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Specific Inhibition of Stat5a/b Promotes Apoptosis of IL-2-Responsive Primary and Tumor-Derived Lymphoid Cells[1]

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Stat5a/b exhibits 96% homology and are required for normal immune function. The present studies examined Stat5a/b function in lymphoid cells by specific and simultaneous disruption of both proteins using novel phosphorothioate-2′-*O*-methoxyethyl antisense oligodeoxynucleotides (asODN). Efficient delivery was confirmed by the presence of fluorescent TAMRA-labeled ODN in ≥55 and 95% in human primary and tumor cell lines, respectively. Acute asODN administration reduced levels of Stat5a (90%) in 6 h, whereas Stat5b required nearly 48 h to attain the same inhibition, suggesting that the apparent turnover rate for Stat5a was 8-fold higher than that for Stat5b. Expression of the closely related Stat3 protein was unchanged after asODN treatment, however. Molecular ablation of Stat5a/b promoted apoptotic cell death in a significant population of primary PHA-activated T cells (72%) and lymphoid tumor cell line (e.g., YT; 74%) within 24 h, as assessed by 1) visualization of karyolytic nuclear degeneration and other generalized cytoarchitectural alterations, 2) enzymatic detection of TdT-positive DNA degradation, and 3) automated cytometric detection of annexin V translocation. Contrary to findings from Stat5a/b-null mice, cell cycle progression did not appear to be significantly affected. Interestingly, IL-2-insensitive and unprimed T cells and Jurkat cells remained mostly unaffected. Finally, evidence is provided that the cytotoxicity associated with Stat5a/b ablation may derive from activation of caspase-8, an initiator protease that contributes to apoptotic cell commitment. We propose that in lymphoid cells competent to activate Stat5a and Stat5b, both proteins preferentially mediate an antiapoptotic survival influence. ***The Journal of Immunology,*** **2003, 171: 3919–3927.**

T cell activation represents a coordinated cascade of molecular events triggered by engagement of the TCR complex (TCR/CD3) via specific Ags (signal 1). TCR-driven signals are then amplified by the costimulatory molecules B7-1/CD28 and CD40/CD154 (signal 2) which, in turn, promote the synthesis and elaboration of cytokines such as IL-2; cytokine-driven signals are ultimately responsible for driving proliferation (signal 3) (1, 2). IL-2-dependent signals are mediated via cytokine binding to the high affinity IL-2Rα and heterodimerization of two members of the hemopoietin receptor superfamily, designated IL-2Rβ and IL-2Rγ (3–5), which respectively recruit and activate the corresponding receptor-associated Janus tyrosine kinases (Jak)[3] 1 and 3 (4). Jaks catalyze tyrosine phosphorylation of the IL-2Rβ chain, allowing for subsequent recruitment and activation of other signaling element, including a key family of transcription factors known as Stats (2–4, 6). Stats are recruited to the activated receptor, where their SH2 domains dock to select phosphotyrosines (7). Whereas Stats are serine phosphorylated (possibly by mitogen-activated protein kinase family members), Jaks, growth factor receptor tyrosine kinases, and numerous *src* kinases catalyze Stat phosphorylation on a conserved tyrosine residue that facilitates dissociation from the receptor, homodimerization/heterodimerization, and subsequent migration to the nucleus to regulate genes controlling cell growth and differentiation (1, 5, 8)

In lymphocytes, the homologous proteins Stat5a and Stat5b are believed to be critical for cellular activity. Stat5a (originally identified as a mammary gland factor) and Stat5b are activated by multiple cytokines in addition to IL-2, including IL-3, IL-5, IL-7, IL-9, and IL-15; various growth factors; as well as prolactin, growth hormone, and erythropoietin (9). Stat5a-deficient female mice exhibit defective lobuloalveolar development and milk production in response to prolactin (10). Similarly, Stat5b-deficient mice exhibit retarded growth profiles comparable with abnormalities found in Laron dwarfism, a disease deriving from defective growth hormone receptor function (11). However, no defects in immune activity are evident in single Stat5 knockouts, whereas mice deficient in both Stat5 genes exhibit impaired immune function. Indeed, although T cells from these animals remain viable, they are incapable of normal proliferation in response to IL-2 (12). Expression of several putative Stat5-dependent genes may confer this phenotype, given that T cells from these Stat5a/b-null mice possess reduced protein levels of cyclins A, D2, D3, and E and cyclin-dependent kinase 6 (12); taken together, these findings suggest a role for Stat5a/b as cell cycle regulators.

Whereas Stat proteins are unphosphorylated and inactive in quiescent cells, constitutively active Stats such as Stat5 are commonly observed in a number of malignancies including human T cell

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[3] Abbreviations used in this paper: γ_c, common γ chain; Jak, Janus tyrosine kinase; ODN, oligodeoxynucleotide; asODN, antisense ODN; msODN, missense ODN; PI propidium iodide; IETD, acetyl-Ile-Glu-Thr-Asp; FasL, Fas ligand; AFC, 7-amino-4-trifluoromethyl coumarin.

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leukemia virus-1, *Src*, v-ABL, EBV-transformed cell lines, and some patient lymphocytes (13–19). These findings support the notion that Stat5 promotes cytoprotective (or antiapoptotic) gene transcription. For T cell growth factors such as IL-2, proliferation-related Stat5 target genes include c-*myc*, *bcl-x*, *bcl2* (20), and *pim-1* (21). These observations suggest that targeted disruption of Stat5a/b in vivo could be therapeutically relevant for opposition of certain T cell-derived pathologies. It is less clear what response would be obtained after inactivation of Stat5 in functionally mature immune cells, however. Fujii et al. (22) have reported that transgenic mice expressing a variant of IL-2Rβc devoid of Stat5a/b binding sites within an IL-2Rβc-null background possess lymphocytes with impaired proliferative responses to IL-2. Similarly, transfection of pro-B cell and myeloid cell lines (23) with C-terminal deletion variants of Stat5 devoid of *trans* activation potential are viable but remain unresponsive to cytokine-driven cell proliferation and cell cycle gene expression (24, 25). Whether these putative dominant-negative effects are due to disruption of Stat5a/b-responsive gene transcription via N-terminal domain scavenging of other cofactors such as CREB-binding protein (26) and *N*-Myc interactor (27), or tether corepressors such as silencing mediator for retinoic acid receptor and thyroid hormone receptor (28) to the promoter of the target gene, remains less certain.

Stats also control the expression and activity of their own negative regulators, including calpain (a calcium-dependent cysteine protease), generalized protein phosphatases, the protein inhibitor of activated Stats, and the cytokine-induced SH2-containing protein, otherwise referred to as suppressor of cytokine signaling (29–35). Thus, inactivation of Stats may also be expected to uncouple their negative regulatory influence over cell activity. As a therapeutic strategy to manage T cell-derived disease, it is presently unknown whether disruption of Stat5a/b in mature lymphocytes will yield an activated and cytokine-unresponsive T cell or some other dysfunctional phenotype. To address these questions, we have specifically and selectively uncoupled Stat5a/b expression in normal and malignant lymphoid cells and assessed the consequent effects on cellular activity.

Materials and Methods

Cell culture and treatment

YT cells and human PBLs were grown in RPMI 1640 supplemented with 10% FCS (Life Technologies, Gaithersburg, MD), 2 mM L-glutamine, and penicillin-streptomycin (50 IU/ml and 50 mg/ml, respectively). Freshly explanted normal human T lymphocytes purified by isocentrifugation (Ficoll; EM Science, Gibbstown, NJ) were PHA activated for 72 h (1 mg/ml) as previously described (1). Electroporation was conducted 48 h after PHA activation of the primary human T lymphocytes. After electroporation of YT and human T lymphocytes, cells were resuspended in fresh medium. For electroporation, YT and human PBLs were pelleted and resuspended in Opti-Mem I (Life Technologies) at 40×10^6 cells in a 400-μl final volume containing the desired concentrations of oligodeoxynucleotides (ODNs). The mixture of cells and ODNs was then pulsed using Bio-Rad Gene Pulser II (Bio-Rad, Hercules, CA) in 2-mm gap cuvets (BTX model 620) at 220 V and 960 μF capacitance.

Antisense ODN (asODN) treatment

Stat5a/b antisense was synthesized using a phosphorothioate backbone with 2′-*O*-methoxyethyl modification of the five terminal nucleotides (underlined) to increase their stability (ISIS 130826) 5′-GGG CCT GGT CCA TGT ACG TG-3′. Missense ODN (ms-ODN) of the same chemistry was synthesized as a mixture of all four bases resulting in a preparation that contains an equimolar mixture of all possible ODNs by ISIS Pharmaceuticals (Carlsbad, CA). These Stat5a/b antisense ODNs are derived from a shared sequence within both human Stat5a and Stat5b transcripts (bp 2210–2230).

Solubilization of membrane proteins, and Western blot analysis

Frozen cell pellets were thawed on ice and solubilized in lysis buffer (1×10^8 cells/ml) as previously described (1). Supernatants were then rotated end over end for 2 h at 4°C with either 5 μl/ml polyclonal rabbit antisera raised against peptides derived from the C termini of human Stat5a (aa 775–794) or Stat5b (aa 777–787), whereas Stat3 rabbit polyclonal Abs were generated against the C termini of human Stat3 (aa 747–762). Proteins bound to Abs were captured by incubation for 30 min with protein A-Sepharose beads (Pharmacia, Piscataway, NJ), sedimented for purification, and eluted by boiling in 2× SDS-sample buffer (20% glycerol, 10% 2-ME, 4–6% SDS, 0. 004% bromphenol blue in 0.125 M Tris, pH 6.8) for 4 min and separated on 7.5% SDS-PAGE under reducing conditions. Proteins were transferred to polyvinylidene difluoride (Immobilon; Millipore, Bedford, MA) as previously described (1). Western blot analysis was performed with Stat5a (1/1000), Stat5b (1/1000), or Stat3 (1/2000) diluted in blocking buffer and used as previously described (1). For identification of caspase activation, equal protein lysates were inactivated in 2× SDS sample buffer and separated on 11% SDS-PAGE under reducing conditions. Proteins were transferred to a polyvinylidene difluoride membrane, and Western blot analysis was performed using anti-caspase-8 (cell signaling) or anti-actin Ab (Santa Cruz Biotechnology, Santa Cruz, CA).

Assessment of oligonucletide uptake

To assess asODN uptake, YT cells were transfected by electroporation with Stat5a/b asODN sequence tagged with TAMRA (Sigma-Genosys, The Woodlands, TX) at their 3′ end or nonlabeled antisense Stat5a/b ODNs. Immediately after electroporation, cells were washed and resuspended in PBS. An aliquot of cells were placed on a glass slide and observed by fluorescent microscopy. TAMRA (570 nm) and phase contrast images were prepared for each specimen.

Cell survival studies

Cell survival was performed by trypan dye exclusion and annexin V-fluorescein and propidium iodide (PI) staining (Boehringer-Mannheim, Mannheim, Germany).

Trypan dye exclusion. An aliquot of human lymphocytes or YT cells was diluted in trypan blue (0.4% solution in PBS; Mediatech, Washington, DC) and visualized using a hemocytometer. Dead cells, appearing blue, were excluded from the measurement; live cells were counted in four quadrants and averaged.

Annexin V-fluorescein and PI staining. Cells were washed (1.0×10^6) with PBS and centrifuged at $200 \times g$ for 5 min. Cell pellets were resuspended in 100 μl of staining solution (for 10 assays, 20 μl of annexin V-fluorescein, and 20 μl of PI in 1000 μl of HEPES buffer) and incubated for 10–15 min at room temperature. Stained cells were then analyzed on a flow cytometer using 488 nm excitation and 515 nm bandpass filter for fluorescein detection and a filter >600 nm for PI detection.

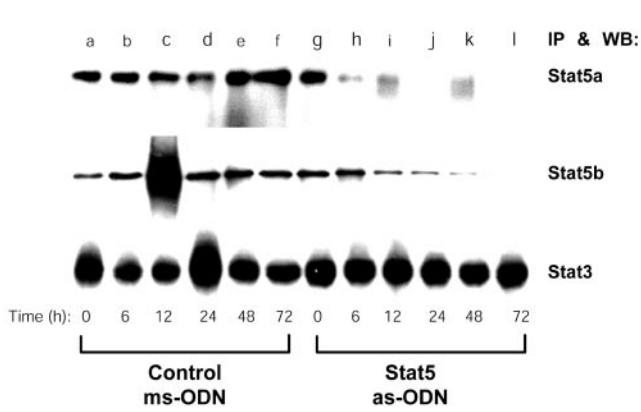

FIGURE 1. Kinetic analysis of Stat5a/b antisense ODN treatment and Stat5a/b protein expression. YT cells were electroporated with 7.5 μM missense ODN (msODN; *lanes a–f*) or Stat5 ODN (*lanes g–l*), and each set was separated into six flasks and cultured within a CO_2 incubator as described in *Materials and Methods*. At various time points (h), cells were harvested (0–72 h), lysed, and immunoprecipitated (IP) with either Stat5a (*upper panel*), Stat5b (*middle panel*), or Stat3 (*lower panel*) Abs and Western blotted (WB) with the corresponding Abs indicated on the right and left. *Middle panel* (*lane c*), Nonreproducible technical error.

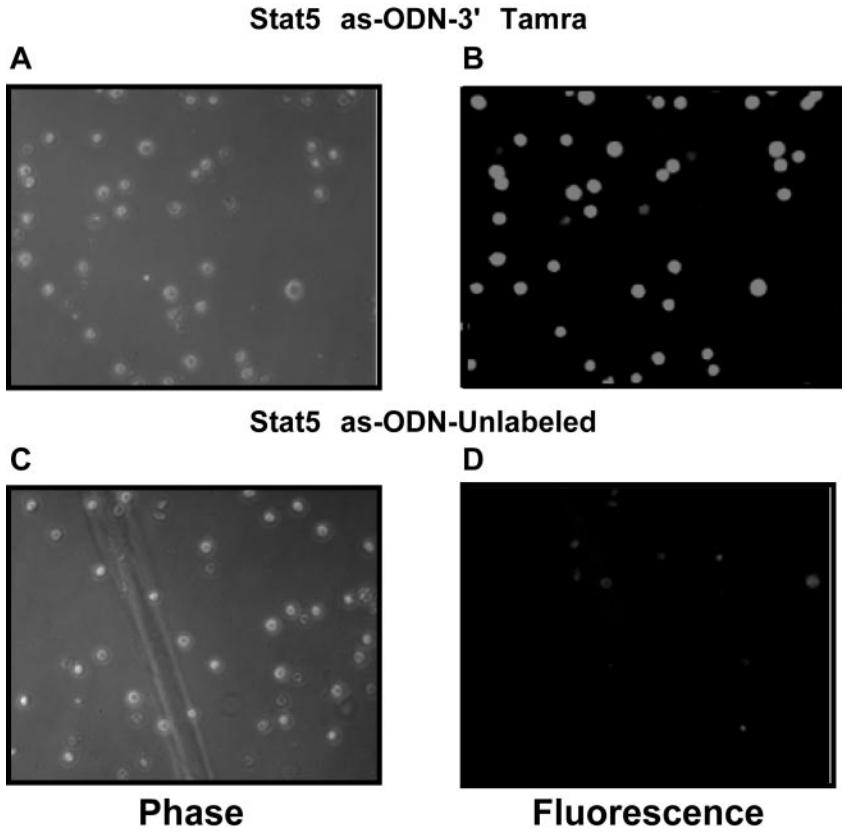

FIGURE 2. Stat5a/b antisense is efficiently delivered into YT cells. YT cells were electroporated with the 3′-TAMRA-labeled Stat5a/b ODN (7.5 μM in 400 μl of Opti-Mem). Soon after electroporation, cells were washed twice and resuspended in PBS. An aliquot of cells was placed on a coverslip and visualized using a fluorescent microscope (emission wavelength, 570 nm; excitation wavelength, 543 nm). *A,* Phase contrast image of YT cells electroporated with the 3′-TAMRA-labeled Stat5a/b antisense and corresponding fluorescent microscope image (*B*). *C,* Phase contrast image of non-TAMRA-labeled Stat5/b antisense electroporated YT cells and background fluorescence (*D*).

Assessment of apoptotic cell death

For TUNEL assays, pelleted cells were resuspended in PBS and fixed in cytocentrifuge preparations according to a slight modification of established procedures (36). For visualization of apoptotic DNA damage, fixed cells were manually 1) treated with ethanol-acetic acid (2:1, v/v) at 20°C for 5 min, 2) stained for broken DNA by treatment with TdT in the presence of FITC-dUTP label (Molecular Probes, Eugene, OR) at 37°C for 60 min, and 3) counterstained for intact DNA with 0.01% PI in sodium citrate at 20°C for 10 min. At least five 50-cell fields were scored for each treatment by fluorescent microscopy at ×40 or ×60 magnification by assessing increased direct fluorescence of end-labeled dsDNA. Fluorescence photomicrographs were obtained by digital image capture. Caspase-8 activity was measured by cleavage of the fluorescent substrate acetyl-Ile-Glu-Thr-Asp (IETD)-7-amino-4-trifluoromethyl coumarin (AFC) (BD Biosciences, San Jose, CA). Briefly, YT cells were electroporated with vehicle, 7.5 μM Stat5a/b asODN, or missense ODN (msODN) as described in all figure legends (1–7). Cells were then harvested after 24 h, lysed, and clarified as described above. Next, cell lysates (20 μg) were spectrofluorometrically assayed for caspase-8 activity according to the manufacturer's recommendation ($n = 3$).

Cell cycle analysis

Serum-starved cells (1×10^6) were washed in PBS and fixed in 70% ethanol overnight. Fixed cells were then washed from the fixative twice and resuspended in PI working solution (50 μg of PI, 20 μg/ml RNase, and 0.5% Tween 20 in PBS). After a 15-min incubation, cells were analyzed on a BD Biosciences FACScan flow cytometer, and the percentage of cells in each phase is calculated using ModFit software (BD Biosciences).

Results

Kinetic profile of specific Stat5a/b, but not Stat3, protein expression

Given the central role of Stat5 in various lymphoid diseases, efforts to disrupt Stat5a/b expression were investigated. Using random genewalk methodology (37), several putative Stat5a/b-specific 2′-*O*-methoxyethyl-modified phosphorothioate asODNs were designed and screened in the YT human lymphoid-derived tumor cell line. Of these, one was found to display substantial selectivity and specificity. This asODN shares extended sequence specificity within both human Stat5a and Stat5b transcripts mapping to bp domains 2210–2230. A dose curve showed that both Stat5a and Stat5b were inhibited after 48 h of treatment at concentrations of 5.0–7.5 μM antisense, but not the missense control (data not shown). To determine the turnover rate of Stat5a/b expression after antisense ablation, YT cells were electroporated with either 7.5 μM msODN control or asODN. After the treatment described above, cells were harvested at various intervals, and Stat5 protein levels were evaluated by Western analysis. As shown in Fig. 1, Stat5a showed greater sensitivity to asODN treatment, as this protein was essentially undetectable after 6 h (Fig. 1, *lane h*). In contrast, Stat5b did not show significant inhibition until ~24 h (Fig. 1, *lane j*) in the same treatment group; moreover, Stat5b expression was still weakly detectable after 72 h (Fig. 1, *middle panel, lane l*) as compared with Stat5a, which was completely inhibited within only 12 h (Fig. 1, *upper panel, lane i*). Treatment with msODN failed to interfere with the expression of either protein (Fig. 1, *lanes a–f*). Moreover, as an additional internal control, the homologous Stat3 protein (Fig. 1, *lower panel; lanes a–l*) isolated from the same cell lysates failed to show sensitivity to either treatment. Antisense Stat5a/b also failed to modify significantly Stat1, -2, or -4 protein levels, whereas Stat6 was not expressed in appreciable amounts in YT cells (data not shown). These results demonstrated that the Stat5a/b asODN was effective for selectively interfering with Stat5a/b protein expression and that Stat5a underwent turnover with more rapid kinetics than did Stat5b.

Efficiency of Stat5a/b antisense introduction into YT cells

To determine whether the observed differences in Stat5a/b expression after antisense treatment resulted from differences in transfection efficiency, delivery of Stat5 asODN labeled with a red fluorophore

(3′-TAMRA) was examined. TAMRA-labeled Stat5 antisense was delivered as described above and visualized by conventional fluorescence microscopy. As presented in Fig. 2*B*, ~100% of treated cells took up labeled ODN as compared with the phase image (Fig. 2*A*); results in Fig. 2*D* exhibited limited background fluorescence in cells expressing the unlabeled ODN-treated cells. Moreover, these images demonstrated that cell viability was maintained after these treatments. Accordingly, it was concluded from these studies that effective inhibition of Stat5a/b protein expression observed in Fig. 2 was the direct result of highly efficient introduction of asODN into intact cells.

Reduction of cell survival by inhibition of Stat5a/b expression in YT cell cultures and primary lymphocytes

Having established that Stat5a/b asODN (7.5 μM) effectively inhibited Stat5a/b protein expression, we next sought to determine the effects of Stat5 ablation on cell survival. For these studies, Stat5a/b-directed asODNs or control msODNs were introduced into YT cells and PHA-activated primary human blood lymphocytes as before. Cell viability was assessed by vital dye-exclusion at 72 h. As shown in Fig. 3*A*, concurrent inhibition of Stat5a and Stat5b expression resulted in a significant reduction in cell viability (90 ± 4%; $p < 0.05$) as compared with msODN at the same concentration. The effects of inhibiting Stat5a/b within PHA-activated primary human blood lymphocytes also showed a significant reduction in cell viability (73 ± 2%; $p < 0.05$) as compared with msODN treatment (Fig. 3*B*). These experiments indicated that Stat5a/b mediates an essential regulatory influence in the survival of IL-2-responsive lymphoid cells.

To characterize directly the cytotoxic consequences of acute Stat5 ablation, YT cells were treated with either Stat5a/b asODN or control msODN constructs during 0–72 h; apoptotic cell death was then assessed along multiple indices. Enzymatic detection of apoptotic DNA degradation after ODN treatment was visualized by fluorescence microscopy in YT cell preparations stained with FITC-dUTP in the presence of TdT (Fig. 4). Substantial apoptotic DNA degradation was evident after 24 h of treatment with Stat5a/b asODN (Fig. 4*C*) as compared with electroporated or control ODN (Fig. 4, *A* and *B*, respectively). The results from three separately scored fields per treatment set are plotted in Fig. 4*D*.

Similarly, automated cytometric detection of annexin V translocation confirmed the apoptotic fate of cells treated with Stat5a/b. Assessment of both cell types by automated cytometric analysis of annexin V-fluorescein and PI provided concordant results. In YT cells and PHA-activated human T cells electroporated in the presence of Stat5a/b asODN (7.5 μM) or control msODN (7.5 μM), Stat5a/b ablation promoted apoptotic cell death; responses observed in non-PHA-activated T cells and Jurkat cells were much less pronounced. By flow cytometric analysis, each quadrant represents the relative proportion of necrotic, necrotic and apoptotic, apoptotic, and healthy cells for quadrants 1, 2, 4, and 3, respectively. As shown in Fig. 5*A*, IL-2-responsive YT cells (Fig. 5, *upper panels*) treated with Stat5a/b asODN showed a significant increase in cell death (Fig. 5, *quadrants 1, 2,* and *4*) as compared with control cells (74% increase in apoptotic cell death vs control). Moreover, PHA-activated primary human blood lymphocytes also showed a significant increase (by 61%) in total cell death. In contrast, nonactivated T cells, which are unresponsive to IL-2 (Fig. 5*B*) were only minimally affected. Stat5a/b antisense was similarly taken up by both primary cultures (~55%; data not shown). The IL-2-insensitive human T cell line, Jurkat, mimicked unactivated T cells and exhibited no significant changes in cell survival (Fig. 5*A*) after the treatment, suggesting that Stat5a/b plays an important role in the survival of cells competent to activate Stat5 by cytokines

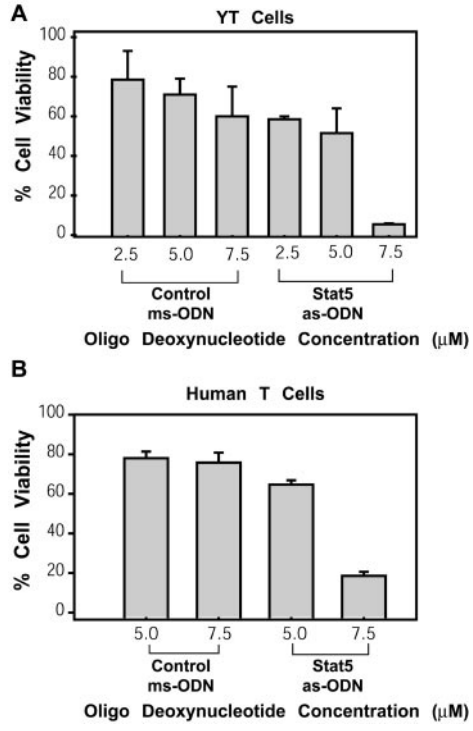

FIGURE 3. Stat5a/b-depleted YT cells and primary human lymphocytes show a significant increase in cell death. YT cells were electroporated with 2.5–7.5 μM Stat5 asODN (*A*), whereas PHA-activated human peripheral lymphocytes (*B*) were electroporated with 5.0–7.5 μM antisense Stat5a/b ODN or missense ODN as described previously. Cell survival was assessed at 72 h by trypan blue dye exclusion. Percent survival in antisense Stat5a/b- or msODN-treated group normalized to electroporated control YT cells void of ODN. Data are from three separate treatment sets.

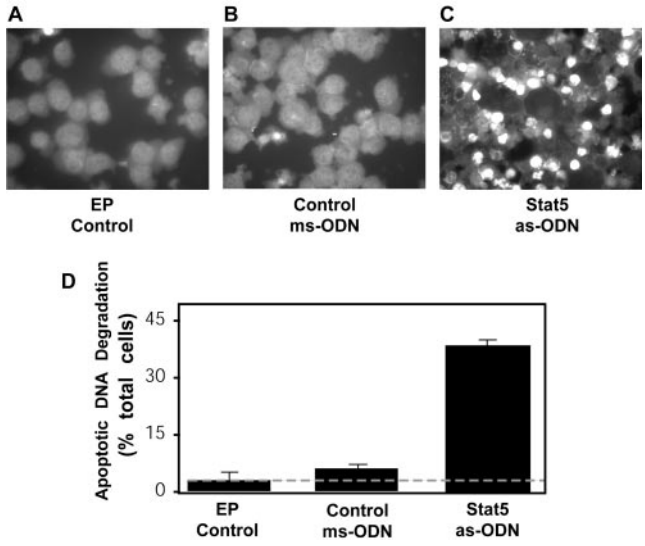

FIGURE 4. Induction of apoptotic cell death by Stat5a/b ablation. YT cells were electroporated (EP) in the absence (control) or presence of a given antisense (msODN, asODN; 7.5 μM), and maintained for 24 h. Fixed cells were then stained with FITC-dUTP in the presence of TdT and apoptotic DNA breakage visualized digitally under fluorescence microscopy as described in *Materials and Methods*. Representative photomicrographs are shown: *A,* electroporated control; *B,* msODN control; *C,* Stat5a/b asODN; *D,* relative occurrence of specific fluorescent labeling. Values reflect mean ± SEM of three scored fields per treatment and are expressed as a percentage of the total treated cell population.

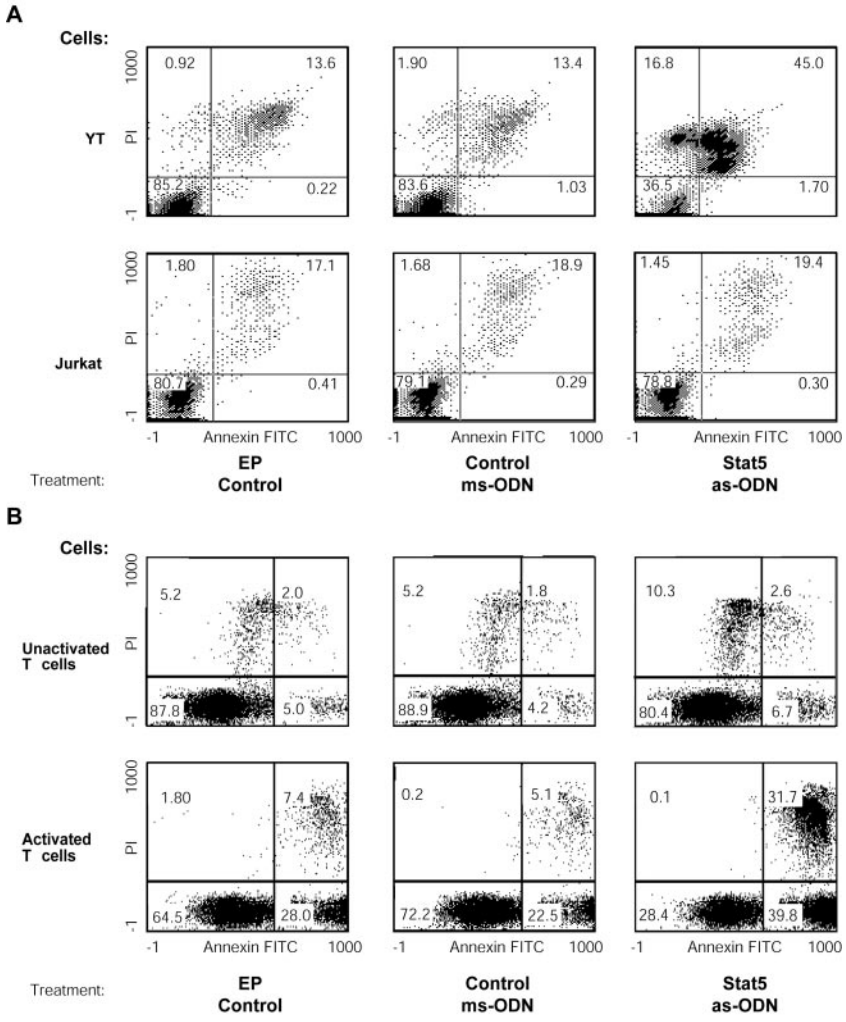

FIGURE 5. FACS of Stat5a/b-depleted YT, primary human T cells, and Jurkat T cells stained with annexin V-fluorescein and PI. *A,* YT or Jurkat cells (*A*) or nonactivated or PHA-activated human T cells (*B*) were electroporated (EP) in the absence (*left*), presence of 7. 5 μM Stat5 asODN (*right*), or 7.5 μM missense ODN (*middle*) as described above. At 24 h postelectroporation, cells were washed in PBS and stained with annexin V-fluorescein and PI. Cells were then analyzed by FACS. Quadrant 1, PI-positive cells showing percent of necrotic cells; quadrant 2, PI- and annexin-positive cells showing percent of apoptotic and necrotic cells; quadrant 3, annexin-positive cells showing percent of apoptotic cells; quadrant 4, annexin- and PI-negative cells showing percent of of live cells.

such as IL-2 or IL-15 for YT cells or T cell growth factors (e.g., IL-2, IL-7, IL-9, IL-15) for activated lymphocytes.

Loss of Stat5a/b expression is unrelated to changes in cell cycle progression

Given the finding that ablation of Stat5a/b expression resulted in cell death, we next examined whether Stat5a/b deficiency promotes altered cell cycle progression (i.e., as reported for Stat5a/b$^{-/-}$ mice which accumulate in or fail to progress through cell cycle) (12). For this purpose, serum-starved, synchronized YT and primary T lymphocytes were treated as described above with 2.5, 5.0, or 7.5 μM Stat5a/b asODN or control msODN. Cells were subjected to FACS and cell cycle progression after 48 h. Neither YT cells (Fig. 6*A*) nor PHA-activated primary T cells (*panel B*) showed skewed accumulation into G_1, S, or G_2 phases of the cell cycle. It was concluded from these findings that Stat5a/b does not play a significant role in cell cycle progression relative to its apparent role in the maintenance of cell survival.

Inactivation of Stat5a/b promotes inactivation of procaspase-8

Given the dramatic induction of apoptotic cell death after molecular ablation of Stat5a/b, we next examined possible mechanisms responsible for apoptotic commitment in Stat5a/b-depleted cells. Previous results have shown that activated T lymphocytes switch from a mitochondrially driven (i.e., intrinsic) to a receptor-dependent (i.e., extrinsic) pathway for apoptotic commitment (38). Apoptosis initiated by Fas ligand (FasL)-FasR requires activation and cleavage of the cysteine initiator protease capspase-8. Active caspase-8 drives activation of the "executioner" enzyme caspase-3 to engage the ultimate commitment to apoptotic cell death (39).

To investigate this event further, YT cells treated with Stat5 asODN or control msODN were assessed for induction of caspase activity, as presented in Fig. 7. Procaspase-8 intermediate products (both 43 and 41 kDa) underwent cleavage within 6 h after Stat5a/b asODN (Fig. 7, *upper panel*). As a procedural control, expression of F-actin (Fig. 7, *lower panel*) verified equivalent protein loading. Next, lysates from Stat5a/b-depleted YT cells were assayed for their ability to cleave the caspase-8 fluorogenic substrate (Ac-IETD-AFC) (Fig. 7*B*). Indeed, caspase-8 activity was increased nearly 3-fold within 24 h as compared with control cells. It was concluded from these observations that depletion of Stat5a/b protein results in coordinate activation of caspase-8, suggesting the involvement of extrinsic mechanisms for apoptotic commitment.

Discussion

The present study demonstrates that the transcription factors Stat5a and Stat5b can act as survival proteins in cells of lymphoid origin. IL-2-responsive PHA-activated human T cells and YT NK cells were susceptible to apoptosis after Stat5a/b depletion as compared with non-IL-2-inducible unprimed human T cells and Jurkat cells. Efficient delivery of Stat5a/b asODN was verified by fluorescent ODN labeling. Selective depletion of Stat5a and Stat5b failed to affect expression of other Stat proteins, confirming the specificity of these molecular manipulations. The turnover rate of Stat5b was

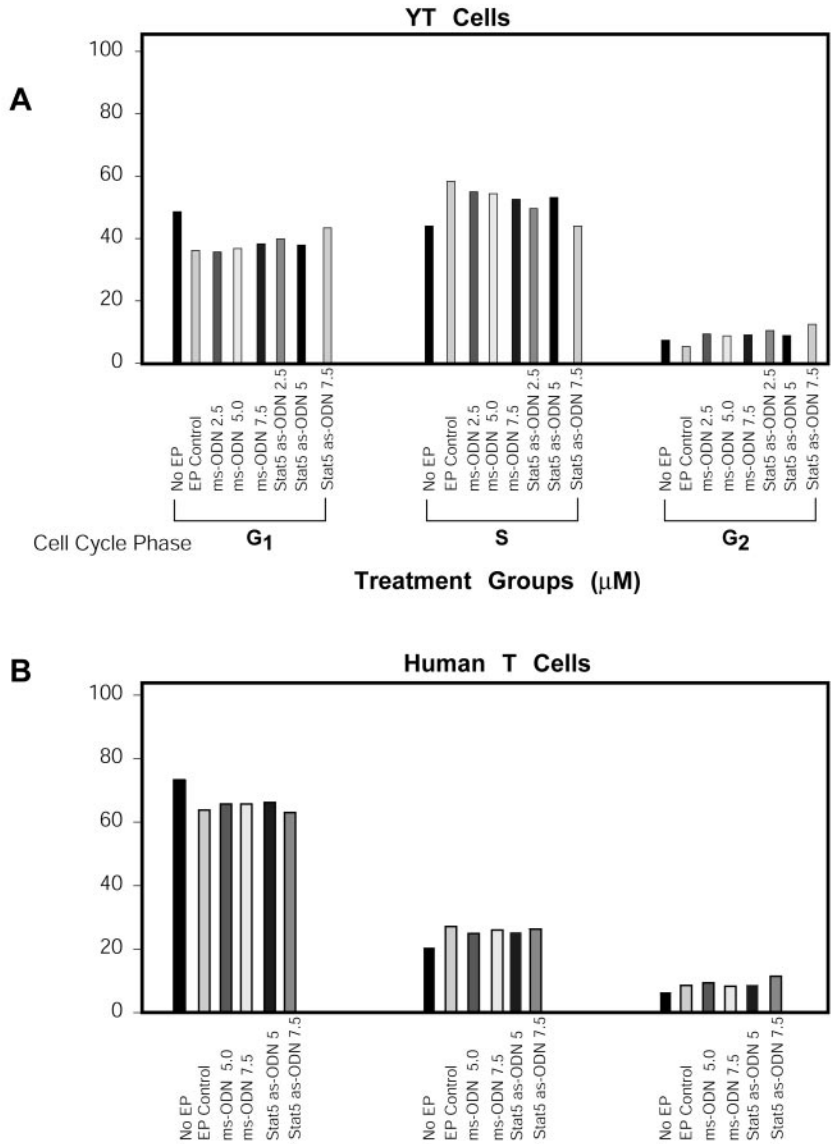

FIGURE 6. Depletion of Stat5a/b does not alter cell cycle progression. YT or human primary lymphocytes were treated with antisense Stat5a/b (2.5–7.5 μM), msODNs (2.5–7.5 μM), no ODNs (EP), or nonelectroporated (No EP). At 72 h postelectroporation, cells were fixed in 70% cold ethanol overnight, stained with PI working solution (50 μg/ml PI, 20 μg/ml RNase, 0.5% Tween 20 in PBS), and analyzed for cell cycle progression by flow cytometry. *Y axis,* percent of cells in each cell cycle, G_1, S, or G_2-M. *A,* YT cells; *B,* human primary lymphocytes.

8-fold slower than that of Stat5a. Nonetheless, the majority of asODN-treated cells ultimately succumbed to apoptosis. The present findings suggest that Stat5a/b plays a definitive role maintaining cell survival, especially in cells responsive to cytokines competent to activate Stat5a/b (e.g., IL-2, IL-7, IL-9). In contrast, these studies fail to substantiate a role for Stat5 in cell cycle progression, which appeared to remain largely unchanged. Lastly, an increase in caspase-8 activity suggests that Stat5 proteins may act as negative regulators of these signaling pathways.

The Stat5a/b proteins are believed to play a key role in various lymphoid diseases, including lymphoid leukemias and lymphomas, and are additionally thought to participate in the regulation of normal immune function (40). Within functional lymphocytes, Stat5a and Stat5b appear to serve a compensatory role in their biological activities. Indeed, single Stat5a- or Stat5b-null mice do not display defects in T cell activity or immune function as compared with dually deficient animals (12). In contrast, it is less clear whether such defects in immune activity are indirectly the result of developmentally related processes. Because our results indicate that Stat5a/b depletion yields apoptosis with cell cycle changes not discernible in either PHA-activated human T cells or YT cells, the present findings support the notion that Stat5a/b$^{-/-}$ murine T cells may recruit an alternative survival pathway to ensure survival of lymphocyte progenitors. These effects may confer a selective advantage over committed lymphoid cells responsive to IL-2 and other growth factors.

Although differences in cell cycle were not prominent (Fig. 6), depletion of Stat5a/b clearly promotes apoptotic cell death. Thus, whereas some observations support the notion that Stat5 proteins may up-regulate apoptosis-related genes such as *Bcl*-x_L (20). However, we failed to detect changes in this protein after Stat5a/b depletion when assayed at various time points over 48 h (data not shown). We therefore investigated other potential mechanisms. Specifically, we assessed the activation of caspase-8 (the primary initiator of FasL-FasR-mediated apoptosis) and caspase-9 (an essential initiator caspase associated with intrinsic, mitochondrially driven cell death pathways) (39, 41–43). Previous results have shown that activation of T lymphocytes produces a switch from a

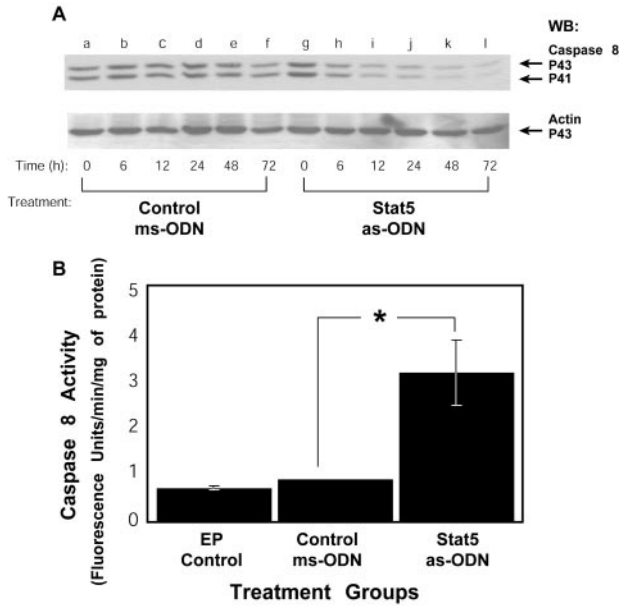

FIGURE 7. Treatment of YT cells with antisense Stat5a/b results in the activation of caspase-8. *A,* YT cells were treated with either 7.5 μM Stat5a/b asODN or msODN as described previously. Cells were then harvested and lysed at the indicated times after electroporation of 0, 6, 12, 24, 48, or 72 h. Western blots (WB) of cell lysate (20 μg) was performed using anti-caspase-8 Abs. Anti-actin Western blot was performed to demonstrate equal protein loading. *B,* Caspase-8 activity was measured as the zero order rate generation of caspase-8 substrate fluorescence of IETD-AFC increase normalized to equivalent protein (30 μg) for each treatment set. Values are means $\pm$ SE; $n = 3$. $*$, $p < 0.05$ of Stat5a/b asODN compared with control msODN treatment set as indicated.

classical apoptotic (mitochondrion-dependent) pathway to a FasL-FasR-dependent mode of apoptosis (38). Apoptosis initiated by the FasL-FasR interaction requires activation and breakdown of procaspase-8 (39). Although the present studies demonstrate activation of caspase-8 (Fig. 7), we could only detect depletion of procaspase-9 forms via Western blot, whereas enzymatic activity could not be substantiated via the fluorogenic substrate assay (data not shown).

Whether Stat5a/b signals control expression of a negative regulator of this pathway remains to be determined. One possibility is that Stat5 proteins have a role in protection from apoptosis by inhibiting activation-induced cell death, a process involving up-regulation of death-inducing receptors such as FasR and/or the type 2 receptor for TNF in response to activation by a diverse spectrum of lethal stimuli including IL-2 (44). Although the Stat5a/b activator IL-2 can play a significant role in the promotion of cell growth, it is nonetheless significant that this cytokine sensitizes these cells to apoptotic stimuli after acute up-regulation of FasR expression, ultimately resulting in suppression of the inhibitor of apoptosis FLIP (44, 45).

It is plausible that Stat5a/b heterodimers or homodimers bind to the promoter regions of survival genes such as *Bcl-2* and BcL-XL, thus initiating transcription of these survival factors (45–48), however neither protein was found to be significantly affected following Stat5a/b depletion. Stat5a/b signaling elements may also cooperate with other signaling pathways to modulate cell survival. Additional possibilities, such as mitogen-activated protein kinase and phosphoinositide 3-kinase, which generates multiple secondary messengers competent to activate protein kinase B-Akt, may also promote transcription of IκB kinase-α. IκB kinase mediates the phosphorylation and degradation of IκB, consequently gating intermolecular release and nuclear translocation of NF-κB (49–51) and resulting in the transcription of prosurvival genes such as c-*myb* (39). NF-κB has also been implicated in protection from apoptosis in response to IL-2 in several malignant cell lines and in primary human T cells (50, 51). Whether phosphoinositide 3-kinase or NF-κB are under direct transcriptional control of Stat5a/b has not been established. Nonetheless, pharmacological interruption of this signaling sequence with rapamycin (to antagonize the 256-kDa serine/threonine kinase mammalian target of rapamycin) results in T cell clonal anergy (52–55).

Although the molecular events regulating T cell survival are less clear, these findings suggest that Stat5a/b-driven signals are essential for T cell survival. Although all Stats have been shown to modulate immune activity, other cellular lymphoid pathologies are known to involve compromised Stat5 activity and immune function. Earlier results from our group have shown that T cells isolated from immunocompromised mammary adenocarcinoma-bearing mice and HIV-infected patients lacked detectable levels of both Stat5a and Stat5b proteins, whereas other Stats were expressed in normal steady state levels (56, 57). Indeed, one of the hallmarks of active HIV infection is progressive loss of $CD4^+$ T cells through apoptosis (58). It is therefore tempting to speculate that spontaneous apoptosis in HIV-infected T cells is directly related to changes in Stat5 expression. Along this line, a recent report by Selliah and Finkel (59) suggests that certain strains of HIV-1 (including NL4–3) inhibit expression of Stat5, whereas the HIV IIIB strain antagonizes Stat5 activation via Jak3. Collectively, these findings suggest that T cell apoptosis and dysfunction may be a path for viral cytopathy. It is also known that primary activated T cells are dependent on γ_c cytokines (e.g., IL-2, IL-7, and IL-9), all of which activate Stat5; consistent with this position, the depletion of γ_c cytokines in T cell clones has been shown to induce T cell anergy (23, 60, 61). Additional evidence suggests that blockade of γ_c with mAbs results in T cell apoptosis by inhibiting expression of *Bcl-2* (62). We have also reported that inhibition of Stat5a/b activity by selective Jak3 inhibitors disrupts Stat5a/b activation and can significantly affect T cell viability within 48–72 h (1, 63) with kinetics corresponding to Stat5a/b depletion. Thus, although this work has focused on the role of Stat5a/b in IL-2-responsive cells, lymphoid progenitors dependent on cytokines competent to activate Stat5a/b might also be susceptible to cell death after Stat5a/b ablation. Lastly, new evidence suggests that cytokines that inhibit T cell proliferation driven by T cell growth factors, such as IFN-α, may do so via acute inhibition of Stat5 activity (64).

In conclusion, the present findings demonstrate that the status of Stat5a/b expression directly influences survival of IL-2-responsive lymphoid cells. This work suggests that targeted inhibition of Stat5a/b expression and/or activity in hyperactive T cell-dependent diseases (e.g., lymphoma, allergy, or alloreactive T cells noted within experimental transplantation settings) may hold therapeutic potential. In addition, these observations suggest that correction of Stat5a/b expression in immunologically debilitating diseases, such as infection by HIV and other viruses that uncouple Stat5a/b signaling, may also be clinically relevant. Certainly, extensive work will be required to validate these hypotheses.

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