High basal STAT4 balanced by STAT1 induction to control type 1 interferon effects in natural killer cells

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The best-characterized type 1 interferon (IFN) signaling pathway depends on signal transducer and activator of transcription 1 (STAT1) and STAT2. The cytokines can, however, conditionally activate all STATs. Regulation of their access to particular signaling pathways is poorly understood. STAT4 is important for IFN-γ induction, and NK cells are major producers of this cytokine. We report that NK cells have high basal STAT4 levels and sensitivity to type 1 IFN-mediated STAT4 activation for IFN-γ production. Increases in STAT1, driven during viral infection by either type 1 IFN or IFN-γ, are associated with decreased STAT4 access. Both STAT1 and STAT2 are important for antiviral defense, but STAT1 has a unique role in protecting against sustained NK cell IFN-γ production and resulting disease. The regulation occurs with an NK cell type 1 IFN receptor switch from a STAT4 to a STAT1 association. Thus, a fundamental characteristic of NK cells is high STAT4 bound to the type 1 IFN receptor. The conditions of infection result in STAT1 induction with displacement of STAT4. These studies elucidate the critical role of STAT4 levels in predisposing selection of specific signaling pathways, define the biological importance of regulation within particular cell lineages, and provide mechanistic insights for how this is accomplished in vivo.

Type 1 IFNs (IFN-α/β) are a family of cytokines with a wide range of antiviral and immunoregulatory effects (1–6). Binding to their heterodimeric IFN-α/β receptor (IFNAR) is understood to activate signal transducer and activator of transcription 2 (STAT2) and STAT1, with their phosphorylation leading to the formation of a transcriptional complex including STAT1–STAT2 heterodimers and induction of interferon-stimulated genes (5, 7, 8). STAT1–STAT1 homodimers can be activated by stimulation of the receptors for either type 1 IFNs or IFN-γ (5, 7) to result in overlapping induction of certain genes (2, 4–6). In addition to STAT1 and STAT2, type 1 IFNs have been reported to conditionally activate all of the STATs, including STAT4 (3, 9). This flexibility may provide an explanation for how their functions are extended from antiviral effects to regulation of immune responses, but the understanding of the mechanisms in place to preferentially access different signaling pathways is limited. Based on the observations that STAT1 levels are induced during viral infections, and that this negatively correlates with type 1 IFN activation of STAT4 in mixed populations ex vivo, this laboratory has proposed that access to either of the two signaling molecules is a consequence of the relative concentration of STAT1 (10). However, the in vivo pathways coordinating these events, their biological importance, and the mechanisms for how changing STAT1 levels acts to limit access to STAT4 remain unknown.

NK cells are major contributors to innate IFN-γ production (11). Because NK cells can produce high levels of IFN-γ, they may be particularly well adapted for making the cytokine (12, 13). Under conditions of cytokine stimulation for IFN-γ production, activation of STAT4 is important for peak responses (14, 15). Type 1 IFNs can induce IFN-γ (3, 9, 10, 13, 16–20). They are not potent inducers of peak NK cell IFN-γ production under immunocompetent conditions of viral infections (14, 15), however, and they have an additional paradoxical function for negatively regulating NK cell IFN-γ responses (13). The inhibitory effect of type 1
IFN may be important for promoting health because IFN-γ can also contribute to cytokine-mediated disease (21–25). The mechanisms for selecting between enhancement and inhibition are not clear, and much remains to be learned about how NK cells are simultaneously predisposed to be good IFN-γ producers, but tightly regulated to protect against detrimental responses. The relationship between type 1 IFNs and access to STATs may be important in the control.

The experiments presented here evaluate the expression and function of STATs within NK cells responding in vivo. They were performed in the context of the lymphocytic choriomeningitis virus (LCMV) infection inducing a dominant type 1 IFN innate cytokine response in mice (12, 26–29). The results demonstrate that NK cells are predisposed to STAT4 pathways and have an unusual sensitivity for type 1 IFN activation of STAT4 because their basal expression of this protein is substantially higher in comparison to other populations. Dramatic increases in STAT1 are, however, elevated in all cells during infection. In the presence of STAT2, type 1 IFNs induce a rapid increase in STAT1. In the absence of STAT2, an early IFN-γ production induces STAT1 with delayed kinetics. Characterization of responses in immunocompetent compared with STAT1− or STAT2−deficient mice identify the importance of elevated STAT1 for inhibition of the type 1 IFN pathway to STAT4. The changes in STAT4 sensitivity are accompanied by a displacement of its association with the type 1 IFN receptor; STAT4 is coprecipitated with IFNAR in NK cells, but not total or non-NK cells, from uninfected mice, but increased STAT1 is coprecipitated with the receptor in all populations after infection. These results define the critical role of basal STAT4 levels in predisposing selection of signaling pathways, and suggest basal STAT4 association with the type 1 IFN receptor as a mechanism for the preferential access. Collectively, the studies precisely map intracellular signaling pathways regulating type 1 IFN effects, and demonstrate how modification of accessibility is shaped to control immune responses.

RESULTS
Expression of STAT4 and STAT1
Flow cytometric techniques were developed to measure intracellular levels of total STAT1 and STAT4 proteins, as well as their active phosphorylated forms, pSTAT1 and pSTAT4. To validate the approach, splenic leukocytes from WT, STAT1−deficient, or STAT4−deficient C57BL/6 mice were isolated and control-treated or treated with a type 1 IFN (recombinant mouse [rm] IFN-β) for 90 min in culture. Isootype staining was also performed as a control. Results were compared with those observed with cell lysates evaluated in Western blot analyses using β-actin measurements for protein loading control. A variety of antibodies reported to be specific for the total or activated STATs were screened. Results obtained with those determined to be the most specific for the application are shown in Fig. 1. Total STAT1 was detected by both Western blot and flow cytometric analyses in WT, but not STAT1−cells, and pSTAT1 was induced after type 1 IFN treatment in only WT populations. There was uniform shift in the intensity of cytoplasmic staining (Fig. 1 A). Total STAT4 was detected by Western blot in WT, but not STAT4− cells, and pSTAT4 was induced after type 1 IFN treatment in only WT populations. It was difficult to detect STAT4 in the mixed populations, however, and pSTAT4−positive cells were a small subset (Fig. 1 B).

NK cells are <5% of splenic leukocytes. To evaluate differential STAT levels in NK cells, populations from WT 129 mice were used to prepare total, NK (enriched to >90% purity by CD49b−positive selection), and non-NK (CD49b−negative) populations. Proteins were extracted for Western blot analysis. Compared with β-actin, NK cells expressed ~10–20-fold more STAT4 protein than total or non-NK cells (Fig. 1 C). Similar differences were observed evaluating intracellular staining of the mixed populations with gating on total viable, CD49b+CD3− NK cells, or CD49b−non-NK cells. Peak STAT4 expression in all but NK cells was low at a fluorescent intensity within 1 log. In contrast, 75% of NK cells had high STAT4 expression, with populations into the second log of intensity (Fig. 1 D). The STAT1 levels were low in the three different populations, but NK cells routinely had the lowest levels using either method of analysis (Fig. 1, C and D). Thus, NK cells have an unusually high basal STAT4 and lower STAT1 expression.

To examine the levels of these signaling molecules during infection, 129 mice were either uninfected or challenged with 2 × 10^4 PFUs of LCMV on day 0 (D0). At D0 or D1.5 and D2.5 after infection, splenic leukocytes were harvested, and the total or NK (CD49b+CD3−) cells were evaluated for intracellular expression of STAT4 compared with STAT1. The STAT4 levels were relatively constant and low in the total leukocyte populations on D0, D1.5, and D2.5. In contrast, the STAT1 levels were dramatically increased with 67–75% of the populations having high expression after infection (Fig. 1 E). The NK cells consistently expressed higher levels of STAT4, but also had dramatic increases in STAT1, from <2 to >70% positive, during infection (Fig. 1 F). Hence, high levels of STAT4 are sustained in NK cells and not in total populations, but STAT1 levels are dramatically induced in all of the cells during early viral infection.

Activation of STAT4 and STAT1
To evaluate the activation of the STATs in vivo, flow cytometric analyses were performed for intracellular expression of pSTAT4 or pSTAT1. Immediately after harvest, there was no evidence of phosphorylated STATs in total (Fig. 1 G) or NK (CD49b+CD3−; Fig. 1 H) cells prepared from uninfected mice (D0). There was a reproducible detection of a small proportion (7%) of pSTAT4-positive NK cells on D1.5 that was not observed in any of the other populations examined. Both total and NK cell populations were ~20% pSTAT1 positive on D1.5 and 10% pSTAT1 positive on D2.5. Thus, all of the examined populations are responding to the conditions of viral infection with activation of STAT1, and a small population of NK cells is responding with activation of STAT4.
To examine cellular responsiveness to type 1 IFN for STAT activation, cells isolated on D0, D1.5, and D2.5 were rested at 37 °C for 4 h without cytokine stimulation to allow for "clearing" of signals delivered in vivo, and then control-treated or treated with IFN-β/H9252 for 90 min. Flow cytometric analyses evaluated pSTAT1 and pSTAT4 (Fig. 2). The total and NK cells were all type 1 IFN responsive, and they maintained this responsiveness during infection. Total populations had relatively uniform shifts in pSTAT1 staining after IFN treatment, with 36–43% of the cells becoming highly positive. The NK cells also responded with pSTAT1, but the populations were more heterogeneous on D0, with 24–53% of the cells becoming positive.

Figure 1. In vivo expression of STAT1 and STAT4. Total STAT1, pSTAT1, total STAT4, or pSTAT4 protein levels were evaluated in splenic cell lysates by Western blot analyses, with β-actin measurements as a loading control, or within individual cells by flow cytometry with antibody isotype control staining. Techniques were validated (A and B) using samples derived from WT, STAT1-deficient, or STAT4-deficient mice. Cells were control treated or treated with rmIFN-β in culture (see Materials and methods). In the histograms of the flow cytometric staining analyses, results presented by dotted lines are isotype controls, gray areas are total STATs or pSTATs in control WT cells, and thick lines are pSTATs in control WT cells treated with IFN-β. The thin lines show results using the respective STAT-deficient cells for total STATs or for pSTATs after treatment with IFN-β. Subset levels of STAT expression were evaluated with splenic leukocytes from uninfected WT mice by Western blot analysis of lysates collected from "total" cells, or subsets separated into NK (CD49b+) and non-NK (CD49b−) cells (C), and histograms of flow cytometric analyses of STAT4 and STAT1 expression in gated total, CD49b+CD3− NK, and non-NK cells (D). The effects of LCMV infection on STAT (E and F) and pSTAT (G and H) levels were evaluated with freshly isolated cells [E and F] with splenic leukocytes derived from uninfected or D1.5- and D2.5-infected WT mice. Histograms present levels in total [E and G] and CD49b+CD3− NK [F and H] cells. Numbers are the means ± the SEM of positive cell percentages compared with the isotype controls from three mice in each group. Data are representative of more than three independent experiments.

Figure 2. Ex vivo responsiveness to type 1 IFN for activation of STAT1 and STAT4. Cells were prepared from uninfected or mice infected with LCMV for 1.5 or 2.5 d. Responsiveness of "rested" cells to type 1 IFN for STAT4 and STAT1 activation was determined using splenic leukocytes incubated without the addition of cytokines for 4 h, and then either unstimulated or stimulated with rmIFN-β for 90 min. Cells were collected and stained to detect intracellular pSTAT4 and pSTAT1 expression within total and NK cells using flow cytometry. Histogram plot graphs (linear y axes of cell number) represent pSTAT1 (top) or pSTAT4 (bottom) staining. Dotted lines show staining with isotype controls. Gray areas or solid lines show staining of untreated cells or IFN-β-treated cells, respectively. Numbers given are means, with SEMs in parentheses, of percentages of positive cells in IFN-β-treated cells, compared with isotype controls. Data are representative of more than three independent experiments.

To examine cellular responsiveness to type 1 IFN for STAT activation, cells isolated on D0, D1.5, and D2.5 were rested at 37 °C for 4 h without cytokine stimulation to allow for “clearing” of signals delivered in vivo, and then control-treated or treated with IFN-β for 90 min. Flow cytometric analyses evaluated pSTAT1 and pSTAT4 (Fig. 2). The total and NK cells were all type 1 IFN responsive, and they maintained this responsiveness during infection. Total populations had relatively uniform shifts in pSTAT1 staining after IFN treatment, with 36–43% of the cells becoming highly positive. The NK cells also responded with pSTAT1, but the populations were more heterogeneous on D0, with
24% becoming highly positive, and the proportions of highly positive cells increasing to 45 and 53% on D1.5 and D2.5, respectively, after infection (Fig. 2, top). Total populations were relatively insensitive for STAT4 activation at all times examined (Fig. 2, bottom). In contrast, there was a dramatic pSTAT4 response of the D0 NK cells; 58% of these cells had high-intensity staining for pSTAT4. The STAT4 response was greatly diminished after infection, with only 5–10% of the NK cell populations prepared on D1.5 or 2.5 of infection demonstrating STAT4 activation. Collectively, the results show that all cells prepared from uninfected and infected mice are able to respond to type 1 IFN with STAT1 activation, but NK cells are uniquely armed to dramatically respond with STAT4 activation. This STAT4 phosphorylation response is diminished during infection.

**Regulation of STAT1 protein expression**

Because both type 1 IFN and IFN-γ have been reported to induce STAT1 in culture (30), their contribution to in vivo STAT1 induction was evaluated. These experiments took advantage of IFNAR (IFNα/βR)-deficient, IFN-γ R-deficient, and double receptor (IFN-α/β/γ R)-deficient compared with WT 129 mice. Mice were uninfected or LCMV infected for 1.5 d. Splenic leukocytes were harvested, and total STAT1 protein levels were determined by Western blot (Fig. 3 A). At D1.5 after infection, STAT1 induction was primarily dependent on type 1 IFN, but not IFN-γ function. Absence of responsiveness to both cytokines resulted in a complete block in STAT1 induction. Flow cytometric studies examining levels within total (Fig. 3 B) and gated CD49b+CD3−NK (Fig. 3 C) cells gave similar results. Thus, the peak early induction of STAT1 is primarily dependent on responsiveness to type 1 IFN.

Type 1 IFN signaling is predisposed to using STAT2 and STAT1, whereas IFN-γ signaling is STAT1 dependent, but STAT2 independent (2, 5, 6). The STAT2 signaling requirement was evaluated by examining STAT1 induction in STAT2-deficient mice compared with WT and STAT1-deficient mice. Infection of WT mice induced STAT1 to high levels, with densities increased by 2.6–3-fold at D1.5

Figure 3. Regulation of STAT1 expression. Total STAT1 protein levels were evaluated in cell lysates by Western blot analyses with β-actin measurements as a loading control (A, D, and G) or within individual cells by flow cytometry, electronically gating on total (B, E, and H) and on CD49b+CD3−NK (C, F, and I) cells. Splenic leukocytes were derived from uninfected or D1.5 LCMV-infected WT, IFNAR-deficient, IFN-γ R−/− deficient mice, or IFN−α/β/γR−/−deficient mice (A–C). Splenic leukocytes were derived from uninfected or 1.5- and 2.5-d LCMV-infected WT, STAT2-deficient, or STAT1-deficient mice (D–F). Splenic leukocytes were derived from uninfected, anti–IFN-γ antibody–treated, or control Ig–treated D2.5 LCMV-infected WT and STAT2-deficient mice (G–I). The numbers in all histograms represent means ± the SEM of three mice in each group of percentages of STAT1-positive cells, compared with the isotype control. Data presented are representative of more than three independent experiments.
and D2.5 (Fig. 3 D). Although infection also induced STAT1 in STAT2-deficient mice, the effect was delayed. The levels were lower at D1.5 than those in WT mice, showing only a 1.6-fold increase compared with D0, but the molecule was induced to comparable levels, with a 2.8-fold increase by D2.5 after infection. Flow cytometric experiments yielded similar results with total (Fig. 3 E) and NK (Fig. 3 F) cells isolated from STAT2-deficient infected mice displaying delayed induction of STAT1. These studies demonstrate that the presence of STAT2 is accompanied by an accelerated induction of STAT1, but a STAT2-independent pathway to STAT1 induction occurs with delayed kinetics.

To examine the contribution of endogenously induced IFN-γ to STAT1 induction in the STAT2-deficient mice, infected mice were control-treated or treated with antibodies neutralizing IFN-γ (Fig. 3, G–I). Although neutralization of IFN-γ only modestly reduced the levels of STAT1 induced on D2.5 after infection of WT mice, there was a 3–5-fold decrease resulting from IFN-γ neutralization in STAT2-deficient mice, and this was also apparent in NK cells. Thus, type 1 IFN provides a dominant pathway and IFN-γ provides an accessory pathway to STAT1 induction, but in the absence of the STAT2-dependent type 1 IFN effect, IFN-γ is critically involved in STAT1 induction.

**IFN-γ and pSTAT4 responses in the context of changing STAT1 levels**

Serum samples were taken at different times after infections and evaluated by ELISA to compare IFN-γ responses in STAT2- to STAT1-deficient and WT mice. Consistent with our previous results (13, 31), immunocompetent mice had only minimal, but STAT1-deficient mice had profound, IFN-γ responses in the circulation on D1.5, D2, D2.5, and D3 after LCMV infection (Fig. 4 A). In STAT2-deficient mice, increased IFN-γ production was observed on D1.5 after infection, but the values returned to near normal by D2.5. Flow cytometric studies performed to evaluate the proportions of NK cells and their expression of IFN-γ demonstrated that the frequencies of NK cells were similar, with all strains showing modest reductions after infection (Fig. 4 B). NK cells only expressed IFN-γ at very low levels during infection of WT mice (Fig. 4 C). In STAT1-deficient mice, however, as many as 10–20% of the NK cells were high-intensity IFN-γ positive at D1.5 and D2.5. In STAT2-deficient mice, ~15% of the NK cells were high-intensity IFN-γ positive at D1.5, but at D2.5 <2% expressed the cytokine, and only with low intensity. Thus, NK cells can be major contributors to early IFN-γ responses during viral infection, but their response is tightly regulated in WT mice and with delayed kinetics in STAT2−, but not STAT1−, mice.

To determine the relationship between total STAT1 levels and IFN-γ expression within individual NK cells, concurrent intracellular expression of these molecules was examined immediately after isolation (Fig. 5 A). During infection of WT mice, the NK cells with dramatically increased STAT1 had only low-level intracellular IFN-γ expression detectable. Much higher levels of IFN-γ were observed in the populations isolated from LCMV-infected STAT1-deficient mice on either D1.5 or D2.5. In STAT2-deficient mice on D1.5, STAT1 expression was still low in most of the NK cells and the induced IFN-γ expression was detectable in these cells as compared with the NK cells already having high STAT1 levels.
STATS AND TYPE 1 IFN EFFECTS ON NK CELLS | Miyagi et al.

Having low STAT1 were the populations expressing pSTAT4. Thus, NK cells can be induced to express high STAT1 and IFN-β/H9253 and/or pSTAT4 in vivo, but those having the highest levels of STAT1 do not express IFN-β/H9253 or pSTAT4; this is particularly clear during infections of STAT2-deficient mice with delayed STAT1 induction.

To directly evaluate the type 1 IFN responsiveness of the cells having different levels of STAT1 conditioned during infection, splenic populations were isolated from uninfected or D2.5 LCMV-infected WT, STAT2-deficient, or STAT1-deficient mice. The cells were incubated in culture for 4 h without stimulation, and then control treated or treated with IFN-β/H9252 for 90 min. Flow cytometric analyses gated on the CD49b/H11001 CD3/H11002 NK cells demonstrated that, if left without cytokine stimulation, all of the NK cells had intracellular pSTAT4 levels returned to baseline values (Fig. 6 compared with Fig. 5B), but maintained total STAT1 levels. In vitro stimulation with IFN-β/H9252 activated STAT4 expression using flow cytometry. Dot plot graphs are logarithmic scale representations of the proportions of cells. Numbers given are means of positive cells in low or high STAT1 staining gates (SEMs are not >3% of results from three different mice). Data are representative of more than three independent experiments.

Figure 5. Characterization of STAT1 levels in NK cells with in vivo–induced IFN-γ and pSTAT4. Splenic leukocytes were prepared on D0, D1.5, and D2.5 of LCMV infection from WT, STAT2-deficient, and STAT1-deficient mice. Populations were stained for CD49b+ and CD3− to identify NK cell subsets, and intracellularly for STAT1 and IFN-γ (A), or STAT1 and pSTAT4 (B). Dot plots represent intracellular STAT1 (log x axes) and IFN-γ (A) or pSTAT4 (B; log y axes) expression in gated CD49b+CD3− NK cells. Three mice per group were used. Numbers within graphs are averages of positive cells in low or high STAT1 staining gates (SEMs are not >2% of results from three different mice). Data are representative of more than three independent experiments.

At D2.5, all of the cells had shifted to express higher STAT1, and there was no detectable IFN-γ expression. Parallel studies with STAT1 and pSTAT4 expression were performed. Remarkably, it was possible to spontaneously detect a strong pSTAT4 response within STAT2-deficient NK cells on D1.5 immediately after isolation and within STAT1-deficient NK cells on D1.5 and D2.5 of infection (Fig. 5B). On D1.5, 16–19% of these cells had a clear induction of pSTAT4, and 10% of the STAT1-deficient NK cells on D2.5 still had pSTAT4. As in the case of IFN-γ expression, the cells having low STAT1 were the populations expressing pSTAT4. Thus, NK cells can be induced to express high STAT1 and IFN-γ and/or pSTAT4 in vivo, but those having the highest levels of STAT1 do not express IFN-γ or pSTAT4; this is particularly clear during infections of STAT2-deficient mice with delayed STAT1 induction.

To directly evaluate the type 1 IFN responsiveness of the cells having different levels of STAT1 conditioned during infection, splenic populations were isolated from uninfected or D2.5 LCMV-infected WT, STAT2-deficient, or STAT1-deficient mice. The cells were incubated in culture for 4 h without stimulation, and then control treated or treated with IFN-β for 90 min. Flow cytometric analyses gated on the CD49b+CD3− NK cells demonstrated that, if left without cytokine stimulation, all of the NK cells had intracellular pSTAT4 levels returned to baseline values (Fig. 6 compared with Fig. 5B), but maintained total STAT1 levels. In vitro stimulation with IFN-β activated STAT4 in NK cells from all uninfected mice; these populations were low for STAT1 expression and dramatically responsive to IFN-β with 48–67% of the cells induced to express pSTAT4. In contrast, the NK cells isolated from mice on D2.5 of infection displayed differences in pSTAT4 induction correlating with total STAT1 levels: 34% of the NK cells isolated from STAT1-deficient mice responded to IFN-β with pSTAT4 expression, but those from WT and STAT2-deficient mice, respectively, were only 10 and 12% pSTAT4 positive (Fig. 6). The smaller proportions of responders were preferentially found in cells...
having lower levels of STAT1, i.e., 7 and 9%. Therefore, type 1 IFN induces activation of STAT4 in NK cells independently of STAT1 or STAT2, but once STAT1 is induced to higher levels, the access to STAT4 is inhibited.

**Type 1 IFN activation of STAT4 and IFN-γ in NK cell in vivo**

To evaluate endogenous type 1 IFN exposure, the cytokines were measured in serum samples. Systemic levels of type 1 IFN bioactivity were observed in all three strains of mice at D1.5 and D2.5 of LCMV infection (Fig. 7 A). ELISA data indicated that both IFN-α and -β were induced (Fig. 7 A). To define the endogenous role of type 1 IFN for NK cell STAT4 activation and IFN-γ production in STAT1- and STAT2-deficient mice induced on D1.5 of infection, IFN-α/β function was neutralized by administration of antibody blocking the receptor. In comparison to samples from control antibody–treated mice, blocking endogenous type 1 IFN function with anti-IFNAR antibody treatments dramatically reduced serum IFN-γ, NK cell IFN-γ expression, and NK cell expression of pSTAT4 in STAT2- (Fig. 7 B) and STAT1-deficient (Fig. 7 C) mice. These results demonstrate the critical in vivo contribution of type 1 IFN to IFN-γ production by, and STAT4 activation in, NK cells stimulated under conditions of reduced STAT1 protein levels.

**Biological consequences of STAT1 and STAT2 deficiencies during LCMV infection**

The consequences of STAT deficiencies for antiviral defense were evaluated in mice infected with LCMV for 2, 3, or 5 d. Spleens were harvested and viral titers were determined in a plaque assay. In comparison to WT mice, an increased susceptibility to infection was observed in both STAT1- and STAT2-deficient mice at all time points (Fig. 8 A). In contrast, disease state as evaluated by changes in body weight was only apparent in STAT1-deficient mice (Fig. 8 B). The absence of STAT1 resulted in weight loss ranging
from 10 to 15% by D3 of infection. During the same time period, the WT and STAT2-deficient mice were gaining weight. Thus, although both STAT1 and STAT2 are required to promote optimal defense against LCMV, STAT1, but not STAT2, is required for sustaining weight as a measurement of health.

Because weight loss is an indicator of cytokine-mediated disease (21), the involvement of IFN-γ was investigated in the infected, STAT1-deficient mice. Mice were treated with antibody neutralizing IFN-γ. In comparison to control treatments, inhibition of IFN-γ provided significant protection from virus-induced wasting at the critical D3 (Fig. 8 C). Because NK cells were major producers of the IFN-γ response, their contribution to disease was evaluated by treating mice with antibodies deleting the cells. NK cell depletion also provided protection from maximal virus-induced wasting (Fig. 8 D). Collectively, these data demonstrate that the absence of either STAT1 or STAT2 results in increased sensitivity to viral infection and increased IFN-γ at the earliest time points, but that sustained IFN-γ production and an NK-cell– and IFN-γ– dependent disease are only induced in the absence of STAT1. Thus, STAT1, in the presence or absence of STAT2, promotes the regulation of NK cell responses and protects against cytokine disease.

**STAT associations with the type 1 IFN receptor**

The aforementioned studies demonstrate that type 1 IFNs can activate STAT4 in NK cells with their high basal levels of this signaling molecule, but not in populations with low STAT4 expression. Moreover, they show that even in the presence of high STAT4, this pathway of activation is lost during viral infection at times correlating with elevated STAT1 expression. STAT1 is associated with the IFNAR before cytokine exposure in cultured fibroblasts (32), but little is known about its association in freshly isolated leukocytes from uninfected and infected mice, and the association of STAT4 with the receptor has not been tested under these conditions. To define the physical interactions between IFNAR and the STATs and advance the mechanism understanding of the loss in STAT4 access, experiments evaluating coprecipitation with the receptor were performed. The antibody used has been shown to immunoprecipitate IFNAR (33). Total, NK (enriched by CD49b-positive selection), and non-NK (CD49b-negative selection) populations isolated from uninfected WT were evaluated. Immunoprecipitations were performed with cell lysates. Total input proteins, as well as precipitated proteins, were characterized by Western blot analysis. Controls were total lysates from IFNAR−, STAT1−, and STAT2−.

**Figure 8.** Role of STAT1 or STAT2 in antiviral defense and disease progression. (A) Spleens, isolated from uninfected or infected WT, STAT1-deficient, or STAT2-deficient mice on the indicated days after LCMV infection, were processed to quantify the viral titers in a plaque assay. Each circle indicates the value of an individual mouse. Black bars represent averages in each group. Data are the accumulated results from more than six independent experiments. Dashed line represents the lower limit of detection (LD). Sample values, which are indicated with §, were below the lower limit of detection. For A and B, white circles are results from WT mice, black circles indicate results from STAT2-deficient mice, and gray circles are results from STAT1-deficient mice. (B) Disease was evaluated by changes in body weights. Uninfected or infected mice were monitored for their body weight once a day from D0 to D5. Percentage of body weight changes compared with D0 value is shown. Data represent the mean ± the SEM from 10 mice in each group. (C) The role for IFN-γ in disease was tested. Anti-IFN-γ antibody–treated or control Ig–treated STAT1-deficient mice were monitored for their body weight changes on D3 after LCMV infection. The percentage of body weight losses is shown. Gray circles represent the values of individual control Ig–treated and gray squares represent anti-IFN-γ antibody–treated mice. Black bars represent averages. Data are from six mice in each group. Differences between control Ig–treated STAT1-deficient mice and anti-IFN-γ antibody–treated STAT1-deficient mice are significant at P < 0.003. (D) The role for NK cells in disease was tested by depleting the cells with anti-AGM1 treatments. Anti-AGM1 antibody–treated or control Ig–treated STAT1-deficient mice were monitored for their body weight changes on D3 after LCMV infection. The percentages of body weight loss are shown. Gray circles represent individual control Ig–treated and gray triangles represent anti-AGM1 antibody–treated mice. Black bars represent averages. Data are from five mice in each group. Differences between control Ig–treated STAT1-deficient mice and anti-AGM1 antibody–treated STAT1-deficient mice are significant at P < 0.05.
STAT4–deficient mice, and control immunoglobulin precipitation (Fig. 9). There was a significant preassociation of STAT4 with IFNAR in NK cells, but not in non-NK or total cells, prepared from uninfected mice (Fig. 9 A, lanes 1–3). To eliminate the possibility of secondary effects induced during positive selection, the experiments were repeated with populations enriching NK cells by negative selection. The STAT4 preassociation with IFNAR was also observed with these NK cells (Fig. 9 B). Only low levels of STAT1 were coprecipitated with the receptor in any of the populations from uninfected mice.

The type 1 IFN response to LCMV can be detected for up to 4 d after infection (26, 29). Because the binding of the IFNAR antibody is inhibited in the presence of the receptor ligand (33), and because the total STAT1 levels are increased for up to 5 d after infection (10, 34) (unpublished data), the experiments to evaluate the consequences of increased STAT1 on STAT4 binding were performed with cells prepared on D5. At this time, STAT4 binding to IFNAR was significantly decreased in NK cells, despite sustained STAT4 levels (Fig. 9 A, lane 5). However, all three populations, including NK cells, had markedly increased associations with the elevated STAT1 (Fig. 9 A, lanes 4–6). Thus, STAT interactions with the type 1 IFN receptor are dramatically regulated in the context of viral infection, and the type 1 IFN receptor on NK cells changes from having a STAT4 to a STAT1 association.

DISCUSSION

These studies demonstrate that high basal STAT4 predisposes NK cell type 1 IFN responses to include STAT4 activation and IFN-γ production, but that the pathway is tightly regulated by type 1 IFN and IFN-γ induction of STAT1. Increased activation of STAT1 and a switch in a STAT4 to a STAT1 association with the type 1 IFN receptor accompany the change in access to STAT4. The results significantly advance the understanding of mechanisms regulating flexibility in the signaling pathways shaping type 1 IFN effects by adding the importance of the role for high basal STAT4 levels and type 1 IFN receptor interactions in promoting particular responses, and for STAT1 induction in interfering with these. In addition, they define the biological relevance of individual branches in the context of in vivo viral infection by showing that both STAT1 and STAT2 are required for optimal viral control, but that the presence of STAT1 has a unique and important function, independent of STAT2, for protecting against IFN-γ–enhanced disease. Collectively, the observations prove that individual STAT levels are used as mechanisms for both shaping fundamental characteristics of cell subsets and conditioning cellular responses during infection.

The high basal levels of STAT4 in Fig. 1 and the STAT4 association with the type 1 IFN receptor on (Fig. 9) NK cells discovered in this study are novel and surprising findings. Although a few examples of matured or activated monocyte/dendritic cells with high STAT4 have been reported (35, 36), the regulation of STAT4 expression has not been defined, and levels have not been studied in freshly isolated cells from control conditions. Likewise, low basal association of STAT1 with the type 1 IFN receptor has been seen in culture studies (32), but the consequences of high STAT4 on IFNAR associations have not been previously examined. The results challenge the model of ligand-driven stimulation for STAT binding to cytokine receptors, which is based largely on the characterization of molecular events within a limited range of cell types in culture. The basal IFNAR association with STAT4 in NK cells was not predicted by existing information, and raises exciting possibilities about how levels of different STATs might act to provide particular cell lineages flexibility in accessing subset type 1 IFN effects with low levels of the cytokines (Fig. 10).

The increased STAT1 association with IFNAR in all populations after STAT1 induction is consistent with existing models, but adds information concerning their sustained interactions. Type 1 IFN activation of STAT4 and IFN-γ expression suggests that NK cells may be an early source of this cytokine, at times preceding STAT1 induction, but that the induction of elevated STAT1 levels, through either the type 1 IFN or IFN-γ receptors, regulates type 1 IFN enhancement of IFN-γ production. Thus, the type 1 IFN pathway to promoting IFN-γ production is inhibited when NK cells sense the product in the environment, and would be under any conditions of IFN-γ exposure. In this way, induction of STAT1 by IFN-γ can be seen as part of a feedback inhibition system. Conversely, however, STAT1 is important in the
Pathways, and IFN-
basal STAT4 respond to type 1 IFN with STAT1 activation and induction.

Type 1 IFN access to STAT4 and IFN-
promote increased expression of STAT1, and this response helps regulate

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Despite their higher basal levels of STAT4, NK cells from the STAT2 − mice ex-
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Although the activation of STAT4 is clearly decreased
and a STAT4 association with IFNAR, is replaced by STAT1
when STAT1 is induced, the precise molecular changes respon-
sible for this remain to be elucidated. There is a possi-
bility that phosphorylation of the receptor’s cytoplasmic tail
driven by cytokine binding results in conditions less favorable
for STAT4 binding, but more favorable for STAT1 binding. However, the levels of STAT1 appear to be a critical ingre-
dient because, in addition to being preferentially activated in
NK cells from all tested uninfected mice (Figs. 2 and 6) even
though STAT1 is present, STAT4 is activated in a type 1 IFN-
dependent manner in NK cells from the STAT2−mice ex-
posed to type 1 IFN at D1.5 of infection (Figs. 5 and 7).

Direct competition between STAT1 and STAT3 for binding to the IFN-γ receptor has been reported (39), but STAT2 is important for type 1 IFN receptor access to STAT1 (5, 6).

Even if STAT2 facilitates type 1 IFN receptor access to STAT1 (5, 6), however, it does not appear to be necessary for blocking type 1 IFN access to STAT4 because it occurs

Use of the STAT2-deficient mice and flow cytometry
to evaluate responses within individual cells greatly facilitated
these studies. The delayed STAT1 induction in the STAT2-
deficient mice made it possible to capture NK cell IFN-γ expression in vivo, and to watch it disappear as the STAT1
levels increase. The NK cell responses in STAT2-deficient mice parallel those in STAT1-deficient mice on D1.5, but those in WT mice once their STAT1 levels are increased on
D2.5 of infection (Figs. 3–5). This is true, even though both
STAT1− and STAT2−deficient mice have increased viral bur-
dens (Fig. 8). The intracellular analysis of STAT1, along with
pSTAT4, allows a precise approach for capturing the type 1 IFN signal proximal to the receptor and evaluating the levels of both within a single cell (Fig. 5). The experiments blocking
type 1 IFN receptor function in vivo clearly demonstrate
that the type 1 IFN activation is dependent on this receptor and its cytokines (Fig. 7). To our knowledge, this is the first definition of the activation of a particular signaling pathway
within individual cells during an in vivo infection. The stim-
ulation of pSTAT1 with short-term IFN-β exposure, using
cells isolated from uninfected or infected mice, demonstrates
that when STAT1 levels are high the block is in access to
STAT4, but not STAT1 (Fig. 2). These results help exclude
a role for the suppressor of cytokine signaling 1 (SOCS1) in
regulating access to STAT4 because this mediator would also
be expected to block STAT1 activation (40). There is, how-
ever, a possibility that SOCS1 may help favor the STAT4
pathway by blocking access to low levels of STAT1 (41, 42),
and that during infection, increasing STAT1 concentrations
act to overcome this suppression and help change STAT pref-
ernces. Characterization of the contributions made by the
various SOCS to the balance between STAT4 and STAT1
activation is beyond the scope of this study, but these mol-
ecules have the potential to help in the selection of type 1 IFN activation pathways.

In agreement with the previously reported sensitivity to
vesicular stomatitis virus replication (43), but in contrast to
the appearance of the LCMV-induced wasting disease in only
the STAT1−deficient conditions reported in this study, the
induction of an optimal antiviral state during LCMV infection
depends on the presence of both STAT1 and STAT2. Because
WT mice have elevated STAT1 and are protected from
the virus during infection, access of type 1 IFN to STAT2-
dependent antiviral effects must remain intact, even when
STAT1 levels are increased. A remarkable observation reported in this study, however, is that the IFN-γ-dependent disease resulting from STAT1 deficiency during LCMV infection must be activated through STAT1-independent pathways. There are indications that the STAT1 regulation of type 1 IFN access to STAT4 might have a parallel in STAT1 regulation of IFN-γ access to STAT3 (39). IFN-γ can activate either STAT1 or STAT3, and there are examples of gene targets for this cytokine that are STAT1-dependent, STAT1-independent, and/or revealed in the absence of STAT1 (44, 45). Thus, changing access to different STAT signaling pathways may also regulate the range of IFN-γ-mediated biological effects.

In contrast to the well-characterized downstream targets of STAT1–STAT2 and/or STAT1–STAT1 signaling for gene expression (5), the understanding of the contributions made by STAT4 signaling is still limited (46). This signaling molecule is clearly important in the activation of IFN-γ, and has been best studied for its role in response to interleukin-12 (14, 15, 47). In both the human and mouse systems, however, type 1 IFN can also activate STAT4 (3, 9, 16, 17, 19, 20), and the signaling molecule contributes to type 1 IFN-mediated enhancement of an endogenous T cell IFN-γ response during LCMV infection (10, 26). Thus, there are likely to be mechanisms in place to promote type 1 IFN access to STAT4, in addition to higher basal STAT4 levels. Ongoing studies in the laboratory are evaluating T cell responses to type 1 IFN and the regulation of a STAT4 contribution to these during viral infections.

In summary, this careful examination of signaling in the context of intact biological responses has resulted in several surprises concerning the regulation of cytokine effects. The data reported demonstrate that regulation of basal levels of particular STATs and their receptor association fundamentally contributes to an important and characteristic function of NK cells: innate IFN-γ production. They also help explain why it is necessary to regulate STAT levels and how this is done in the context of an in vivo viral infection. The picture emerging is one whereby flexible use of cytokines, receptors, and signaling molecules has evolved to orchestrate complex biological responses. As defined by this work, the sorting of signaling molecules and their concentrations to shape the consequences of exposure to the same cytokines provide mechanisms for extending the value of a limited set of genes.

**MATERIALS AND METHODS**

**Mice.** Specific pathogen-free WT 129SvEv mice were purchased from Taconic. Breeding pairs of mice mutated in the IFNAR-γ, IFN-γR-α, or IFN-α/β/γR-γene (48, 49) on the 129 background were either originally purchased from B&K Universal Limited or were provided by J. Durbin (Children’s Hospital and Research Institute, Columbus, OH). Breeding pairs of the STAT2-deficient 129 mice were a gift from C. Schindler (Columbia University, New York, NY) (43); breeding pairs of the STAT1-deficient 129 mice (50) were purchased from Taconic. For additional experiments evaluating specificity of staining and Western blots, breeding pairs on the C57BL/6 background of STAT4-deficient mice (47) were obtained from M. Kaplan (Indiana University School of Medicine, Indianapolis, IN), and of STAT1-deficient (51) mice were obtained from J. Durbin. Mice were bred and maintained in isolation facilities at Brown University through brother-sister mating. All mice used in experiments were 5–12 wk old. Animals obtained from sources outside of Brown University were housed in the animal care facility for at least 1 wk before use. Handling of mice and experimental procedures were conducted in accordance with institutional guidelines for animal care and use.

**Virus, infections, and in vivo treatment protocols.** All experiments were initiated on D0. Mice were either not infected or i.p. infected with 2 × 10⁶ PFUs of LCMV Armstrong strain clone E350 (13, 26). For in vivo neutralization of IFN-γ, 1 mg of anti–IFN-γ antibody (XM1G12; prepared and purchased from BioExpress) or the same amount of Rat IgG control antibody (Sigma-Aldrich) was injected i.p. 4 h before infection. For experiments on in vivo blocking of IFNAR function, 2.5 mg of monoclonal anti–mouse IFNAR antibody, or the same amount of isotype control antibody, was injected i.p. 4 h before infection. The anti–IFNAR antibody and the control antibody were gifts from R.D. Schreiber (Washington University School of Medicine, Saint Louis, MO) (52). In vivo NK cell depletion was performed with antibody against asialo-ganglios N-tetraacetylganglioside (AGM1) as previously described (13, 53, 54). Responses were compared with animals receiving normal rabbit immunoglobulin.

**Sample preparations.** On the indicated days after the initiation of experiments, serum samples, spleen tissue homogenates, and splenic leukocytes were prepared as previously described (10, 13, 15, 31, 55).

**Flow cytometric analyses.** To detect intracellular IFN-γ, STAT1, STAT4, pSTAT1, and pSTAT4, approaches were developed as modifications of published studies (13, 15, 26, 34, 56, 57). In brief, in the STAT1, pSTAT1, and pSTAT4 studies, cells were first labeled for surface staining with FITC-conjugated anti–mouse IgG1 antibody (AB5-1). Antibodies were purchased from BD Biosciences. The PE-conjugated STAT1 was a customized preparation. Cold pure methanol incubation was used for permeabilization. A PerCP-conjugated anti–CD3ε antibody (145–2C11) was used after intracellular staining because methanol treatment diminished the intensity of this stain. Corresponding isotypes were used as controls. For the test of colocalization of STAT1 with IFN-γ, cells were stained with FITC-conjugated anti–CD49b antibody (DX5). A PE-conjugated anti–STAT1 (clone #42) (34), Alexa Fluor 647-conjugated anti–STAT4 pY693 (38/p-Stat4), and PE-conjugated anti–STAT1 pY701 antibody (4a) were used. For the STAT4 studies, biotin–conjugated anti–CD49b antibody (DX5) was used with streptavidin–APC, and the anti–STAT4 antibody (clone #8) was used with FITC–conjugated anti–mouse IgG1 antibody (AB5-1). Antibodies were purchased from BD Biosciences. The PE-conjugated STAT1 was a customized preparation. Cold pure methanol incubation was used for permeabilization. A PerCP-conjugated anti–CD3ε antibody (145–2C11) was used after intracellular staining because methanol treatment diminished the intensity of this stain. Corresponding isotypes were used as controls. For the test of colocalization of STAT1 with IFN-γ, cells were stained with FITC–conjugated anti–CD49b antibody (DX5) and PerCP-conjugated anti–CD3ε antibody (145–2C11), fixed, and permeabilized with Cytofix/Cytoperm (BD Biosciences), incubated with DNase (Sigma-Aldrich) at 37°C for 1 h, and stained with PE–conjugated anti–STAT1 antibody and APC–conjugated anti–IFN-γ antibody (XM1G12) or corresponding isotype controls. Samples were acquired using a FACSCalibur (BD Biosciences), with the CellQuest Pro software. Laser outputs were 15 mW at 488- and 635-nm wavelengths. At least 100,000 events were collected within the leukocyte gate for analysis. Specificity of staining for STAT1 and pSTAT1 was demonstrated using cells isolated from STAT1-deficient mice (50, 51), and for STAT4 and pSTAT4 using cells isolated from STAT4-deficient (47) mice. Isotype control antibodies included mouse IgG2a, mouse IgG2b, and mouse IgG1, all of which were obtained from BD Biosciences.

**NK cell enrichment.** Populations were processed to isolate NK cells by positive selection of CD49b-positive cells or by depletion of non-NK cells, respectively using anti–CD49b magnetic beads or the NK Cell Isolation kit (Miltenyi Biotec) according to the manufacturer’s instructions. The splenic leukocytes were incubated on polystyrene plates for 90 min at 37°C before being used to exclude macrophages and increase the purity of selected cells. The separated populations were analyzed by flow cytometry immediately after.
selection. The purity of the fraction of both CD49b-positive and -negative cells in positive selection was >80–90%. NK cells were >50% of the population of the negatively selected cells.

**Cell lysates, Western blotting, and immunoprecipitations.** Cells were incubated on polystyrene plates for 90 min at 37°C and harvested. Collected cells or selected cells were lysed, processed, and analyzed as previously described (34). Monoclonal anti-STAT1 antibody (clone #42) and monoclonal anti-STAT4 antibody (clone #8) were purchased from BD Biosciences. Rabbit polyclonal anti-β-actin antibody obtained from Abcam was used as a loading control. Specificity for STAT1 and pSTAT1 was demonstrated using samples from STAT1-deficient mice (50, 51), and for STAT4 and pSTAT4 using samples from STAT4-deficient (47) mice. For immunoprecipitations, cells were washed with cold PBS and lysed in lysis buffer (20 mM Tris, pH 7.6, 200 mM NaCl, 3 mM EDTA, 10% glycerol, 1% Triton X-100, protease inhibitor cocktail, DTT, and Na3VO4), as previously reported (33). Precipitated proteins were resolved on SDS-PAGE and transferred to PVDF membrane, followed by incubation with anti-STAT4, STAT1, and IFNAR antibodies, respectively. Reactive bands on Western blots were detected with anti–mouse IFNAR antibody purchased from Leinco Technologies (33).

**Standard sandwich ELISA.** Colorimetric changes of enzyme substrates were detected at 405-nm wavelength using a SpectraMax 13. Biron, C.A., and G.C. Sen. 2007. Innate immune responses to viral infection. In Field Virology. Fifth Edition. D.M. Knipe and P.M. Howley, editors. Walter Kluwer/Lippincott, Williams & Wilkins, Philadelphia. 249–278 pp.

**In vitro stimulation of splenic leukocytes.** Splenic leukocytes from uninfected or virus-infected mice were resuspended to 2 × 106 cells/ml in RPMI 1640 containing 10% FBS. Cells were incubated for 4 h at 37°C before testing for responsiveness to IFN-β for STAT1 or STAT4 activation. Recombinant murine IFN-β was used at a 10,000 U/ml concentration. For cells left unstimulated, media were added. Cells were incubated at 37°C for 90 min, and then collected for the analysis of flow cytometry. The IFN-β was a gift from Biogen Idec (specific activity of 2 × 104 U/mg).

**IFN-γ measurements.** IFN-γ levels in serum were determined by standard sandwich ELISA, as previously described (13). Colorimetric changes of enzyme substrates were detected at 405-nm wavelength using a SpectraMax 250 Reader (Molecular Devices). The limit of detection for the assay was between 10 and 20 pg/ml.

**Type 1 IFN evaluation.** Active IFN-α/β in serum was evaluated as protection against vesicular stomatitis virus–induced cytopathic effects (34, 60). Protection was scored 2–3 d after challenge by visual examination for reduction in cytopathic effects. 1 U/ml of IFN-α/β is defined as the dilution at which 50% protection from vesicular stomatitis virus–mediated lysis occurs. The limit of detection was 4 U/ml. High levels of IFN-γ did not interfere with the assay. For IFN-α or IFN-β in serum, the mouse IFN-α ELISA kit and the mouse IFN-β ELISA kit from PBL Biomedical Laboratories were used. The limits of detection for the assay were 12.5 or 15.6 pg/ml, respectively.

**Plaque assay.** LCMV viral titers in spleen homogenates were determined as previously described (31). Frozen organs were homogenized, and debris was removed by centrifugation. Serially diluted supernatants were incubated in duplicate on Vero cell monolayers for 1.5 h at 37°C. LCMV standards and LCMV viral titers in spleen homogenates were determined

**Evaluation of the body weight.** Uninfected or infected mice were weighed by the precision electronic balance Navigator (Ohaus Corporation) once a day at the same time on every day during infection from D0 (before infection) to D5.

**Statistical analysis.** Statistical analyses were performed in Excel 11.2.5 (Microsoft) using two-tailed Student’s t test where indicated.

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