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STAT1 Affects Lymphocyte Survival and Proliferation Partially Independent of Its Role Downstream of IFN-γ

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Lymphocytes derived from mice deficient in STAT1 showed reduced apoptosis and enhanced proliferation in vitro. To understand the involvement of STAT1 in the observed reduction in apoptosis, we examined the levels of caspase and bcl-2 family genes that are involved in cell survival and/or apoptosis. The levels of caspase 1 and 11, two enzymes involved in both cytokine protein processing and induction of apoptosis, were reduced in STAT1 −/− cells compared with wild-type. However, the levels of bcl-2 genes were comparable in both mice. STAT1 −/− cells also displayed an enhanced proliferation following TCR stimulation. This hyperproliferation could not be ascribed completely to the loss of IFN-γ-mediated antiproliferation. First, similar phenotypes were also observed in fibroblasts and pre-B cells derived from STAT1 −/− mice, which do not produce IFN-γ. Second, comparisons with cells lacking the gene for IFN-γ or with cells treated with neutralizing Abs to IFN-γ only partially mimicked the STAT1 −/− phenotype. Interestingly, the kinetics of degradation of p27kip1, a CDK inhibitor, following TCR ligation were faster, and, concomitantly, the up-regulation of CDK2 kinase activity and protein levels were increased in stimulated T cells of STAT1 −/− mice relative to those of wild-type mice. Furthermore, STAT1 −/− animals were more susceptible to carcinogen-induced thymic tumors, a possible consequence of altered T cell growth and/or survival. These results demonstrate an essential role for STAT1 for lymphocyte survival and proliferation that is only partially dependent on IFN-γ signaling. The Journal of Immunology, 2000, 164: 1286–1292.

Survival, apoptosis, and proliferation of lymphocytes are major processes critical to the maintenance of homeostasis in the immune system during development and responses to antigenic stimuli. The determination of survival and/or apoptosis of lymphocytes is mainly governed by two families of genes, namely bcl-2 and caspases. Apoptosis of lymphocytes operates during the process of negative selection in the thymus and during clonal deletion in the periphery, and can be triggered by many stimuli, for instance, UV or gamma irradiation, staurosporine, steroid hormones, FasL, and TNF-α. Although the mechanisms for triggering apoptosis by these reagents are distinct, they are all mediated by a group of functionally related proteases, termed caspases. Caspases are present in cells as inactive proenzymes and are activated by proteolytic processing into two subunits in response to apoptotic stimulation. Fourteen mammalian caspases have been identified thus far, and these enzymes are involved both in apoptosis and in cytokine protein processing. Modulation of both the levels and activities of caspases can affect cell survival.

Bcl-2 family, consisting of both proapoptotic and antiapoptotic members, acts as a checkpoint upstream of caspases and mitochondrial dysfunction. The proapoptotic members, such as bcl-2-associated x protein (bax), bcl-2 antagonist/killer (bak), bcl-xL, bcl-2-associated death promoter (bad), and bcl-xS can form homodimers or heterodimers with the antiapoptotic members, including bcl-2, bcl-xL, myeloid cell leukemia sequence 1 (mcl-1), and bfl-1, through conserved domains bcl-2 homology (BH1), BH2, and BH3. The overall ratio of the proapoptotic molecules to antiapoptotic molecules determines the susceptibility to a death stimulus. Within the thymus, bcl-2 is expressed in nearly all CD4+ or CD8+ single positive thymocytes but not CD4+CD8+ double positive thymocytes, possibly leaving them more susceptible to apoptosis. In addition, bcl-2 is highly expressed in mature peripheral T cells in the spleen and lymph nodes. Conversely, bcl-xL is highly expressed in the immature double positive thymocytes but absent from mature single positive thymocytes. Similarly, bcl-2 and bcl-xL have a reciprocal pattern of expression in B cell compartment where bcl-2 is high in pro-B cells in the bone marrow and mature B cells in the periphery. Conversely, bcl-xS is high in pre-B cells but down-regulated in mature B cells. The reciprocal expression patterns of bcl-2 and bcl-xL indicate that their tight regulation is likely critical for providing survival signals for lymphocytes during development.

Proliferation of T lymphocytes occurs in central lymphoid organs during positive selection as well as in the periphery during clonal expansion in response to foreign Ags. Peripheral T lymphocytes are normally resting and require two sequential mitogenic signals to reenter the cell cycle and proliferate from this quiescent state. The first signal is provided by stimulation of TCR, which induces the synthesis of proteins necessary for progression from G0 to G1. TCR stimulation alone, however, is not sufficient to promote the activation of G1 cyclin-CDK2 complexes, at least in part due to inhibition by p27kip1, a CDK inhibitor. The second

Department of Pathology and Kaplan Cancer Center, New York University School of Medicine, New York, NY 10016

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2 Address correspondence and reprint requests to Dr. David E. Levy, Department of Pathology, New York University School of Medicine, 550 First Avenue, New York, NY 10016. E-mail address: levyd01@med.nyu.edu

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3 Abbreviations used in this paper: bak, bcl-2 antagonist/killer; GKO, IFN-γ knock-out; BH, bcl-2 homology; mAPO, mouse apoptosis; IAP, inhibitor of apoptosis.
mitogenic signal, which can be provided by IL-2 or other mitogenic cytokines, leads to inactivation and degradation of the CDK inhibitor, allowing progression into S phase (17, 18).

Proliferation of T cells in response to Ag is mediated primarily by an autocrine/paracrine growth pathway. The principle autocrine cytokines for T cells are IL-2 and IL-4 (19). Activated T cells transiently increase the transcription of several genes, including IL-2 and IL-2 receptors, that are essential for T cell proliferation and function. The up-regulated IL-2R α-chain (CD25) associates with constitutively produced IL-2R β- and γ-chains to form high affinity receptors that are capable of stimulating T cell proliferation in response to physiologically relevant concentrations of IL-2 (20). IL-2 signaling subsequently leads to activation of several STAT proteins. STAT5, activated by IL-2, plays a critical role in IL-2-mediated induction such that STAT5→ cytopsins show impaired induction of high affinity receptors and markedly reduced proliferation in response to IL-2 (21, 22). Although it has been suggested that STAT5 might be activated in response to Ag, such activation is probably completely dependent on cytokine stimulation (23). Similarly, STAT6 is required for IL-4-dependent lymphocyte proliferation. STAT6→ cytopsins show reduced proliferation in response to IL-4, due in part to increased protein levels of the CDK inhibitor p27Kip1 following IL-4 stimulation (24). In contrast, STAT1, a major mediator of IFN-γ, was reported to induce the CDK inhibitor p21WAF1 in fibroblast cells, in response to IFN-γ, and was suggested to be involved in cell growth arrest (25). However, the role of p21 in the cell cycle regulation of lymphocytes is controversial, since p21 is also induced during the proliferative response to TCR stimulation (18).

In this report, we studied the role of STAT1 in primary T cell physiology, using mice lacking the STAT1 gene. These experiments showed that STAT1 modulates lymphocyte survival/apoptosis and proliferation. Moreover, although some of these effects can be ascribed to the action of IFN-γ, an IFN-γ-independent action of STAT1, possibly mediated by IFN-α, was also revealed by these studies.

Materials and Methods

Animals

STAT1−/− mice lacking STAT1 (26), IFNAR−/− mice lacking IFN type I receptors (27), IFNγR−/− mice lacking IFN type II receptors (28), and GKO mice lacking IFN-γ (29) were compared with wild-type mice of the same strain background and were housed under specific pathogen-free conditions. All work with animals was approved by the Institutional Animal Care and Use Committee of New York University.

In vitro survival assay

Freshly prepared splenocytes of wild-type or STAT1−/− mice were cultured in 12-well plates for different periods of time. Viable cells were scored and enumerated by trypsin blue exclusion. The in vitro cultured splenocytes were fixed with cold 70% ethanol overnight, stained with propidium iodide at 40 μg/ml for 30 min at room temperature, and analyzed by flow cytometry.

RNase protection assay

RNA probes were prepared using mouse apoptosis (mAPO)-1 and mAPO-2 template sets according to the manufacturer’s instructions (PharMingen, San Diego, CA). For mAPO-1, 3 x 106 cpm of mixed probes specific for mouse caspase 1 (ICE), caspase 2 (ICDH2), caspase 3 (YAMA, CPP32), caspase 6 (Mch2), caspase 7 (Mch3), caspase 8 (FLICE, Mch-5), caspase 11, caspase 12, caspase 14, and two housekeeping genes, L32 and GAPDH, were hybridized at 56°C overnight with 20 μg of total RNA prepared from splenocytes. For mAPO-2, 2.5 x 106 cpm of mixed probe specific for mouse bcl-W, bfl-1, bcl-x, bak, bax, bcl-2, bad, and two house-keeping genes, L32 and GAPDH, were hybridized at 56°C overnight with 15 μg of total RNA prepared from splenocytes. Unhybridized ssRNA was then digested with a mixture of RNases A and T1, and protected RNA was resolved in 6% sequencing gels followed by autoradiography and quantitation by phosphor imager.

Enrichment and stimulation of T lymphocytes and Western blot

Freshly prepared splenocytes of wild-type or STAT1−/− mice were stained with rat anti-B220 Ab (Caltag, South San Francisco, CA) followed by two washes with washing buffer (0.2% BSA containing 1% PBS) then incubated with anti-rat Ig-coupled Dynalbeads (Dynal, Oslo, Norway) for 30 min at 4°C. Purified T cell were left untreated or stimulated with plate-bound 2C11 anti-CD3 Ab (10 μg/ml) plus IL-2 (50 U/ml) for 5 h or 24 h or were subject to immunoprecipitation with anti-CD2 Ab-conjugated protein A-Sepharose. Following three washes with lysis buffer and 1 wash with kinase buffer (50 mM Tris (pH 7.5), 10 mM MgCl2, 1 mM DTT), the beads were resuspended in 20 μl of kinase buffer and incubated with histone H1 (5 μg/reaction) and [γ-32P]ATP (20 μCi) at 30°C. The reactions were boiled in sample buffer, resolved by 12% SDS-PAGE, transferred to nitrocellulose membranes, and detected by autoradiography.

Histone H1 kinase assay

In vitro kinase assay for CD2 was performed as described (17). In brief, equal amounts of whole cell extracts prepared from purified T cells left untreated or stimulated with plate-bound 2C11 anti-CD3 Ab (10 μg/ml) plus IL-2 (50 U/ml) for 5 h or 24 h were subject to immunoprecipitation with anti-CD2 Ab-conjugated protein A-Sepharose. Following three washes with lysis buffer and 1 wash with kinase buffer (50 mM Tris (pH 7.5), 10 mM MgCl2, 1 mM DTT), the beads were resuspended in 20 μl of kinase buffer and incubated with histone H1 (5 μg/reaction) and [γ-32P]ATP (20 μCi) at 30°C. The reactions were boiled in sample buffer, resolved by 12% SDS-PAGE, transferred to nitrocellulose membranes, and detected by autoradiography.

In vitro proliferation assays

Lymphocytes (1 x 105) were seeded on anti-CD3 (2C11)-coated 96-well plates (2 μg/ml) in the presence of human IL-2 (50 U/ml). [3H]Thymidine (1 μCi) was added 24 h before harvest. Where indicated, IFN-γ (500 unit/ml) (Boehringer Mannheim, Indianapolis, IN) or affinity-purified XM12 anti-IFN-γ (50 μg/ml) Ab was added into culture medium at the beginning of the treatment with anti-CD3 Ab.

Fibroblasts were cultured in DMEM supplemented with 10% calf serum. Viable cells from parallel cultures were counted at the indicated times by trypsin blue exclusion. Cell cycle parameters were determined by flow cytometry of propidium iodide-stained cells harvested from asynchronously growing cultures (31, 32), and the mean percentage of cells in S phase was averaged for 2 STAT1−/− and three wild-type cell lines, each measured in triplicate.

Thymic tumor induction

Groups of 30 wild-type or STAT1−/− mice were injected i.p. with N-nitroso-N-methylurea (75 mg/kg), as described (33). Mice that died from disease were found at necropsy to have multifocal thymic tumors, often with visible liver metastases (data not shown).

Results

Enhanced survival of STAT1−/− lymphocytes

The majority of circulating peripheral lymphocytes are quiescent and consist of both naive and memory cells. Although the life span of these two populations differs, both can be measured in weeks or months. However, the persistence of resting cells is dependent on survival signals received from the environment through both cytokine and Ag receptors (34). Removal of quiescent lymphocytes from the microenvironment of lymph nodes or spleen results in rapid induction of apoptosis. We investigated the involvement of STAT1 in this process. Lymphocytes from wild-type and STAT1−/− mice were cultured in the absence of added cytokines or growth factors. Following 4 days of culture, only about 25% of wild-type lymphocytes survived, indicating significant induction of apoptosis. In contrast to wild-type cells, lymphocytes from STAT1−/− mice showed enhanced survival (Fig. 1A), with about 60% of these cells still viable after 4 days, indicating an approximately 2-fold increase in cell survival in the absence of STAT1.

The enhanced survival of STAT1−/− cells correlated with decreased apoptosis. In vitro cultured lymphocytes were analyzed for induction of apoptotic cells by flow cytometry. After a 24-h culture, 34% of wild-type cells had initiated apoptosis, as determined by a hypodiploid DNA content, which increased to nearly 50% of
the cells by 48 h (Fig. 1B). In contrast, an approximate 30% decrease in the apoptotic rate was observed for STAT1\(^2/2\) lymphocytes, resulting in survival of greater than 75% of the cells after 24 h, and this difference was maintained after 48 h with greater than 65% of STAT1\(^2/2\) cells still alive. Interestingly, a small but reproducible increase in cell proliferation was also observed in the STAT1\(^2/2\) population, as judged by the fraction of cells in mitosis with a 4n DNA content. The decreased apoptotic rate measured in STAT1\(^2/2\) cells may explain the observed enhanced survival; however, it did not appear to result from a defect in Fas-dependent killing since cells from both strains of mice were equally susceptible to anti-CD3-induced cell death (data not shown).

**Decreased caspase 1 and caspase 11 mRNA but not bcl-2 gene family in STAT1\(^2/2\) lymphocytes**

Members of the caspase and bcl-2 families play pivotal roles in cell survival and/or apoptosis. We first examined the levels of caspase family mRNA in resting lymphocytes from wild-type and STAT1\(^2/2\) mice to see whether changes in their levels might explain the increased survival or decreased apoptosis observed in mutant cells. Total RNA prepared from spleen cells of wild-type or STAT1\(^2/2\) mice was hybridized with RNase protection probes specific for nine different caspases. As shown in Fig. 2A, the mRNA levels of both caspase 1 and caspase 11 were significantly reduced in STAT1\(^2/2\) cells whereas other caspases were expressed at comparable levels in both STAT1\(^2/2\) and wild-type cells. STAT1 is activated by both type I and type II IFN; therefore, caspase mRNA levels were also examined in animals specifically deficient in signaling by each IFN family. The levels of caspase 11 were decreased in lymphocytes from animals deficient in either receptor. Caspase 1 mRNA was only significantly reduced in IFNGR\(^2/2\) cells. Similar to the effect of STAT1 loss, expression of other caspases was largely unaffected by impaired IFN signaling. Therefore, the basal levels of caspase 1 and 11 appear to rely on IFN-\(\alpha\) and IFN-\(\gamma\) for full expression and caspase 1 requiring only IFN-\(\gamma\).

Several bcl-2-related genes were expressed in the spleens of both wild-type and mutant mice. Bfl-1 was the most prominent species among the bcl-2 family genes expressed that might be...
IFN-γ is secreted by activated T lymphocytes and is known to inhibit proliferation of wild-type lymphocytes, proliferation of wild-type and STAT1−/− cells was compared with those from mice missing the IFN-γ gene (GKO). As would be expected given the antiproliferative capacity of IFN-γ, GKO lymphocytes also exhibited higher growth than wild-type, particularly after prolonged incubation (Fig. 3A). Interestingly, however, growth of GKO cells, while greater than wild-type, did not equal the growth of STAT1−/− cells, suggesting that their growth was still limited by a STAT1-dependent process not mediated by IFN-γ.

Further evidence for both IFN-γ-dependent and -independent regulation of lymphocyte proliferation was obtained by culturing cells with added cytokine or with neutralizing Ab to IFN-γ. Addition of IFN-γ to cultures of GKO cells reduced their proliferation to levels equivalent to wild-type cells (Fig. 3B), demonstrating that their enhanced growth was due to absence of IFN-γ-mediated inhibition. As expected, addition of IFN-γ to cultures of STAT1−/− cells had no effect on their proliferation. Likewise, neutralization of secreted IFN-γ with Ab increased the proliferation of wild-type cells equal to the growth of GKO cells (Fig. 3C) but had no effect on the growth of either GKO or STAT1−/− cells. These results demonstrate that IFN-γ secreted by proliferating wild-type lymphocytes inhibits their growth, especially after extended culture when it accumulates above some minimal threshold level. GKO cells, unable to secrete IFN-γ, or STAT1−/− cells, unable to respond to it, are unaffected by this autocrine inhibition. However, the growth of STAT1−/− cells is further deregulated in a manner independent of the action of IFN-γ since these cells proliferated more than cells deprived of IFN-γ.

**STAT1 affects molecules that regulate the cell cycle**

Since TCR ligation induces resting T cells to undergo cell cycle progression, we investigated whether molecules involved in the regulation of the cell cycle were affected in the absence of STAT1. To avoid the interference of nonproliferating lymphocytes, mainly B cells, freshly isolated splenocytes were enriched for T cells by removing B220-positive cells using anti-B220 Ab and magnetic beads. The levels of CDKs, CDK inhibitors, and cyclins were measured by in vitro kinase assay using histone H1 as a substrate.

**FIGURE 3.** Increased proliferation of STAT1−/− lymphocytes is partially independent of IFN-γ. Splenocytes from STAT1−/−, GKO, and wild-type mice (C57BL/6J) were incubated with anti-CD3 Ab precoated (2 μg/ml) plates in the absence (A) or presence (B) of IFN-γ (500 U/ml) or of anti-IFN-γ Ab (50 μg/ml) (C) for different times, as indicated. Proliferation was measured by [3H]thymidine incorporation for the final 24 h.

**FIGURE 4.** A, STAT1 affects levels of p27k1p1 and CDK2 in stimulated T cells. Nuclear extracts prepared from T cell-enriched populations that were left untreated (W/O) or stimulated with anti-CD3 (10 μg/ml) plus IL-2 (50 U/ml) were subject to immunoblot with Abs to p27 (first panel), CDK2 (second panel), or cyclin A (third panel). Equal protein loading was revealed by Coomassie blue staining in the fourth panel. B, Rapid increase of CDK2 kinase activity in STAT1−/− cells. Whole cell extracts prepared from cells as described in A were subject to immunoprecipitation with anti-CDK2 Ab followed by in vitro kinase assay using histone H1 as a substrate.

Enhanced proliferation of STAT1−/− lymphocytes

IFN-γ is secreted by activated T lymphocytes and is known to have a potent antiproliferative effect. We asked whether disruption of STAT1 might relieve the inhibitory effect of IFN-γ and lead to enhanced proliferation. Splenocytes from wild-type and STAT1−/− mice were treated with anti-CD3 Ab, and their proliferation in vitro was monitored by [3H]thymidine incorporation. Compared with the proliferation profile of wild-type lymphocytes, STAT1−/− lymphocytes showed enhanced growth (Fig. 3A). To test the potential involvement of IFN-γ in the growth regulation of wild-type cells, proliferation of wild-type and STAT1−/− cells was compared with those from mice missing the IFN-γ gene (GKO). As would be expected given the antiproliferative capacity of IFN-γ, GKO lymphocytes also exhibited higher growth than wild-type, particularly after prolonged incubation (Fig. 3A). Interestingly, however, growth of GKO cells, while greater than wild-type, did not equal the growth of STAT1−/− cells, suggesting that their growth was still limited by a STAT1-dependent process not mediated by IFN-γ.

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48 h. The kinetics of reduction of nuclear p27 were significantly enhanced in STAT1−/− cells relative to wild-type cells (Fig. 4A, first panel). After stimulation for 24 h, nuclear p27 in STAT1−/− cell decreased to a minimal level, whereas around 50% of the protein remained in wild-type cells, suggesting that STAT1−/− cells were released from p27 inhibition into the cell cycle earlier or more efficiently than wild-type cells. The mRNA and protein levels of another CDK inhibitor, p21WAF1, however, were similar in both wild-type and STAT1−/− cells after stimulation (data not shown). The protein levels of nuclear CDK2 were significantly increased in both wild-type and STAT1−/− cells after stimulation for 48 h (Fig. 4A, second panel). Interestingly, however, there was about 4-fold more nuclear CDK2 protein in STAT1−/− cells than in wild-type cells (Fig. 4A, second panel). The induced levels cyclin A, E, and D3 were comparable between wild-type and STAT1−/− cells (Fig. 4A, third panel, and data not shown).

Because p27 is known to be a key negative regulator of resting T lymphocytes by binding to CDK2 and inhibiting cell cycle progression (18), the faster removal of p27 in STAT1−/− cells following CD3 stimulation prompted us to investigate levels of CDK2 catalytic activity in STAT1−/− cells. Whole cell extracts were prepared from enriched T lymphocyte populations from spleens of wild-type and STAT1−/− mice that were left untreated or stimulated for 5 h or 24 h. The catalytic activity of immunoprecipitated CDK2 was measured using histone H1 as a substrate. As shown in Fig. 4B, increased histone kinase activity was observed 24 h after stimulation of wild-type cells. In contrast, STAT1−/− cells showed increased activity at 5 h of stimulation. At 24 h, the activity was increased significantly by almost 2-fold that of wild-type cells. An equivalent amount of CDK2 protein was observed in both wild-type and STAT1−/− cells at 24 h (Fig. 4A, second panel), suggesting that the increased CDK2 kinase activity at this early time point in STAT1−/− cells was due to a diminished inhibitory effect of p27 (Fig. 4A, first panel). These results suggest that a more rapid decrease of a CDK inhibitor followed by a higher increase of a CDK protein contribute to enhanced proliferation of T cells devoid of STAT1.

Enhanced proliferation and survival of T lymphocytes in vitro prompted us to examine possible in vivo consequences of the lack of STAT1-mediated growth control. Although no major alterations in the steady-state abundance of lymphocytes were observed, STAT1−/− mice showed increased susceptibility to carcinogen-induced thymic tumor induction. Groups of wild-type and STAT1−/− mice were challenged with N-nitroso-N-methylurea and monitored for tumor induction. Tumor induction occurred earlier and more frequently in STAT1−/− mice than in control mice treated with carcinogen. The mean latency for tumor induction in wild-type mice was 139 ± 4 days whereas mean latency occurred in STAT1−/− mice after 118 ± 4 days (Fig. 5A). The tumor cells were mainly immature T lymphocytes in both wild-type and STAT1−/− mice (data not shown, and Ref. 33). All animals succumbing to disease showed similar thymic tumors at necropsy, with no gross differences in tumor histology between mutant and wild-type mice (data not shown).

Enhanced proliferation in the absence of STAT1 was not limited to T cells. Abelson virus-transformed pre-B cells derived from STAT1−/− mice also exhibited enhanced proliferation relative to wild-type cells (Fig. 5B). In addition, fibroblast lines established from STAT1−/− embryos proliferated more rapidly that wild-type cells, exhibiting a doubling time of ~14 h vs 22 h for wild-type cells. A significantly higher fraction of cells in S phase was observed for asynchronously growing cultures of STAT1−/− cells compared with that of wild-type cells (Fig. 5C). Because neither of these cell types is capable of producing IFN-γ (37), these results again point to STAT1-dependent growth regulation that is independent of the action of IFN-γ.

**Discussion**

We have demonstrated that several functions and properties of lymphocytes were altered in the absence of STAT1. These included enhanced survival, decreased apoptosis, and increased proliferation in vitro, suggesting a critical role for STAT1 in regulating the immune response. To some degree, the STAT1-dependent processes uncovered in lymphocytes could be explained by the known actions of IFN-γ or IFN-α that signal through STAT1 and exert broad activities on lymphocyte functions. Overlap between loss of STAT1 and loss of IFN receptors suggests a role for constitutive IFN signaling in the maintenance of lymphocyte function even in the absence of inflammation. Interestingly, however, evidence of IFN-γ-independent functions in lymphocytes that are nonetheless still dependent on STAT1 was also obtained. It is likely that some of these effects reflect an important role for IFN-α/IFN-β in lymphocyte functions normally ascribed to IFN-γ (38) but the possibility also exists that some STAT1-dependent mechanisms are completely independent of IFN signaling. For instance, we have found that STAT1 can regulate the constitutive expression...
of MHC class I by an IL-7-dependent but IFN-independent pathway (39) and that STAT1 can regulate the proliferation and differentiation of chondrocytes in response to fibroblast growth factor (FGF) (40).

An example of overlap between basal and IFN-induced regulation of gene expression is caspase 1. Caspase 1 has been shown to be involved in IFN-γ-induced apoptosis in a STAT1-dependent manner (41, 42). We found that caspase 1, as well as its upstream activator caspase 11, was also affected under basal, non-IFN-induced conditions by the absence of either IFN receptors or STAT1, suggesting that the levels of these proteins are maintained by constitutive signaling through the IFN pathway. Caspase 1 and caspase 11 belong to the same subfamily of caspase genes and share a similar substrate specificity. Gene targeting experiments have revealed that both caspase 1 and caspase 11 are primarily important for the regulation of inflammatory immune responses, and caspase 1−/− and caspase 11−/− mice are highly resistant to endotoxic shock (4, 5, 43). Because caspase 1 is activated through a direct physical interaction with caspase 11, caspase 11-deficient cells are resistant to apoptosis induced by caspase 1 overexpression, and the production of IL-1α and IL-1β is blocked after LPS stimulation, which is a critical event mediated by caspase 1 during toxic shock (5). The decreased level of caspase 1 and caspase 11 in STAT1−/− lymphocytes may contribute to the reduced apoptosis observed. However, similar to caspase 1−/− or caspase 11−/− cells, mutation of STAT1 did not protect thymocytes from gamma-irradiation- or dexamethasone-induced cell death (data not shown), confirming that the major avenues leading to lymphocyte apoptosis are independent of STAT1. Recently, a family of inhibitor of apoptosis (IAP) proteins were found to suppress cell death perhaps by binding directly to caspases and inhibit their function (44). Although the role of STAT1 in the regulation of IAP proteins is not clear, an involvement of IAP in enhanced survival of STAT1−/− lymphocytes cannot be excluded.

A dependence on STAT1 for production of caspase 1, 2, and 3 has been observed in mutagenized human fibrosarcoma U3A cells (45). Interestingly, it was reported that STAT1 function did not require tyrosine phosphorylation or dimerization for maintenance of caspase gene expression, suggesting a non-cytokine-dependent role for STAT1 in these cells. In contrast to this report, we found reduced levels of caspase 1 and 11, but not of 2 and 3, in STAT1−/− murine lymphocytes. Moreover, we found that loss of IFN responsiveness, either type I or type II, also led to reduced levels of caspase 1 and 11, suggesting a standard, cytokine-dependent role for STAT1 in this process. The differences between mouse lymphocytes and human fibrosarcomas for regulation of caspase gene expression by STAT1 remain to be determined. Interestingly, the levels of bcl-2-related genes were not altered in the lymphocytes lacking STAT1, although it has been shown that IFN-γ up-regulates bak in an adenocarcinoma cell line (42).

The in vitro proliferation of lymphocytes from wild-type, GKO, and STAT1−/− mice in the presence or absence of exogenous IFN-γ or of anti-IFN-γ Abs confirmed the known role for IFN-γ in regulation of cell growth. However, the differences between disruption of STAT1 and selective modulation of IFN-γ responsiveness revealed that additional mechanisms beyond loss of IFN-γ-mediated antiproliferation contribute to the enhanced proliferation of STAT1−/− cells. GKO lymphocytes unable to secrete IFN-γ grew more rapidly than wild-type cells but still slower than STAT1−/− cells. Moreover, wild-type cells treated with neutralizing Ab against IFN-γ proliferated similarly to GKO cells but still more slowly than STAT1−/− cells. Hyperproliferation of STAT1−/− lymphocytes was not due to an abnormal regulation of IL-2Rα-chain expression required for high affinity receptor expression as seen in STAT5-deficient mice (21, 22) since normal levels of IL-2Rα were expressed in the absence of STAT1 (data not shown). The fact that the hyperproliferation was also observed in other cell types, e.g., fibroblasts and transformed pre-B cells, which do not express IL-2R or IFN-γ, suggests that multiple STAT1-dependent processes are involved.

The faster down-regulation of the p27 CDK inhibitor and higher up-regulation of CDK2 in STAT1−/− cells after stimulation pinpointed altered levels of cell cycle modulators as an underlying mechanism of enhanced proliferation. p27 is highly expressed in T cells and is rapidly eliminated upon mitogenic stimulation, leading to progression of the cell cycle from quiescence (G0) to S phase (17, 18). An enhanced mitogenic activation has also been documented in thymocytes deficient in p27 (46). Thus, the faster kinetics of degradation of p27 protein in STAT1−/− cells may contribute to the promotion of T cell proliferation at early stages of stimulation. Indeed, the CDK2 kinase activity was found to be higher in STAT1−/− lymphocytes than that of wild-type at 24 h of stimulation whereas protein levels of CDK2 were comparable between these two mice (Fig. 4, A and B), suggesting that an earlier release of inhibition of p27 may explain an enhanced proliferation rate at this time (Fig. 3A). CDK2, one of the targets of p27 CDK inhibitor, is necessary for entry into S phase (47). The increased levels of nuclear CDK2 in STAT1−/− cells after 48 h stimulation would greatly facilitate cell cycle progression (Figs. 3 and 4), providing an important driving force for proliferation. Although a direct role of STAT1 in the regulation of CDK inhibitors and CDKs remains to be determined, IFN-α has been shown to suppress IL-2-mediated decline of p27 and up-regulation of the protein levels of several CDKs, including CDK2 in T lymphocytes (48).

Although decreased apoptosis and enhanced proliferation were observed in STAT1−/− lymphocytes in vitro, mutant animals were nonetheless capable of maintaining normal lymphoid homeostasis in vivo. Moreover, no significant increase in spontaneous lymphoproliferative disease occurred in these animals. One likely reason for this difference is that in vitro growth conditions exaggerate the loss of normal growth regulation. For instance, rapid lymphocyte apoptosis occurs in vitro for cells that would survive if left in a lymphoid organ. Presumably, survival signals provided by the organ microenvironment override the induction of apoptosis observed in vitro.

A potential in vivo correlate of altered lymphocyte growth control was observed in chemical carcinogen-treated mice. STAT1−/− mice displayed a higher and more rapid incidence of thymic tumor induction than their wild-type counterparts. A similar observation of increased incidence of fibrosarcomas induced by the carcinogen methylcholanthrene has been reported by Schreiber and colleagues (49). They provided evidence that at least part of the increased susceptibility to methylcholanthrene was due to impaired IFN-γ responsiveness of the induced tumors, suggesting a defect in immune surveillance. However, given the altered growth and survival parameters of STAT1−/− lymphocytes in culture, defects in intrinsic growth control may also have contributed to the observed increased tumor incidence. For instance, it has recently been reported that STAT1-dependent genes are expressed in cultured epithelial cells at confluence and during senescence, again suggesting a general role for this pathway in cellular growth control independent of classical inducers of IFN (50). These results suggest that STAT1 contributes to cellular growth control in both direct and indirect pathways. Impairment of these controls may contribute to immune dysfunction and malignancy.
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References

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