Modulation of STAT1 protein levels: a mechanism shaping CD8 T-cell responses in vivo

M. Pilar Gil, Rachelle Salomon, Jennifer Louten, and Christine A. Biron

Type 1 interferons (IFNs) are induced during a variety of infections, most notably viral infections; are used therapeutically to treat cancers, viral infections, and multiple sclerosis; and are being developed as vaccine adjuvants. Gene expression analyses using cell lines have shown that hundreds of genes can be differentially regulated by IFN-α/β, and certain biologic effects attributed to these cytokines are paradoxical. Thus, there must be sophisticated mechanisms controlling the downstream consequences of exposure to the cytokines.

Receptor binding of IFNα/β elicits phosphorylation of the transcription factors STAT1 and STAT2. Heterodimers of phosphorylated STAT1 and STAT2 can help promote gene expression through the IFN-stimulated responsive elements. Other STAT family members have been implicated in the signaling activated by type 1 IFNs, but the best understood of the IFN-mediated effects are through STAT1. This transcription factor is critical for the antiproliferative effects of type I and II IFNs. STAT1 is also an important intermediary in the activation of many of the biologic responses at early times after in vivo challenge with viruses or treatments with IFNs. In contrast, other effects of IFNs are inhibited by the presence of STAT1, but revealed under STAT1-negative conditions. Interestingly, STAT1 expression is induced as a result of STAT1 activation, and the protein levels of STAT1 are elevated during viral infection. Observations suggest that regulation of STAT1 protein levels may provide a mechanism to naturally modify the effects of type 1 IFNs.

The studies presented here were undertaken to evaluate IFN effects on T-cell proliferation and to investigate how the antigen-specific cells required for defense can escape the growth inhibitory effects mediated by the cytokines in vivo. Responses to lymphocytic choriomeningitis virus (LCMV) infections of mice were characterized because the conditions are associated with well-defined and extended production of type 1 IFNs, as well as striking expansion of antigen-specific CD8 T cells. Moreover, previous work from our group has shown that at the times overlapping with IFNα/β production, STAT1 protein expression is dramatically elevated. The results of our new work demonstrate that STAT1 is an important contributor to the early regulation of nonspecific CD8 T-cell expansion during infection. Moreover, they show that, although STAT1 protein expression is induced in all populations, including CD4 T cells, responding CD8 T-cell subsets have relatively less STAT1 and breakthrough to proliferate at times overlapping with viral replication and the IFNα/β response. These events precede the return of other populations to low STAT1 levels. Thus, the antiproliferative effects exerted by type 1 IFNs may provide conditions that limit nonspecific T-cell expansion early during immune responses, but the required antigen-specific CD8 T cells can curtail these effects by selectively expressing reduced STAT1 levels. The results have profound implications concerning...
the secondary consequences of manipulating in vivo IFN concentrations and functions.

Materials and methods

Mice and in vivo manipulations

Specific pathogen-free wild-type C57BL/6 mice were purchased from Taconic Laboratory Animals and Services (Germantown, NY). STAT1-deficient mice initially on the 129 background were crossed onto the C57BL/6 background for more than 5 generations, and colonies were established in the animal care facilities at Brown University. Experimental groups were age matched, and all mice used in experiments were 8 to 12 weeks of age. Animals obtained outside of Brown University were housed for at least 1 week before use. Handling of mice and experimental procedures were in accordance with institutional guidelines for animal care and use. Experiments were initiated on day 0, with mice either not infected or infected intraperitoneally with 2 × 10⁶ plaque-forming units (PFUs) LCMV Armstrong strain, clone E350. Where indicated, IFN-α was measured in a viral plaque assay with vero cells as described.²³

Splenocyte preparations and CD8 T-cell enrichment

On indicated days following initiation of experiments, mice were killed and spleens were harvested. Leukocytes were prepared as previously described.²¹ In certain experiments, CD8 T cells were isolated by negative selection using magnetic-activated cell sorting (MACS) enrichment kits and the program DepleteS on the autoMACS instrument (Miltenyi Biotec, Auburn, CA). The purity of enriched samples was greater than 90%.

CFSE proliferation analysis

Leukocytes were resuspended to 1 × 10⁶ cells/mL in PBS containing 5% FBS, and CFSE labeling was performed as described²⁴ with some modifications. Briefly, CFSE was rapidly mixed with the cells to a final concentration of 5 μM and incubated for 5 minutes at room temperature. After 2 washes with 10 volumes of PBS–5% FBS, the CFSE-labeled leukocytes were incubated in the absence or presence of cytokines. In all experiments, the concentration of cells was 1 × 10⁶ cells in 200 μL RPMI–10% FBS. Cells were incubated in 96-well plates at 37°C for the times indicated. A final concentration of 20 ng/mL IL-2, IL-7, and IL-15 resulted in 50% protection from the BrdU Flow Kit protocol (BD Biosciences), using the solutions and protocol from the BrdU Flow kit to detect nuclear and cytoplasmic STAT1. The approach was developed as a modification of reported staining of human monocytes.²² Studies of cells isolated from the STAT−/− mice demonstrated specificity of staining. Isotype control antibodies were used in all the analyses. The mean fluorescence intensities of the isotype and STAT1−/− controls were lower than the first log of intensity with narrow uniform peaks.

IFN evaluation

Serum was obtained from mice anesthetized with isoflurane (Abbott Laboratories, Abbott Park, IL) as previously described.²⁶ Active IFNαβ was evaluated as protection against vesicular stomatitis virus (VSV)–induced cytopathic effects. Protection was scored at 2 to 3 days after challenge by visual examination for reduction in cytopathic effects. One unit per milliliter of IFNαβ is defined as the dilution at which 50% protection from VSV-mediated lysis occurs. The limit of detection was 8 U/mL. For IFNα enzyme-linked immunosorbent assay (ELISA), the primary antibody was a rat anti–mouse IFNα mAb (F-18; HyClone, Logan, UT); a goat anti-rabbit IgG (DakoCytomation, Glostrup, Denmark); the secondary antibody was a horseshad peroxidase–conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Baltimore, PA); the substrate was ABTS Peroxidase Substrate (KPL, Gaithersburg, MD) and the standard, a recombinant mouse IFNαA from PBL Biomedical Laboratories. Colorimetric changes of enzyme substrates were detected at 405 nm wavelength using a SpectraMax 250 reader (Molecular Devices, Sunnyvale, CA). The limit of detection was 1500 pg/mL. For certain experiments, the mouse IFNα ELISA Kit from PBL Biomedical Laboratories was used. The limit of detection for this assay was 31.25 pg/mL.

Cell lysates and Western blot analysis

Total splenocytes, enriched CD8 T cells, and non-CD8 cells, as well as CD8 T cells sorted for high or low CFSE staining were lysed in a solution containing 50 mM Tris HCl pH 7.5, 0.3M NaCl, 0.5% Triton X-100, 2 mM EDTA, 0.4 mM Na3VO4, and a cocktail of protease inhibitors (Roche Diagnostics, Indianapolis, IN). Protein (30 μg) was separated on SDS-PAGE gels (gradient 4%–20% LongLife MicroGels; Gradient, Hawthorne, NY), following the Gradipore protocol for blotting. Monoclonal anti-STAT1 antibody (clone 1) was purchased from BD Transduction Laboratories (Lexington, KY); rabbit polyclonal anti-STAT4 antibody (C-20) obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and rabbit polyclonal anti-β-actin from Abcam (Cambridge, United Kingdom) were used as a loading control. Blot images were acquired in a BioRad (Hercules, CA) GelDoc and analyzed using the public domain National Institutes of Health (NIH) Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Real-time PCR

RNA from sorted CD8 T cells was extracted with the RNAeasy kit from Qiagen (Valencia, CA), using on column digestion with DNase I (Qiagen). One-step real-time reverse transcriptase–polymerase chain reaction (RT-PCR) was performed with Quantitect Probe RT-PCR kit (Qiagen) following the amplification parameters given in the manufacturer protocol. Sequences of STAT1 primers and FAM-labeled probe were as follows: forward primer, 5′-AACGGAACTGATCATCA; reverse primer, 5′-CCGGGACACTC- CATCAAAC; and probe, 5′-CAGCACCAAGACACC. Fold changes were calculated relative to the level of β-actin, determined using the QuantiTect Min Actb Assay (Qiagen). Reactions were carried out using Applied Biosystems (Foster City, CA) 7300 Real Time PCR system. To calculate the relative expression of target and reference genes, amplification
efficiencies were determined. Results were plotted as ratio of STAT1 expression between proliferating and nonproliferating cells, and as amplification curves of ΔRn (an indicator of the signal generated by the PCR) against cycle numbers. The Ct values, indicating the earliest cycle for detection, are given in the legends.

Results
Role for STAT1 in IFN-mediated inhibition of CD8 T-cell proliferation

The STAT1 effects on CD8 T-lymphocyte proliferation were evaluated in vitro with responses to exogenous growth factors. Splenic leukocytes derived from STAT1-competent or -deficient mice were labeled with carboxy-fluorescein diacetate, succinimidyl ester (CFSE) and incubated with IL-2, IL-7, or IL-15. All of these cytokines use the common cytokine-receptor γ-chain to induce cell proliferation and have well-described effects on CD8 T-cell proliferation. Flow cytometric analyses of proliferation, as measured by dilution of CFSE staining, demonstrated that presence of the growth factors resulted in 40% to 70% of the wild-type CD8 T cells dividing after 5 days in culture (Figure 1A). As expected, addition of the type 1 IFN, IFNα, dramatically inhibited this proliferation. Although IL-2, IL-7, or IL-15 also supported the division of STAT1-deficient cells, IFNα failed to inhibit their proliferation (Figure 1A). These results confirm the role of STAT1 as a key mediator in the ability of IFNs to regulate proliferation of CD8 T lymphocytes.

To examine the STAT1-dependent effects on proliferation in vivo, the CD8 T-lymphocyte proliferation elicited in response to LCMV infection was examined. For these experiments, the thymidine analog, BrdU, was injected into STAT1-competent and -deficient mice. This approach marks populations replicating their DNA. Uninfected (day 0) mice or mice infected with LCMV for 1, 2, 3, or 4 days received injections of BrdU 2 hours prior to killing (Figure 1B). These periods of infection overlap with peak type 1 IFN induction. Although overall splenic cell yields were not dramatically altered in wild-type as compared with STAT1-deficient mice and both groups responded to infection with the production of type 1 IFNs, CD8 T cells had deregulated proliferation in STAT1-deficient mice (Figure 1B). In contrast to the minimal expansion of, and low BrdU incorporation by, wild-type CD8 T lymphocytes, there was a greater than 2-fold expansion, and approximately 10% of these cells in the STAT1-deficient mice were labeled with BrdU. The expanding subsets were not specific for the LCMV immunodominant epitopes because they did not bind class I MHC molecules presenting these determinants (data not shown). Thus, STAT1 is critical for inhibiting nonspecific CD8 expansion at early times after challenge in vivo.

Differential regulation of STAT1 protein levels in responding CD8 T cells

The aforementioned results demonstrate that high type 1 IFNs and STAT1 protein levels can be beneficial in limiting nonspecific T-cell proliferation. However, antigen-specific T cells need to be expanded under these conditions. In uninfected spleens, approximately 10% of the leukocytes are CD8 T cells having a diverse, nonspecific repertoire for antigen. By day 5 after infection, the CD8 T cells comprise up to 15% of the spleen and a low frequency of these can be identified as virus specific. The subset can represent greater than 50% of the leukocytes and is primarily virus specific by day 8 after infection. The day-5 and -6 time points are key because they overlap with periods of CD8 T-cell effector function, declining viral burdens, and type 1 IFN responses. Previous studies from our group have demonstrated that STAT1 levels are induced in total splenic leukocytes at days 2 to 5 after LCMV infection. To extend this characterization to particular cell subsets, STAT1 levels were determined by Western blot analysis of proteins extracted from CD8 T cells, as compared with non-CD8 and total splenic leukocyte populations isolated from uninfected (day 0) or day 3, 5, or 8 LCMV-infected mice. Remarkably, although STAT1 protein was modulated in all the populations tested (Figure 2), being low on days 0 and 8 and induced to high levels on days 3 and 5 after infection, the kinetics of induction were dramatically altered in wild-type as compared with STAT1-deficient mice. This approach marks populations replicating their DNA. Uninfected (day 0) mice or mice infected with LCMV for 1, 2, 3, or 4 days received injections of BrdU 2 hours prior to killing (Figure 1B). These periods of infection overlap with peak type 1 IFN induction. Although overall splenic cell yields were not dramatically altered in wild-type as compared with STAT1-deficient mice and both groups responded to infection with the production of type 1 IFNs, CD8 T cells had deregulated proliferation in STAT1-deficient mice (Figure 1B). In contrast to the minimal expansion of, and low BrdU incorporation by, wild-type CD8 T lymphocytes, there was a greater than 2-fold expansion, and approximately 10% of these cells in the STAT1-deficient mice were labeled with BrdU. The expanding subsets were not specific for the LCMV immunodominant epitopes because they did not bind class I MHC molecules presenting these determinants (data not shown). Thus, STAT1 is critical for inhibiting nonspecific CD8 expansion at early times after challenge in vivo.

![Figure 1. Contribution of STAT1 to regulation of CD8 T-cell proliferation. (A) CD8 T cells from wild-type or STAT1-deficient mice proliferate in response to growth factors in culture, but STAT1 is required for the antiproliferative effects mediated by type 1 IFN. Total splenic populations were prepared from uninfected mice, CFSE-labeled on day 0, and cultured for 5 days in the absence or presence of IFNα (1 × 10^4 U/mL), with or without IL-2, IL-7, or IL-15 (20 ng/mL each). Flow cytometry histogram plots of gated CD8 T cells are shown. Numbers are proportions of cells dividing as assessed by dilution of CFSE. (B) STAT1 contributes to the negative regulation of early CD8 T-cell proliferation in vivo during LCMV infection. Wild-type and STAT1-deficient mice were infected with LCMV and followed for 4 days after infection, and CD8 T-cell proliferation was measured by BrdU incorporation in vivo. Proportions and numbers of total, as well as BrdU-positive CD8 T cells, are shown. Active IFNα/β in serum was measured by bioassay and serum IFNα by ELISA. All data are means ± SDs. Results for both panels are representative of at least 3 independent experiments.](image-url)
were still detectable at these times. Hence, STAT1 levels are limited in CD8 T cells at times when elevated expression of the transcription factor is maintained in other subsets, and a subset of the population is being activated in the presence of virus and type 1 IFN to mediate viral clearance.

Differential STAT1 expression in proliferating CD8 T cells

Two different approaches were developed to selectively evaluate STAT1 levels in CD8 T cells induced to proliferate during the infection. The first took advantage of the observation that splenic leukocytes harvested on day 5 after LCMV challenge continue to develop, without addition of exogenous factors, during in vitro culture. Cells obtained from uninfected, day 5, or day 8 LCMV-infected mice were labeled with CFSE and evaluated for proliferation at days 1, 2, 3, 4, and 5 after culture. Extensive ex vivo proliferation could only be demonstrated with the cells isolated on day 5 (Figure 3; data not shown). The CD4 T-cell subset had only modest proliferation under these conditions, but the CD8 T-cell subset continued to proliferate such that greater than 30% of the cells had diluted out the CFSE by day 3 after culture, and the recovery of this subset almost doubled. Interestingly, the addition of exogenous IFNα, at the concentrations blocking the cytokine-supported expansion of cells from uninfected mice (Figure 1), was unable to abolish the expansion of the in vivo–activated cells (Figure 4A; data not shown). Thus, the proliferation was relatively insensitive to IFN-mediated inhibition. To assess differences in STAT1 levels between the proliferating and nonproliferating cells, leukocytes isolated on day 5 after infection were CFSE labeled and cultured for 3 days ex vivo. CD8 T cells were prepared by magnetic bead purification and sorted into CFSE-low and -high populations.
(Figure 4A-B). Western blot analyses of the proteins extracted from the different cell populations showed that the CD8 had less STAT1 than the non-CD8 populations and that the proliferating had very low amounts of STAT1 compared with the nonproliferating CD8 T cells (Figure 4C). A similar difference was observed analyzing STAT1 mRNA expression: it was lower in the proliferating cells and higher in the nonproliferating CD8 T cells with a difference of 3.5 points (Figure 4D). These results demonstrate that STAT1 is differentially expressed such that proliferating cells have relatively low and nonproliferating cells have relatively high levels. They suggest that selection of T-cell subsets for expansion is dependent on low STAT1 expression.

To directly examine STAT1 protein levels within individual cells immediately after isolation, a variety of antibodies were screened for their ability to fluorescently label total STAT1 protein intracellularly. The approach required optimizing conditions for cytoplasmic and nuclear staining of total STAT1 using a phycoerythrin-conjugated anti–mouse STAT1 antibody. Isotype control antibodies and cells derived from STAT1-/- mice were used as negative controls to demonstrate specificity of staining (see “Materials and methods”). The results of staining for STAT1 protein in total leukocyte populations complemented the results of the Western blot analysis and extended them to evaluate expression within individual cells. Low but detectable levels of STAT1 were expressed with relative uniformity in populations from uninfected mice, expression was dramatically elevated with greater than 87% of cells expressing high levels on days 5 and 6 after infection, and decreased on days 7 and 8 (Figure 5). As expected, the CD8 but not the CD4 T-cell subset was expanding in vivo starting on day 6 after infection (Figure 5). Gating for analysis of the CD4 T-cell population demonstrated that the majority of these cells had STAT1 levels similar to those observed in total populations (Figure 5). In contrast, the CD8 T-cell population also had STAT1 being induced but a subset had lower expression on day 6 as compared with total or CD4 T cells, that is, 47% versus 11% and 10%. Moreover, the levels of expression were lower in a proportion of the T cells throughout the peak induction of STAT1 with bright staining at only 53% on day 6, 26% on day 7, and 15% on day 8 as compared with approximately 90%, 70%, and 40% to 60% of the total and CD4 T-cell populations (Figure 5). Thus, 2 different populations of CD8 T cells were developing, one with induced STAT1 levels equivalent to those achieved in total and CD4 T-cell populations and another with lower STAT1 levels.

To further explore the subset of the proliferating cells in vivo, splenic leukocytes were harvested from uninfected or LCMV-infected mice who received injections of 5-bromo-2'-deoxyuridine (BrdU) 2 hours prior to killing, and fluorescently labeled with anti-CD8 or anti-CD4, anti-BrdU, and anti-STAT1. As expected, the proportions of CD4 T cells were not dramatically increasing on days 6, 7, and 8 after infection (Figure 6A). Only 12% of these cells were incorporating the DNA precursor, BrdU, on day 6, but they were contained within the CD4 T-cell subset having relatively low levels of STAT1. Analysis of the expanding CD8 T cells demonstrated that greater than 40% of these were incorporating BrdU on day 6 after infection, and they were preferentially represented in the higher proportion of STAT1-low cells (Figure 6B). Thus, as compared with total cells and CD4 T cells, CD8 T cells are expanded to higher proportions and numbers during infection, have higher proportions of the low STAT1 subset, and the populations replicating their DNA during the 2 hours immediately prior to harvest are found in the low STAT1 subset.

**Differential STAT1 expression in antigen-specific CD8 T cells**

To examine STAT1 levels within the antigen-specific CD8 T-cell subset, a fluorescently labeled MHC class I Db tetramer conjugated to the LCMV peptide GP33-41 was also used for staining. This LCMV peptide is an immunodominant viral determinant, and Db GP33-41 is bound by T-cell receptors specific for the complex. Splenic leukocytes harvested from uninfected or LCMV-infected mice who had received injections of BrdU 2 hours prior to being killed were stained with anti-CD8, anti-BrdU, anti-STAT1, and Db GP33-41. The antigen-specific CD8 T-cell subset was preferentially found in the low STAT1 subset on day 6 after infection, representing about 10% of the CD8 T cells (Figure 6B). Analysis of the Db GP33-41-positive and BrdU-positive or -negative subsets showed that all of the antigen-specific cells were low for STAT1 (Figure 6C). In contrast, analysis of the Db GP33-41–negative subset showed that STAT1-low and -high populations were present (Figure 6C). These results suggest that the greater higher proportions of the STAT1-low, BrdU-positive cells, that is, 40%, contained antigen-specific cells for other immunodominant viral peptides or cells in cycle having down-regulated antigen receptors. Although the proportions cannot be precisely quantitated, these data conclusively show that the in vivo–proliferating, antigen-specific CD8 T cells display a phenotype characterized by low STAT1 expression.

**Discussion**

The data presented here show that STAT1 is an important factor in regulating inappropriate CD8 T-cell proliferation in vivo at early...
levels and reduced sensitivity to IFN-mediated antiproliferative effects. The dichotomy of the magnitude of expression allows antigen-specific CD8 T-cell expansion, even in the presence of type 1 IFNs, to defend against infection. Thus, a fine-tuning of cytokine effects by regulation of total levels of intracellular signaling molecules is defined.

The modulation of STAT1 protein levels following viral infection within antigen-specific CD8 T cells is reported here for the first time. The studies show that the ability to proliferate in the presence of type 1 IFNs is a consequence of less total protein rather than protein absence. Although earlier studies with STAT1-deficient cells suggested that the presence or absence of the molecule might be the factor determining the consequences of cytokine exposure, the in vivo titration of the levels observed here provides a more biologically conservative mechanism, with the potential to maintain a cell’s ability to access antiviral gene targets for defense while limiting the antiproliferative effects of IFN exposure. The titration of expression is more than about 2 logs of fluorescence intensity when evaluated within individual cells by FACS analysis, and intensity changes with the kinetics of infection in all populations (see Figure 5). Consistent with the observed dynamic increases and decreases in expression, careful timing was required to demonstrate the difference, with ex vivo cells expanded in culture, by Western blot analysis (see Figure 4). This may explain why earlier attempts by others have failed to detect reduced STAT1 within total proliferating CD4 T-cell populations by Western blot analysis. Alternatively, because the proportions of CD4 T cells with low STAT1 levels were much smaller (Figures 5-6), the difference in STAT1 levels may provide a mechanism preferentially favoring antigen-specific CD8 T-cell proliferation in the context of high type 1 IFN induction. Current studies in our laboratory are extending the characterization of STAT1 levels to other cell types and advancing the understanding of the pathways regulating STAT1 expression in antigen-specific CD8 T cells.

The results add to and extend earlier work from our group demonstrating that type 1 IFNs promote IFN-γ production by CD8 T cells, and that the presence of STAT1 negatively regulates the induction of IFN-γ by IFN-α/β. Taken together, these studies indicate that cells are controlling responses to the cytokines, ranging from inhibition of proliferation to induction of IFN-γ, based on access to intracellular signaling pathways. The new work also provides a biologic context for doing this during a competent immune response. The data complement studies from others showing that the type 1 IFNs, as well as another cytokine using STAT1 for receptor signaling, IFN-γ, can act to increase the abundance of CD8 T cells during viral infections and in response to antigen stimulation in culture. In contrast to the early inhibition of proliferation reported here (Figure 1), these reports demonstrate that another consequence of IFN exposure may act later during the development of an immune response to enhance the numbers of antigen-specific cells. The balance is likely to be one more example of how cell conditioning to express different levels of STAT molecules may change responses to the cytokines in paradoxical ways. Ongoing experiments are evaluating the possible modification of gene targets after IFN treatment of populations conditioned to express various levels of STAT1 in vivo, and the requirements for other STAT molecules in the expression of individual genes (M.P.G. and C.B., unpublished observations). By providing the biologic context for the regulation of STAT expression, these experiments extend and complete the work characterizing how different STAT molecules play particular roles in accessing type 1 IFN effects in T cells.
The results have profound implications concerning the consequences of therapeutic intervention to regulate IFN levels and functions. They suggest that treatments with IFNs to protect against viral infections and cancer may differentially regulate immune function depending on the cellular levels of total STAT1 protein. If the cytokines are administered under conditions whereby all of the T cells respond with high expression of STAT1, the treatments may interfere with the expansion of antigen-specific cells for defense. However, neutralization of endogenous IFN function to protect against autoimmune diseases may result in conditions promoting the activation and expansion of nonspecific cells as well as limit the antimicrobial defense mechanisms activated by the cytokine. Clearly, learning how to control the levels of STAT1 within cells and using this during cytokine manipulation may help promote appropriate immune responses and allow access to the antimicrobial defense functions as needed.

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References


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