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CUTTING EDGE

Cutting Edge: Role of STAT1, STAT3, and STAT5 in IFN-αβ Responses in T Lymphocytes

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Engagement of the IFN-αβ receptor initiates multiple signaling cascades, including activation of the STAT. In this study, we demonstrate that IFN-αβ, although antiproliferative in wild-type CD4$^+$ or CD8$^+$ T cells, act as strong mitogens on their STAT1$^{-/-}$ counterparts. Furthermore, IFN-αβ exert little effect on apoptosis in wild-type cells, but are potent survival factors in the absence of STAT1. The antiapoptotic response in the absence of STAT1 is predominantly mediated by STAT3, and to a lesser extent by STAT5A/B. In contrast, the mitogenic IFN-αβ response gained through the absence of STAT1 is only marginally affected when STAT5A/B expression is also abrogated, but is completely dependent on STAT3 activation. These findings provide the first evidence for a function of STAT3 and STAT5A/B in the IFN-αβ response, and support a model in which the IFN-αβ receptor initiates both pro- and antiapoptotic responses through STAT1, and STAT3 and STAT5A/B, respectively. The Journal of Immunology,* 2005, 174: 609–613.

Type I IFNs, including IFN-α and IFN-β, represent a class of cytokines that exhibit an unique pleiotropy of biological effects, encompassing growth inhibition, antiviral defense, and immunomodulatory properties (1). Moreover, IFNs have been shown to mediate survival and development of cells of hemopoietic origins (2, 3). Binding to their specific cell surface receptors, IFNAR1 and IFNAR2, leads to the tyrosine phosphorylation and activation of the STAT (4–6). Among these, STAT1, STAT2, and IFN regulatory factor 9 cooperatively form the DNA binding protein complex ISGF3, which is required for up-regulation of IFN-stimulated genes. In cells lacking STAT1 or STAT2, type I IFNs fail to elicit an antiviral state or an antiproliferative response (7). STAT1$^{-/-}$ mice display increased susceptibility to viral infections, because IFN is unable to mediate expression of genes necessary to establish an antiviral state (8, 9). In addition, the lack of IFN-mediated up-regulation of MHC expression results in an inability to mount an effective immune response to bacterial infections (10). STAT1$^{-/-}$ mice also display an increased incidence of tumor formation, presumably a consequence of impaired tumor surveillance (11, 12), and are more susceptible to autoimmune disease (13).

Although the antiviral and antiproliferative effects of IFN, and the role of STAT1 and STAT2 therein, have been studied extensively, the mechanism by which type I IFNs act to modulate the development and function of cells of hemopoietic origin is still unclear. Moreover, even though STAT3 and STAT5 are strongly activated by type I IFNs, very little is known about the contributions of these STAT family members to the biological effects of IFN-$\alpha\beta$.

In this study, we report that type I IFNs acquire potent antiapoptotic and mitogenic effects in T cells lacking STAT1. These unexpected effects of IFN-$\alpha\beta$ are predominantly mediated by STAT3, and to a lesser extent by STAT5, and thus provide novel evidence for an important function of these STAT proteins in the IFN response.

Materials and Methods

Animals

Wild-type (WT)[2] and STAT1$^{-/-}$ 129/SvEv mice were purchased from Taconic Farms STAT5A/B$^{-/-}$ mice were kindly provided by Dr. J. Ihle (St. Jude's Children's Research Hospital, Memphis, TN). All mice used in these experiments were bred and maintained in accordance with University of California, San Diego (UCSD), Animal Care Facility regulations.

Reagents and Abs

Anti-CD4 and -CD8 mAb, and annexin V were obtained from BD Pharmingen. Anti-B220 mAbs were from eBioscience. Anti-CD3e mAb (clone 2C11) and IL-2 were kindly provided by S. Hedrick (UCSD). IL-7 (J558) was kindly provided by R. Rickert (UCSD). Production of murine rIFN-β was previously described, and murine rIFN-γ was purchased from PeproTech. IL-6 was from R&D Systems. CFSE was obtained from Molecular Probes. Phosphospecific STAT1 and STAT3 Abs were from New England Biolabs, and STAT5A and phosphospecific STAT5A/B Abs were obtained from Upstate Biotechnology. The cell-permeable STAT3 inhibitor peptide was purchased from Calbiochem.

Preparation of cells

Cells from lymphoid organs were maintained in RPMI 1640/10% FBS. CD4$^+$ T cells were isolated through negative depletion. Briefly, macrophages were separated by adherence to tissue culture dishes. The remaining cells were labeled with biotinylated anti-CD8 and anti-B220 mAb, incubated with anti-biotin magnetic beads, and loaded onto MACS separation columns (Miltenyi Biotec). This procedure yielded >95% CD4$^+$ T cells.

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[2] Abbreviation used in this paper: WT, wild type.

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Survival assay

Lymphocytes were cultured in the absence or presence of IFN-α, IFN-β, IL-6, or IL-7. On days indicated, cells were collected, and apoptosis was assessed by staining with annexin V and 7-aminoactinomycin D (BD Pharmingen).

T cell stimulation and proliferation assay

Lymphocytes were labeled with CFSE (5 μM) and stimulated by addition of soluble anti-CD3 (50 ng/ml) or in the presence of plate-bound anti-CD3 (200 ng/ml) along with IL-2 or IFNs and unlabeled APCs in 96-well plates. After 72 h, the extent of proliferation was evaluated by flow cytometric analyses.

Western blot analysis

Cells were lysed in 20 mM HEPES (pH 7.4), 1% Triton X-100, 100 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM sodium vanadate, and 1 mM PMSF. Lysates were centrifuged at 13,000 × *g*, and protein concentration was determined using Bradford reagent (Bio-Rad). Resolved proteins were detected after transfer with the indicated primary Abs, followed by incubation in HRP-conjugated secondary Abs (Zymed) and ECL (Amersham Biosciences).

Results

Mice lacking STAT1 are highly susceptible to autoimmune disease due to a lack of functional $CD4^+CD25^+$ regulatory T cells (13), but have been reported to otherwise display no apparent abnormalities in T lymphocyte development (8, 9).

Indeed, when we examined the T cell populations from thymus, spleen, and lymph nodes of WT and $STAT1^{-/-}$ mice at 8–10 wk of age, we could not detect any significant differences in the $CD4^+$, $CD8^+$, $CD4^+CD8^+$, and $CD4^-CD8^-$ T cell compartments (data not shown). This analysis supported the notion of normal T cell development in $STAT1^{-/-}$ mice.

Inhibition of resting T cell apoptosis by IFN-β in $STAT1^{-/-}$ mice

Previous studies reported that $STAT1^{-/-}$ lymphocytes display a reduced rate of apoptosis when cultured in the absence of cytokines, presumably due to decreased basal levels of different caspases (3). However, when we cultured purified $CD4^+$ T cells rather than total splenocytes in cytokine-free medium, we found that T cells derived from $STAT1^{-/-}$ mice displayed comparable, if not slightly increased, cell death compared with cells from age-matched WT animals (Fig. 1*A*). Furthermore, we were unable to detect any significant differences in caspase 3 expression levels between the two cell populations (not shown).

Next, we sought to determine whether the presence of STAT1 contributed to cytokine-mediated survival effects on peripheral T cells. IL-7 is well recognized as a potent T cell survival factor. Indeed, analysis of both WT and $STAT1^{-/-}$ $CD4^+$ splenocytes (Fig. 1*B*) or thymocytes (Fig. 1*C*) cultured in presence of IL-7 displayed dramatic decreases in apoptosis. In contrast, IL-6, reported to promote activated T cell survival (2), had no effect on the survival of either WT or $STAT1^{-/-}$ resting $CD4^+$ T cells derived from thymus (Fig. 1*C*) or spleen (not shown). Similarly, IFN-γ, which exclusively activates STAT1, had no effect on survival of either WT or $STAT1^{-/-}$ T cells (Fig. 1*C*). Unexpectedly, when $STAT1^{-/-}$ $CD4^+$ resting T cells were cultured with IFN-β, this cytokine promoted cell survival in a dose-dependent manner nearly as effective as IL-7 (Fig. 1, *B* and *C*). Strikingly, IFN-β was unable to elicit such an antiapoptotic response in WT $CD4^+$ T cells (Fig. 1, *B* and *C*). IFN-α, which uses the same cell surface receptor, exerted survival effects comparable with those of IFN-β (data not shown).

This antiapoptotic effect of type I IFNs, which only occurred in the absence of STAT1, was not restricted to $CD4^+$ T cells, but could also be observed in $STAT1^{-/-}$ $CD8^+$ T cells (Fig.

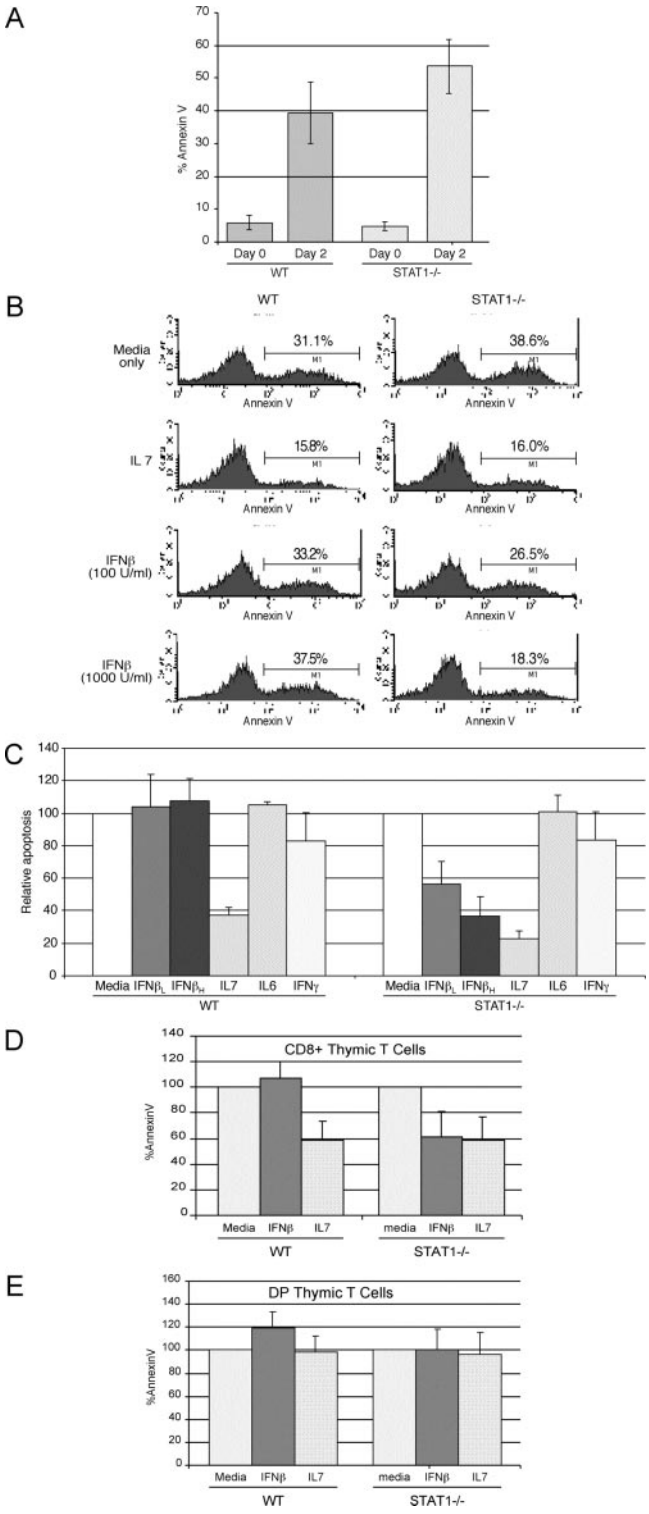

FIGURE 1. IFN-β selectively inhibits apoptosis in $STAT1^{-/-}$ T cells. *A*, $CD4^+$ splenocytes were cultured in medium for 48 h, and the apoptotic subpopulation was identified by flow cytometry after staining with cell surface markers and annexin V. *B*, $CD4^+$ splenocytes were cultured in the absence or presence of IFN-β (100 U/ml (IFN-β_L) or 1000 U/ml (IFN-β_H)) for 48 h, or treated with IL-7 (1:500). Apoptotic cells were detected as described in *Materials and Methods*. *C*, $CD4^+$ thymocytes were cultured in the presence of IFN-β (100 U/ml or 1000 U/ml), IL-7 (1:500), IL-6 (50 ng/ml), or IFN-γ (2 ng/ml) for 48 h, and apoptotic cells were detected as described above. Cell death in medium without cytokines was set to 100%. *D* and *E*, Same as in *C*, except $CD8^+$ or $CD4^+CD8^+$ thymocytes were treated with 100 U/ml IFN-β. The average results or a representative of four individual experiments is shown.

1*D*). However, IFN-$\alpha\beta$ did not promote survival of WT or STAT1$^{-/-}$ CD4$^+$CD8$^+$ double-positive thymocytes (Fig. 1*E*). IFN-$\alpha\beta$ also did not support survival of STAT1$^{-/-}$ CD19$^+$ B cells, further demonstrating the cell type specificity of the antiapoptotic effects (data not shown).

Mitogenic effects of IFN-$\alpha\beta$ in STAT1$^{-/-}$ T cells

Having observed STAT1-independent responses toward IFN-β stimulation in resting T cells, we decided to investigate the effects of the cytokine on activated T cells. CFSE-labeled, CD4$^+$ T cells derived from lymph nodes of WT and STAT1$^{-/-}$ mice were stimulated with plate-bound anti-CD3 Ab (clone 2C11), in the absence or presence of IL-2 (50 U/ml). Flow cytometric analysis after 3 days of culture revealed that STAT1$^{-/-}$ CD4$^+$ T cells respond to anti-CD3 stimulation with slightly increased proliferation when compared with WT cells. This hyperresponsiveness is even more pronounced when the cells are stimulated via the Ag receptor in the presence of IL-2 (Fig. 2). This enhanced proliferative response was also apparent in CD8$^+$ T cells stimulated with soluble anti-CD3 Ab in the absence or presence of IL-2 (data not shown).

The role of STAT1 in the antiproliferative effects of type I IFNs has been well documented (14). As such, IFN-β potently inhibits the proliferation of WT T cells activated with anti-CD3 with or without IL-2 (Fig. 2). Intriguingly, when IFN-β was added to activated STAT1$^{-/-}$ CD4$^+$ T cells, we found that IFN-β had not only lost its antiproliferative effects, but rather had acquired potent mitogenic properties, resulting in an enhanced proliferation compared with anti-CD3 treatment alone (Fig. 2). IFN-β treatment alone did not induce proliferation (not shown). Similar results were observed when STAT1$^{-/-}$ CD8$^+$ T cells were stimulated with anti-CD3 in the presence of IFN-β, or when the cells were exposed to IFN-α (not shown). These data illustrate that type I IFNs induce STAT1-dependent and -independent pathways, resulting in drastically different biological responses.

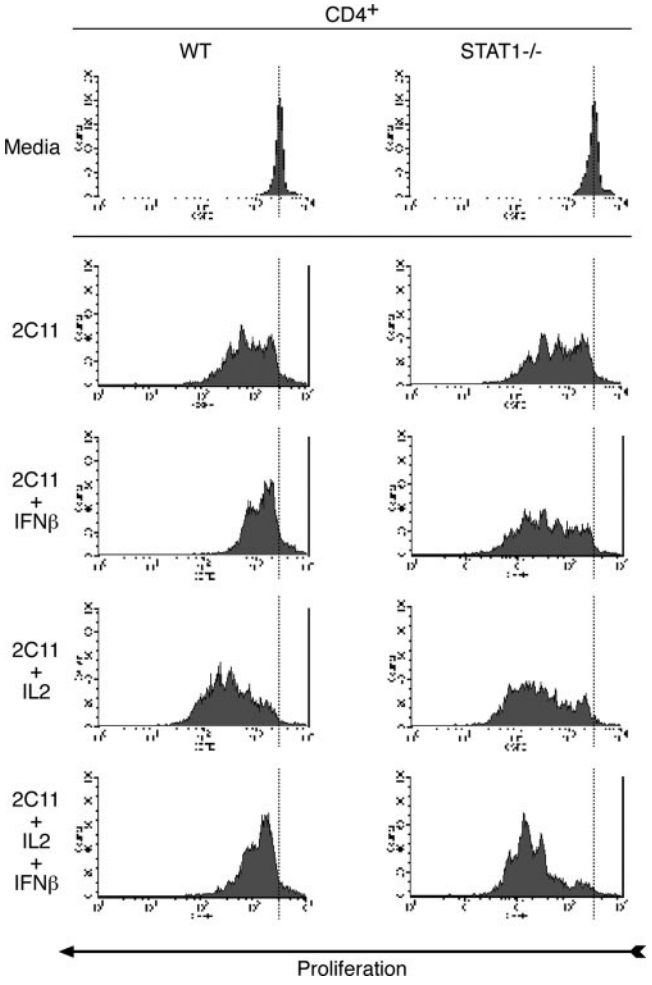

FIGURE 2. Enhanced proliferation of STAT1$^{-/-}$ T cells in response to IFN-β. CD4$^+$ T cells derived from peripheral lymph nodes were labeled with 5 μM CFSE and cultured for 72 h with plate-bound anti-CD3 (200 ng/ml) and APCs in the absence or presence of IFN-β (1000 U/ml), IL-2 (50 U/ml), or both.

Inhibition of apoptosis in resting and activated STAT1$^{-/-}$ T cells

We next sought to determine whether the antiapoptotic effects of IFN-β on STAT1$^{-/-}$ T cells exist not only on resting, but also on activated T cells by simultaneously analyzing cell proliferation and apoptosis. Treatment of WT CD4$^+$ splenic T cells with IFN-β in the presence of anti-CD3 inhibited proliferation only slightly; however, activation-induced cell death was substantially increased. In contrast, addition of IFN-β to anti-CD3-treated STAT1$^{-/-}$ CD4$^+$ T cells resulted not only in their enhanced proliferation, but also reduced the number of apoptotic cells (Fig. 3). As such, ~55% of STAT1$^{-/-}$ CD4$^+$ T cells were viable and had undergone cell divisions in response to anti-CD3 stimulation in the presence of IFN-β, whereas >50% of WT cells succumbed to activation-induced cell death. Similar effects of IFN-β were observed when cells were costimulated with anti-CD3 and anti-CD28. These results clearly demonstrate that type I IFNs mediate their survival effects in the absence of STAT1 in resting as well as activated T cells.

Role of STAT3 and STAT5 in IFN-β signaling

The observation that IFN-β provides survival and mitogenic signals only in the absence of STAT1 is rather intriguing, given the fact that STAT1 activation is generally appreciated as a crucial requirement for many of the physiological functions of IFNs. To determine which pathway(s) account(s) for the antiapoptotic effect of IFN-β in STAT1$^{-/-}$ T cells, we analyzed several signaling pathways known to influence cell growth and survival.

PI3K and its downstream target Akt/protein kinase B were shown to be activated by type I IFNs (15, 16); however, wortmannin, a potent inhibitor of PI3K, failed to abolish the antiapoptotic effect of IFN-β on STAT1$^{-/-}$ CD4$^+$ T cells (data not shown). IFN-$\alpha\beta$ have also been reported to increase the levels of antiapoptotic members of the bcl-2 family in B cells, but not in T cells (17, 18). Indeed, Western blot analysis of IFN-β-treated purified CD4$^+$ T cells revealed no difference in the levels of bcl-2 and bcl-x_L proteins (data not shown). Similarly, we were unable to detect any activation of the NF-κB pathway.

Type I IFNs activate several other members of the STAT family besides STAT1 (4–6). STAT2 activation is essential for the formation of ISGF3; however, this transcription complex also requires STAT1. Furthermore, as STAT2 nuclear translocation is impaired in STAT1-deficient cells, it seemed unlikely that STAT2 accounts for the antiapoptotic and mitogenic effects of IFN-$\alpha\beta$ in STAT1$^{-/-}$ T cells. However, we found that

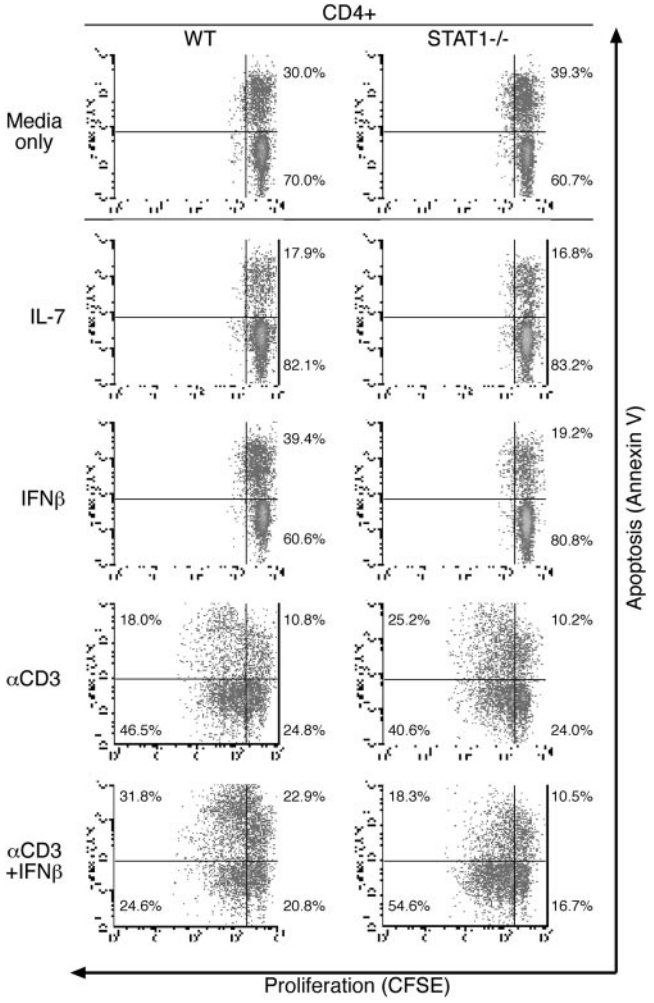

FIGURE 3. Effects of IFN-β on activated STAT1$^{-/-}$ T Cells. CD4$^+$ T cells derived from peripheral lymph nodes were labeled with 5 μM CFSE and cultured with APCs and the indicated treatments. After 72 h, cells were stained with annexin V, and cell proliferation and apoptosis were analyzed by flow cytometry. A representative of four independent experiments is displayed.

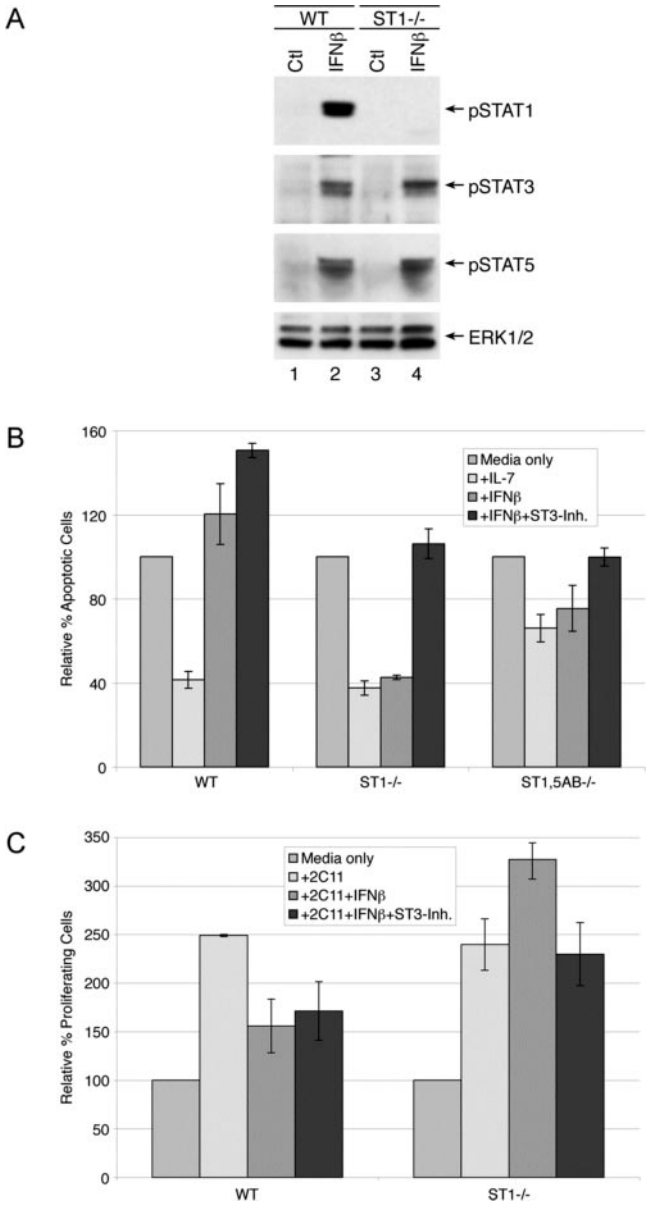

FIGURE 4. STAT3 and -5 mediate STAT1-independent IFN-β responses. *A*, CD4$^+$ splenic T cells were purified from age-matched WT and STAT1$^{-/-}$ mice, and treated with 10,000 U/ml IFN-β for 30 min. Resolved whole-cell lysates were probed with anti-phospho-STAT1, anti-phospho-STAT3, and anti-phospho-STAT5 Abs. The blots were subsequently probed with anti-ERK1/2 antisera to ensure equal protein loading (anti-phospho-STAT1 blot reprobe is shown). *B*, CD4$^+$ splenic T cells were purified from age-matched WT, STAT1$^{-/-}$, and STAT1/5A/5B$^{-/-}$ mice. Cells were cultured with APCs in the presence or absence of IFN-β (1000 U/ml) or IL-7 (1:500), and the STAT3-specific inhibitory peptide was added to the cultures 1 h before stimulation as indicated. After 48 h, the degree of apoptosis was analyzed by annexin V staining, and cell death in medium without cytokines was set to 100%. *C*, Same as *A*, except cells were cultured for 72 h with plate-bound anti-CD3 (200 ng/ml) in the absence or presence of IFN-β (1000 U/ml), and the STAT3-specific inhibitory peptide as indicated. Cell proliferation in medium without stimuli was set to 100%. The average results of four independent experiments are shown.

IFN-β can lead to rapid tyrosine phosphorylation of STAT3 and STAT5 in WT as well as STAT1$^{-/-}$ T cells (Fig. 4*A*). Importantly, these STAT family members are recognized for their contributions to the mitogenic and survival effects of many other cytokines.

To explore the role of STAT5 in the STAT1-independent IFN-β responses, we crossed STAT5A/5B$^{-/-}$ mice (19) to the STAT1$^{-/-}$ animals to generate STAT1/5A/5B triple-deficient mice. To circumvent the difficulties associated with the lethal phenotype of STAT3 deficiency (20), we decided to use a STAT3-specific inhibitor to elucidate its contribution to IFN-β signaling. Turkson et al. (21, 22) reported the use of a cell-permeable phosphopeptide corresponding to the STAT3 tyrosine phosphorylation site to specifically disrupt STAT3 dimer formation and DNA binding.

The additional absence of STAT5A/B in STAT1$^{-/-}$ mice results in a partial loss of the antiapoptotic effects of IFN-β when compared with mice lacking only STAT1 (Fig. 4*B*). Addition of the STAT3 inhibitor peptide to WT cells slightly increased the number of apoptotic cells in the presence of IFN-β (Fig. 4*B*), but did not effect cell viability in the absence of IFN-β (data not shown). Strikingly, administration of the STAT3 inhibitor to STAT1$^{-/-}$ or STAT1/5A/5B$^{-/-}$ CD4$^+$ T cells completely prevented the antiapoptotic effects of IFN-β (Fig. 4*B*).

Analysis of the mitogenic responses of the CD4$^+$ T cells revealed that the additional STAT5A/B deficiency was of no

consequence for the mitogenic effects of IFN-β due to STAT1 deficiency (data not shown). Addition of the STAT3 inhibitor peptide to IFN-β-treated WT cells did not affect the STAT1/2-mediated antiproliferative effects of the cytokine (Fig. 4*C*), thereby supporting the specificity of the inhibitor. In contrast, the mitogenic responses elicited by IFN-β in STAT1$^{-/-}$ CD4$^+$ T cells were completely abolished in the presence of the inhibitor (Fig. 4*C*).

The results shown in Fig. 4*B* suggest that STATs 3 and 5 cooperate to yield the STAT1-independent, antiapoptotic IFN-β responses. To test whether these STATs could potentially form heterodimers, we performed coimmunoprecipitation experiments with lysates from untreated and IFN-β-stimulated CD4$^+$ T cells. Indeed, we found that STAT3 and STAT5 form a heterodimer as a consequence of exposure of the cells to IFN-β (not shown).

In summary, our results support a model in which IFN-$\alpha\beta$ trigger two opposing pathways: a STAT1-dependent, dominant signaling cascade accounts for the antiproliferative effects of these cytokines and their proapoptotic properties. In contrast, activation of STATs 3 and 5 by IFN-$\alpha\beta$ promotes cell proliferation and survival. However, the latter outcomes are apparent only under conditions where STAT1 expression is abrogated.

It remains to be determined whether a complete lack of STAT1 expression is required to reveal the antiapoptotic and mitogenic effects of IFN-$\alpha\beta$, or if the inhibition of STAT1 activation and function in T cells is sufficient to allow for these altered IFN responses. Our findings described here are of likely clinical relevance, because many tumor cells harbor defects in STAT1 activation or expression (11, 12), and type I IFNs are frequently used in the treatment of hemopoietic malignancies. Similarly, numerous viral proteins are known to interfere with STAT1 expression and function (23–26). Under such circumstances, IFN-$\alpha\beta$ might potentially elicit cellular responses that are detrimental to their therapeutic benefit.

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