p65 DNA binding (Fig. 4B). We found no effect of IFN-γ priming on both readouts of NF-κB activation, indicating that global NF-κB activity remains unaltered upon IFN-γ priming.

We next determined p65 recruitment to the promoters of specific IFN-γ–repressed genes by performing p65 ChIP, followed by PCR. We found decreased p65 recruitment to the *Il1b* and *Cxcl1* promoters (Fig. 4C, 4D), which is in line with the decreased transcriptional activation of these genes described above. p65 recruitment to the *Tnf* promoter (Fig. 4E), however, was unaltered by IFN-γ priming, which fits as *Tnf* expression is not repressed by IFN-γ priming. For the *Il6* promoter, there were no detectable amounts of p65 enrichment measured (data not shown), suggesting p65 independence.

IFN-γ priming results in epigenetic changes on promoter/enhancer sites of inhibited genes

To explain the decreased p65 recruitment to repressed genes, we sought site-specific explanations for how the first (IFN-γ) stimulus can lead to repression of the second (LPS) stimulus. Thus, we further hypothesized that IFN-γ affects LPS-induced chromatin remodeling of promoter or enhancer regions of repressed *Il1b*, *Il6*, and *Cxcl1* genes. Indeed, we found that IFN-γ inhibits LPS-induced histone 3 lysine 9 and 14 acetylation on the *Il1b* and *Il6* promoter and the *Cxcl1* enhancer (Fig. 4F–H). Again, histone acetylation of the *Tnf* promoter was not changed upon IFN-γ priming (Fig. 4I). Analysis of ChIP-sequencing data of IFN-γ–treated macrophages (24) also showed changes in the histone acetylation status of *Il1b* and *Cxcl1* enhancers already after 4 h of IFN-γ treatment in murine macrophages (Supplemental Fig. 4A, 4B). These data indicate that chromatin remodeling occurs rapidly after stimulation with IFN-γ. Moreover, in macrophages lacking STAT1, IFN-γ did not decrease the acetylation status of these sites (Supplemental Fig. 4C, 4D), showing that this chromatin-remodeling mechanism is STAT1 dependent.

IFN-γ priming of macrophages hampers neutrophil recruitment

As pathway analysis revealed a defect of IFN-γ–primed macrophages in the recruitment of inflammatory cells, namely leukocytes, myeloid cells, and phagocytes (Fig. 1D, 1E), we assessed the potential of IFN-γ–primed macrophages to recruit neutrophils. We performed a Transwell migration assay in which we used supernatant of primed macrophages to recruit labeled neutrophils. We found that LPS stimulation of macrophages potentiates the recruitment of neutrophils, but that prior IFN-γ priming decreases this recruitment (Fig. 5A). In subsequent in vivo studies, we injected LPS in the peritoneum of mice, with or without preinjection of IFN-γ (18 h prior to the LPS injection) and analyzed cellular recruitment after 6 h. We found that IFN-γ priming decreased the number of neutrophils attracted to the peritoneum by injection with LPS (Fig. 5B), without affecting the proportions of neutrophils in blood and spleen (Fig. 5C, 5D). Also, in the peritoneal fluid we found a decreased expression of CXCL1 (Fig. 5E), and a trend toward less IL-6 (Fig. 5F) in the IFN-γ–pretreated mice. These data fit with the pathway analysis predictions and the observed decrease in expression of neutrophil migratory factors *Cxcl1* and *Cxcl2* (Fig. 1C).

Discussion

The present study was designed to investigate the effects of IFN-γ priming on the LPS-induced gene expression repertoire of mac-

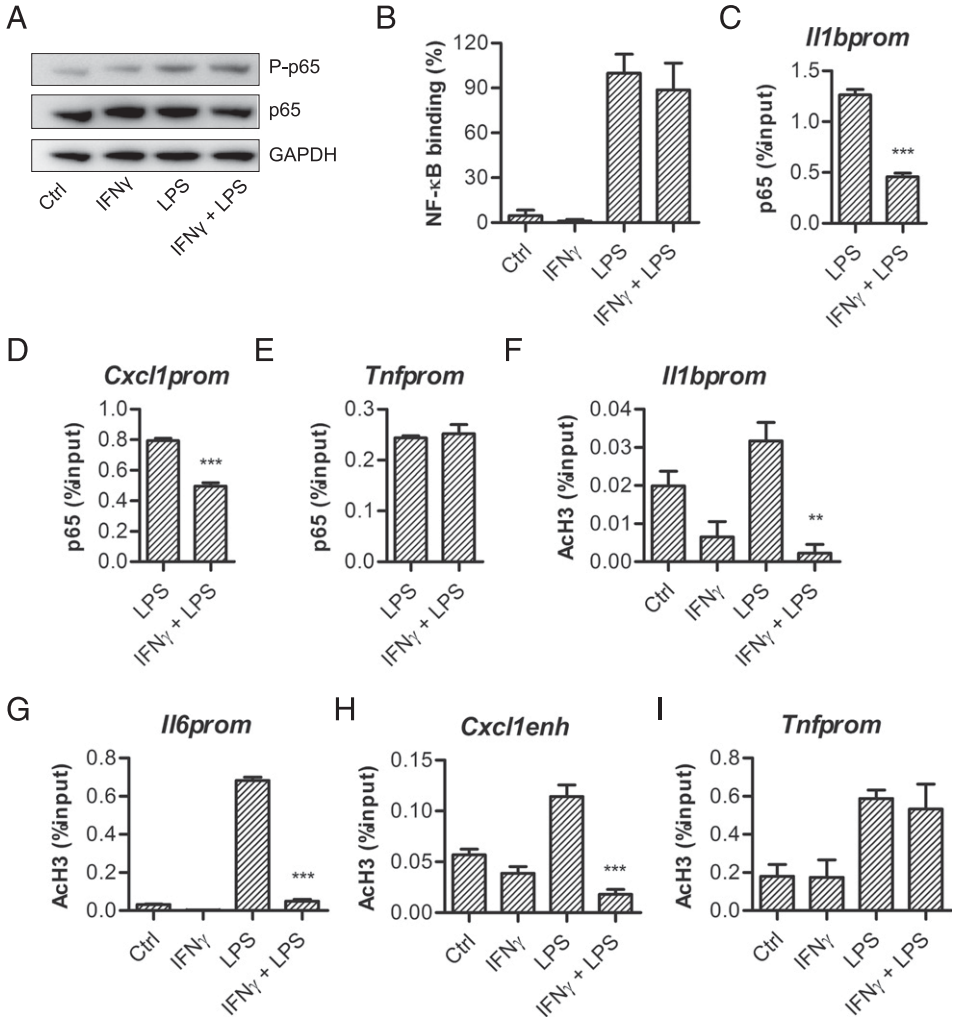

FIGURE 4. Global NF-κB activity is unaltered, whereas NF-κB recruitment to promoters of repressed genes is attenuated upon IFN-γ priming. (**A**) Western blot analysis for P-Ser^{536} p65 NF-κB revealed no difference in the LPS-induced NF-κB phosphorylation after IFN-γ priming. (**B**) IFN-γ priming does not influence LPS-induced NF-κB p65-binding capacities in a TransAM activity assay. (**C–E**) ChIP for p65 was performed on 40 million fixed cells per condition; ChIP-PCR was used to determine p65 binding to *Il1b* (C), *Cxcl1* (D), and *Tnf* (E) promoters. (**F–I**) ChIP for AcH3 (K9/14) was performed on 12 million fixed cells per condition, and ChIP-PCR was used to determine enrichment on the *Il1b* (F) and *Il6* promoter (G), the *Cxcl1*enhancer (H), and *Tnf* promoter (I). Enrichment is presented as a percentage of input. For p65 ChIPs, unpaired *t* test was performed for statistical analysis. One of at least two representative experiments with triplicates was shown. Error bars indicate SEM. For AcH3 ChIPs, one-way ANOVA with Bonferroni test was used for statistical analysis, $^{**}p < 0.01$, $^{***}p < 0.001$, IFN-γ + LPS compared with LPS.

rophages. Current literature mainly focuses on how IFN-γ potentiates LPS-induced gene expression. For several typical M1 marker genes, it was very elegantly shown how STAT1, the transcription factor activated by IFN-γ, helps NF-κB to increase inflammatory gene transcription (9, 27, 28). Although in our study we also found LPS-induced genes, including typical M1 markers

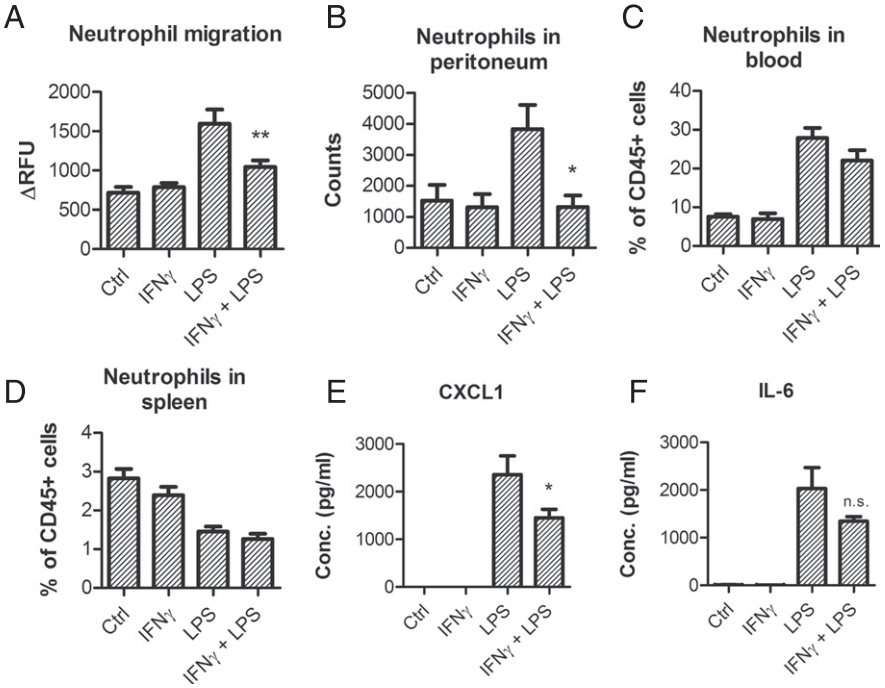

FIGURE 5. Macrophage IFN-γ priming decreases LPS-induced neutrophil recruitment. (**A**) Neutrophils were isolated from bone marrow of female C57BL/6 mice. They were fluorescently labeled, and chemotaxis in response to macrophage supernatant was measured. Migration of neutrophil toward the supernatant is presented here as the difference in relative fluorescence units (RFU). The presented data shown here represent the mean of three independent experiments. (**B–F**) C57BL/6 mice (6 per group) were injected i.p. with LPS, with or without a pretreatment with IFN-γ. Six hours after LPS injection, the peritoneum was flushed and also spleen cells and blood were harvested at sacrifice. (B) IFN-γ priming decreases LPS-enhanced neutrophil ($CD45^+CD11B^+Ly6G^+$ cells) recruitment to the peritoneum. IFN-γ priming does not alter the proportions of neutrophils in the blood (C) or in the spleen (D). The LPS-induced levels of CXCL1 (E) and IL-6 (F) in the peritoneum were attenuated by IFN-γ priming. Error bars indicate SEM. One-way ANOVA with Bonferroni test was used for statistical analysis, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with LPS only.

like *Nos2* and *Il12b*, to be upregulated, we identified an even larger proportion of LPS-induced genes that were repressed by prior IFN-γ priming. Pathway analysis revealed that especially cellular movement is negatively affected by IFN-γ priming. Among the downregulated genes were *Il6*, *Il1b*, *Cxcl1*, and *Cxcl2*, which are crucial for Th17 responses and neutrophil migration (29, 30). We hereby demonstrate that IFN-γ priming not only potentiates proinflammatory responses, as recently described in human macrophages (31), but may also contribute to the resolution of inflammation. It also negatively affects LPS-induced genes, like genes involved in cell recruitment. Based on our data, we can conclude that IFN-γ is a multifaceted cytokine that can alter the course of an inflammatory response by regulating the transcription of inflammatory genes in macrophages in both positive and negative directions. IFN-γ is a pleiotropic cytokine that can affect many different (immune) cell types (32), but for our studies we chose to focus on the specific regulation of macrophage responses.

By transcription factor DNA-binding motif analysis, we found the repression by IFN-γ priming was predicted to be dependent on STAT1. STAT1 is known for its proinflammatory characteristics through induction of antiviral products, increasing the expression of IFN regulatory factors and facilitating NF-κB–dependent gene expression (33–36). Furthermore, it was shown that STAT1 is required for the induction of the LPS-induced gene program that depends on autocrine signaling by type I IFNs (i.e., inducible NO synthase) (37). In this study, we demonstrate that IFN-γ–mediated inhibition of the *Il6* gene expression is also STAT1 dependent. Moreover, we show that STAT1 is a transcriptional repressor of *Il1b* and *Cxcl1* expression. Reports describing the effect of IFN-γ on IL-1β production are conflicting. Several previous reports demonstrated a decrease of IL-1β production by IFN-γ via STAT1 (38-40), whereas others found that STAT1 is necessary for inflammasome activation and thereby IL-1β production (41). Moreover, in human macrophages IFN-γ potentiates IL-1β production (38).

The repression of CXCL1 by IFN-γ was previously described to be dependent on IFN-β signaling in postinfluenza bacterial pneumonia (26). Therefore, we assessed the effect of IFN-γ priming in LPS-activated IFN-$\beta^{-/-}$ macrophages. In IFN-$\beta^{-/-}$ macrophages, CXCL1 expression was still inhibited by IFN-γ priming, indicating that the priming effects are not due to increased IFN-β production. IFN-$\beta^{-/-}$ macrophages do produce more CXCL1 in response to LPS, confirming that autocrine IFN-β signaling does inhibit CXCL1 production (26).

Pathway analysis performed on the IFN-γ–inhibited genes revealed an overrepresentation of genes involved in NF-κB signaling, TLR/pattern recognition receptor signaling, and chemokine signaling. Although we did not observe any differences by IFN-γ priming on global NF-κB activity, we found a marked decrease in LPS-induced p65 recruitment to the *Il1b* and *Cxcl1* promoters. For the *Il6* promoter, we found no detectable amounts of p65 enrichment, which suggests a different transcription factor, for example, AP-1 (42), at play.

As global NF-κB activity was unaltered, but p65 recruitment to repressed genes was decreased, we sought site-specific explanations for how the first (i.e., IFN-γ) stimulus can lead to repression of the second (LPS) stimulus. We therefore hypothesized that IFN-γ affects LPS-induced epigenetic changes on promoter or enhancer regions of IFN-γ–repressed genes. Indeed, we found that IFN-γ inhibits LPS-induced chromatin remodeling, determined in this work based on the histone acetylation status, on the *Il1b* and *Il6* promoter and the *Cxcl1* enhancer. This might be caused by altered histone deacetylase or histone acetyl transferase activity, but may also be a consequence of histone remodeling due to modifications on other histone residues (43). Moreover, we found that the reduction in histone acetylation was dependent on STAT1, indicating that STAT1 represses the recruitment of the transcriptional machinery at these specific genes. As a result, p65 recruitment is attenuated and gene transcription is repressed. The mechanism whereby IFN-γ exerts these anti-inflammatory mechanisms is distinct from other anti-inflammatory mechanisms, such as activation of suppressor of cytokine signaling pathways (44), or, in the case of IL-10, posttranscription inhibition of inflammatory genes by decreasing mRNA stability (45).

Finally, as pathway analysis revealed a defect of IFN-γ–primed macrophages in the recruitment of inflammatory cells, we assessed the potential of these macrophages to recruit neutrophils. We found that IFN-γ priming decreased LPS-induced neutrophil recruitment both in vitro and in vivo. It has been reported previously that IFN-γ (and also type I IFNs) produced during viral infections in mice suppresses innate protection against subsequent bacterial pathogens (26, 46–49). Also, in humans, postinfluenza patients are more susceptible to secondary bacterial infections, particularly pneumococcal pneumonia. It is known that most influenza-related deaths are not primarily due to the influenza infection itself (50, 51), but rather due to a second hit, a bacterial infection, to which the influenza patient is sensitized. Based on these and our current data, one could propose that the inflammatory program of macrophages that have been primed by IFN-γ, produced during virus-induced T cell responses, is mainly focused on clearing viral pathogens. Hereby, macrophages shut down the transcriptional program necessary for neutrophil-mediated bacterial clearance.

Concluding, we showed that IFN-γ priming of macrophages represses a specific transcriptional program, including *Il6*, *Il1b*, *Cxcl1*, and *Cxcl2*, which are crucial mediators of Th17 responses and neutrophil migration (29, 30). We found that the repression was dependent on STAT1, resulting in decreased chromatin accessibility due to epigenetic modifications. Our data thus show that IFN-γ priming shifts the inflammatory repertoire of macrophages by repression of a specific subset of genes, which may consequently alter host defense responses.

Acknowledgments

We thank Anton Tool for technical support, Dr. Mathieu and Dr. Gysemans for providing bone marrow of the STAT1$^{-/-}$ mice, and Dr. Kalinke for providing bone marrow from IFN-β–deficient mice.

Disclosures

The authors have no financial conflicts of interest.

References

1. Gordon, S., and F. O. Martinez. 2010. Alternative activation of macrophages: mechanism and functions. *Immunity* 32: 593–604.
2. Martinez, F. O., and S. Gordon. 2014. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep.* 6: 13.
3. McLaren, J. E., and D. P. Ramji. 2009. Interferon gamma: a master regulator of atherosclerosis. *Cytokine Growth Factor Rev.* 20: 125–135.
4. Schroder, K., P. J. Hertzog, T. Ravasi, and D. A. Hume. 2004. Interferon-gamma: an overview of signals, mechanisms and functions. *J. Leukoc. Biol.* 75: 163–189.
5. Nathan, C. F., H. W. Murray, M. E. Wiebe, and B. Y. Rubin. 1983. Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J. Exp. Med.* 158: 670–689.
6. DiDonato, J. A., M. Hayakawa, D. M. Rothwarf, E. Zandi, and M. Karin. 1997. A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. *Nature* 388: 548–554.
7. Takeda, K., and S. Akira. 2004. TLR signaling pathways. *Semin. Immunol.* 16: 3–9.
8. Schroder, K., M. J. Sweet, and D. A. Hume. 2006. Signal integration between IFN-gamma and TLR signalling pathways in macrophages. *Immunobiology* 211: 511–524.
9. Farlik, M., B. Reutterer, C. Schindler, F. Greten, C. Vogl, M. Müller, and T. Decker. 2010. Nonconventional initiation complex assembly by STAT and NF-kappaB transcription factors regulates nitric oxide synthase expression. *Immunity* 33: 25–34.
10. Negishi, H., Y. Fujita, H. Yanai, S. Sakaguchi, X. Ouyang, M. Shinohara, H. Takayanagi, Y. Ohba, T. Taniguchi, and K. Honda. 2006. Evidence for li-

censing of IFN-gamma-induced IFN regulatory factor 1 transcription factor by MyD88 in Toll-like receptor-dependent gene induction program. *Proc. Natl. Acad. Sci. USA* 103: 15136–15141.
11. Durbin, J. E., R. Hackenmiller, M. C. Simon, and D. E. Levy. 1996. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell* 84: 443–450.
12. Dunning, M. J., M. L. Smith, M. E. Ritchie, and S. Tavaré. 2007. beadarray: R classes and methods for Illumina bead-based data. *Bioinformatics* 23: 2183–2184.
13. Reimers, M., and V. J. Carey. 2006. Bioconductor: an open source framework for bioinformatics and computational biology. *Methods Enzymol.* 411: 119–134.
14. Cairns, J. M., M. J. Dunning, M. E. Ritchie, R. Russell, and A. G. Lynch. 2008. BASH: a tool for managing BeadArray spatial artefacts. *Bioinformatics* 24: 2921–2922.
15. Shi, W., A. Oshlack, and G. K. Smyth. 2010. Optimizing the noise versus bias trade-off for Illumina whole genome expression BeadChips. *Nucleic Acids Res.* 38: e204.
16. Kauffmann, A., R. Gentleman, and W. Huber. 2009. arrayQualityMetrics—a bioconductor package for quality assessment of microarray data. *Bioinformatics* 25: 415–416.
17. Barbosa-Morais, N. L., M. J. Dunning, S. A. Samarajiwa, J. F. Darot, M. E. Ritchie, A. G. Lynch, and S. Tavaré. 2010. A re-annotation pipeline for Illumina BeadArrays: improving the interpretation of gene expression data. *Nucleic Acids Res.* 38: e17.
18. Smyth, G. K. 2004. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.* 3: e3.
19. van Lieshout, M. H., B. P. Scicluna, S. Florquin, and T. van der Poll. 2014. NLRP3 and ASC differentially affect the lung transcriptome during pneumococcal pneumonia. *Am. J. Respir. Cell Mol. Biol.* 50: 699–712.
20. Scicluna, B. P., C. van 't Veer, M. Nieuwdorp, K. Felsmann, B. Wlotzka, E. S. Stroes, and T. van der Poll. 2013. Role of tumor necrosis factor-α in the human systemic endotoxin-induced transcriptome. *PLoS One* 8: e79051.
21. Ho Sui, S. J., J. R. Mortimer, D. J. Arenillas, J. Brumm, C. J. Walsh, B. P. Kennedy, and W. W. Wasserman. 2005. oPOSSUM: identification of over-represented transcription factor binding sites in co-expressed genes. *Nucleic Acids Res.* 33: 3154–3164.
22. Heinz, S., C. Benner, N. Spann, E. Bertolino, Y. C. Lin, P. Laslo, J. X. Cheng, C. Murre, H. Singh, and C. K. Glass. 2010. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol. Cell* 38: 576–589.
23. Hoeksema, M. A., M. J. Gijbels, J. Van den Bossche, S. van der Velden, A. Sijm, A. E. Neele, T. Seijkens, J. L. Stöger, S. Meiler, M. C. Boshuizen, et al. 2014. Targeting macrophage histone deacetylase 3 stabilizes atherosclerotic lesions. *EMBO Mol. Med.* 6: 1124–1132.
24. Ostuni, R., V. Piccolo, I. Barozzi, S. Polletti, A. Termanini, S. Bonifacio, A. Curina, E. Prosperini, S. Ghisletti, and G. Natoli. 2013. Latent enhancers activated by stimulation in differentiated cells. *Cell* 152: 157–171.
25. Kuijpers, T. W., N. A. Maianski, A. T. Tool, K. Becker, B. Plecko, F. Valianpour, R. J. Wanders, R. Pereira, J. Van Hove, A. J. Verhoeven, et al. 2004. Neutrophils in Barth syndrome (BTHS) avidly bind annexin-V in the absence of apoptosis. *Blood* 103: 3915–3923.
26. Shahangian, A., E. K. Chow, X. Tian, J. R. Kang, A. Ghaffari, S. Y. Liu, J. A. Belperio, G. Cheng, and J. C. Deng. 2009. Type I IFNs mediate development of postinfluenza bacterial pneumonia in mice. *J. Clin. Invest.* 119: 1910–1920.
27. Burke, S. J., B. L. Updegraff, R. M. Bellich, M. R. Goff, D. Lu, S. C. Minkin, Jr., M. D. Karlstad, and J. J. Collier. 2013. Regulation of iNOS gene transcription by IL-1β and IFN-γ requires a coactivator exchange mechanism. *Mol. Endocrinol.* 27: 1724–1742.
28. Levy, D. E. 2010. NF-kappaB-ISGF3 transcription factor cooperation: coincidence detector or memory chip? *Immunity* 33: 1–2.
29. Bettelli, E., T. Korn, and V. K. Kuchroo. 2007. Th17: the third member of the effector T cell trilogy. *Curr. Opin. Immunol.* 19: 652–657.
30. Oliveira, S. H., C. Canetti, R. A. Ribeiro, and F. Q. Cunha. 2008. Neutrophil migration induced by IL-1beta depends upon LTB4 released by macrophages and upon TNF-alpha and IL-1beta released by mast cells. *Inflammation* 31: 36–46.
31. Xue, J., S. V. Schmidt, J. Sander, A. Draffehn, W. Krebs, I. Quester, D. De Nardo, T. D. Gohel, M. Emde, L. Schmidleithner, et al. 2014. Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. *Immunity* 40: 274–288.
32. Dalton, D. K., S. Pitts-Meek, S. Keshav, I. S. Figari, A. Bradley, and T. A. Stewart. 1993. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science* 259: 1739–1742.
33. Hoeksema, M. A., J. L. Stöger, and M. P. de Winther. 2012. Molecular pathways regulating macrophage polarization: implications for atherosclerosis. *Curr. Atheroscler. Rep.* 14: 254–263.
34. Takaoka, A., H. Yanai, S. Kondo, G. Duncan, H. Negishi, T. Mizutani, S. Kano, K. Honda, Y. Ohba, T. W. Mak, and T. Taniguchi. 2005. Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature* 434: 243–249.
35. Krausgruber, T., K. Blazek, T. Smallie, S. Alzabin, H. Lockstone, N. Sahgal, T. Hussell, M. Feldmann, and I. A. Udalova. 2011. IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. *Nat. Immunol.* 12: 231–238.
36. Liu, J., S. Cao, L. M. Herman, and X. Ma. 2003. Differential regulation of interleukin (IL)-12 p35 and p40 gene expression and interferon (IFN)-gamma-primed IL-12 production by IFN regulatory factor 1. *J. Exp. Med.* 198: 1265–1276.
37. Ohmori, Y., and T. A. Hamilton. 2001. Requirement for STAT1 in LPS-induced gene expression in macrophages. *J. Leukoc. Biol.* 69: 598–604.
38. Masters, S. L., L. A. Mielke, A. L. Cornish, C. E. Sutton, J. O'Donnell, L. H. Cengia, A. W. Roberts, I. P. Wicks, K. H. Mills, and B. A. Croker. 2010. Regulation of interleukin-1beta by interferon-gamma is species specific, limited by suppressor of cytokine signalling 1 and influences interleukin-17 production. *EMBO Rep.* 11: 640–646.
39. Schindler, R., P. Ghezzi, and C. A. Dinarello. 1989. Interferons as inhibitors of interleukin 1 induced interleukin 1 synthesis. *Lymphokine Res.* 8: 275–280.
40. De Boer, M. L., J. Hu, D. V. Kalvakolanu, J. D. Hasday, and A. S. Cross. 2001. IFN-gamma inhibits lipopolysaccharide-induced interleukin-1 beta in primary murine macrophages via a Stat1-dependent pathway. *J. Interferon Cytokine Res.* 21: 485–494.
41. Joshi, V. D., D. V. Kalvakolanu, W. Chen, L. Zhang, T. J. Kang, K. E. Thomas, S. N. Vogel, and A. S. Cross. 2006. A role for Stat1 in the regulation of lipopolysaccharide-induced interleukin-1beta expression. *J. Interferon Cytokine Res.* 26: 739–747.
42. Jang, S., K. W. Kelley, and R. W. Johnson. 2008. Luteolin reduces IL-6 production in microglia by inhibiting JNK phosphorylation and activation of AP-1. *Proc. Natl. Acad. Sci. USA* 105: 7534–7539.
43. Quintin, J., S. C. Cheng, J. W. van der Meer, and M. G. Netea. 2014. Innate immune memory: towards a better understanding of host defense mechanisms. *Curr. Opin. Immunol.* 29: 1–7.
44. Yoshimura, A., T. Naka, and M. Kubo. 2007. SOCS proteins, cytokine signalling and immune regulation. *Nat. Rev. Immunol.* 7: 454–465.
45. Rajasingh, J., E. Bord, C. Luedemann, J. Asai, H. Hamada, T. Thorne, G. Qin, D. Goukassian, Y. Zhu, D. W. Losordo, and R. Kishore. 2006. IL-10-induced TNF-alpha mRNA destabilization is mediated via IL-10 suppression of p38 MAP kinase activation and inhibition of HuR expression. *FASEB J.* 20: 2112–2114.
46. Robinson, K. M., S. M. Choi, K. J. McHugh, S. Mandalapu, R. I. Enelow, J. K. Kolls, and J. F. Alcorn. 2013. Influenza A exacerbates *Staphylococcus aureus* pneumonia by attenuating IL-1β production in mice. *J. Immunol.* 191: 5153–5159.
47. Kudva, A., E. V. Scheller, K. M. Robinson, C. R. Crowe, S. M. Choi, S. R. Slight, S. A. Khader, P. J. Dubin, R. I. Enelow, J. K. Kolls, and J. F. Alcorn. 2011. Influenza A inhibits Th17-mediated host defense against bacterial pneumonia in mice. *J. Immunol.* 186: 1666–1674.
48. Didierlaurent, A., J. Goulding, S. Patel, R. Snelgrove, L. Low, M. Bebien, T. Lawrence, L. S. van Rijt, B. N. Lambrecht, J. C. Sirard, and T. Hussell. 2008. Sustained desensitization to bacterial Toll-like receptor ligands after resolution of respiratory influenza infection. *J. Exp. Med.* 205: 323–329.
49. Sun, K., and D. W. Metzger. 2008. Inhibition of pulmonary antibacterial defense by interferon-gamma during recovery from influenza infection. *Nat. Med.* 14: 558–564.
50. O'Brien, K. L., M. I. Walters, J. Sellman, P. Quinlisk, H. Regnery, B. Schwartz, and S. F. Dowell. 2000. Severe pneumococcal pneumonia in previously healthy children: the role of preceding influenza infection. *Clin. Infect. Dis.* 30: 784–789.
51. Williams, D. J., M. Hall, T. V. Brogan, R. W. Farris, A. L. Myers, J. G. Newland, and S. S. Shah. 2011. Influenza coinfection and outcomes in children with complicated pneumonia. *Arch. Pediatr. Adolesc. Med.* 165: 506–512.