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Different STAT Transcription Complexes Drive Early and Delayed Responses to Type I IFNs

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IFNs, which transduce pivotal signals through Stat1 and Stat2, effectively suppress the replication of Legionella pneumophila in primary murine macrophages. Although the ability of IFN-γ to impede L. pneumophila growth is fully dependent on Stat1, IFN-αβ unexpectedly suppresses L. pneumophila growth in both Stat1- and Stat2-deficient macrophages. New studies demonstrating that the robust response to IFN-αβ is lost in Stat1-Stat2 double-knockout macrophages suggest that Stat1 and Stat2 are functionally redundant in their ability to direct an innate response toward L. pneumophila. Because the ability of IFN-αβ to signal through Stat1-dependent complexes (i.e., Stat1-Stat1 and Stat1-Stat2 dimers) has been well characterized, the current studies focus on how Stat2 is able to direct a potent response to IFN-αβ in the absence of Stat1. These studies reveal that IFN-αβ is able to drive the formation of a Stat2 and IFN regulatory factor 9 complex that drives the expression of a subset of IFN-stimulated genes, but with substantially delayed kinetics. These observations raise the possibility that this pathway evolved in response to microbes that have devised strategies to subvert Stat1-dependent responses. The Journal of Immunology, 2015, 195: 210–216.

Legionella pneumophila, the causative agent of Legionnaires’ disease, continues to account for 4–20% of U.S. cases of community-acquired pneumonia (1). Its ability to subvert the host’s immune response is dependent on the Icm/Dot type IV secretion system, which serves to inject numerous effector proteins into host cells (reviewed in Refs. 2–4). Even though L. pneumophila is a human pathogen, it can infect murine macrophages. This model has been exploited to identify several L. pneumophila pathogen-associated molecular patterns, as well as their corresponding pattern recognition receptors (3). These pattern recognition receptors then direct the production of potent inflammatory mediators, including TNF-α, IL-1β, and type I IFNs (IFN-Is).

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Abbreviations used in this article: BMM, bone marrow–derived murine macrophage; ChIP, chromatin immunoprecipitation; GAS, IFN-γ activation site; iBMM, immortalized BMM; IFN-I, type 1 IFN; iNOS, inducible NO synthase; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; ISGF, IFN-stimulated gene; IRF-1, IFN regulatory factor 1; IRF-9, IFN regulatory factor 9; LMP2, IFN regulatory factor 2; Mx-1, IFN-inducible antiviral protein; Noi, IFN-αβ receptor; Noi-S, Noi-deficient macrophages; Noi-Stat1, Stat1-deficient macrophages; Noi-Stat2, Stat2-deficient macrophages; Oas, 2′-5′ oligoadenylate synthetase; PCR, polymerase chain reaction; pkR, p53-regulated; P6, pyridone 6; Socs, suppressor of cytokine signaling; WT, wild-type.

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Legionella pneumophila

IFNs, initially identified for their potent antiviral activity, mediate their biological responses through the STAT family of transcription factors. They direct the rapid and robust expression of a large family of IFN-stimulated genes (ISGs; reviewed in Refs. 5–9). Type 1 IFNs (IFN-Is; IFN-αβ) mediate their response through the IFN-α receptor and two associated JAKs, Jak1 and Tyk2, where the activity of Jak1 is dominant (10). This culminates in the recruitment and subsequent JAK-dependent phosphorylation of Stat1 and Stat2. Once activated, these STATs form either Stat1-Stat1 or Stat1-Stat2 dimers. The Stat1 homodimers directly bind to IFN-γ activation site (GAS) enhancers to drive a rapid expression of target genes (e.g., IFN regulatory factor [IRF]-1, LMP2, and Stat1) (11, 12), whereas the Stat1-Stat2 heterodimers associate with IRF-9 to form ISG factor (ISGF) 3. This transcription factor binds to the IFN-αβ–stimulated responsive element (ISRE) to direct the rapid expression of a distinct set of genes, which include Mx-1, Oas, inducible NO synthase (iNOS), ISG15, IIf2, IIf3, Ddxs58 (a.k.a., RIG-1), Dusp1, Dusp2, and Bst2, as well as many other genes (6–8). Of note, this group of genes also includes the suppressor of cytokine signaling (Socs) 1, responsible for rapidly downregulating JAK activity (12, 13). Intriguingly, there is also compelling evidence that basally secreted IFN-Is play an important role in host homeostasis, including directing basal Stat1 and Stat2 expression (11, 12, 14). In contrast, type II IFN (IFN-γ) expression is more restricted and mediates its response through a signaling cascade, consisting of the IFN-γ receptor, Jak1, Jak2, and Stat1 (5, 8, 15). Analogous to IFN-Is, IFN-γ–activated Stat1 homodimers induce the expression of GAS-driven genes.

Even though type I and II IFNs both effectively suppress L. pneumophila replication in macrophages, their responses are mediated by distinct signaling pathways (16–18). The ability of IFN-γ, but not IFN-1, to suppress L. pneumophila growth is abrogated in Stat1−−/− macrophages. IFN-Is also retain their ability to effectively suppress bacterial growth in Stat2−−/−/C211 macrophages (17). Similar observations have been reported for measles, lymphocytic choriomeningitis, and Dengue viruses (19, 20). To explore these observations, Stat1-Stat2 double-knockout macrophages were generated. Unexpectedly, IFN-Is lost their ability to suppress L. pneumophila...
growth in these macrophages, suggesting that Stat1 and Stat2 function redundantly in their ability to mediate this response. Although the mechanism by which Stat1 independently signals is well understood (i.e., Stat1 homodimers) (5), the mechanism by which Stat2 signals independently of Stat1 has not been fully characterized (19–25). The current study exploits primary Stat1−/− macrophages to explore this Stat2-only pathway. Genetic and biochemical studies reveal that activated Stat2 associates with IRF-9, whereupon it binds to the ISRE element to drive the gene expression of target genes. The kinetics of this response is both delayed and dependent on persistent ligand stimulation. These observations raise the possibility that this pathway potentially evolved to function as a backup response in the setting of either an inherited or acquired loss of Stat1 (26–28).

**Materials and Methods**

**Legionella pneumophila**

The *L. pneumophila* JR32 (restriction-defective Philadelphia-1, streptomycin-resistant) strain was grown in AYE broth or on CYE plates, as previously described (17). C57BL/6J bone marrow–derived murine macrophages (BMMs) were infected with a *L. pneumophila* strain that was also Fla− (Flagellin deficient) (17).

**Mice**

The 129 and C57BL/6J mice were purchased from Jackson ImmunoResearch Laboratories and bred in a specific pathogen-free facility. Homozygous Stat1−/−, Stat2−/−, IRF-9−/−, and Stat1−/−Stat2−/− double-knockout mice were either from 129 (Fig. 1) or C57BL/6J backgrounds, as previously reported (11, 29–31). The Columbia University Institutional Animal Care and Use Committee approved all animal studies in New York, and the institutional ethics committee at the University of Vienna determined that all studies carried out in Vienna were in accordance with Austrian law (permit GZ 680 205/67-BrGt/2003).

**Cell culture**

Primary murine macrophages were prepared by culturing bone marrow cells in RPMI 1640 or DMEM (Invitrogen-Life Technologies, Grand Island, NY), 10% FCS (Hyclone, Logan, UT), Penn/Strep (Life Technologies), and 20% L929 conditioned media for day 7–10 cultures, as previously reported (17, 32). Cells were stimulated with murine IFN-α/β (1000 U/ml; PBL, Piscataway, NJ), which is active on human and murine cells; murine IFN-β (250 U/ml; PBL); or murine IFN-γ (50 U/ml; PBL). In some IFN-1–treated cells, the JAK inhibitor tetracyclic pyridone 6 (P6; 2 μM; Calbiochem, La Jolla, CA) was added 1 or 4 h prior to harvest. Some macrophages were immortalized with a v-myc/v-raf–expressing retrovirus (33).

**Growth curves**

In vivo bacterial growth was evaluated by infecting (multiplicity of infection = 0.25) day 6 BMMs (2.5 × 10^6 BMMs per well of 24-well plate) with postexponential phase *L. pneumophila*, as reported (17). All infections were carried out in triplicate and verified through at least three independent studies.

**Biochemical studies**

Whole-cell extracts were prepared from IFN-α/β− or IFN-β−–treated cells and evaluated by immunoblotting with Abs specific for Stat1 (Santa Cruz Biotechnology, Dallas, TX) (11, 12), phospho-Stat1 (Cell Signaling, Beverly, MA), Stat2 (11, 12), phospho-Stat2 (UBI/EMD-Millipore, Temecula, CA), phospho-Stat1 (Cell Signaling), Jak1 and phospho-Jak1 (UBI/EMD-Millipore), and tubulin (Sigma-Aldrich, St. Louis, MO), as previously reported (11, 12, 34). For EMSA, whole-cell or nuclear extracts were prepared and evaluated, as previously described (17, 34). Briefly, extracts (1–4 μl) were incubated with a [32P]dATP–labeled (5′) γ-32P-dATP, 6000 Ci/mMol; PerkinElmer, Waltham, MA) dsOAS oligonucleotide probe with binding buffer. In some studies, extracts were preincubated (30 min, 4°C) with 1–2 μl Abs (11, 12, 21) or competed with a 7-fold excess of cold dsOAS oligonucleotide simultaneously to the addition of radiolabeled probe.

**RT-PCR**

Total RNA was prepared from day 6 BMMs before or after IFN-α/β− treatment with the Nucleospin RNA kit (Macherey-Nagel, Düren, Germany), according to manufacturer’s instructions, or by TRIzol (Fig. 4; Invitrogen, Carlsbad, CA). A quantity amounting to 200 ng (2 μg in Fig. 4) of total RNA was reverse transcribed (Moloney murine leukemia virus; Invitrogen) (17, 34). cDNA was PCR amplified by either standard or quantitative approaches with SYBR Green master mix (Promega, Madison, WI; or Applied Biosystems, Foster City, CA) on a real-time thermocycler (Stratagene MX3005p or Eppendorf Mastercycler EP Realplex) with gene-specific primers (see Supplemental Table I). Expression was normalized to either a GAPDH or β-actin control.

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChIP) assays were performed essentially as described (19). Briefly, BMMs were fixed in 1% formaldehyde and lysed in 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 1% SDS, and sonicated and diluted with 9 parts 50 mM Tris-HCl (pH 8.0), 167 mM NaCl, 1.1% Triton X-100, and 0.11% sodium deoxycholate. A total of 10 μg sonicate was then immunoprecipitated (protein G–agarose beads; Pierce Thermo Scientific, Rockford, IL) with a murine-specific Stat2 Ab and eluted at 65°C. Eluted DNA was treated with RNase A and proteinase K, and then

**FIGURE 1.** *L. pneumophila* growth in Stat1−/− and Stat2−/− BMMs. *L. pneumophila* JR32–Lp: multiplicity of infection = 0.25 growth was evaluated by a colony-forming assay (24, 48, or 72 h postinfection) in 129 (WT) control, (A) Stat1−/− (B) Stat2−/−, and (C) Stat1/Stat2−/− double-knockout BMMs in the 129 background (2.5 × 10^5/well of 24-well plate), as previously described (17). Some BMMs were pretreated with a single dose of IFN-α/β (1000 U/ml) or IFN-γ (50 U/ml) 2 h prior to infection. Please note that (A) and (B) were previously published (17) and are included solely for comparison’s sake. Studies are representative of more than three independent experiments. Similar results were obtained in the C57BL/6J background.
purified with QIAquick PCR Purification kit (Qiagen, Valencia, CA) prior to SYBR Green PCR base Q-PCR on the Light-Cycler 480 PCR System (Roche, Indianapolis, IN) for 45 cycles (see Supplemental Table I for primers). The percentage of input DNA was determined by comparing cycle threshold value obtained with immunoprecipitated DNA and cycle threshold value obtained from input DNA.

**Generation of lentiviral particles and knockdown of IRF-9 in immortalized BMMs**

pGIPZ lentiviral short hairpin RNA vectors specific for murine IRF-9 (clone V2LM5, 167172; see Supplemental Table I) and a proprietary control (RH5436e) were obtained from Open Biosystems (Huntsville, AL). Fresh, filtered (0.45-μm) lentivirus, prepared as previously reported (36), was used to infect immortalized wild-type (WT) and Stat1−/− BMMs. Positive populations were selected and maintained on puromycin (5 μg/ml; Thermo-Fisher).

**Results**

**IFN-Is direct a Stat2-dependent suppression of L. pneumophila growth**

Classic studies identified a Stat1-Stat2 heterodimer as critical in transducing the biological response to IFN-Is (5, 11, 37). Unexpectedly, however, recent studies revealed that IFN-1 (IFN-α/β) was able to robustly suppress *L. pneumophila* growth in both Stat1−/− and Stat2−/− macrophages, whereas the ability of IFN-γ to suppress growth was completely dependent on Stat1 (17). To more rigorously explore this unexpected finding, Stat1-Stat2 double-knockout (Stat1−/−Stat2−/−) mice were generated. As previously reported (17), IFN-α effectively suppressed *L. pneumophila* growth in WT, Stat1−/−, and Stat2−/− macrophages in both the 129 (Fig. 1A, 1B) and C57BL/6J backgrounds (data not shown). Intriguingly, this response was lost in Stat1−/−Stat2−/− macrophages, in both the 129 (Fig. 1C) and C57BL/6J backgrounds (data not shown). The robust IFN-α-dependent activation of Stat3 in Stat1−/−Stat2−/− macrophages excluded the possibility that Stat3 might play an important role in the suppression of *L. pneumophila* growth (Supplemental Fig. 1A). Likewise, previous studies in Stat1−/− and Stat2−/− macrophages had excluded significant roles for other IFN-I–stimulated mediators, like p38 and phosphoinositide 3 kinases in the suppression of *L. pneumophila* growth (17, 38) (see also Supplemental Fig. 1A). These observations were consistent with a number of other reports (19, 20, 23) and raised the intriguing possibility that Stat1 and Stat2 are redundant in their ability to suppress *L. pneumophila* in response to IFN-Is. Because IFN-I–dependent Stat1 homodimer formation and transcriptional activity have been well documented (11, 12), subsequent studies focused on how Stat2 may signal independently of Stat1 (i.e., in Stat1−/− macrophages).

**Stat2 is able to direct ISRE-driven gene expression in Stat1−/− cells**

Studies exploring the mechanism by which IFN-Is suppress *L. pneumophila* had excluded an important role for iNOS, an IFN target gene (17, 34). However, they also surprisingly revealed that IFN-I–dependent iNOS expression was equivalent in WT and Stat1−/− BMMs at 24 h of stimulation (17). This observation suggested that Stat2 could direct iNOS expression independently of Stat1, consistent with other recent in vitro studies on IFN-I–dependent ISG expression (i.e., *Apobec3g*, *Adar1*, *Nox2*, and *Oas*) (20, 22–25). Moreover, our contemporaneous studies on Dengue virus–infected WT and Stat1−/− BMMs revealed overlapping patterns of ISG expression (19).

To further investigate these findings, expression of two well-characterized ISRE-driven ISGs, ISG-15 and Mx-1, was carefully evaluated by quantitative PCR in WT, Stat1−/−, Stat2−/−, and IRF-9−/− BMMs (7, 8, 11, 35). As anticipated, both genes were rapidly induced by IFN-I in WT BMMs (Fig. 2A). Consistent with previous studies, both genes were difficult to detect in Stat1−/− BMMs early after IFN-I stimulation (i.e., by 4 or 8 h), and they were essentially undetectable in Stat2−/− and IRF-9−/− BMMs (Fig. 2A) (11, 29, 39). Remarkably, however, at later time points both genes were robustly induced by IFN-I in Stat1−/− BMMs. Similar observations were made with other well-known ISRE-driven ISGs (i.e., *Ddx58*, *Ifit2*, *Ifit3*, *Dusp1*, *Dusp2*, *Adar*, *Bst2*, and *Oas2*; see Supplemental Fig. 1B).

**FIGURE 2.** Delayed kinetics of IFN-α-stimulated gene expression and Stat2 activation. (A) The kinetics of IFN-α/β (1000 U/ml)-dependent Mx-1 and ISG-15 expression was evaluated by quantitative PCR in C57BL/6J (WT), Stat1−/−, Stat2−/−, and IRF-9−/− BMMs. Similar results were obtained with IFN-β treatment and BMMs from the 129 background. (B) Whole-cell extracts (WCEs) were prepared from day 6 (d6) C57BL/6J (WT) (top), Stat1−/− (middle), and Stat2−/− (bottom) BMMs after stimulation with IFN-α/β (1000 U/ml), as indicated. Extracts were fractionated and immunoblotted with Abs specific for phospho-Stat1 (pSt1; Cell Signaling), phospho-Stat2 (pSt2; UB1), and tubulin (Sigma-Aldrich). The same extracts were refractionated by SDS-PAGE and immunoblotted for total-Stat1 (tStat1; Santa Cruz) and total-Stat2 (tStat2) (11). These results are representative of more than three independent experiments. Similar results were obtained with IFN-β treatment and in the 129 background.
To determine whether this delayed expression correlated with IFN-I–dependent STAT activation, extracts from WT, Stat1−/−, and Stat2−/− BMMs were evaluated by immunoblotting with Abs specific for the activated (i.e., tyrosine-phosphorylated) isoforms of Stat1 and Stat2. As expected, IFN-I stimulated the rapid, robust, and transient activation of Stat1 and Stat2 in WT BMMs (Fig. 2B, top panel) (12, 37). Yet, in Stat1−/− BMMs, significant quantities of phospho-Stat2 did not begin to accumulate until after prolonged IFN-I stimulation (i.e., 12, 18, and 24 h; Fig. 2B, middle panel). Moreover, this delayed activation correlated with the belated, IFN-I–dependent nuclear accumulation of Stat2 in the Stat1−/− BMMs (see Supplemental Fig. 2). Consistent with previous results (11, 12), IFN-I–treated Stat2−/− BMMs exhibited a rapid (i.e., 0.5 h; see Fig. 2B, bottom panel), albeit modest Stat1 activation that corresponded closely with the expression of GAS-driven target genes (e.g., IRF-1 and Stat1; Supplemental Fig. 1B) (11, 12). Intriguingly, there was also a second peak of phospho-Stat1, correlating with the delayed Stat2 activation observed in Stat1−/− BMMs, as well as a prolonged pattern of GAS-driven gene expression (see Supplemental Fig. 1B). These observations highlight the correlation between the delayed kinetics of Stat2 activation and ISG expression in IFN-I–stimulated Stat1−/− BMMs.

**Stat2 directs the formation of ISRE-binding complex in Stat1−/− cells**

To explore the possibility that Stat2 independently directs the expression of ISRE-driven genes in Stat1−/− BMMs, EMSAs and ChIP studies were carried out. As previously reported for WT BMMs, IFN-I–stimulated robust activation of ISGF3 (Stat1:Stat2:IRF-9) DNA-binding activity (see Fig. 3A) (11, 12, 37). Consistent with the preceding phospho-immunoblotting results, ISGF3 activity peaked early (0.5–2 h; data not shown) in WT cells (12), after which it rapidly decayed. In contrast, the ISRE DNA-binding activity observed in IFN-I–treated Stat1−/− BMMs was negligible at early time points (e.g., 0.5 h), but became pronounced by 16 h (Fig. 3A), reflecting the pattern of Stat2 phosphorylation (Fig. 2B). Moreover, this complex exhibited a distinct and slower mobility that did not appear to correlate with the faster IRF-1 ISRE-binding complex observed in IFN-I–treated cells (40). Further excluding IRF-1 was its limited expression in Stat1−/− BMMs. For ChIP studies, IFN-α–treated C57BL/6J (WT), Stat1−/−, Stat1−/− Stat2−/− BMMs, as above, were cross-linked, sonicated, and immunoprecipitated with a Stat2-specific Ab and interrogated by quantitative PCR. Error bars represent the SEM, and asterisks denote statistically significant differences (*p < 0.05, **p < 0.01, ***p < 0.0005).
BMMs (see Supplemental Fig. 1B). Of note, the slower migrating DNA-binding complex observed in the Stat1−/− BMMs was reminiscent of one identified when Stat2 and IRF-9 were overexpressed in U3A cells (21). Moreover, this slower migrating complex was supershifted by Abs specific for murine Stat2 and IRF-9, as was the case for bona fide ISGF3 in WT BMMs (Fig. 3A) (11, 12, 41).

Next, ChIP studies were employed to determine whether components of this novel complex were recruited to the promoters of bona fide ISRE-driven genes, as we recently reported for Dengue virus–infected cells (19). Although limitations with the IRF-9 Ab precluded directly evaluating recruitment of this component, the Stat2 Ab highlighted effective Stat2 recruitment to Mx-1 and ISG15 promoters at both 0.5 and 16 h of IFN-I stimulation in WT BMMs. In contrast, in Stat1−/− BMMs Stat2 was only recruited to these two promoters after 16 h of IFN-I stimulation. These observations are not only consistent with prior reports suggesting a Stat2–IRF-9 complex may drive the expression of some genes (21–23, 25), but also demonstrated that this more slowly migrating ISRE-binding complex is recruited to the promoters of bona fide ISGs.

To confirm that IRF-9 was required for the delayed expression of ISGs observed in Stat1−/− BMMs, IRF-9 was knocked down in immortalized BMMs (iBMMs) through lentiviral-mediated short hairpin IRF-9 RNA (Fig. 4A). Consistent with studies on IRF-9−/− BMMs (Fig. 2A), Mx-1 failed to be expressed in WT iBMMs in which IRF-9 had been knocked down (Fig. 4A). More importantly, IRF-9 was required for the IFN-I–dependent expression of both Mx-1 and ISG-15 in Stat1−/− iBMMs (Fig. 4A). An additional analysis in Stat1/Stat2 double-knockout BMMs revealed that Stat2 is absolutely required for IFN-I–dependent gene expression in Stat1-deficient macrophages (Fig. 4B). These data provide additional support for the model that, absent Stat1, a Stat2–IRF-9 complex directs the expression of ISRE-driven genes, albeit with substantially delayed kinetics.

**Activation of the Stat2–IRF-9 complex requires continuous IFN-I–dependent signaling**

Next, studies were undertaken to explore why Stat2 was activated with such delayed kinetics in Stat1−/− BMMs. Because the basal level of Stat2 was reduced in Stat1−/− BMMs (Fig. 2), the first set of studies probed Stat2 t1/2 through cyclheximide-dependent turnover (Supplemental Fig. 3). These studies revealed that Stat2 protein had a considerably shorter t1/2 in Stat1−/− than WT BMMs. However, Stat2 levels increased steadily upon IFN-I treatment in both WT and Stat1−/− BMMs, although the response was delayed in Stat1−/− BMMs (e.g., Fig. 5B). Consistent with this, the level of Stat2 transcripts increased more rapidly in WT than Stat1−/− BMMs upon IFN-I treatment (Supplemental Fig. 1B), suggesting that resting Stat1−/− cells may not express sufficient, stable levels of Stat2 to enable a rapid and robust signaling response.

A second set of studies explored whether the ligand-dependent (data not shown) and delayed Stat2 activation observed in Stat1−/− BMMs was associated with a prolonged activation of Jak1, the dominant JAK in IFN-I response (10). As previously reported, Jak1 activity, interrogated through phospho-immunoblotting, was rapidly induced in IFN-I–stimulated WT BMMs. But this activation was quite transient, a response that has been attributed to the IFN-I–dependent expression of an important negative regulator, Socs1 (12, 13, 42). In contrast, Jak1 phosphorylation was more robust and prolonged in the Stat1−/− BMMs, indicating an extended duration of activity (Fig. 5A).

To determine whether this enhanced Jak1 activity was important for the delayed activation of Stat2 in Stat1−/− BMMs, these cells were treated with a potent JAK inhibitor, P6. As anticipated, the addition of P6 1 h prior to harvest after a short IFN-I treatment (i.e., 0.5 or 1 h) of WT BMMs led to a substantial reduction in STAT phosphorylation, demonstrating this drug effectively blocked JAK-dependent activation (Fig. 5B, top panels). Likewise, a 4-h pulse of P6 prior to harvesting IFN-I–treated WT cells at 18 or 22 h had little effect because the signal had already decayed. However, in Stat1−/− BMMs, the 4-h P6 pulse significantly impaired the delayed activation of Stat2 (Fig. 5B, bottom panels). Consistent with this, the addition of P6 in the final 4 h of IFN-α stimulation led to a significant reduction in ISG-15 and Mx-1 gene expression in Stat1−/− BMMs, as well as WT BMMs (Fig. 5C). These studies confirmed that prolonged Stat2 activation in Stat1−/− BMMs was dependent on prolonged and ligand-dependent Jak1 activity.

A final set of studies explored whether a defect in Socs1 expression might account for prolonged Jak1 activity in IFN-I–treated Stat1−/− BMMs. In contrast to the rapid and robust expression profile in WT BMMs, Socs1 expression was substantially reduced in IFN-I–stimulated Stat1−/− BMMs, but less dramatically in the
Stat2−/− and IRF-9−/− BMMs failed to robustly induce Socs1 expression, an important negative regulator of IFN-I–stimulated JAK activation (12, 13). Third, a dose-response study revealed that the amounts of IFN-I required to achieve equivalent levels of phospho-Stat2 in WT and Stat1−/− BMMs led to equivalent levels of target gene expression at early time points in WT BMMs versus later time points in Stat1−/− BMMs, respectively (Supplemental Fig. 3B). These observations are consistent with a model, in which over time, accumulating Stat2 protein and prolonged JAK activity reach the threshold of phospho-Stat2 required for IFN-I–dependent signaling. However, it is certainly possible that rate-limiting concentrations of IRF-9, the second component of the signaling complex, may also contribute to this delay. Additional models could be considered, including a specific restriction in nuclear translocation (44), elevated phosphatase activity (45), or an enhanced capacity for phospho-Stat2 degradation in Stat1−/− BMMs.

The ability of a Stat2–IRF-9 complex to drive the expression of a subset of ISGs was presaged by several cell line–based, overexpression studies. The first such study demonstrated that with ectopic overexpression in HEK-293 cells, activated Stat2 could dimerize and, with the addition of ectopic IRF-9, form a more slowly migrating ISRE-binding complex (21). Additional overexpression studies in Stat1-deficient U3A cells revealed a physical interaction between IRF-9 and activated Stat2 (22). Moreover, this complex, along with the unusually prolonged Stat2 activation kinetics observed in NB4 cells, was ascribed a role in IFN-I–stimulated RIG-G expression. Likewise, Stat1 and Stat2 knockdown studies in Hep3B cells highlighted a more important role for Stat2 (versus Stat1) in the IFN-I–dependent induction of several ISGs, including APOBEC3G (23); but IRF-9 knockdown was associated with only a partial block of ISG expression. Remarkably, a parallel knockdown of Stat1 in HEK-293 cells revealed an essential role for this STAT in the IFN-I–dependent expression of the same ISGs (23). Similarly, two studies in primary cells, including our own with Dengue virus, revealed a more important role for Stat2 than Stat1 in the innate response to this virus.
response to several viruses, but did not elucidate the signaling pathways responsible for this effect (19, 20). Finally, a more recent study has ascribed a more important role for Stat2 in the ability of IFN-β and TNF-α to synergistically stimulate the expression of DUOX2 NADPH oxidase in two epithelial lines (25).

Confirming several earlier overexpression studies, our results demonstrate that, in primary Stat1−/− cells, Stat2 can associate with IRF-9 to drive the delayed expression of a subset of ISGs important in the innate response to L. pneumophila with IRF-9 to drive the delayed expression of a subset of ISGs important in the innate response to L. pneumophila, Dengue virus, as well as potentially other viruses. Although we find little evidence of a significant quantity of activated Stat2−IRF-9 complex in WT BMMs, it is intriguing to speculate that this pathway evolved as a backup response to defend against pathogens that impede Stat1 activity (e.g., Paramyxovirus) (27). It is also possible that, during severe immune stress, Stat1 may become functionally deficient, providing an additional setting in which the Stat2−IRF-9 pathway could provide a backup response. In future studies, it will be interesting to explore whether this delayed pathway serves to afford a more durable IFN-I response during chronic microbial challenges, or potentially to integrate responses between IFN-I and TNF-α (26).

Disclosures

The authors have no financial conflicts of interest.

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