Activation of Tyk2 and Stat3 Is Required for the Apoptotic Actions of Interferon-β in Primary Pro-B Cells*

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The growth-inhibitory effects of type 1 interferons (IFNs) (IFNαβ) are complex, and the role of apoptosis in their antigrowth effects is variable and not well understood. We have examined primary murine interleukin-7-dependent bone marrow-derived pro-B cells, where IFNβ, but not IFNα, induces programmed cell death (PCD). IFNβ-stimulated apoptosis is the same in pro-B cells derived from wild type and Stat1−/− mice. However, in pro-B cells from Tyk2−/− mice, where there is normal activation of Stat1 and Stat2, IFNβ-stimulated PCD is not observed. Loss of B cells in lymphocytic choriomeningitis virus-infected mice has been shown to be mediated through the expression of IFNα/β (1). In wild type mice infected with lymphocytic choriomeningitis virus, there is a greater loss of B cells in the bone marrow and spleen than in Tyk2−/− mice infected with the virus, suggesting that the expression of this kinase plays an in vivo role in IFNαβ-mediated PCD. In contrast to IFNβ-stimulated tyrosine phosphorylation of Stat1 and Stat2, Stat3 tyrosine phosphorylation is defective in Tyk2−/− pro-B cells, suggesting that this Stat family member is required for apoptosis. In support of this hypothesis, inhibition of Stat3 activation in wild type B cells reverses the apoptotic effects of IFNβ. Furthermore, expression of a constitutively active form of Stat3 in Tyk2−/− B cells partially restores IFNβ-stimulated PCD. These results demonstrate an important role of Tyk2-mediated tyrosine phosphorylation of Stat3 in the ability of IFNβ to stimulate apoptosis of primary pro-B cells. IFNβ and IFNα can stimulate distinct sets of genes and selectively regulate different biological responses (2).

Interferon-stimulated gene factors mediate gene induction by binding to enhancers found within the promoters of type 1 IFN-induced early response genes (3, 4). Src homology 2 domain-containing transcription factors called Stats are required for the activation of genes by IFNs. These transcription factors are covalently modified by tyrosine phosphorylation and subsequently translocate to the nucleus such that they interact with enhancers needed for interferon-stimulated gene expression. The Jak family of tyrosine kinases is also an integral component in these signaling cascades (5). Expression of both Jak1 and Tyk2 is required for type 1 IFN activation of Stats in human cells, whereas in mouse cells, the actions of type 1 IFNs are only partially dependent upon Tyk2 (6, 7) in that Stat1 and Stat2, but not Stat3, are tyrosine-phosphorylated in Tyk2-null cells incubated with IFNα. The biological consequences of type 1 IFN activation of Stat3 in most cells are not clear.

Type 1 IFNs are inhibitors of cell growth and are presently used for the treatment of leukemias and other malignancies, such as melanomas and renal cell carcinomas. Although it is clear that type 1 IFN activation of the Jak/Stat pathway is required for their antiproliferative effects (8), it is not clear what role, if any, this signaling cascade plays in determining whether type 1 IFNs stimulate cell cycle arrest, a slowing of the cell cycle without accumulation in a single phase of the cell cycle, or induction of programmed cell death (PCD). In Daudi cells, a human Burkitt lymphoma B cell line, cells are arrested at the G0/G1 phase of the cell cycle after treatment with IFNα, whereas in the human leukemia Jurkat cell line, IFNα causes a slowing of the cell cycle without inducing cell cycle arrest or apoptosis (9). Daudi cells show a suppression of the DNA binding activity of the E2F transcription factor as well as decreased levels of phosphorylation of the retinoblastoma protein (10, 11).

A variety of hematopoiesis-derived T and B cell lines, certain melanomas, and other cell lines derived from solid tumors have been reported to undergo apoptosis when incubated with type 1 IFNs (12–16). The best described system to examine type 1 IFN-stimulated apoptosis of primary cells is murine IL-7-dependent bone marrow-derived B cells. These pro-B cells show both inhibited cell growth as well as apoptosis when incubated with a combination of virally produced IFNα and IFNβ for 24–48 h. Similar effects of this mixture of cytokines are seen in several IL-7-dependent murine pro-B cell lines (16). Two reports using mice support in vitro studies concerning the ability of type 1 IFN to inhibit the expression of pro-B cells in vivo. IFNα treatment of newborn mice inhibits the development of both T and B cell populations, and infection of mice with lymphocytic choriomeningitis virus (LCMV)
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Table 1

| Mouse genotype | IFNβ | IFNγ |
|----------------|------|------|
| Wild type      | 28 ± 4 | 22 ± 3 |
| Stat1/–        | 24 ± 5 | 0 |
| Stat5a/–, Stat5b/– | 40 ± 7 | 0 |
| Tyk2/–         | 0 | 24 ± 3 |

Cells were incubated with or without IFNβ (1000 units/ml) or IFNγ (10 ng/ml) for 36 h prior to staining with annexin V. Annexin V from untreated cells was subtracted from cells incubated with IFNs. The background staining ranged from 2 to 10%. Data are presented as mean ± S.D. values of four independent experiments.

Expression of Tyk2 Is Required for IFNβ-mediated Apoptosis of Murine Pro-B Cells—To examine the role of Tyk2 in IFNβ-stimulated apoptosis, we studied primary bone marrow-derived IL-7-dependent pro-B cells, since they have been shown previously to undergo PCD in the presence of a combination of IFNα and IFNβ (16). Furthermore, in Stat1−/− pro-B cells, the antigrowth effects of IFNβ are intact, whereas in Tyk2−/− cells, the antigrowth effects of IFNβ are lost (19, 23). These reports, however, did not address the issue of type 1 IFN-stimulated apoptosis in pro-B cells. To examine whether IFNβ-stimulated PCD in pro-B cells requires the expression of Stat1 and Tyk2, we isolated IL-7-dependent pro-B cells from bone marrow of Tyk2−/−, Stat1−/−, and Stat1,5a,b−/− mice. Since the genetic background of each knock-out mouse varied, B cells from wild type littermates were used to confirm that IFNβ-mediated apoptotic responses were intact. IFNβ-stimulated apoptosis in pro-B cells isolated from mice with different genetic backgrounds did not significantly differ (data not shown). Cells were incubated with or without IFNβ (1000 units/ml) or IFNγ (10 ng/ml) for 36 h. IL-7 was present during all incubations. Apoptosis was assayed by staining with annexin V. (Table 1). Both IFNβ and IFNγ stimulated apoptosis in pro-B cells from wild type mice. Induction of PCD by IFNβ was also observed in cells from mice that do not express Stat1 or Stat1,5a,b. In contrast, IFNγ was not able to stimulate PCD in cells from Stat1−/− or Stat1,5a,b−/− mice. Cells isolated from mice that do not express Tyk2 showed normal induction of apoptosis in the presence of IFNγ, but IFNβ-induced apoptosis was absent. Similar results were observed using terminal dUTP nick-end labeling assays (data not shown). Interestingly, the antiproliferative actions of IFNβ are present...
but are not as pronounced in Tyk22/− pro-B cells when compared with wild type pro-B cell counterparts. The difference in the antigrowth effects of IFNβ in wild type compared with Tyk22/− cells is approxi-
mately the percentage of annexin V-positive cells seen in wild type cells incubated with IFNβ (Table 2). These results differ from those of Shi-
moda et al. (19), who observed no antiproliferative effects of type 1 IFNs
in bone marrow cells from Tyk2−/− mice. However, they were not isolat-
ing the same population of bone marrow cells used in these assays.

Previous studies examining the actions of type 1 IFNs on PCD of
pro-B cells have used either a combination of IFNα and IFNβ isolated
from virally infected cells or recombinant IFNα (16, 19, 23). We wanted
to examine whether there were any differential actions of IFNα and
IFNβ on apoptosis of pro-B cells. Cells isolated from wild type mice
were incubated with or without recombinant murine IFNα or recom-
binant IFNβ for 48 h, and numbers of cells were counted or cells were
stained with annexin V (Table 3). Cells incubated with either IFNβ
or IFNα displayed about 50 and 34% fewer cells, respectively, than
untreated cells. However, only cells treated with IFNβ were annexin
V-positive. Although this finding was somewhat surprising, since most
of the actions of IFNα and IFNβ appear to be similar, there are a number
of reports suggesting that the actions of these two IFNs can be selective
with regard to the activation of immediate early genes as well as their
antiviral and antiproliferative actions (for a review, see Ref. 2).

To confirm that the lack of Tyk2 expression is responsible for resis-
tance to IFNβ-induced PCD, Tyk22/− cells were reconstituted with
murine Tyk2, encoded in the retroviral vector MSCV-IRES-GFP (27).

| TABLE 2 | Antigrowth and apoptotic actions of IFNβ in murine IL-7-dependent pro-B cells |
|---------|---------------------------------------------------------------------------------|
| Cells were incubated with or without IFNβ (1000 units/ml) for 48 h prior to
| staining with annexin V or counting. Annexin V staining in untreated cells was
| subtracted from cells treated with IFNs. Data are shown as mean ± S.D. |
| Apoptosis | Cell count (growth inhibition) |
|---------|---------------------------|
| Wild type | % 22 ± 2 65 ± 5 |
| Tyk22/− | % 4 ± 3 36 ± 3 |

| Table 3 | Antigrowth and apoptotic actions of IFNβ and IFNα in murine IL-7-dependent pro-B cells |
|---------|---------------------------------------------------------------------------------|
| Cells were incubated with or without IFNα or IFNβ (1000 units/ml) for 48 h prior to
| staining with annexin V or counting. Annexin V staining in untreated cells was
| subtracted from cells treated with IFNs. Data are shown as mean ± S.D. |
| Apoptosis | Cell count (growth inhibition) |
|---------|---------------------------|
| IFNβ | % 34 ± 5 51 ± 5 |
| IFNα | % 2 ± 4 39 ± 3 |

IFNβ-stimulated apoptosis of Tyk22/− pro-B cells, which express Tyk2.
Tyk22/− pro-B cells were infected with retroviruses that encode Tyk2-GFP or GFP. Cells
were selected for expression of GFP and incubated with or without IFNβ for 48 h and
stained with annexin V. WT, wild type.

IFNβ-induced Apoptosis of Pro-B Cells Requires Activation of Stat3—
Previous studies have indicated that there is a selective defect in IFNα-stimulated tyrosine
phosphorylation of Stat3 in Tyk22/− bone marrow-derived macrophages, whereas tyrosine
phosphorylation of Stat3 is normal in cells incubated with IFNγ, IL-12, IL-6, and IL-10 (6, 7).
We wanted to confirm these findings in primary B cells that were used for the
apoptosis assays. Cell extracts were prepared from wild type, Stat1−/−, Stat1, 5a/b−/−, and Tyk2−/− pro-B cells that had been incu-
bated with or without IFNβ (1000 units/ml) for 20 min. Immunoblots were probed with phosphotyrosine-specific Stat1 antisera (Fig. 2A
top panel) and also with Stat1 antisera to ensure equal loading of
protein (Fig. 2A, bottom). As reported by Shimoda et al. (6) and
Karaghiosoff et al. (7), using primary bone marrow-derived macro-
phages, we detected comparable levels of IFNβ-stimulated tyrosine-
phosphorylated Stat1 in pro-B cells from wild type and Tyk2−/− mice. We
next examined IFNβ-stimulated tyrosine phosphorylation of Stat3
(Fig. 2B, top). Whereas IFNβ-stimulated tyrosine phosphorylation of
Stat3 was observed in wild type, Stat1−/−, and Stat1, 5a/b−/− pro-B cells,
we were unable to detect any tyrosine phosphorylation of Stat3 in
Tyk2−/− pro-B cells. IFNβ-stimulated tyrosine phosphorylation of Stat3 in the
Tyk2-null B cells was examined after a variety of incubation
times (10–60 min) with the cytokine to ensure that there was no change
in the kinetics of activation. These findings correlate with an absence of
IFNα/β-stimulated tyrosine phosphorylation of Stat3 in bone marrow-derived macrophages isolated from Tyk2-null mice (7).

As mentioned earlier, in 2FTHG cells that do not express Tyk2 or express a kinase-inactive Tyk2, IFNα-stimulated tyrosine phosphorylation of Stat3 and IFNα + vanadate-induced PCD are not observed (20, 21). The loss of IFNβ-stimulated tyrosine phosphorylation of Stat3 in Tyk2−/− pro-B cells and its activation in all other B cells where IFNβ stimulates PCD suggests that Tyk2 may mediate some or all of its apoptotic actions through changes in the phosphorylation state of Stat3. To directly address this issue, we infected primary bone marrow-derived B cells from wild type mice with constructs that express either GFP or constitutively active (CA) or dominant negative (DN) Stat3 (27). CA-Stat3 contains two substitutions of cysteines in its Src homology 2 domain, which results in spontaneous dimerization of the protein (28). Pools of cells that stably express GFP were obtained by FACS, and these cells were incubated without or with IFNβ for 48 h. The amount of apoptosis was then determined by annexin V staining (Table 4). Pro-B cells that express either GFP or GFP-CA STAT3 showed approximately a 50% increase in annexin V-positive cells following treatment with IFNβ compared with untreated samples. However, pro-B cells that expressed GFP-DN STAT3 showed only a 20% increase in annexin V-positive staining compared with untreated samples. The basal apoptosis in each pool of cells was approximately the same (5%). To determine the amount of tyrosine-phosphorylated Stat3, each of the pools of infected cells was incubated with IFNβ for 30 min. Cell lysates were then subjected to Western blotting with phosphotyrosine-specific Stat3 antiserum (Fig. 3, middle). Cells expressing MSCV-GFP showed induction in tyrosine phosphorylation of Stat3 after incubation with IFNβ (Fig. 3, compare lanes 1 and 2). This induction is decreased about 50% in cells expressing DN-STAT3 (Fig. 3, compare lanes 2 and 4), whereas cells expressing CA-STAT3 show a basal level of tyrosine-phosphorylated Stat3 that is further increased by incubation of cells with the cytokine (Fig. 3, compare lanes 5 and 6). The partial decrease in tyrosine-phosphorylated Stat3 in cells expressing the dominant negative form of the protein correlates with the partial but significant decrease in IFNβ-stimulated apoptosis seen in these cells. The fact that elevated levels of tyrosine-phosphorylated Stat3 in cells expressing the constitutively active form of the protein show neither enhanced basal nor IFNβ-stimulated apoptosis indicates that activation of Stat3 on its own is not sufficient to program the apoptotic response. To determine whether expression of the DN-Stat3 was causing a nonspecific effect on IFNβ signaling, we probed the same blot with antiserum that recognizes tyrosine-phosphorylated Stat2 (Fig. 3, top). IFNβ-stimulated phosphorylation of Stat2 was the same in all three cell lines. To ensure equal protein loading of the samples, we also probed the blot for actin (Fig. 3, bottom), which was comparable for all samples.

As an independent confirmation of the role of Stat3 in the apoptotic actions of IFNβ, we also incubated wild type primary pro-B cells with a cell-permeable peptide that has been shown to block the actions of tyrosine-phosphorylated Stat3 (26). This peptide selectively prevents binding of tyrosine-phosphorylated Stat3 to DNA (26). Pro-B cells were incubated with the Stat3 inhibitor peptide (500 μM) for 1 h prior to being incubated with or without IFNβ for an additional 48 h. Apoptotic cells were then measured by staining with annexin V (Fig. 5). Treatment of primary pro-B cells with IFNβ induced 27% more annexin V-positive cells compared with untreated samples. Incubation of cells with the Stat3 inhibitor peptide completely reversed the apoptotic actions of IFNβ. Incubation of cells with only the inhibitor peptide without IFNβ had no effect on the basal number of annexin V-positive cells, which in this particular experiment was 11% (data not shown). Incubation of cells with a nonspecific peptide at the same concentration as the inhibitor peptide did not alter the apoptotic actions of IFNβ (data not shown).

These results confirm our findings that expression of DN Stat3 prevents IFNβ-stimulated apoptosis of pro-B cells and provides an independent assay for the requirement of IFNβ-stimulated tyrosine phosphorylation of Stat3 to induce PCD in these cells.

Expression of Constitutively Active Stat3 in Tyk2−/− Cells Restores IFNβ-stimulated Apoptosis—If the inability of IFNβ to induce tyrosine phosphorylation of Stat3 is responsible for the lack of apoptosis in Tyk2−/− pro-B cells, one would predict that expression of constitutively active Stat3 in these cells would restore this response. We therefore infected Tyk2−/− pro-B cells with either wild type or a constitutively active form of Stat3. GFP-expressing cells were isolated by FACS and incubated with or without IFNβ for 48 or 72 h prior to staining with annexin V (Fig. 4A). Tyk2−/− cells that express wild type Stat3 show no IFNβ-induced PCD when incubated 48 or 72 h with this cytokine. However, Tyk2−/− cells that express CA-Stat3 displayed IFNβ-stimulated PCD after 72 h. Interestingly, induction of apoptosis in the Tyk2−/− cells expressing CA-Stat3 is delayed compared with wild type pro-B cells incubated with IFNβ, since we observed PCD in wild type cells after 48 h (or less) of incubation with the cytokine. The reasons for this delay are not clear, but it may be due to a Tyk2-dependent, Stat3-independent pathway that is activated by IFNβ, which is needed for the

| TABLE 4 |
| IFNβ-stimulated apoptosis is decreased in cells that express dominant negative Stat3 |
| Wild type pro-B cells were infected with GFP retroviruses that express either GFP alone or GFP and DN or CA Stat3. Stable pools of GFP-expressing cells were selected and incubated with or without IFNβ for 48 h prior to staining with annexin V. The percentage of annexin V-positive cells from untreated cells was subtracted from each sample. The levels of apoptosis in untreated cells ranged from 3 to 6% and were not significantly different in those lines expressing Stat3 DN or Stat3 CA. |

| MSCV-GFP apoptosis | MSCV-GFP Stat3 DN apoptosis | MSCV-GFP Stat3 CA apoptosis |
|---------------------|-----------------------------|-----------------------------|
| %                   | %                           | %                           |
| 50 ± 6              | 20 ± 2                      | 48 ± 4                      |

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FIGURE 3. IFNβ-stimulated tyrosine phosphorylation of Stat3 but not Stat2 is diminished in pro-B cells that express DN-Stat3. Wild type B cells were infected with retroviruses that encode for GFP, DN-Stat3-GFP, or CA-Stat3-GFP. Cells were selected for expression of GFP and incubated with or without IFNβ (1000 units/ml) for 30 min. Cell extracts were immunoblotted with phosphotyrosine-specific antiserum or actin (bottom) as a control for equal protein loading.

| TABLE 5 |
| A Stat3 inhibitor peptide prevents IFNβ-induced apoptosis of pro-B cells |
| Cells were incubated with or without 500 mM inhibitor peptide and with or without IFNβ (1000 units/ml) for 48 h prior to staining with annexin V. Annexin V staining in the absence of IFNβ treatment was subtracted from cells incubated with IFNβ. Data are presented as mean ± S.D. values of two independent experiments. |

| IFNβ apoptosis | IFNβ + Stat3 peptide apoptosis |
|----------------|--------------------------------|
| %              | %                             |
| 27 ± 4         | 0 ± 1                         |
more accelerated PCD seen in wild type cells. In Tyk2−/− pro-B cells, constrictively active Stat3 is also tyrosine-phosphorylated when the cells are left untreated (Fig. 4B, lane 7). IFNβ does stimulate tyrosine phosphorylation of CA-Stat3 in Tyk2−/− cells but not to the levels seen in wild type cells that express CA-Stat3 (Fig. 3, lanes 5 and 6). IFNβ also stimulates tyrosine phosphorylation of wild type Stat3, which is overexpressed in Tyk2−/− cells (Fig. 4B, lanes 5 and 6) but not to the same extent as in Tyk2−/− cells (Fig. 4B, lanes 7 and 8). Note that the levels of expression of wild type Stat3 and CA-Stat3 in Tyk2−/− pro-B cells are approximately the same. We have no proof of why CA-Stat3 is tyrosine-phosphorylated in response to IFNβ in Tyk2−/− cells. However, this Stat3 spontaneously dimerizes, which may make it a better substrate for another tyrosine kinase that has a low affinity for Stat3 but can phosphorylate the protein when it is in a dimerized configuration.

Tyk2−/− Mice Are Relatively Resistant to LCMV-induced Depletion of Hematopoietic Cells—LCMV-induced bone marrow aplasia occurs in mice that do not express the IFNαR1 subunit of the type 1 IFN receptor (1). The actions of LCMV thus appear to be a direct effect of type 1 IFN expression.

We have examined the effects of LCMV infection on expression of nucleated cells and B220+ B cells in the bone marrow and spleen in wild type and Tyk2−/− mice. There were no significant differences in the numbers of nucleated cells in the bone marrow and spleen of uninfected wild type and Tyk2−/− mice (Table 6). Wild type and Tyk2−/− mice (3 mice/group) were given an intravenous dose of 2×10⁶ plaque-forming units of LCMV-Clone 13. Three days postinfection, bone marrow and spleen cells were harvested and analyzed for total nucleated cells (Table 7). There was an ~84% decrease in the number of nucleated cells in the bone marrow of wild type mice infected with LCMV compared with uninfected mice. Interestingly, in LCMV-infected Tyk2−/− mice, there was only a 49% loss of total nucleated cells compared with uninfected mice. The numbers of nucleated splenic cells from LCMV-infected Tyk2−/− mice showed no significant changes compared with uninfected Tyk2−/− mice, whereas wild type mice infected with the virus showed ~50% fewer cells compared with uninfected mice.

We also examined the numbers of B220+ cells (reflective of B lineage) in bone marrow and spleen of wild type and Tyk2−/− mice before and after LCMV infection (Fig. 5). The number of B220+ cells was not altered in the spleens of LCMV-infected Tyk2−/− mice, whereas wild type-infected mice showed a 40% decrease in this population of cells. Similar results were seen in the bone marrow, where there was a 25% loss of B220+ cells in infected Tyk2−/− mice and a 66% loss of cells in wild type infected mice. The fact that there is a decrease in B220+ cells in the bone marrow of Tyk2−/−-infected mice may be a reflection of the antiproliferative effects of type 1 IFNs (see Table 2) on actively dividing cells, which would not be present in the spleen. The decrease in B220+ cells as well as nucleated cells are consistent with the previously published decreases in LCMV-infected wild type mice (1). The fact that Tyk2−/− mice show a significant resistance to LCMV-induced lymphoid hypoplasia, particularly in B lineage cells, is consistent with our in vitro data using primary IL-7-dependent B cells.

### DISCUSSION

The mechanisms by which type 1 IFNs inhibit cell growth are variable and complex. Growth inhibition may or may not result in cell death, and many of the reported studies used transformed cells from different origins, which may account for these variable effects. In this report, we have examined IFNβ-induced apoptosis of bone marrow-derived...
The primary findings of this report indicate that IFNβ-stimulated tyrosine phosphorylation of Stat3 together with the expression of Tyk2 are essential signaling components required for IFNβ-induced apoptosis of pro-B cells. Although active Stat3 has been generally regarded as having a role in the regulation of cell proliferation, our results indicate that there is an antiproliferative effect of type 1 IFNs in Tyk2-null mice infected with LCMV. This is consistent with the finding that there is an antiproliferative effect of type 1 IFNs in Tyk2−/− pro-B cells, but it is less pronounced than in wild type cells, since these cells also undergo PCD (see Table 2).

Now that we have established a role of both Tyk2 and Stat3 in IFNβ-induced PCD of primary B cells in vitro and the role of Tyk2 in vivo, we can identify the downstream targets of Tyk2 and Stat3. Preliminary results indicate that the apoptotic actions of IFNβ in pro-B cells are mediated by a mitochondria-dependent, caspase-independent pathway (data not shown). Proteins such as apoptosis-inducing factor or HtAr2, which can induce apoptosis in the absence of caspase activation through mitochondria-dependent events, are two such proteins that need to be evaluated to see if they contribute to the apoptotic actions of IFNβ. We are also determining whether there are changes in a variety of mitochondrial functions that are regulated by Tyk2 activation of Stat3. Such information will provide us with targets for the apoptotic actions of IFNβ.

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