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Early Inhibition of IL-1β Expression by IFN-γ Is Mediated by Impaired Binding of NF-κB to the IL-1β Promoter but Is Independent of Nitric Oxide

Tatjana Eigenbrod, Konrad A. Bode, and Alexander H. Dalpke

The significance of bacterial RNA recognition for initiating innate immune responses against invading pathogens has only recently started to be elucidated. Bacterial RNA is an important trigger of inflammasome activation, resulting in caspase-1-dependent cleavage of pro–IL-1β into the active form. It was reported previously that prolonged treatment with IFN-γ can inhibit IL-1β production at the level of both transcription and Nlrp3 inflammasome activation in an NO-dependent manner. As a result of the delayed kinetics of NO generation after IFN-γ stimulation, these effects were only observed at later time points. We report that IFN-γ suppressed bacterial RNA and LPS induced IL-1β transcription in primary murine macrophages and dendritic cells by an additional, very rapid mechanism that was independent of NO. Costimulation with IFN-γ selectively attenuated binding of NF-κB p65 to the IL-1β promoter, thus representing a novel mechanism of IL-1β inhibition by IFN-γ. Transcriptional silencing was specific for IL-1β because expression of other proinflammatory cytokines, such as TNF, IL-6, and IL-12p40, was not affected. Furthermore, by suppressing IL-1β production, IFN-γ impaired differentiation of TH17 cells and production of neutrophil chemotactic factor CXCL1 in vitro. The findings provide evidence for a rapid immune-modulating effect of IFN-γ independent of NO.

Interleukin-1β is a prototypic proinflammatory cytokine mediating a plethora of effects, including induction of fever, mobilization of immune cells from the bone marrow, and upregulation of adhesion factors and chemokines, which finally results in neutrophilic influx at the site of inflammation (1). Moreover, IL-1β promotes differentiation of T cells into the TH17 lineage (2, 3). IL-1β is mainly synthesized by monocytes, macrophages, and dendritic cells (DCs) in an NF-κB–dependent pathway (4–6) following activation by a variety of pathogen-associated molecular patterns (PAMPs). IL-1β is transcribed as an inactive 31-kDa precursor protein, pro–IL-1β, which is cleaved into its active form by caspase-1. Caspase-1 is an inflammatory caspase that is activated by autophosphorylation with a macromolecular complex called the inflammasome. The best-characterized inflammasome is the Nlrp3 inflammasome that consists of the NLR family member Nlrp3, the adapter molecule Asc, and (pro) caspase-1 (7). The Nlrp3 inflammasome can be activated by a panel of different stimuli with TLR ligands or inflammatory cytokines providing the first or so-called “priming” signal and a second signal that can be delivered in the form of ATP, pore-forming bacterial toxins, or crystalline substances (7–9). Another trigger for Nlrp3 inflammasome activation is bacterial RNA (bRNA), which might serve as both signal 1 and signal 2 for inflammasome activation (10–13). Although the critical roles for bacterial DNA and viral RNA in the induction of innate immune responses have been acknowledged for long time, the importance of bRNA recognition has only recently started to be unraveled. It was demonstrated that intracellular delivery of bRNA induced secretion of type I IFNs, as well as proinflammatory cytokines, including TNF, IL-12p40, or IL-1β (10, 14–17). In plasmacytoid DCs, bRNA-induced production of type I IFNs is mediated by TLR7 (15), whereas the murine receptor TLR13 was identified only recently to sense bRNA in both bone marrow–derived macrophages (BMDMs) and DCs by recognizing a unique nucleotide motif present in the bacterial 23S rRNA (18, 19). Activation of TLR13 triggers the recruitment of the common TLR adapter molecule MyD88, resulting in activation of NF-κB and transcription of NF-κB–dependent cytokines (19). Moreover, bRNA was shown to play a key role in innate immune defense against a variety of Gram-positive bacteria by representing the major PAMP of those pathogens (14).

Although the innate immune system was thought to initiate unspecific responses, it now turns out that, depending on the invading pathogen, different components of innate immunity are activated. TH cells are involved in this orchestration through the secretion of cytokines. Among those, IFN-γ has important functions. The main target cells of IFN-γ are macrophages and DCs, in which IFN-γ stimulates phagocytosis, Ag processing, and presentation and enhances the production of reactive oxygen species, as well as NO, ultimately resulting in improved elimination especially of intracellular pathogens (20). Further, IFN-γ enhances TH1 cell development. Despite those clearly proinflammatory features, IFN-γ was described to suppress the generation of active IL-1β. Two different mechanisms have been implicated in this process. On the one hand, it was reported that IFN-γ inhibits IL-1β at the level of transcription (21–23). On the other hand, a very recent publication demonstrated that IFN-γ can inhibit Nlrp3 inflammasome activation, thus preventing cleavage of pro–IL-1β into the active form (24). Mechanistically, a critical role for IFN-

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Abbreviations used in this article: BMDM, bone marrow–derived macrophage; bRNA, bacterial RNA; CHI, chromatin immunoprecipitation; DC, dendritic cell; DC-CM, dendritic cell–conditioned medium; iNOS, inducible NO synthase; o.n., overnight; PAMP, pathogen-associated molecular pattern; WT, wild-type.

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γ-induced NO was demonstrated in both cases (22, 24). Because NO production occurs with delayed kinetics (25–27), these effects were observed most prominently after long-term stimulation. We describe in this article that IFN-γ mediates the downregulation of bRNA and LPS-induced IL-1β via an additional, very rapid mechanism that is independent of NO. IFN-γ suppresses the transcription of IL-1β, but not of TNF, IL-6, and IL-12p40, by selectively inhibiting binding of NF-κB p65 to the IL-1β promoter. In addition, IFN-γ-dependent suppression of IL-1β attenuated effector functions of this cytokine, as shown by impaired differentiation of Th17 cells and CXCL1 production in target cells.

Materials and Methods

Reagents

RPMI 1640 containing stable glutamine and DMEM was purchased from Biochrom (Berlin, Germany), FCS, sodium pyruvate, Lipofectamine 2000, and TRIZol reagent were from Life Technologies (Darmstadt, Germany). Utrapure LPS from Salmonella minnesota was provided by U. Seydel (Forschungszentrum Borstel, Sülfeld, Germany). Silica and ATP were purchased from Sigma (Munich, Germany). All recombinant cytokines, as well as rIL-1R antagonist, were purchased from PeproTech (Hamburg, Germany). Anti-CD3 and anti-CD28 were from BD (Heidelberg, Germany). Actinomycin D was from Calbiochem (Merck Millipore, Darmstadt, Germany), and L-NIL dihydrochloride was from Biotrend (Köln, Germany).

Mouse strains

SOCS1−/−/IFNγ−/− and IFNγ−/− mice (28) were obtained from Martin Bogdan (University Hospital Erlangen, Erlangen, Germany), and inducible NO synthase (iNOS)-deficient mice (30) were obtained from Falk Weih (Fritz Lipmann Institute, Jena, Germany). Wild-type (WT) C57BL/6 mice were main-

tained in the animal facility of the University of Heidelberg. All animal studies were approved by the local authorities.

Cell isolation and differentiation

Bone marrow GM-CSF–derived myeloid DCs and BMDMs were prepared from 8–12 wk-old mice. For differentiation of DCs, 8 × 10^5 bone marrow cells were seeded into 15-cm cell culture plates in differentiation medium (RPMI 1640 containing stable glutamine, supplemented with 10% FCS, 1% penicillin/streptomycin, 0.05 mM 2-ME). Cell culture supernatants of a plasmacytoma X63 cell line producing murine GM-CSF were added as a source of GM-CSF. Immature DCs (CD11c+, B220−) were harvested at day 8. For generation of BMDMs, bone marrow cells were seeded into 15-cm Petri dishes in DMEM supplemented with 30% L929 supernatant, 10% FCS, and 1% penicillin/streptomycin. Mesothelial cells were prepared as described previously (31). Briefly, the peritoneum and intact i.p. organs were digested with 0.25% trypsin-EDTA solution for 60 min at 37°C. Intact tissues and tissue debris were discarded, and the remaining cells were resuspended in DMEM containing stable glutamine supplemented with 15% heat-inactivated FCS, 1 mM sodium pyruvate, and antibiotics and cultured overnight (o.n.). The next day, nonadherent cells were removed, and fresh media were added. Mesothelial cells were used at passage two.

Cell stimulation and transfection

DCs were stimulated in RPMI 1640 supplemented with 10% FCS, and macrophages were stimulated in DMEM supplemented with 10% FCS. For ELISA experiments, DCs were stimulated at 2 × 10^5/well in a 96-well plate, and BMDMs were stimulated at 2 × 10^5/well in a 48-well plate. For Western blot and quantitative PCR experiments, cells were stimulated at 5 × 10^4/well in a 48-well plate (DCs) or 24-well plate (BMDMs). Unless specified otherwise, cells were transfected with 5 μg/ml bRNA derived from Staphylococcus aureus complexed with Lipofectamine 2000 at a ratio of 1 μL Lipofectamine 2000/1 μg RNA. IFN-γ was added at the time of bRNA transfection at a concentration of 20 ng/ml, unless stated otherwise. In some experiments, cells were incubated with LPS (100 ng/ml) for 1 h, followed by stimulation with silica for 5 h. For actinomycin D experiments, cells were transfected for 4 h with bRNA prior to addition of actinomycin D at a concentration of 5 μg/ml. Cells were harvested at the indicated time after the addition of actinomycin D for RNA isolation. Mesothelial cells were plated at a concentration of 5 × 10^4/well in a 48-

well plate and stimulated after o.n. adherence with serial dilutions of DC-conditioned medium (DC-CM), prepared as described below.

DC-CM

For Th17-differentiation experiments, DC-CM was prepared by o.n. stimulation of DCs with bRNA in the presence or absence of IFN-γ. For stimulation of primary mesothelial cells, DC-CM was prepared by stimulation with CpG oligodeoxynucleotide (1 μM) for 1 h, followed by incubation with silica for 5 h. Cell culture supernatants were collected, centrifuged at 1500 rpm for 5 min to remove residual cells, and stored at −80°C for later use.

In vitro differentiation of Th17 cells

Spleens were removed from 8–12-wk-old mice and passed through a 100-

μm cell strainer, and erythrocytes were lysed. Afterwards, splenocytes were centrifuged, resuspended in RPMI 1640 supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin, and plated on a 96-

well plate at a final concentration of 1 × 10^5/ml in the presence of plate-

bound anti-CD3 (2 μg/ml), soluble anti-CD28 (5 μg/ml), rTGF-β (10 ng/ml), rL-23 (5 ng/ml), and anti-IFN-γ (2 μg/ml). Conditioned DC medium, prepared as described above, was added, and IL-17 levels in cell-free culture supernatants were measured by ELISA after 4 d. Where indicated, spleno-

cytes were also treated with IL-1RA to inhibit IL-1β–induced signaling.

Bacterial culture and preparation of total bRNA

S. aureus and Streptococcus pyogenes were grown in Luria–Bertani medium or brain heart infusion broth, respectively, and harvested within the mid log phase growth. After a digestion step with lysosome (1 h at 40 mg/ml), bRNA from S. aureus was isolated using TRIzol reagent, according to the manufacturer’s protocol. The obtained RNA underwent a further purification step using the RNeasy mini kit (Qiagen, Hilden, Germany), including an on-

column DNA digestion, according to the manufacturer’s instructions. Purity of the bRNA preparations was validated by determining the 260/230 nm and 260/280 nm extinction ratio by NanoDrop (Thermo Scientific).

Isolation of mammalian RNA for quantitative real-time PCR

Total RNA from cells was isolated using the peqGold Total RNA kit, including on-column DNA digestion (Peqlab, Erlangen, Germany). RNA was transcribed into cDNA using the high-capacity cDNA reverse-transcription kit (Applied Biosystems, Woolsont, U.K.), according to the manufacturer’s instructions. Quantitative real-time PCR was performed with SYBR Green (Applied Biosystems) using a standard protocol. Identity of amplicons was checked by melting curve analysis, and no reverse transcriptase or no template controls were included. Analyses were performed in duplicates. Primer sequences for quantitative real-time PCR were as follows: β-actin, forward TGG GGG CCT CAA AGG AAA GAG 9 -3 ; reverse 9 -CTT TGG TTC TTC TGC TTC-3 ; pro–IL-1β, forward 9 -GAG AAA CTC TGC TGT CCT CAC CCA-3 ; reverse 9 -AAA GGC AGA GTC TTC GGT-3 ; pro–IL-12 p40, forward 9 -GAT CTC TGG TTC TGC TTC CCA-3 ; reverse 9 -ACA GTG CTC TGT CAC CCA-3 ; pro–IL-16, forward 9 -AAA CTT GGG GTT TGG TTC TCC-3 ; reverse 9 -GGA CAC AGG AGA GAG GAG-3 ; pro–IL-18, forward 9 -GGA CAC AGG AGA GAG GAG-3 ; reverse 9 -CAA CAA TGG AAC TGG AAA-3 . All primers were custom synthesized by MWG-Biotech (Ebersberg, Germany).

Immunoblotting and cytokine measurements

For Western blot analysis of IL-1β expression, complete cell culture supernatants were collected, together with the cells, in a lysis buffer containing 1% Nonidet P-40, supplemented with protease inhibitors leupeptin, aprotinin, and pepstatin A (1 μg/ml) and 4-(2-aminoethyl)benz-

zensulfonylfluorid (1 mM). Proteins were separated by 12% SDS-PAGE without prior precipitation, and membranes were probed with a goat anti-mouse IL-1β Ab (M-20; Santa Cruz Biotechnology). For Western blot analysis of MAPK and NF-κB activation, BMDMs were starved in DMEM without FCS for 1 h prior to stimulation. Membranes were probed with Abs against IκBα, phosphorylated IκBα, phosphorylated p38, phosphory-

lated JNK, phosphorylated ERK, NF-κB p65, NF-κB p65 phospho–Ser586, NF-κB p65 phospho–Ser68, NF-κB p65 phospho–Ser468 (all from Cell Signaling Technology), NF-κB p65 phospho–Ser311 or NF-κB p65 phospho–Ser276 (Assay Biotech). For cytokine measurement, levels of murine IL-1β, TNF, IL-6, IL-12p40 (BD), IL-17, and CXCL1 (Bio-Systems, Wiesbaden, Germany) were detected in cell-free supernatants by ELISA, according to the manufacturer’s instructions.

Griess assay for detection of NO2−

For detection of NO2− that is formed by spontaneous oxidation of NO under physiological conditions, Griess assay of cell-free culture super-

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natants was performed (32). Absorption was measured at 550 nm, and 
NO2− concentration in the samples was calculated using a standard curve 
derived from serial dilution of NaNO2.

Quantification of NF-κB activation
For quantification of NF-κB activation, BMDMs were starved in DMEM 
without FCS for 1 h prior to transfection with bRNA in the absence or 
presence of IFN-γ. Cells were harvested, and nuclear extracts were pre-
pared using the Nuclear Extraction Kit from Active Motif (Carlsbad, CA).
Afterwards, quantification of NF-κB subunit activation was performed 
using the TransAM NF-κB family transcription factor assay kit (Active 
Motif), according to the manufacturer’s instructions. A total of 5 μg nu-
clear extracts was used for each NF-κB subunit investigated.

Chromatin immunoprecipitation
Chromatin immunoprecipitation (ChIP) was performed, as described pre-
viously (33), with an Ab recognizing NF-κB p65 (C-20; Santa Cruz 
Biotechnology). The abundance of the DNA fragment in the i.p. samples 
was expressed as a percentage of input and was calculated using the fol-
lowing equation: 2^[-ΔΔCt]/(2^[-ΔΔCt]input + 2^[-ΔΔCt]) × 100 ChIP primers for the 
IL-1β promoter were described previously (34); sense 5′-CCC CTA AGA 
ATT CCC ATC AAG C-3′, antisense 5′-GAG CTG TGA AAT TTT CCC 
TTG G-3′. ChIP primers for the IL-6 promoter were sense 5′-CCC ACC 
CTC CAA CAA AGA TT-3′ and antisense 5′-GCT CCA CAG CAG AAT 
GCA TTA-3′.

Nuclease-protection assay
Chromatin accessibility in murine BMDMs stimulated with bRNA in the 
absence or presence of IFN-γ was assessed by nuclease protection assay 
using the EpiQ Chromatin Kit Data Analysis Tool provided by the supplier.

Statistical analysis
Statistical significance between groups was determined by the two-tailed 
Student t test. Differences were considered significant for p < 0.05.

Results
Costimulation with IFN-γ selectively suppresses secretion of 
IL-1β but does not interfere with release of TNF, IL-6, 
and IL-12p40
Given the emerging role of bRNA in initiating innate immune 
responses, we set out to investigate how IFN-γ influences bRNA-
mediated cytokine production in BMDMs and DCs. To this end, 
cells were transfected with purified bRNA, because lipofection 
facilitates its delivery into the endosome where TLR13 is 
localized (19). Simultaneous administration of IFN-γ at the time of 
bRNA transfection dose-dependently attenuated IL-1β secretion 
by both DCs and BMDMs (Fig. 1A, 1B). The same was observed 
for IL-1β secretion induced by classical inflammasome activator 
Silica in LPS-primed cells (Fig. 1B, right panel). Strikingly, co-
stimulation with IFN-γ did not inhibit bRNA-induced production of 
other proinflammatory cytokines, including TNF and IL-6, and 
it enhanced IL-12p40 secretion (Fig. 1C, 1D), which is in line with 
the long-known priming effects of IFN-γ on macrophages (20).
We (10) and other investigators (14) showed previously that innate 
immunity activation by streptococci is mediated mainly through 
recognition of their RNA. Similar to the data using transfected 
bRNA, costimulation with IFN-γ inhibited IL-1β release in re-
sponse to S. pyogenes infection but potentiated TNF (data not 
shown) and IL-12p40 production (Fig. 1E). IFN-γ signaling is 
known to activate SOCS-1, which acts as a negative-feedback 
hitbor to terminate IFN-γ-dependent responses. Therefore, the 
influence of SOCS-1 on IFN-γ-mediated IL-1β inhibition was tested.

Because Socs1−/− mice are embryonically lethal, cells derived from 
Socs1−/−/Ifng−/− mice were investigated. In line with the role of 
SOCS-1 as a negative regulator of IFN-γ signaling, SOCS-1/IFN-γ 
double-deficient DCs were more sensitive to IL-1β suppression by 
exogenously added IFN-γ than were IFN-γ-deficient control cells 
(Fig. 1F).

IFN-γ inhibits IL-1β transcription without affecting mRNA 
stability
It was next investigated at which level inhibition of IL-1β pro-
duction occurred. Although a minor suppression of bRNA-
mediated caspase-1 activation was detected (data not shown), 
the more prominent regulation was observed at the level of pro-
IL-1β protein expression (Fig. 2A). To determine whether this 
effect was due to transcriptional or translational regulation, pro-
IL-1β mRNA was measured in a time-course experiment. Si-
multaneous administration of IFN-γ suppressed bRNA and LPS-
induced expression of pro–IL-1β mature mRNA, as well as pro– 
IL-1β premRNA in both DCs and BMDMs (Fig. 2B–D). Notably, 
suppression of IL-1β transcription by IFN-γ had already occurred 
at the earliest times investigated (i.e., within 30 min). Downregu-
lation of pro–IL-1β premRNA indicated that the regulation oc-
curred at the level of transcription. To rule out an influence of
IFN-γ on pro–IL-1β mRNA stability, cells were treated with the transcriptional inhibitor actinomycin D 4 h after stimulation with bRNA. During the follow-up period, no difference in the degradation rate of pro–IL-1β mRNA was detected in IFN-γ-treated cells compared with control cells (Fig. 2E). In the next step, it was investigated how the timing of IFN-γ application (i.e., before or after transfection of bRNA) influenced the suppressive effect on IL-1β transcription. Treatment with IFN-γ for 2 h prior to bRNA transfection was slightly more efficient in downregulating IL-1β mRNA than was concomitant stimulation. Notably, when IFN-γ was added 1 or 2 h after transfection of bRNA, pro–IL-1β premRNA levels declined rapidly compared with those observed upon simultaneous stimulation (Fig. 2F).

**Inhibition of IL-1β secretion by costimulation with IFN-γ is independent of NO**

It was reported that IFN-γ can inhibit IL-1β production through intermediate production of NO (22, 24). However, NO synthesis is known to be a late event after IFN-γ stimulation (25–27). Indeed, in the current study, NO was detectable only at later time points (24 h) in bRNA+IFN-γ–treated cells, as assessed by Griess assay (Fig. 3A). Thus, it was unlikely that NO was involved in the immediate effects of IFN-γ on IL-1β production that we observed. In line with these data, iNOS inhibitor NIL did not exert any effect on IL-1β transcription in cells that were stimulated simultaneously with bRNA and IFN-γ for 2–6 h (Fig. 3B). Likewise, NIL did not antagonize inhibition of IL-1β secretion in cells that were transfected o.n. with bRNA in the presence of IFN-γ (Fig. 3C). Only when cells were pretreated for 24 h with IFN-γ prior to o.n. transfection of bRNA did inhibition of NO production by NIL reverse IFN-γ–mediated downregulation of IL-1β secretion (Fig. 3D), confirming the results of previous studies (22, 24). Of note, under these conditions, downregulation of IL-1β was accompanied by increased NO concentrations and vice versa (compare Fig. 3D and 3E). Similar results were obtained in inos−/− cells. iNOS deficiency had no influence on IL-1β mRNA expression at early time points (Fig. 3F) and showed only marginal effects on IL-1β secretion after o.n. stimulation (Fig. 3G). Again, a prominent contribution of NO was only seen upon long-term IFN-γ stimulation (Fig. 3G). In conclusion, these data clearly demonstrate that IFN-γ-induced NO is not involved in the early suppressive effects of IFN-γ on IL-1β.

**Costimulation with INF-γ does not interfere with general MAPK and NF-κB activation**

To determine the mechanism underlying IFN-γ–mediated suppression of IL-1β transcription, it was next investigated whether IFN-γ interfered with activation of NF-κB and MAPKs. To this end, BMDMs and DCs were stimulated with bRNA in the presence or absence of IFN-γ, and cell lysates were immunoblotted with Abs recognizing activated forms of NF-κB, p38, ERK, and JNK. bRNA induced rapid phosphorylation and degradation of IκBα, as well as phosphorylation of the investigated MAPKs. Activation of these signaling pathways was not impaired upon co-stimulation with IFN-γ (Fig. 4A, 4B). To exclude that treatment with IFN-γ might affect nuclear translocation of activated NF-κB or interfere with activation of selective NF-κB subunits that cannot be detected by IκBα phosphorylation and degradation, cells...
Expression of pro–IL-1\(\beta\) WT and iNOS–deficient mice were stimulated as indicated. (PCR. Levels of secreted IL-1\(\beta\) Unstimulated control; ns, not significant. at least three independent experiments. * represent the mean

were stimulated as before, and nuclear extracts were prepared. Activation of NF-κB subunits in the nuclear extracts was investigated using the TransAM NF-κB transcription factor assay kit, which quantifies DNA binding of NK-κB subunits to a consensus sequence. However, as depicted in Fig. 4C, IFN-γ did not impair NF-κB subunit activation by bRNA.

IFN-γ inhibits binding of NF-κB p65 to the IL-1β promoter

Because no general effects of IFN-γ on NF-κB activation were detected, despite NF-κB being an important activator of pro–IL-1β transcription, the hypothesis was tested that IFN-γ might interfere with recruitment of NF-κB p65 to the IL-1β promoter. To this end, BMDMs were transfected with bRNA or stimulated with LPS in the presence or absence of IFN-γ, and ChIP for NF-κB p65 was performed. As expected, both bRNA and LPS enhanced p65 binding to the IL-1β promoter. Notably, treatment with IFN-γ inhibited p65 recruitment to the IL-1β promoter induced by both stimuli (Fig. 5A, left panel). This inhibition was specific, because IFN-γ did not interfere with NF-κB p65 recruitment to the IL-6 promoter (Fig. 5A, right panel), which is in line with the missing effect of IFN-γ on IL-6 production (Fig. 1C, 1D).

We next aimed to unravel the mechanism responsible for the reduced IL-1β promoter binding of p65 in IFN-γ–stimulated cells. It is well established that nuclear functions of NF-κB are regulated by posttranslational modifications, including phosphorylation of serine residues. Thus, a possible mechanism underlying the observed phenotype is that IFN-γ induces posttranslational modifications of p65, selectively disabling it from IL-1β promoter binding. To test this hypothesis, BMDMs were transfected with bRNA or LPS in the presence or absence of IFN-γ, and cell lysates were immunoblotted with Abs specifically recognizing the indicated p65 serine phosphorylation sites. However, costimulation with IFN-γ did not alter bRNA- or LPS-induced phosphorylation of p65 at the four residues investigated (Fig. 5C, 5D).

It was reported that RelB was critical for transcriptional silencing of IL-1β in LPS-tolerized THP-1 cells. To test whether RelB was also relevant for the specific suppression of p65 recruitment to the IL-1β promoter observed in the current study, cells derived from RelB−/− mice were investigated. WT and RelB-deficient cells showed a similar increase in pro–IL-1β transcription upon stimulation with bRNA and LPS and displayed a similar level of transcriptional suppression by IFN-γ (Fig. 5D, 5E). The data obtained on the mRNA level were confirmed by IL-1β protein secreted into cell culture supernatants (Fig. 5F).

Next, a nuclease-protection assay was performed to investigate whether treatment with IFN-γ affects chromatin accessibility of the IL-1β promoter. In line with other reports, the IL-1β promoter was highly accessible, even in unstimulated cells, and it showed only marginal changes upon stimulation with bRNA in the presence or absence of IFN-γ (Fig. 5G). Thus, it can be concluded that IFN-γ selectively inhibits recruitment of NF-κB p65 to the IL-1β promoter.
addition, IL-1 is the recruitment of neutrophils to the site of inflammation. In to inhibit IL-1–induced signaling. A main function of Th17 cells release was observed upon treatment of splenocytes with IL-1RA receiving bRNA-conditioned medium. A similar inhibition in IL-17 DCs produced lower amounts of IL-17 compared with those re-

CXCL1 secretion. In line with an important role for IL-1

Next investigated how this reduction in IL-1 affects Th17 differentiation. As depicted in Fig. 6A, spleno-

promoter, but this effect is independent of RelB, chromatin ac-

accessibility at the IL-1β promoter, or the serine phosphorylation status of p65.

IL-1β–dependent suppression of IL-1β inhibits differentiation of Th17 cells and production of chemotactic factor CXCL1

IL-1β–mediated enhancement of T cell differentiation into the Th17 lineage was demonstrated both in vitro and in vivo. Because IFN-γ suppressed IL-1β production in the current study, it was next investigated how this reduction in IL-1β affects Th17 differenti-

tion in vitro. To this end, splenocytes were stimulated with DC-CM, prepared as described in Materials and Methods, in the presence of plate-bound anti-CD3, soluble anti-CD28, TGF-β, IL-

23, and an IFN-γ–blocking Ab to eliminate the direct effects of IFN-γ on Th17 differentiation. As depicted in Fig. 6A, spleno-

cytes incubated with DC-CM derived from bRNA+IFN-γ–treated DCs produced lower amounts of IL-17 compared with those receiving bRNA-conditioned medium. A similar inhibition in IL-17 release was observed upon treatment of splenocytes with IL-1RA to inhibit IL-1–induced signaling. A main function of Th17 cells is the recruitment of neutrophils to the site of inflammation. In addition, IL-1β is a strong inducer of CXCL1, a major neutrophil-

attracting chemokine. Therefore, it was tested how IFN-γ–dependent inhibition of IL-1β expression influences CXCL1 secretion by primary murine mesothelial cells that produce large amounts of this chemokine in response to IL-1 stimulation (Fig. 6B) (10). Mesothelial cells were incubated with serial dilutions of DC-CM derived from the stimulation of DCs with CpG-silica in the absence or presence of IFN-γ. CpG was chosen because mesothelial cells do not express CpG receptor TLR9 (35), thus eliminating the potential interference of TLR-induced versus DC-CM–induced CXCL1 secretion. In line with an important role for IL-1β in CXCL1 induction, mesothelial cells stimulated with DC-CM from CpG-silica+IFN-γ–treated cells, containing low amounts of IL-1β, showed reduced CXCL1 release compared with CpG-silica DC-

CM–treated control cells (Fig. 6C).

Discussion

Much progress has been made in unraveling the crucial role of bRNA recognition in initiating innate immune responses against invading pathogens. Sensing of bRNA seems to be of special im-

FIGURE 5. IFN-γ silences IL-1β transcription by inhibiting binding of NF-κB p65 to the IL-1β promoter. (A) BMDMs were transfected with bRNA or stimulated with LPS in the presence or absence of IFN-γ, as indicated. ChIP assay was performed from nuclear extracts with an anti-NF-κB p65 Ab, and recruitment to the IL-1β promoter (left panel) or IL-6 promoter (right panel) was measured by quantitative real-time PCR. Abundance of the DNA fragment in the i.p. samples was expressed as a percentage of input. A no-Ab control (no Ab) was used to control unspecific binding. Values represent the mean ± SD of triplicate wells. BMDMs were stimulated with bRNA (B) or LPS (C) in the presence or absence of IFN-γ. Cellular extracts were immunoblotted with Abs recognizing NF-κB p65 or the indicated NF-κB p65 phosphorylation site. BMDMs (D, E) or DCs (F) derived from WT or RelB-deficient mice were treated as before, and expression of pro–IL-1β premRNA (D, E) or IL-1β secretion into culture supernatants (F) was analyzed. Results are representative of at least three independent experiments. (G) Chromatin accessibility at the GAPDH and IL-1β promoter was assessed by nuclelease-protection assay. Values represent pooled data from three independent experiments (± SD). *p < 0.05, **p < 0.01. co, Unstimulated control; Nig, nigericin.

FIGURE 6. IFN-γ–induced downregulation of IL-1β suppresses differen-
tiation of Th17 cells and diminishes CXCL1 release in target cells. (A) Primary splenocytes were stimulated with DC-CM derived from bRNA or bRNA+IFN-γ–stimulated cells in the presence of plate-bound anti-CD3 (2 μg/ml), soluble anti-CD28 (5 μg/ml), rTGF-β (10 ng/ml), rIL-23 (5 ng/ml), anti–IFN-γ (2 μg/ml), and IL-1RA (5 μg/ml). IL-17 production was determined in cell-free culture supernatants by ELISA. Primary murine mesothelial cells were stimulated with rIL-1β at 1000, 300, 100, 30, or 10 pg/ml (B) or incubated with serial dilutions of DC-CM derived from CpG-silica or CpG+silica+IFN-γ (DC-CM-g)–stimulated DC. (C) CXCL1 production was measured after 6 h by ELISA. Values represent the mean ± SD of duplicate wells. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01. co, Unstimulated control.
to Gram-positive bacteria (14). Moreover, bRNA can activate the Nlrp3 inflammasome, resulting in caspase-1-mediated cleavage of pro–IL-1β into the biologically active form (10, 11, 13).

It was reported that IFN-γ can downregulate IL-1β production in response to LPS stimulation and infection with *Mycobacterium tuberculosis*, as well as in models of autoimmune diseases, like collagen-induced arthritis, both in vitro and in vivo (21–24, 36, 37). Several reports indicated that IFN-γ-mediated suppression of LPS-induced IL-1β occurred at the level of transcription (21–23). Although most studies did not further analyze the underlying mechanism, De Boer et al. (22) demonstrated that this transcriptional silencing was crucially dependent on IFN-γ-induced NO, because pharmacological inhibition of iNOS reversed the IFN-γ–mediated phenotype. Furthermore, LPS itself can trigger NO synthesis, and it was suggested that LPS-induced NO could act as a negative-feedback inhibitor to limit LPS-mediated IL-1β transcription, thus preventing overshooting immune responses (26, 38). The exact mechanism accounting for NO-mediated impairment of IL-1β transcription remains poorly defined, but NO-dependent S-nitrosylation of NF-κB p50 was proposed in one publication (38). Still, it is unclear how S-nitrosylation of NF-κB could specifically inhibit transcription of IL-1β but not other proinflammatory cytokines. More recently, it was shown that IFN-γ can suppress IL-1β production on an additional level (i.e., by interference with inflammasome assembly). IFN-γ–induced inhibition of caspase-1 activation was caused by NO-mediated S-nitrosylation of Nlrp3 and, therefore, was specific to the Nlrp3 inflammasome without affecting other inflammasomes (24). Accordingly, IFN-γ−/− and NOS2−/− mice showed enhanced caspase-1 activation and IL-1β secretion in an in vivo model of *M. tuberculosis* infection (24). However, generation of NO is a rather late event and does not occur in the first hours after stimulation (Fig. 3A) (25–27). This is in line with the notion that suppression of inflammasome activation was most prominent when cells were treated with IFN-γ for 24 h prior to *M. tuberculosis* infection (24), and LPS-induced IL-1β secretion was increased by iNOS inhibition after 20 h, but not 6 h, of incubation (26). In the current study, we confirmed NO-dependent inhibition of IL-1β release in cells that were exposed to IFN-γ for 24 h prior to transfection of bRNA.

Additionally, we demonstrate in this study that IFN-γ attenuates IL-1β production in murine BMDMs and DCs by another, very fast mechanism involving impaired recruitment of NF-κB p65 to the IL-1β promoter. This inhibition was specific, because other proinflammatory cytokines, including TNF, IL-6, and IL-12p40, were not affected. The remarkably rapid kinetics of transcriptional IL-1β silencing indicated a direct mechanism that was independent of inducible mediators, such as NO. Indeed, pharmacological inhibition of NO synthesis did not interfere with these instantaneous effects, and NO was not detected in culture supernatants at early time points when IL-1β suppression was already observed. This additional direct pathway of IL-1β suppression might be important for ensuring that IFN-γ can exert immune modulating, anti-inflammatory functions without delay. Although the current study focused primarily on bRNA as an emerging PAMP, downregulation of IL-1β was not stimulus specific and also occurred upon treatment with LPS and infection with *S. pyogenes*. In conclusion, our data add another layer of complexity to IFN-γ–dependent IL-1β regulation by unraveling an immediate NO-independent transcriptional silencing, in addition to the previously reported delayed, NO-dependent regulation of both IL-1β transcription and Nlrp3 inflammasome activation. In addition, IL-1β suppression can be elicited by IFN-γ, as well as by type I IFN (37, 39, 40). In BMDMs, stimulation with IFN-β attenuated IL-1β transcription by intermediate generation of IL-10, which, in turn, suppressed IL-1β transcription in a STAT3-dependent manner. In addition, IFN-β inhibited caspase-1 activation by an IL-10–independent mechanism (39). A recent study extended these findings by demonstrating that IFN-β–induced NO was involved in this inhibitory effect, again through S-nitrosylation of Nlrp3 (41).

In resting monocytes and macrophages, the IL-1β promoter is packaged into a nucleosome-free, highly accessible chromatin structure that remains largely unchanged upon stimulation, whereas the IL-1β promoter is inaccessible in undifferentiated promyelocyte cells, as well as in primary B and T cells (6, 34, 42). Moreover, several transcription factors, including IRF8, Pu.1, nonphosphorylated STAT1, and CEBPβ, were described to be constitutively bound to the IL-1β promoter and/or enhancer elements, enabling rapid gene transcription upon cellular activation (6, 34, 43–45). Recruitment of NF-κB is one of the crucial steps for initiating IL-1β transcription after stimulation (4–6, 46). Mechanistically, we demonstrated that rapid, NO-independent suppression of IL-1β by IFN-γ was due to selective impairment of NF-κB p65 binding to the IL-1β promoter. However, the underlying pathways remain unclear. In line with the selective inhibition of IL-1β, but not other proinflammatory cytokines, such as IL-6, TNF, and IL-12p40, IFN-γ did not interfere, in general, with cytosolic activation and nuclear translocation of NF-κB. The function of NF-κB is known to be regulated by a variety of posttranscriptional modifications, including phosphorylation, acetylation, and ubiquitination, which can either enhance or inhibit transcriptional activity, in part, in a gene-specific manner. Notably, p65 phosphorylation at different serine residues plays an important role in fine-tuning NF-κB activity (47–50). Thus, it was possible that IFN-γ suppresses IL-1β expression by altering the modification pattern of NF-κB p65. LPS stimulation caused phosphorylation of NF-κB p65 at Ser729, Ser311, Ser468, and Ser336, whereas bRNA induced phosphorylation at Ser468 and Ser336, and, to a lesser extent, Ser729 and Ser311. However, no differences in the phosphorylation pattern were observed upon treatment with IFN-γ. Nevertheless, because this represents only a subset of known modifications, it does not exclude that IFN-γ might affect modifications that were not investigated in this study.

It is known that IL-1β is transcriptionally silenced in LPS-tolerized cells, despite normal cytosolic activation and accumulation of NF-κB p65 in the nucleus, thus resembling the data presented in this study. NF-κB subunit RelB was found to be both necessary and sufficient for mediating this phenotype. Mechanistically, RelB induced formation of facultative heterochromatin by recruitment of H3K9 methyltransferase G9a and HP1, thus preventing NF-κB p65 binding to the IL-1β promoter (51–53). However, RelB was dispensable for the selective inhibition of IL-1β transcription in response to IFN-γ, because RelB–deficient and WT cells displayed a similar suppression of IL-1β upon IFN-γ treatment. Using a nuclease-protection assay, it was further excluded that IFN-γ had a relevant effect on chromatin accessibility that could hinder NF-κB p65 binding to the IL-1β promoter. Thus, further investigations are necessary to elucidate the underlying pathway in detail.

IL-1β was linked to the promotion of Th17 development in models of autoimmune diseases and bacterial and fungal infections, as well as in inflammatory and steady-state conditions in the intestine (2, 3, 54, 55). In line with this, we show in this study that IFN-γ–induced suppression of IL-1β resulted in impaired generation of IL-17–producing cells in vitro. In addition, IFN-γ was recently reported to negatively regulate transcription of IL-23, another Th17-promoting cytokine (56). Thus, interference with IL-1β and IL-23 expression might contribute to the well-known inhibitory effect of the Th1 cytokine IFN-γ on Th17 differentiation.
tion (57). An important effector function of Th17 cells is the recruitment of neutrophils to the site of infection. Although IL-17 does not directly act on neutrophils, it enhances secretion of neutrophil-attracting chemokines, such as CXCL1 and CXCL8, in neighboring cells, in part by prolonging the half-life of their mRNA (58, 59). Likewise, both IL-1α and IL-1β are strong inducers of CXCL1 (31, 60, 61). Accordingly, attenuation of IL-1β secretion by IFN-γ impaired CXCL1 production in target cells. Similar immune-modulatory functions were proposed for IFN-γ in vivo in models of persistent M. tuberculosis infection in which tissue damage that can be detrimental to the host was caused by the inflammatory response rather than by the bacterium itself. In this setting, IFN-γ showed anti-inflammatory properties by suppressing IL-1β and IL-17 production, thus inhibiting neutrophil recruitment and tissue damage (24, 62).

In summary, we describe a novel, very rapid, and NO-independent mechanism of IL-1β inhibition by IFN-γ. IFN-γ is known to orchestrate Th responses through DC regulation, thus strengthening Th1 induction by enhancing IL-12 while limiting Th2 differentiation. As we showed in this study, IFN-γ also impairs Th17 differentiation and production of neutrophil-attracting CXCL1 by a mechanism involving suppression of IL-1β.

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Disclosures

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References

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