

Interferons Direct an Effective Innate Response to *Legionella pneumophila* Infection^{*S}

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Courtney R. Plumlee[‡], Carolyn Lee[§], Amer A. Beg[¶], Thomas Decker^{||}, Howard A. Shuman[§], and Christian Schindler^{§**1}

From the Departments of [‡]Biological Sciences, [§]Microbiology and Immunology, and ^{**}Medicine, Columbia University, New York, New York 10032, the [¶]Moffitt Cancer Center, University of Southern Florida, Tampa, Florida 33612, and the ^{||}University of Vienna, A-1030 Vienna, Austria

Legionella pneumophila remains an important opportunistic pathogen of human macrophages. Its more limited ability to replicate in murine macrophages has been attributed to redundant innate sensor systems that detect and effectively respond to this infection. The current studies evaluate the role of one of these innate response systems, the type I interferon (IFN-I) autocrine loop. The ability of *L. pneumophila* to induce IFN-I expression was found to be dependent on IRF-3, but not NF- κ B. Secreted IFN-I is then in turn suppress the intracellular replication of *L. pneumophila*. Surprisingly, this suppression is mediated by a pathway that is independent of Stat1, Stat2, Stat3, but correlates with the polarization of macrophages toward the M1 or classically activated phenotype.

Legionella pneumophila, the causative agent of Legionnaires' Disease, remains an important cause of human morbidity (1). Like other pulmonary pathogens, *L. pneumophila* subverts host immunity through its capacity to replicate within macrophages. Genetic studies have mapped *L. pneumophila* virulence and immune evasion to the Dot-Icm type IV secretion system (reviewed in Refs. 2–4). Subsequent studies identified "effector" proteins, which the Dot-Icm system injects into host cells, enabling the bacteria to avoid lysosomal targeting and establish a unique replicative compartment (5).

The ability of murine macrophages to resist *L. pneumophila* infection has been attributed to several redundant innate sensor and response systems. For example, the unique susceptibility of macrophages from A/J mice to *L. pneumophila* infection has been mapped to *Naip5/Birc1e* (2, 6). Subsequent studies determined that the Naip5 sensor functioned upstream of Ipaf in its capacity to recognize *L. pneumophila* flagellin, culminating in inflammasome activation (7–10). The inflammasome directs the caspase 1-dependent secretion of interleukin (IL)-1 β and IL-18,

as well as promoting pyroptosis (7, 11, 12). Additional studies have implicated TLR2 and MyD88, as well as a subsequent secretion of TNF, in the innate response to *L. pneumophila* (9, 13–17).

Both type I (e.g. IFN- α/β) and type II (IFN- γ) IFNs play an important role in the innate response to intracellular microbes (18). Although IFN- γ 's ability to classically activate macrophages toward an M1 phenotype has been intimately associated with antibacterial activity, more recent studies have also ascribed antibacterial activity to IFN-I (19–22).

Characterization of IFN's antiviral activity led to the identification of the IFN- α receptor (IFNAR), the IFN- γ receptor (IFNGR), and the JAK-STAT signaling paradigm, where STATs (Signal Transducers and Activators of Transcription) are transcription factors and JAKs (Janus Kinases) the receptor-associated kinases that activate them (23). Specifically, IFN- γ directs the formation of a single transcription factor, the Stat1 homodimer, whereas IFN-I directs the activation of both Stat1 homodimers and ISGF-3 (IFN-stimulated gene factor 3; Stat1 + Stat2 + IRF9). Recent studies have underscored an important role for IRF-3 and IRF-7 in promoting activation of an IFN-I autocrine loop. This entails a sequential expression of IFN- β and IFN- α , and is dependent on IFNAR. This autocrine loop plays an important role in innate immunity (23–25).

Both IFN- γ and IFN-I suppress *L. pneumophila* growth in murine macrophages (13, 26, 27). Our studies highlight a role for the IFN-I autocrine loop in the innate response to this bacterium. The ability of *L. pneumophila* to induce IFN-I expression was found to depend on IRF-3, yet be independent of the flagellin-Naip5 axis, as well as p50/cRel. Additionally, in contrast to the critical role Stat1 plays in the antibacterial response of IFN- γ , the ability of IFN- α to suppress *L. pneumophila* growth was found to be independent of both Stat1 and Stat2. Finally, the Stat1-independent protection afforded by IFN- α correlated with the induction of classically activated M1 macrophage markers.

EXPERIMENTAL PROCEDURES

Mice—129, C57Bl/6J and A/J strains of mice were purchased from Jackson Laboratories and bred in a specific pathogen free

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¹ To whom correspondence should be addressed: Columbia University, HHSC 908, 701 West 168th St., New York, NY 10032. Tel.: 212-305-5380; E-mail: cws4@columbia.edu.

² The abbreviations used are: IL, interleukin; IFN, interferon; JAK, Janus kinases; STAT, Signal Transducers and Activators of Transcription; WT, wild

type; MOI, multiplicity of infection; GFP, green fluorescent protein; IFNAR, IFN- α receptor; IFNGR, IFN- γ receptor; BMM, bone marrow-derived murine macrophages.

facility. Homozygous Stat1(−/−), Stat2(−/−), IFNAR1(−/−), IFNGR1(−/−), and Stat3-fl/fl × Rosa26-ER(T2)-Cre (Stat3-cko) mice were either in a 129 or C57Bl/6J background, as noted (19, 28–32). p50/cRel double knock-out (dko), IRF-3(−/−), and TBK-1/TNFR1 dko mice have been previously described (33–36). Columbia University review committees approved all relevant studies.

Cell Culture—Primary murine macrophages were prepared by culturing bone marrow (BM) cells in RPMI 1640 (Invitrogen), 10% fetal calf serum (Hyclone, Logan, UT), P/S (GIBCO), and 20% L929-conditioned medium for 5–7 days, as previously reported (32, 37). L-cell-conditioned medium was collected from L929 cells (ATCC, Manassas, VA) cultured in Dulbecco's modified Eagle's medium (GIBCO) plus 10% fetal calf serum for 7 days. Cells were stimulated with IFN- $\alpha_{A/D}$ (1000 units/ml; PBL, Piscataway, NJ), active on both human and murine cells, or murine IFN- γ (50 units/ml; PBL). Some cells were pretreated with LY294002 (50 μ M; 30 min; Calbiochem, La Jolla, CA) or SB203580 (50 μ M; 30 min; Calbiochem). Stat3 conditional knock-out (cko) BM cells were treated with 250 nM 4-hydroxytamoxifen (Sigma-Aldrich) on culture days 1 and 5 to delete Stat3.

L. pneumophila—The *L. pneumophila* strain JR32 (restriction-defective Philadelphia-1, streptomycin-resistant) and dotA strains in the JR32 background were grown in AYE broth or on CYE plates, previously described (38, 39). GFP-*L. pneumophila* was created through the introduction of pXDC-31, and Fla− (Flagellin-deficient) strain through standard techniques (38–40).

Growth Curves—*Ex vivo* bacterial growth was evaluated by infecting (MOI = 0.25) day 6 bone marrow-derived murine macrophages (BMMs) with post-exponential phase *L. pneumophila* in 24-well plates (2.5×10^5 BMMs per well; (41)). Specifically, BMMs were infected with bacteria resuspended in RPMI. The zero hour, collected after 2 h of absorption, as well as all other time points were collected after washing infected BMMs with sterile water (10 s) and then lysing them in 1 ml of sterile water (5 min). Recovered bacteria were serially diluted in water and plated on CYE agar. Three days later, colony counts were enumerated. All infections were carried out in triplicate, and verified in at least three independent studies.

RT-PCR—Total RNA was prepared from day 6 BMMs before or after *L. pneumophila* infection (MOI = 10) through lysis in Trizol (Invitrogen, Carlsbad, CA). 3–5 μ g of total RNA was reverse-transcribed (M-MLV; Invitrogen), as previously reported (32, 37). For semi-quantitative analysis, cDNA was amplified for 28 cycles, fractionated on a 0.8% agarose gel and stained with ethidium bromide. For quantitative (Q) analysis, cDNA was amplified in an ABI Prism 7500 with SYBR green master mix (Applied Biosystems, Foster City, CA) and specific primers (see supplemental Table S1). Gene expression was normalized to a β -actin control. For each primer set, Ct values and standard curves were generated by plotting log DNA concentration versus Ct values from 1:5 serial dilutions with SDS1.9.1 software (Applied Biosystems).

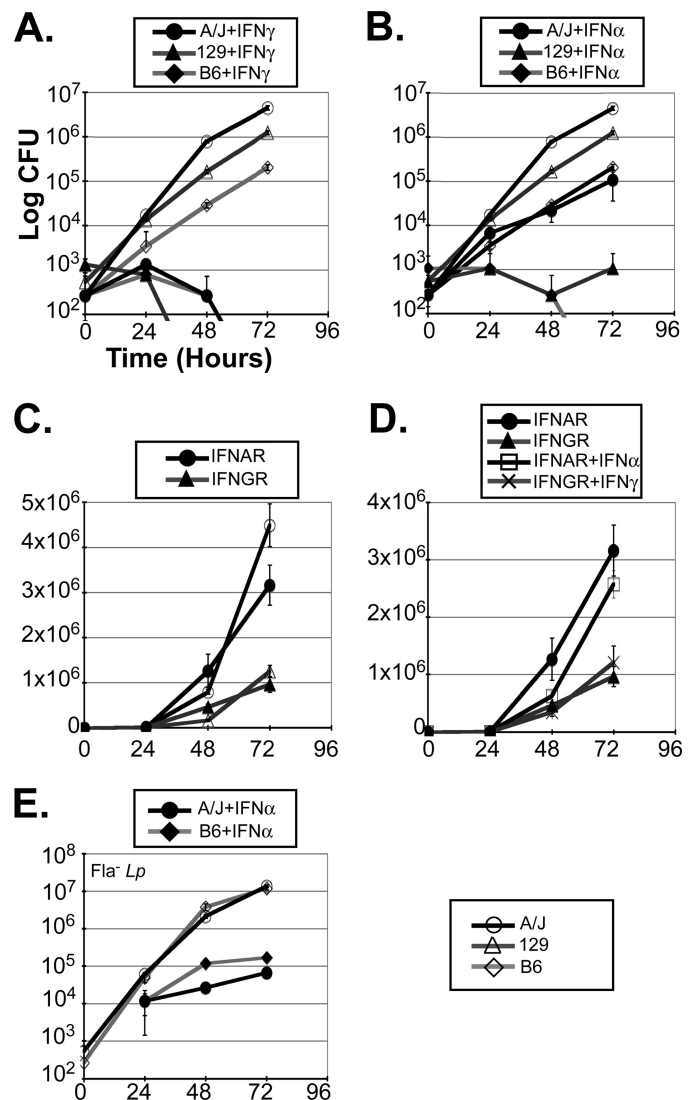


FIGURE 1. *L. pneumophila* infection is sensitive to IFNs. *L. pneumophila* (JR32; WT) growth was evaluated over 3 days ($t = 0, 24, 48$, and 72 h) by colony forming unit (CFU) assay of infected (MOI = 0.25) day 6 BMMs (2.5×10^5 /well of 24-well plate) from A/J, 129-SvEv (129) and C57Bl/6J (B6) mice (A–E). In some studies A/J, 129 and C57Bl/6J BMMs were pretreated with a single dose of IFN- γ (A, 50 units/ml) or IFN- $\alpha_{A/D}$ (B, 1000 units/ml) 2 h prior to *L. pneumophila* infection. C and D, control studies revealed significantly enhanced *L. pneumophila* replication in IFN- α receptor (IFNAR) knock-out BMMs ($p < 0.0097$ versus 129 BMMs), but not in IFN- γ receptor (IFNGR) knock-out BMMs ($p > 0.1$ versus 129 BMMs). E, Fla− *L. pneumophila* growth was evaluated in A/J and C57Bl/6J BMMs with or without IFN- $\alpha_{A/D}$ (1000 units/ml) pretreatment. Differences in *L. pneumophila* growth were statistically significant in A/J, C57Bl/6J, and 129 cells ($p < 0.005$), when compared with IFN- α -treated samples. Each infection was carried out in triplicate. Data are representative of more than three independent experiments.

FACS Analysis—Cells were stained with different combinations of fluorochrome-coupled antibodies (32). Anti-CD86 (GL1-PE), anti-I-A^b (AF6–120), and anti-Streptavidin-APC were from BD Pharmingen (San Diego, CA). BM macrophage cultures were infected with fluorescent bacteria. After 1–2 days of growth, cells were stained, and data collected on an LSRII (Beckton Dickinson, Franklin Lakes, NJ). Flow data were analyzed with FlowJo software (TreeStar, Ashland, OR), as previously reported (32).

Biochemical Studies—Whole cell extracts were prepared from IFN- $\alpha_{A/D}$ (1000 u/ml; PBL)-treated cells and evaluated

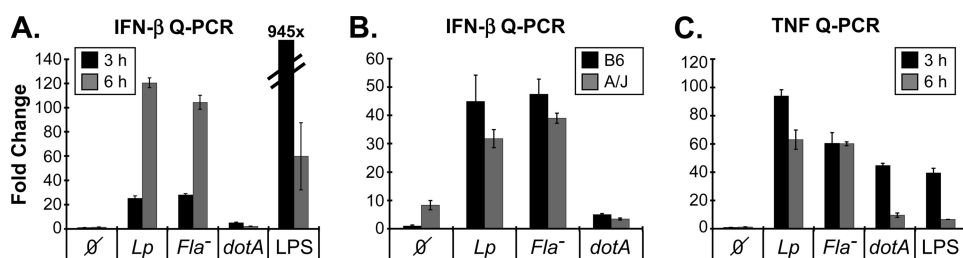


FIGURE 2. Cytokine expression during *L. pneumophila* infection. A, fold induction of IFN- β expression was evaluated by Q-PCR ($t = 3$ & 6 h; see supplemental Table S1 for primers) on RNA recovered from C57Bl/6J BMMs infected (MOI = 10) with wild type (JR-32; *Lp*), *Fla* $^-$, or *dotA* *L. pneumophila*. Expression was normalized to β -actin. Stimulation with LPS ($1 \mu\text{g/ml}$) served as a positive control. B, fold induction IFN- β expression 6-h after *L. pneumophila* (JR-32 = *Lp*; *Fla* $^-$; or *dotA*) infection of C57Bl/6J and A/J BMMs, as in panel A. C, fold induction TNF expression ($t = 3$ and 6 h; see supplemental Table S1 for primers) evaluated by Q-PCR, as in panel A. Results are each representative of three independent experiments.

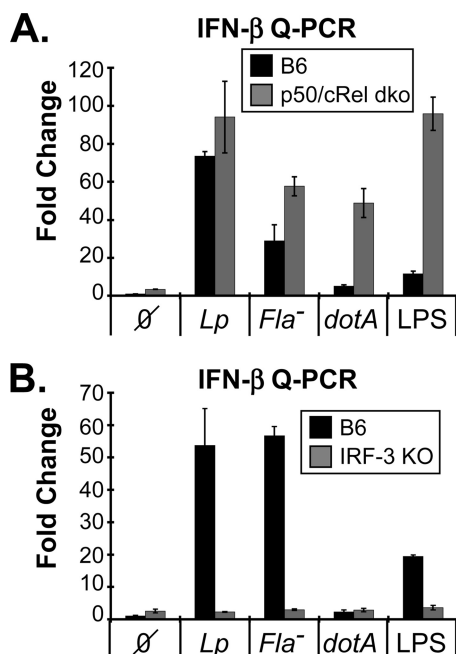


FIGURE 3. *L. pneumophila* induced IFN-I expression in p50/cRel and IRF-3 knock-out BMMs. A, fold induction IFN- β expression in *L. pneumophila* infected C57Bl/6J and p50/cRel dko BMMs 6-h after infection, as in Fig. 2. B, fold induction IFN- β expression in *L. pneumophila*-infected C57Bl/6J and IRF3($-/-$) BMMs 6-h after infection, as above. Both sets of data are representative of three independent studies.

by immunoblotting, as reported (20). Membranes were probed with antibodies specific for Phospho-Stat3 (Cell Signaling Tech., Beverly, MA), Stat3 (Cell Signaling Tech.), and Stat1 (20).

NO Assay—48 h supernatants were collected from BMMs and evaluated for NO production by a modified Griess Assay (37). Macrophages were pretreated with IFN- $\alpha_{A/D}$ (1000 units/ml, 2 h; PBL), murine IFN- γ (50 units/ml, 2h; PBL) and/or infected with *L. pneumophila* (MOI = 1). In some cases, cells were treated with L-NIL (100 μM , Sigma) or L-NMMA (1 mM, Sigma) at 30 min after infection.

RESULTS

IFN- α and IFN- γ Protect Macrophages from Infection with *L. pneumophila*—To more carefully explore the ability of IFNs to suppress *L. pneumophila* replication in mice, BMMs were

prepared from three distinct murine backgrounds, A/J, 129 and C57Bl/6J, as well as IFNGR and IFNAR knock-out mice (28). As anticipated, *L. pneumophila* replicated more effectively in A/J than C57Bl/6J BMMs. 129 macrophages exhibited an intermediate phenotype (see Fig. 1, A and B and Refs. 13, 26, 27). Consistent with previous reports, IFN- γ pretreatment rendered all three genotypes, but not IFNGR($-/-$) BMMs, resistant to infection (Fig. 1, A and D and Refs. 26, 42, 43). IFN- α also significantly

suppressed *L. pneumophila* growth in all three genotypes, but not in IFNAR($-/-$) macrophages (Fig. 1, B and D). Although IFN- α tended to be a bit less potent, (Fig. 1, A versus B, see also Figs. 4 and 7), it effectively suppressed the growth of both wild type and flagellin null (*Fla* $^-$, panel E) *L. pneumophila*, in all genotypes (panels B and D; data not shown). This provided evidence that the response to IFN-Is is independent of Naip5 and the inflammasome (13). Intriguingly, *L. pneumophila* replicated significantly more efficiently in IFNAR($-/-$) than the parental 129 macrophages (Fig. 1C), suggesting that *L. pneumophila* may stimulate IFN-I autocrine activity (see below and Refs. 13, 27). The lack of an analogous response in IFNGR($-/-$) BMMs, likely reflects the inability of macrophages to secrete autocrine IFN- γ (data not shown).

***L. pneumophila* Infection Stimulates the Secretion of IFN-I**—To determine whether *L. pneumophila* stimulated autocrine IFN-I production, IFN- β expression was evaluated by a sensitive Q-PCR assay (22, 44). Both WT (denoted *Lp*) and *Fla* $^-$ *L. pneumophila* stimulated a robust 50–100-fold induction of IFN- β in C57Bl/6J macrophages (Fig. 2A). This response was maximal at 6 h after infection. However, *dotA* mutants, defective in type IV secretion and intracellular replication, did not stimulate IFN- β expression (Fig. 2A). Similar results were obtained in A/J macrophages, indicating that the flagellin-Naip5 axis does not contribute to this response (Fig. 2B and Ref. 13). Control studies revealed that WT (*Lp*) and *Fla* $^-$ *L. pneumophila* stimulated strong TNF induction at both 3 and 6 h, whereas the response to *dotA* was considerably more transient (Fig. 2C). Moreover, this was associated with considerably lower quantities of secreted TNF (supplemental Fig. S1A and Ref. 13, 45)). Likewise, only WT *L. pneumophila* stimulated robust IL-1 β secretion (supplemental Fig. S1B). These results underscore the need for sustained intracellular growth to stimulate a strong inflammatory response (Fig. 2 and supplemental Fig. S1). Moreover, differences in the pattern and kinetics of cytokine expression suggested that IFN- β and TNF expression are directed by distinct *L. pneumophila* sensor systems, which are also independent of the Naip5-IpaF axis.

***L. pneumophila* IFN- β Expression Is IRF-3-dependent**—Two transcription factors, NF- κ B and IRF-3, play an acute role in stimulating the expression of IFN- β (35, 46–49). Electrophoretic mobility shift assays (EMSA) revealed that p50 and cRel are the major NF- κ B transcription factors activated by LPS

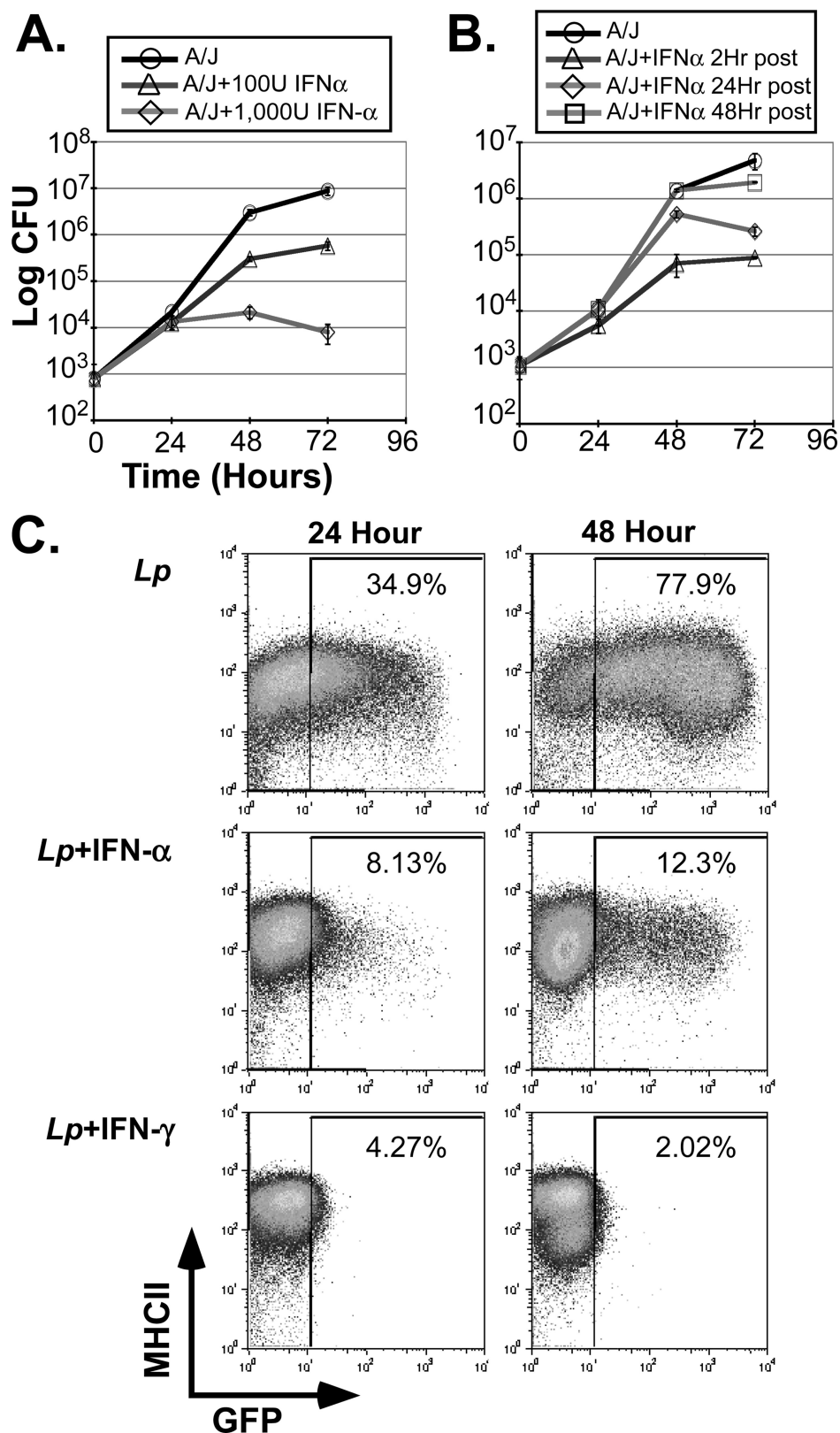


FIGURE 4. Characterization of IFN- α -dependent response to *L. pneumophila* infection. A, *L. pneumophila* (JR-32) growth was evaluated in d6 A/J BMMs, after 2 h of pretreatment with 100 units/ml or 1000 units/ml of IFN- $\alpha_{A/D}$, as in Fig. 1. B, *L. pneumophila* growth was evaluated in A/J BMMs treated with IFN- $\alpha_{A/D}$ (1000 units/ml) either at 2, 24, or 48 h after infection, as indicated. C, GFP-Fla-*L. pneumophila* growth in d6 C57Bl/6J BMMs was evaluated by FACS, 24 and 48 h after infection (MOI = 1). Macrophages were pretreated (2 h before infection) with one dose of IFN- $\alpha_{A/D}$ (500 units/ml), or IFN- γ (50 units/ml). Cells were harvested, stained for MHC-II (I-A^b + anti-Streptavidin-APC; BD Pharmingen) and analyzed with LSRII and FlowJo software. Each study is representative of more than three independent experiments.

and *L. pneumophila* in murine macrophages (supplemental Fig. S2, A–C). Moreover, dotA-dependent NF- κ B activation was considerably more transient than with either WT or Fla-*L. pneumophila* (supplemental Fig. S2C). To explore the potential role NF- κ B subunits may play in IFN-I expression, p50 and cRel double knock-out BMMs were exploited (35). Upon infection with *L. pneumophila*, p50/cRel double knock-out macrophages exhibited a pattern of IFN- β expression that was analogous to control C57Bl/6J cells (Fig. 3A). Curiously, the p50/cRel double knock-out macrophages displayed increased IFN- β induction when they were infected with dotA or stimulated with LPS, underscoring a potentially complex counter regulatory role recently highlighted for NF- κ B (13, 45, 50, 51). Moreover, *L. pneumophila* replicated normally in p50/cRel double knock-out BMMs (supplemental Fig. S2D). These observations indicate that NF- κ B does not significantly contribute to *L. pneumophila*-dependent IFN-I response.

Next, the potential role IRF-3 plays in directing the *L. pneumophila* dependent expression of IFN- β was evaluated. This analysis exploited IRF3(-/-) BMMs, thereby avoiding the more limited sensitivity of biochemical assays for IRF-3 activity (34). As above, WT and Fla-*L. pneumophila* both stimulated a robust induction of IFN- β expression in control C57Bl/6J BMMs (Fig. 3B). However, IFN- β expression was absent in IRF3(-/-) BMMs, underscoring the important role this transcription factor plays in regulating the IFN-I autocrine loop (14, 22, 34, 49). Notably, *L. pneumophila*-dependent TNF expression was not affected by the loss of IRF-3 (supplemental Fig. S2E).

IFN- α Stimulates a Unique Innate Immune Response—Next, we turned our attention to understanding how IFN-Is suppress *L. pneumophila* growth. Pretreatment with either 100 or 1000 units/ml of IFN- α rendered A/J BMMs resist-

ant to *L. pneumophila* growth, in a dose-dependent manner (Fig. 4A). Similar results were obtained in 129 or C57Bl/6J macrophages (not shown). Moreover, IFN- α treatment impeded *L. pneumophila* replication, even when applied 24-h after infection, suggesting that intracellular bacteria represent the critical target of IFN-I activity (Fig. 4B). Consistent with this, IFN- α pretreatment did not affect the uptake of GFP-labeled *L. pneumophila* (not shown). Likewise, when GFP-*L. pneumophila* were exploited in a FACS-based assay, their replication was sensitive to IFN treatment (Fig. 4C). Consistent with growth curve studies (see Fig. 1), a modest level of GFP-*L. pneumophila* replication was observed in IFN- α -treated BMMs, whereas IFN- γ completely suppressed GFP-*L. pneumophila* growth at 24 and 48 h (Fig. 4C).

Stat1 has been ascribed a critical role in directing the antimicrobial activity associated with IFN- γ , or “classically activated” (i.e. M1) macrophages (19–21, 52, 53). To determine whether Stat1 played a similarly important role in the ability of IFN- α to suppress *L. pneumophila* replication, BMMs from Stat1(–/–) mice in the 129 background were evaluated. As anticipated, *L. pneumophila* growth was resistant to IFN- γ treatment in Stat1(–/–) BMMs (Fig. 5A). In striking contrast, *L. pneumophila* remained sensitive to IFN- α treatment in Stat1(–/–) BMMs. Likewise, IFN- α was able to effectively suppress bacterial growth in Stat2(–/–) BMMs (Fig. 5B), excluding an important role for either Stat1 homodimers or ISGF-3 (Stat1 + Stat2 + IRF-9, Ref. 23) in this response. Next, a potential role for Stat3, which is also activated by IFN-Is (54), was evaluated. IFN- α was still able to suppress *L. pneumophila* growth in Stat3-cko BMMs, albeit with the slower kinetics associated with the parental C57Bl/6J background (Fig. 5C). These results reveal that IFN-Is do not direct their protective response toward *L. pneumophila* through canonical Stat1-, Stat2-, or Stat3-dependent pathways, but rather through a distinct pathway.

IFNs Activate M1 Macrophages—The antibacterial response to IFN- γ correlates directly with the development of M1 or “classically activated” macrophages (52); see Fig. 6), whereas polarization toward alternatively activated M2 macrophages had no effect on *L. pneumophila* growth (data not shown). As previously reported, this response was associated with an up-regulation of MHC-II and CD86 surface expression, as well as induction of iNOS (53, 55). Likewise, the ability of IFN- γ to direct MHC-II, CD86, and iNOS expression was fully dependent on Stat1 (see Fig. 6, A and B). In contrast, the equally effective ability of IFN- α to induce these M1 markers was fully independent of Stat1 (Fig. 6B). Moreover, IFN- α directed M1 polarization was also independent of Stat2 (not shown). The close correlation between IFN-I stimulated, STAT-independent suppression of *L. pneumophila* growth and M1 polarization, suggests that the classical activation of macrophages is important for this innate response. These observations also highlight an unexpected functional dichotomy in the ability of type I and II IFNs to polarize macrophages toward the M1 phenotype and suppress *L. pneumophila* growth.

The relatively rapid, IFN- α -stimulated and Stat1-independent induction of iNOS, a characteristic M1 marker, provided an opportunity to explore the potential role noncanonical signaling pathways may play in IFN-I response. Based on previous

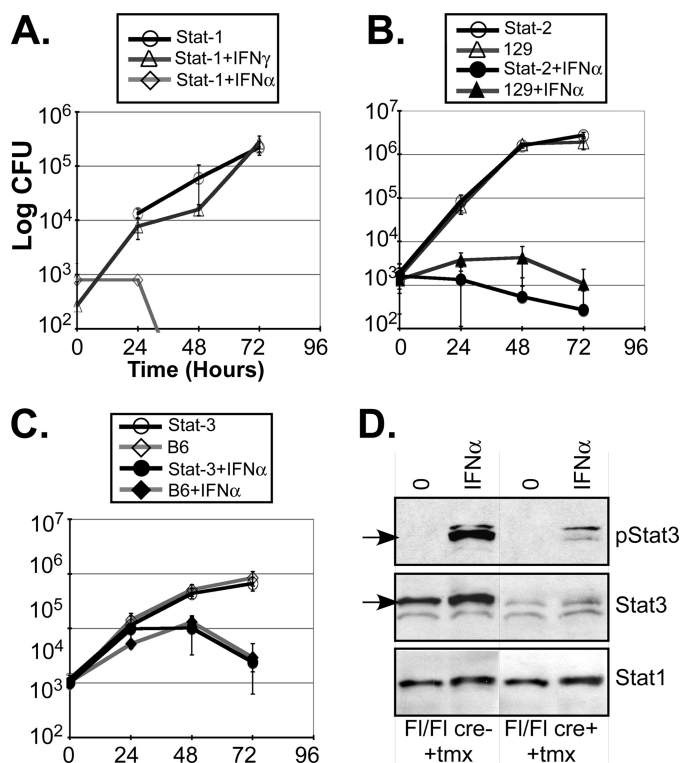


FIGURE 5. The role of Stat1, Stat2, and Stat3 on IFN suppression of *L. pneumophila* growth. A, *L. pneumophila* (JR-32; Lp) growth was evaluated in d6 Stat1(–/–) BMMs, as in Fig. 1, either with or without IFN- α /D (1000 units/ml) or IFN- γ (50 units/ml) pretreatment (2 h before infection). B, *L. pneumophila* (JR-32; Lp) growth was evaluated in d6 Stat2(–/–) and 129 BMMs after IFN- α /D pretreatment, as in panel A. C, *L. pneumophila* (JR-32; Lp) growth was evaluated in d6 Stat3-cko and C57Bl/6J BMMs (see below), after IFN- α /D pretreatment, as in panel A. D, Stat3-cko macrophages were generated through the addition of 250 nM tamoxifen to day 1 and day 5 BM cultures from Stat3-fl/fl \times Rosa26-ER(T2)-Cre (Stat3-cko) mice. Control BMMs were generated from Stat3-fl/fl mouse. Whole cell extracts were prepared after IFN- α /D (1000 units/ml; 30 min) treatment and evaluated by immunoblotting with antibodies for phospho-Stat3, Stat3, and Stat1. These results are each representative of more than three independent experiments.

evidence of a role for p38 and PI-3 kinases in STAT-independent IFN-I response (56, 57), the ability of IFN- α to induce iNOS expression was evaluated in the presence of two specific inhibitors, SB203580 and LY94002, respectively (Fig. 6C and Ref. 56). Neither inhibitor blocked IFN- α stimulated expression of iNOS, raising the possibility that these two kinases also do not contribute to the IFN-I-dependent M1 polarization associated with *L. pneumophila* suppression. Consistent with this possibility, biochemical studies suggest that IFN-I does not effectively activate these kinases in primary murine macrophages (supplemental Fig. S4).

Nitric Oxide Accounts for Phenotypic Differences between Type I and II IFNs—NO production has not been ascribed a significant role in the innate response to *L. pneumophila* infection (26, 58). To determine whether NO might contribute to the noncanonical IFN-I response associated with *L. pneumophila* suppression, additional studies were carried out. As previously reported, *L. pneumophila* failed to effectively stimulate NO production (26, 58). However, pretreatment of cells with IFN- γ and to a lesser extent with IFN- α led to a robust induction of NO (Fig. 7A). Moreover, this was blocked through the addition

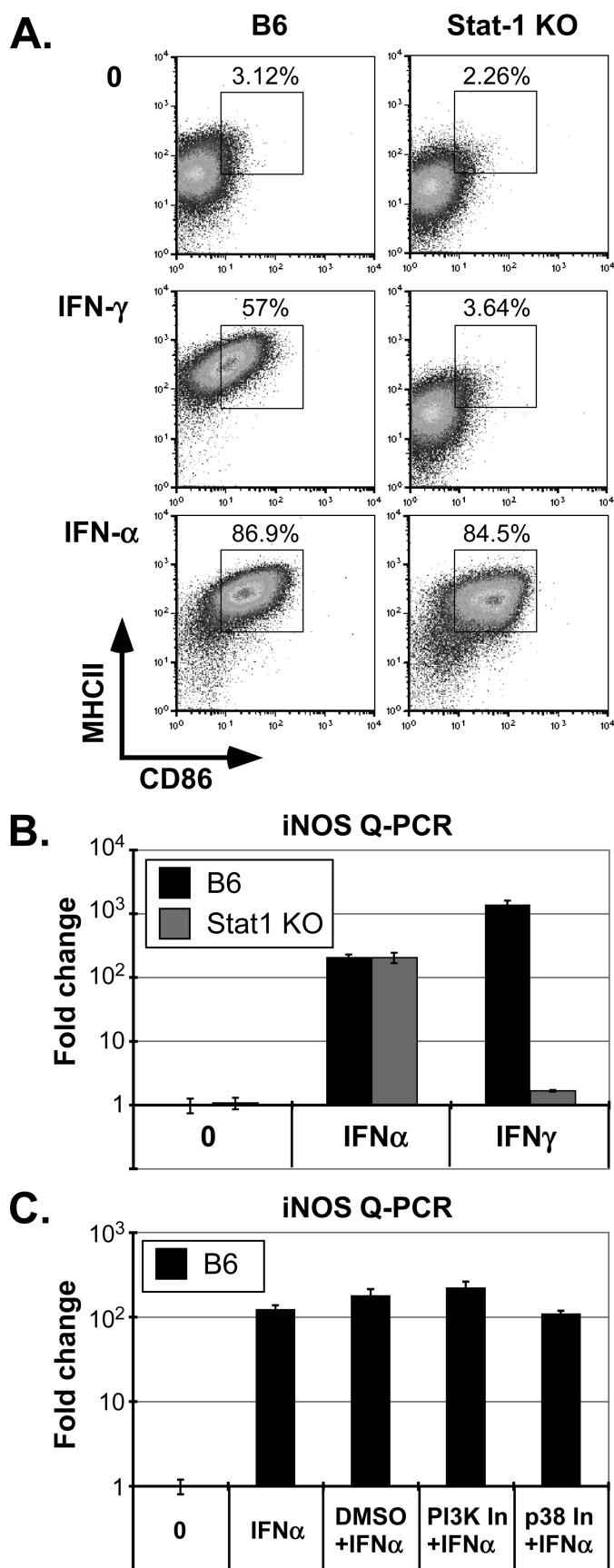


FIGURE 6. IFN- α directs STAT independent M1 polarization of macrophages. A, IFN- $\alpha_{A/D}$ (500 units/ml; 48 h)- or IFN- γ (50 units/ml; 48 h)-treated C57Bl/6J and Stat1(-/-) d6 BMMs were stained for MHC-II (I-A^b +

of either the iNOS-specific inhibitor, L-NIL, or a more general NOS inhibitor, L-NMMA (Fig. 7A). These observations raised the possibility that the more robust NO response stimulated by IFN- γ might account for the modestly enhanced ability of IFN- γ (versus IFN- α) to suppress *L. pneumophila* growth (e.g. Figs. 1, 4C, and 7B). Consistent with this, the level of intracellular *L. pneumophila* replication was modestly increased when NO production was blocked in IFN-pretreated macrophages (Fig. 7B). Moreover, the change in *L. pneumophila* growth was considerably more striking in IFN- γ -pretreated macrophages, suggesting that NO production is likely to account for the more effective ability of IFN- γ (versus IFN- α) to suppress *L. pneumophila* replication. These studies also suggest that M1-associated responses that are independent of iNOS play a far more important role in the ability of IFNs to impede *L. pneumophila* growth.

DISCUSSION

Pattern recognition sensors systems evolved to detect the presence of pathogen associated molecular patterns (PAMPs) and initiate effective innate responses (24, 59, 60). Exploiting the murine system, investigators have identified two cytosolic sensors, Naip5/Birc1e and Ipaf, which specifically initiate an innate response to *L. pneumophila* flagellin (2, 7–9, 11). This entails activation of the inflammasome, leading to caspase1 dependent secretion of IL-1 β and IL-18, as well as the induction of pyroptosis (7, 11, 12). Curiously however, IL-1 β and IL-18 do not appear to directly regulate *L. pneumophila* growth in macrophages (3, 13). Moreover, the Naip5-Ipaf sensor system does not seem to participate in the secretion of other cytokines that have been associated with *L. pneumophila* infection (13, 27).

Evidence that *L. pneumophila* replicated more robustly in IFNAR1(-/-) BMMs raised the possibility that the IFN-I auto-crine loop may contribute to the innate response in WT macrophages, as recently suggested (13, 27). Consistent with this, both WT and Fla- *L. pneumophila* potently stimulated IFN- β expression, analogous to what has been reported for *L. monocytogenes*, *F. tularensis*, as well as other microbial pathogens (34, 44, 61, 62). Moreover, this response is largely independent of TLRs (13, 14), IPS-1/MAVs (63), the Naip5-Ipaf-flagellin axis (Fig. 2 and Ref. 13), IFNAR1, and Stat1 (supplemental Fig. S3A). Mechanistic studies determined that *L. pneumophila* stimulated IFN- β expression was dependent on IRF-3, but not p50/c-Rel (Fig. 3). Consistent with this, IFN- β expression also appeared to be dependent on TBK-1, the kinase that activates IRF-3 (see supplemental Fig. S3B and Ref. 44). Previous RNAi-based studies in human epithelial cells, which do not support robust *L. pneumophila* growth, have also implicated IRF-3, as well as IPS-1 in *L. pneumophila*-stimulated IFN-I expression (64).

anti-Streptavidin-APC) and anti-CD86-PE and evaluated by FACS, as in Fig. 4. Percentages of double positive cells, delineated by box are indicated. B, fold induction iNOS expression was evaluated by Q-PCR (see supplemental Table S1 for primers) from IFN- $\alpha_{A/D}$ (1000 units/ml; 24 h) or IFN- γ (50 units/ml; 24 h)-treated d6 C57Bl/6J and Stat1(-/-) BMMs, as in Fig. 2. C, fold induction iNOS expression was evaluated in IFN- $\alpha_{A/D}$ (1000 units/ml; 10 h) stimulated C57Bl/6J BMMs, as in panel A. Macrophages were either pretreated with DMSO (solvent control), a PI3K inhibitor (LY294002, 50 μ M; 30 min), or a p38 inhibitor (SB203580, 50 μ M; 30 min). Each study is representative of at least three independent experiments.

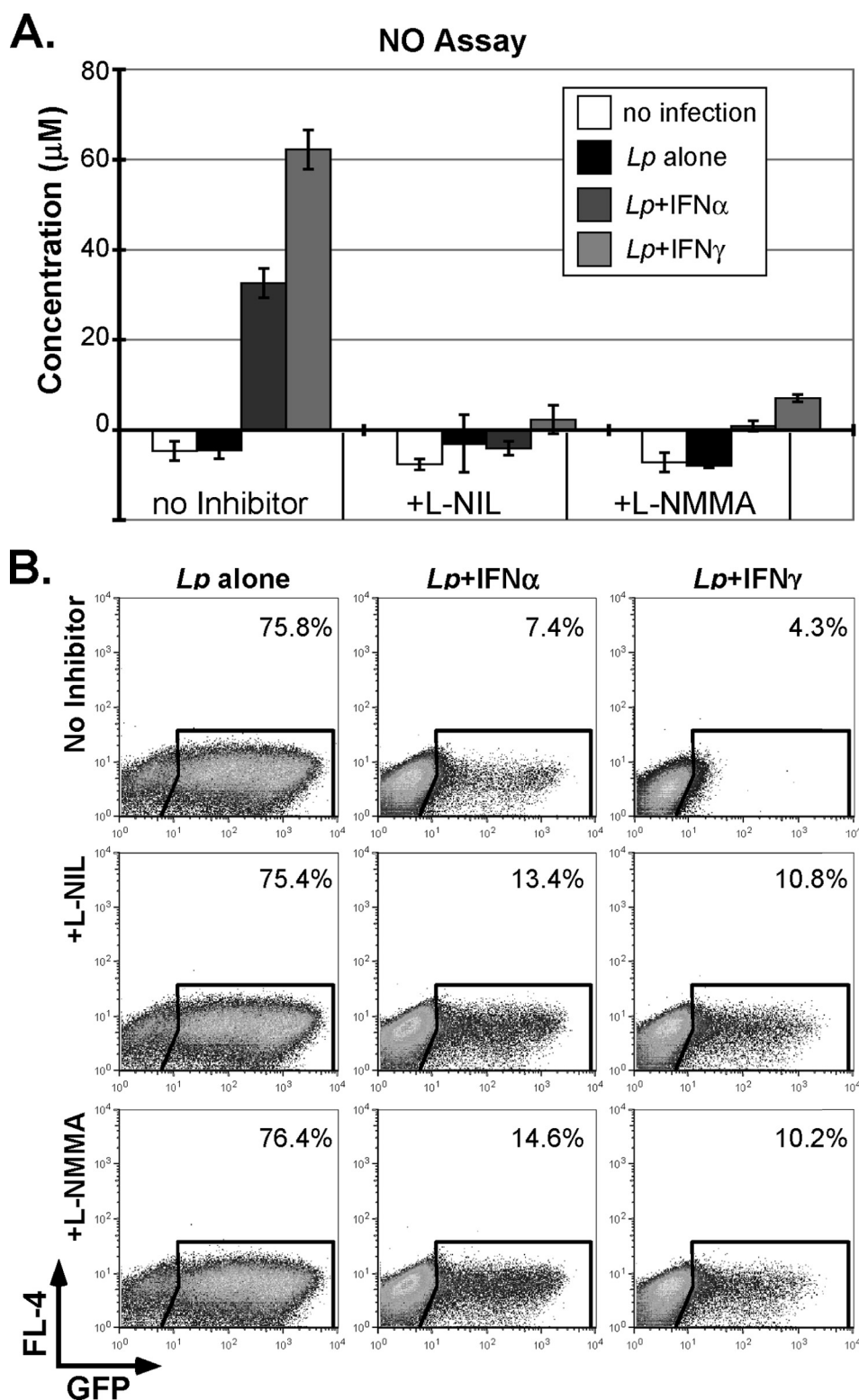


FIGURE 7. NO production in IFN-stimulated and *L. pneumophila*-infected macrophages. A, culture supernatants were evaluated for NO production 48 h after infection of day 6 C57Bl/6J BMMs with *L. pneumophila* (GFP Fl α –, MOI = 1). In some cases, native, IFN- α_{AD} – or IFN- γ -pretreated BMMs (as in Fig. 4C) were also treated with L-NIL (100 μ M) or L-NMMA (1 mM) at 30 min after infection. B, C57Bl/6J BMMs, treated as in panel A, were evaluated 48 h after infection by FACS, as detailed in Fig. 4. The percentage of GFP-positive cells is indicated. These studies are representative of three independent experiments.

Genetic studies excluding a role for IPS-1 in *L. pneumophila*-dependent IFN- β expression raised the possibility that novel host receptors may sense the cytoplasmic accumulation of bac-

terial nucleic acids (14, 63, 65–69). However, the μ g quantities of bacterial nucleic acids required to stimulate IFN- β expression and our inability to detect even traces of *L. pneumophila* nucleic acids in the cytoplasm of infected macrophages (data not shown) do not support this model. Thus, the identification of the innate sensor that promotes activation of the IFN-I autocrine loop during a *L. pneumophila* infection remains an important goal for future studies.

Studies exploring how IFNs antagonize *L. pneumophila* growth revealed a correlation with polarization of macrophages to the inflammatory M1, but not M2 phenotype (Figs. 4 and 6; not shown, Ref. 52). Curiously, even though Stat1 was shown to play a critical role in both IFN- γ -dependent M1 polarization and *L. pneumophila* suppression, this was not the case for IFN- α . Rather, the ability of IFN-I to impede *L. pneumophila* growth was found to be independent of Stat1, Stat2, and Stat3. Intriguingly, IFN-I-dependent M1 polarization was also found to be independent of these canonical STAT signaling pathways (Fig. 5), raising the possibility that these two responses are functionally related. Additional studies exploiting the more rapid kinetics of IFN- α -stimulated iNOS expression, an archetypal M1 response, revealed a similar noncanonical IFN-I dependent signaling pathway. Moreover, the relatively rapid kinetics of IFN-I stimulated iNOS expression provided an opportunity to largely exclude an important role two well characterized STAT independent signals, p38 and PI-3 kinases, in this noncanonical IFN-I response. Consistent with the possibility that these 2 kinases may not contribute to M1 polarization or *L. pneumophila* suppression, biochemical studies failed to reveal robust activation of p38 and PI-3 kinases in IFN- α treated macrophages. Future studies will employ additional knock-out models to more rigorously explore the nature of IFN-I stimulated signals in M1 polarization and the suppression of *L. pneumophila* replication.

In summary, the innate response to *L. pneumophila* infection entails the activation of multiple pattern recognition systems that direct the secretion of several inflammatory cytokines, including IL-1 β , IL-18, TNF, and IFN- β (7, 11, 13). Although it seems likely that these pathways synergize to mount an effective innate response to *L. pneumophila*, IFN-I remains the single most effective cytokine in suppressing *L. pneumophila* growth (12, 13).

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Note Added in Proof—Two recently published studies suggest that RNA polymerase III directs a RIG-I-IPS-1-dependent response to microbial DNA, including that of *L. pneumophila*, that culminates in IFN- β expression (70, 71).

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