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Interleukin-4-mediated Protection of Primary B Cells from Apoptosis through Stat6-dependent Up-regulation of Bcl-xL*

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Apoptosis is an integral aspect of B lymphocyte development and homeostasis and is regulated by the engagement of antigen costimulatory and cytokine receptors. Although it is well established that interleukin 4 (IL-4) is a potent anti-apoptotic cytokine for B lymphocytes, little is known about the IL-4-induced molecular events regulating cell survival. Stat6 is rapidly activated after IL-4 stimulation, but its role in B lymphocyte apoptosis has not been explored. In this report we demonstrate that Stat6 is a critical signaling molecule for IL-4 in protecting primary B cells from passive and Fas-induced cell death. We show that expression of the Bcl-2 family member, Bcl-xL, is induced maximally by IL-4 and anti-IgM/IL-4 in a Stat6-dependent manner. Additionally, we demonstrate that *bcl-xL* transcription is likely to be directly activated through a Stat6 binding site in the *bcl-xL*-flanking region. Finally, reconstitution of Stat6-deficient splenic B cells with Bcl-xL was able to protect those cells from Fas-induced cell death. These results suggest that the anti-apoptotic activity of IL-4 in B cells is mediated through the activation of Stat6 and subsequent transcription of Bcl-xL.

Both resting and activated mature B lymphocytes require cytokine or antigenic survival signals for viability and die passively by neglect in their absence (for review, see Ref. 1). Primed B lymphocytes can also be actively programmed to die through engagement of cell surface Fas by encountering Fas ligand (FasL) on activated T cells. These highly regulated processes are thought to be important for removing autoreactive B cells, terminating an immune response, and maintaining a state of equilibrium.

The activation or suppression of apoptosis is regulated in part by the members of the Bcl-2 family, proteins found in various cytoplasmic membranes including mitochondrial membranes (for review, see Refs. 2 and 3). Several members, including Bcl-2 and Bcl-xL, are antagonistic to apoptotic stimuli and are thought to function by preserving mitochondrial membrane integrity and preventing cytochrome *c* release into the cytoplasm. Other Bcl-2 family members, such as Bax and Bad, promote apoptosis. These pro-apoptotic proteins are thought to antagonize the function of Bcl-2 and Bcl-xL through heterodimer formation. Consequently, the relative levels of intracellular pro- *versus* anti-apoptotic Bcl-2 family proteins are critical in determining the viability of a cell. Clearly it is of interest to understand how the expression of these proteins is regulated in response to external stimuli.

The cytokine IL-4[1] has been demonstrated to be a potent cofactor for B and T lymphocyte proliferation and differentiation (for review, see Ref. 4). Additionally, its role as an anti-apoptotic factor has been studied extensively. IL-4 has been demonstrated to prevent cell death by neglect of resting T and B lymphocytes after growth factor withdrawal in culture (5, 6). It can also prevent apoptosis of B cells induced by Ig cross-linking and glucocorticoids and render activated B cells insensitive to Fas ligation (7–9). However, the molecular events induced by IL-4 that regulate cell survival are poorly understood.

Signal transducer and activator of transcription 6 (Stat6) is a critical mediator of IL-4 signaling (for review, see Ref. 10). Stat6 is a latent cytoplasmic transcription factor recruited specifically to the IL-4 receptor and activated by phosphorylation after IL-4 stimulation. Activated Stat6 homodimers are capable of translocating to the nucleus where they can influence the transcription of IL-4-responsive genes. The importance of Stat6 in IL-4 signal transduction has been demonstrated in Stat6-deficient lymphocytes, which are unable to proliferate normally in response to IL-4, are defective in their ability to activate IL-4 responsive genes, and are unable to undergo IL-4-dependent Th2 differentiation (11–13). However, Stat6 does not appear to be required for the anti-apoptotic effects of IL-4 in resting or activated T cells or in myeloid cell lines (5, 14–16). This raises the question of whether Stat6 is a required intermediary for the anti-apoptotic effect of IL-4 on B cells.

In this study we demonstrate that in contrast to T lymphocytes, Stat6 signaling is involved in the anti-apoptotic activities of IL-4 in primary B cells that were committed to die by either growth factor withdrawal or by Fas ligation. We find that Stat6-deficient B cells are defective in their ability to maximally induce expression of the anti-apoptotic Bcl-2 family member, Bcl-xL, in response to IL-4 stimulation. Additionally, we have identified a Stat6-responsive element upstream of the *bcl-xL*, gene suggesting that Stat6 is directly responsible for activating the IL-4-induced transcription of this anti-apoptotic factor. Finally, reconstitution of Bcl-xL expression in Stat6-

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[1] The abbreviations used are: IL-4, interleukin 4; Stat6, signal transducer and activator of transcription 6; IRS-2, insulin receptor substrate 2; GFP, green fluorescent protein; GAS, γ-activating sequence.

deficient primary B cells renders the cells resistant to Fas-induced cell death.

EXPERIMENTAL PROCEDURES

Mice—Stat6-deficient and insulin receptor substrate 2 (IRS-2)-deficient mice were generated and maintained as described previously (13, 17).

Lymphocyte Culture—Naïve Th cells were purified from lymph nodes by cell sorting (Mo Flo) using anti-CD4 and anti-CD62L (BD PharMingen) to 98% purity. Splenic B lymphocytes were purified to 95% purity using Macs magnetic beads specific for B220 (Miltinyi Biotec) per the manufacturer's instructions. Both T and B lymphocytes were cultured at 1–2 $\times$ 10^6 cells/ml in RPMI 1640 supplemented as described previously (13). Recombinant IL-4 (Peprotech) was added to the indicated cultures at a concentration of 10 ng/ml. Anti-IgM (Jackson Laboratories) was added to the indicated cultures at a concentration of 5 μg/ml. Anti-CD40 (BD PharMingen) was added to the indicated cultures at a concentration of 5 μg/ml.

Immunoblot Analysis—Whole cell extracts were prepared by lysing cells in 50 mM Tris, 0.5%Nonidet P-40, 5 mM EDTA, 50 mM NaCl and clearing the lysates by microcentrifugation. 4 μg of protein were separated on a 10% polyacrylamide gel and transferred to an Optitran membrane (Schleicher & Schuell). The immunoblots were blocked for 1 h at room temperature in 5% dry milk in TBST (50 mM Tris, pH 7.5, 100 mM NaCl, 0.03% Tween 20) and incubated with either Bcl-xL- or Bcl-2-specific antibodies (Santa Cruz Biotechnology) diluted 1:1000 in blocking buffer overnight at 4 °C. The blots were washed with TBST and incubated with either horseradish peroxide-conjugated anti-mouse or anti-rabbit antibody (Santa Cruz Biotechnology) in blocking buffer for 1 h at room temperature. After washing the blots with TBST, detection was carried out using enhanced chemiluminescence (ECL, Amersham Biosciences) according to manufacturer's instructions.

Northern Analysis—Total RNA was isolated using TRIzol RNA isolation reagent (Invitrogen). The RNA was separated on a 1.5% agarose, 6% formaldehyde gel and transferred to GeneScreen (PerkinElmer Life Sciences) membrane. The membrane was hybridized with radiolabeled cDNA probes for *bcl-xL*, *bcl-2*, and γ-actin. The relative expression of *bcl-xL* compared with *bcl-2* was determined by densitometry using a ChemiImager 4000 (AlphaInotech).

Propidium Iodide Analysis—Lymphocytes were cultured as described in the figure legends, pelleted by centrifugation, and fixed in 40% EtOH. The cells were treated with RNase A (50 μg/ml) for 45 min at 37 °C and subsequently stained with 700 mM propidium iodide in 3.8 $\times$ 10^{-2} M sodium citrate. Analysis was performed on a FACScan flow cytometer (BD PharMingen).

Transient Transfections and Luciferase Assays—5 $\times$ 10^6 B lymphoma cells were combined with 10 μg of both the reporter and a Stat6 expression plasmid (18) in 0.4 ml supplemented RPMI medium. The cells were transfected using a Bio-Rad electroporator (280 V, 975 microfarads) and placed on ice for 10 min. The individual transfectants were then split into two cultures and cultured overnight in 2.5 ml of supplemented RPMI medium. Recombinant IL-4 (10 ng/ml) was added to the indicated cultures 24 h after transfection, and the cells were harvested after an additional 24 h. Luciferase assays were performed using the luciferase assay system per the manufacturer's instructions (Promega). The 4$\times$Stat6 gene constructs were generated by synthesizing double-stranded oligos that span the *bcl-xL* Stat6 site and cloning them into the *Bgl*II site of pTkluc. The sequence of the *bcl-xL* Stat6 wild type oligo is 5′-GATCCCCCGGTCTTCTTCAGGGGAAACTGAGGCCG-GCTTCA-3′ and the sequence of the *bcl-xL* Stat6mut oligo is 5′-GAT-CCCCCGGTCTTCTAGAGGGCTAACTGAGGCCGGCTTCA-3′. The *bcl-xL* promoter region was cloned using primers 5′-CTAAACCCATA-CCTCCGGGA-3′ and 5′-GCGCAAGCTTGGGCTCAACCAGTCCATTG-TC-3′, digested with *Hin*dIII, and cloned into pGL2 (Promega). Single copies of the 40-bp wild type and mutant Stat6 sites were cloned upstream of the promoter into the *Bgl*II site.

Retroviral Transduction of B Cells—The GFP-RV bicistronic vector was obtained from K. Murphy (19), and the Phoenix-Eco packaging cell line was obtained from G. Nolan (20). The *bcl-xL* cDNA was ligated with *Xho*I linkers and cloned into the *Xho*I cloning site of GFP-RV. Transfection of the packaging cell line was performed using Effectene (Qiagen), and viral supernatants were harvested 48–72 h later. Lipopolysaccharide-activated (25 μg/ml, 24 h) purified splenic B cells were retrovirally transduced by incubation of 1 $\times$ 10^7 B cells at 1 $\times$ 10^6/ml with an equal volume of viral supernatant, 8 μg/ml Polybrene, and 25 μg/ml lipopolysaccharide. The cultures were centrifuged at 500 $\times$ *g* for 40 min at room temperature. Infections were repeated 24 h later with a resulting transduction efficiency as assessed by GFP expression of 10–35%. GFP+ cells were sorted by fluorescence-activated cell sorter (BD PharMingen) 48 h later and cultured in 5 μg/ml anti-CD40 and lipopolysaccharide for 48 h. Anti-Fas (BD PharMingen) was added at 0.06 μg/ml to the cultures, and cells were analyzed for DNA content by propidium iodide analysis.

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared from BJAB B cells cultured in the presence or absence of IL-4 for 1 h as described previously (21). 1 μg of nuclear extracts were used in electrophoretic mobility shift assay as described previously (22). Double-stranded oligos spanning the 40-bp of *bcl-xL* genomic sequence described under "Transient Transfections and Luciferase Assays" were used in the assay. The Stat6 and Stat5 antibodies were obtained from Santa Cruz Biotechnology.

RESULTS

Stat6 Signaling Contributes to IL-4-mediated Rescue of Primary B Cells from Apoptosis—To examine the contribution of Stat6 in regulating cell death in lymphocytes, we utilized mice that were genetically deficient for this protein (13). We initially studied the requirement for Stat6 in the rescue of B and T cells from death after cytokine withdrawal. Purified splenic B or T cells from Stat6-deficient and wild type mice were cultured in the presence or absence of IL-4 overnight and subsequently analyzed for the presence of apoptotic cells by propidium iodide staining. As previously reported, a significant proportion of both wild type and Stat6-deficient T and B lymphocytes were found to be subdiploid after culture without cytokine (Fig. 1) (5, 6). In agreement with previously published reports, IL-4 served as an effective anti-apoptotic factor for resting T cells, rescuing 62% of those cells from apoptosis, regardless of Stat6 expression (5, 14) (Fig. 1*A*). The addition of IL-4 to the wild type B cell culture also rescued ~62% of those cells from apoptosis (Fig. 1*B*). In contrast to T cells, however, the addition of IL-4 to the Stat6-deficient B cell culture resulted in only a partial rescue from apoptosis (Fig. 1*B*).

IRS-2 is a second, well characterized signaling mediator that directly binds to the IL-4 receptor (23). *In vitro* transfection studies of IL-4 receptor mutants in myeloid cells suggests that IRS-2 is involved in regulating mitogenic and anti-apoptotic signals from IL-4 (24, 25). The role IRS-2 plays in regulating IL-4 responses in primary B cells has not been reported. To determine whether IRS-2 plays a role in protecting primary B lymphocytes from apoptosis after IL-4 stimulation, we repeated these studies in B lymphocytes from IRS-2-deficient mice (17). In contrast to what we find for Stat6, B lymphocytes purified from IRS-2-deficient mice were still efficiently rescued from apoptosis by IL-4 (Fig. 1*C*). These results suggest that Stat6, but not IRS-2, plays an important role in the rescue of primary B cells from apoptosis induced by growth factor withdrawal.

It has been previously demonstrated that splenic B cells stimulated through CD40 up-regulate Fas expression and become susceptible to Fas-induced apoptosis *in vitro*, whereas treatment of CD40-stimulated B cells with either IL-4 or anti-IgM results in resistance to Fas-mediated cell death (8, 26). To determine whether Stat6 is required for IL-4-induced Fas resistance, we purified splenic B cells from both wild type and Stat6-deficient mice and cultured the cells for 48 h in the presence of stimulatory antibodies to CD40. Fluorescence-activated cell sorter analysis of these cells indicated that cell surface Fas expression was induced similarly on both cell populations (data not shown). As expected, the subsequent culture of these cells with antibodies to Fas resulted in signaling for cell death, and combined treatment of the wild type cells with IL-4 in conjunction with anti-CD40 resulted in protection from Fas-induced apoptosis (40% rescue from apoptosis) (Fig. 2). In contrast, IL-4 had no effect on the viability of CD40-stimulated Stat6-deficient B cells after treatment with anti-Fas (Fig. 2). Both wild type and Stat6-deficient cells were equally protected

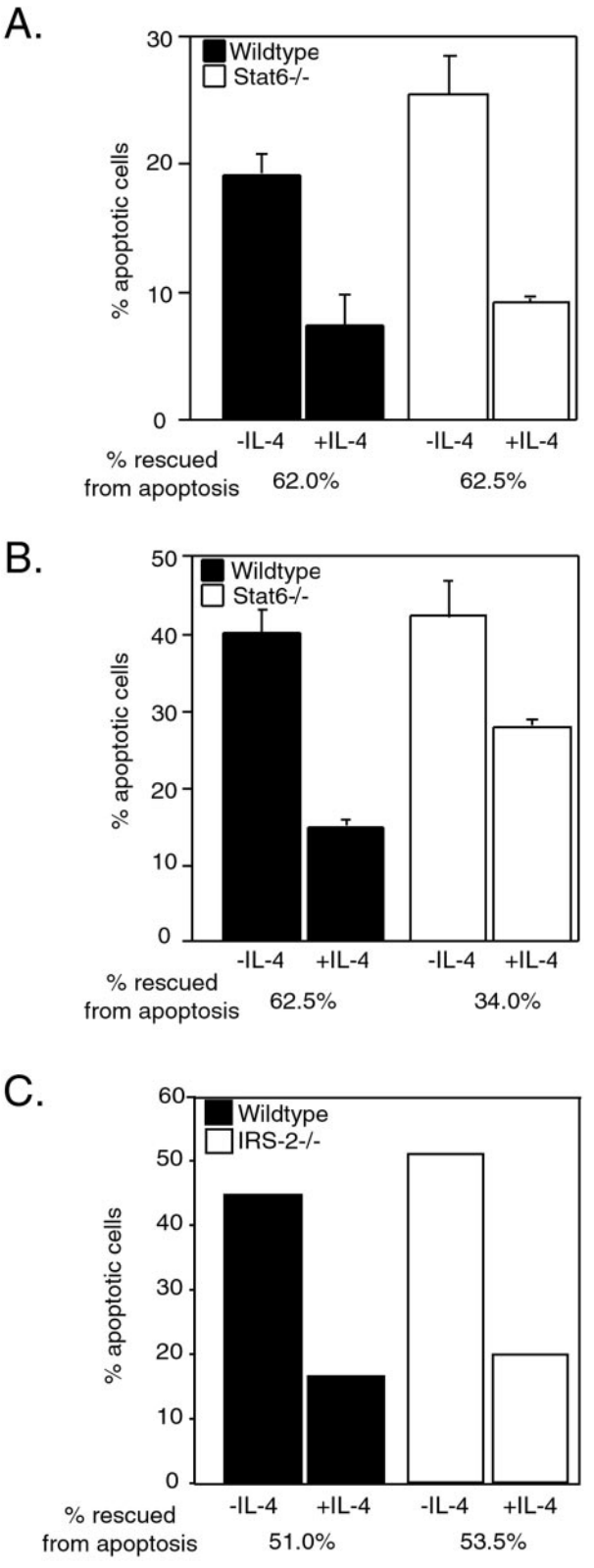

FIG. 1. **Stat6 is required for IL-4 to block passive cell death of B cells *in vitro*.** Purified naïve CD4+ T cells (*A*) or splenic B cells (*B* and *C*) were cultured for 18–24 h in the presence or absence of 10 ng/ml IL-4. The resulting populations were analyzed for apoptosis by propidium iodide staining. The data represent the average of two experiments and are representative of four independent experiments.

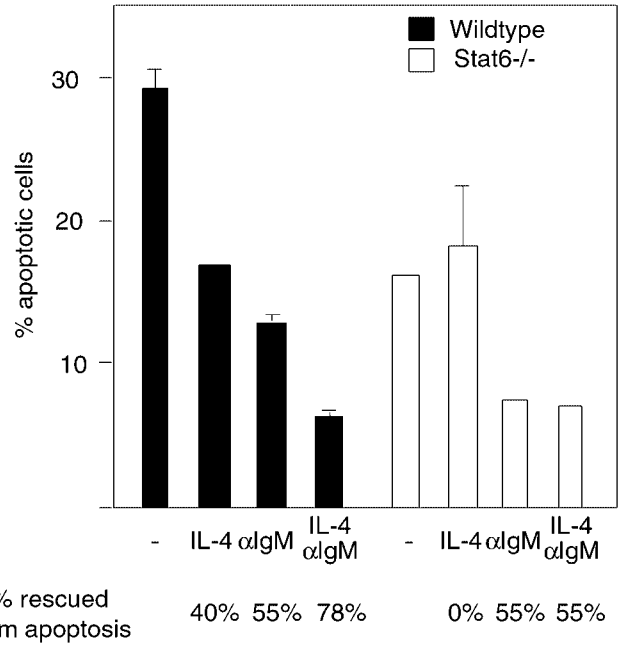

FIG. 2. **Stat6 is required for IL-4 to block Fas-induced cell death of B cells.** Purified splenic B cells were stimulated with anti-CD40 for 48 h. IL-4 and anti-IgM were added for the final 12 h of the culture where indicated. The cells were then induced to undergo Fas-mediated apoptosis by the inclusion of anti-Fas antibody (0.06 μg/ml) overnight. The cells were subsequently analyzed for apoptosis by propidium iodide staining. The results shown are in duplicate and representative of four independent experiments.

from apoptosis by the addition of anti-IgM to the cell cultures (55% rescue from apoptosis), indicating that the defect observed in the Stat6-deficient B cells is specific to IL-4 signaling (Fig. 2). Additionally, wild type B cells treated with both IL-4 and anti-IgM were afforded even greater protection from Fas-induced apoptosis than were B cells treated with either alone, whereas Stat6-deficient B cells did not respond with the same effect (Fig. 2). These results were duplicated in a related experimental system using CD40L to up-regulate Fas expression and Fas ligand (FasL)-dependent Th1 cell-mediated cytotoxicity to kill the cells as previously described (26) (data not shown). The above results indicate that Stat6 signaling is involved not only in IL-4-induced rescue from passive cell death by growth factor withdrawal but is also absolutely required for IL-4-induced protection from Fas-mediated apoptosis in B cells.

Stat6 Is Required for Maximal IL-4-induced Bcl-xL Expression—Based on the above observations, we presumed that IL-4-dependent Stat6 activation induces the transcription of a factor that protects splenic B cells from apoptosis. It has been shown in a number of systems that the expression of anti-apoptotic Bcl-2 family members is modulated by the addition of mitogens and growth factors. For example, Bcl-2 expression in T cells has been demonstrated to be induced by IL-2 and T cell receptor engagement, whereas A1, another anti-apoptotic Bcl-2 family member, has been shown to be induced by Rel proteins after mitogen stimulation in lymphocytes (27–29). Bcl-xL expression in particular has been shown to be up-regulated by a number of different cytokines in a variety of cell types (27, 30). Furthermore, granulocyte-macrophage colony-stimulating factor, IL-3, and Epo were all shown to induce Bcl-xL transcription through the cytokine receptor-associated Stat5 protein (31, 32). These observations made Bcl-xL an attractive candidate for a potential Stat6-dependent, IL-4-inducible gene in primary B cells.

To determine whether IL-4 induces Bcl-xL expression in a Stat6-dependent manner, we analyzed Bcl-xL protein and mRNA expression from wild type and Stat6-deficient B cells treated in culture with IL-4. We found that the inclusion of IL-4

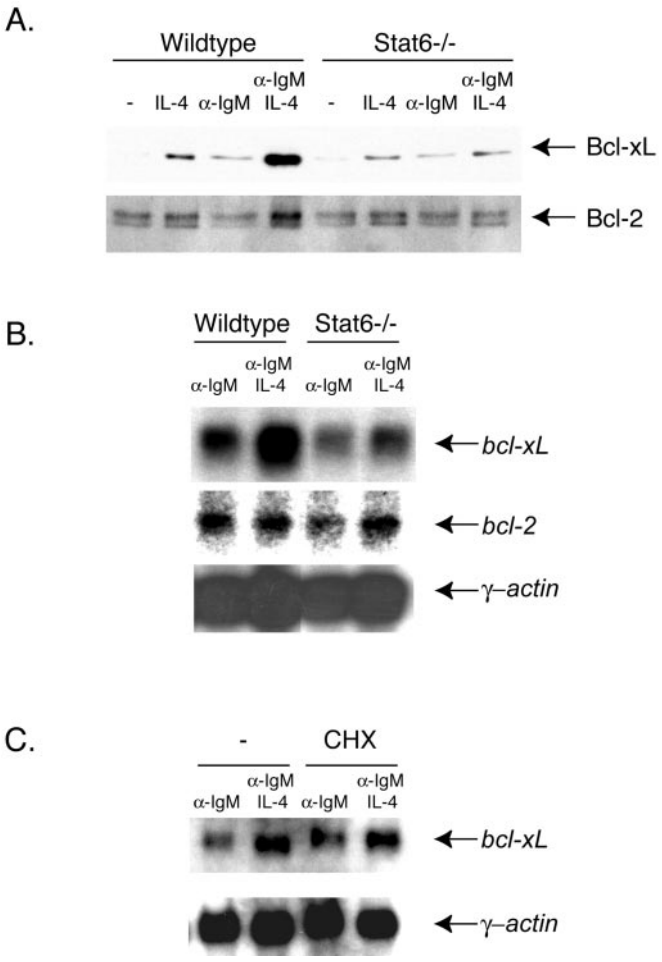

FIG. 3. **IL-4-induced Bcl-xL expression is Stat6-dependent.** *A*, purified splenic B cells were cultured in the presence or absence of IL-4 and/or anti-IgM. Total protein extracts were prepared from the cultures at 48 h and analyzed for Bcl-xL and Bcl-2 protein expression by immunoblot. The results shown are representative of three independent experiments. *B*, total RNA was prepared from 7 h cultures and analyzed for the presence of *bcl-xL*, *bcl-2*, and γ-actin transcripts by Northern analysis. *bcl-xL* mRNA expression was induced 3.3- and 1.3-fold by IL-4 in wild type and Stat6-deficient cells, respectively. The results shown are representative of three independent experiments. *C*, wild type B cells were stimulated with anti-IgM or anti-IgM/IL-4 for 2 h. Cyclohexamide 10 μg/ml (*CHX*) was included 30 min before stimulation in the indicated cultures.

in the wild type B cell cultures resulted in a small but reproducible increase in Bcl-xL protein expression (Fig. 3*A*). IL-4 could significantly induce Bcl-xL expression in both wild type and Stat6-deficient B cells, but maximal induction only occurred in the presence of Stat6, suggesting that both Stat6-dependent and -independent pathways are involved in Bcl-xL expression. Anti-IgM stimulation led to a comparable increase in Bcl-xL protein and mRNA expression in both wild type cells and in Stat6-deficient cells (Fig. 3, *A* and *B*). Notably, the addition of both IL-4 and anti-IgM resulted in a synergistic induction of Bcl-xL expression in normal B cells. In contrast, the maximal induction of Bcl-xL by IL-4 and anti-IgM was especially compromised in Stat6-deficient B cells (Fig. 3, *A* and *B*). In agreement with previously published results, Bcl-2 protein and message levels were minimally affected by these treatments (Fig. 3*A*) (33). We also found that the induction of *bcl-xL* mRNA occurred as early as 2 h after IL-4 stimulation in anti-IgM-treated wild type B cells and was not inhibited by cycloheximide (Fig. 3*C*). These results suggest that IL-4 is capable of rapidly inducing *bcl-xL* mRNA and protein expression and that maximal induction is dependent on the presence of Stat6.

Stat6 Directly Activates a bcl-xL Regulatory Element—The above results imply that a consequence of IL-4 treatment of B cells is the induction of Bcl-xL expression and that this induction is dependent on the activation of Stat6. Furthermore, the activation of Bcl-xL expression in response to IL-4 is quite rapid and does not require the *de novo* synthesis of additional proteins. These observations suggest that Bcl-xL may be a direct transcriptional target of Stat6. To formally test this possibility we analyzed the murine Bcl-xL genomic sequence for the presence of a Stat6-responsive element.

Most Stat proteins recognize a promoter element defined as the γ-activating sequence (GAS), which is composed of a palindromic sequence separated by three nucleotides (TTCNNNGAA) (10, 34). Stat6 is unusual in that it only recognizes the GAS at low affinity and demonstrates instead a marked preference for a variant sequence with a four-base pair spacer (TTCNNNNGAA) (18). In fact, most naturally occurring IL-4-responsive promoters analyzed to date contain Stat6-responsive elements with the four-base pair spacer (35, 36). Previous studies of the *bcl-xL* genomic sequence revealed several functional GAS elements in the first intron of the Bcl-xL gene that are regulated by Epo- and IL-3-activated Stat5 (Fig. 4*A*) (31, 32, 37, 38). Our analysis of the genomic sequence of the *bcl-xL*-flanking region revealed in addition a perfect Stat6-responsive element 928 bp upstream of the first major transcriptional start site and 1600 bp upstream of the starting methionine (Fig. 4*A*) (37). The 40 base pairs surrounding this Stat6 site was multimerized, and the compound element was cloned upstream of a minimal thymidine kinase promoter driving the expression of a luciferase reporter gene. The activity of this element was tested by transiently transfecting M12 B cells with the reporter construct in the presence and absence of IL-4. We found that in the absence of IL-4 the reporter construct was essentially inactive, but in the presence of IL-4 reporter gene activity was induced ~20-fold (Fig. 4*B*). This robust IL-4-induced reporter activity was dependent on an intact Stat6 response element since specific mutations introduced into our reporter construct that destroy the palindrome resulted in a complete loss of IL-4 induction (Fig. 4, *A* and *B*).

We also tested the ability of the Stat6 site to provide IL-4 inducibility to the native *bcl-xL* promoter. A bcl-xL promoter fragment, extending 576 bp upstream of the starting methionine and including the two Stat5 sites described previously, was cloned upstream of the luciferase reporter gene along with the *bcl-xL* Stat6 site (32). A single Stat6 site was able to reproducibly provide IL-4-induced reporter gene activity to the *bcl-xL* promoter (Fig. 4*C*). The IL-4-induced reporter activity was also dependent on an intact Stat6 site and was not able to directly activate transcription through the Stat5 sites (Fig. 4*C* and data not shown). Additionally, using electrophoretic mobility shift assay analysis, we were able to detect an IL-4-induced protein complex from B cell nuclear extracts bound to the *bcl-xL* Stat6 site (Fig. 4*D*). The formation of this complex was reduced when the nuclear extracts were preincubated with antibodies to Stat6, but not to Stat5, suggesting that Stat6 directly binds to an element in the *bcl-xL*-flanking region. These results combined with the findings above strongly suggest that Stat6 is a direct activator of a *bcl-xL* regulatory element in B cells.

Ectopic Bcl-xL Expression Rescues Stat6-deficient B Cells from Fas-induced Apoptosis—The studies above suggest that Bcl-xL is a potential downstream target of Stat6 and may be in part responsible for the protection of IL-4-treated B cells from apoptosis. It has previously been demonstrated that overexpression of a Bcl-xL transgene in developing B cells increased

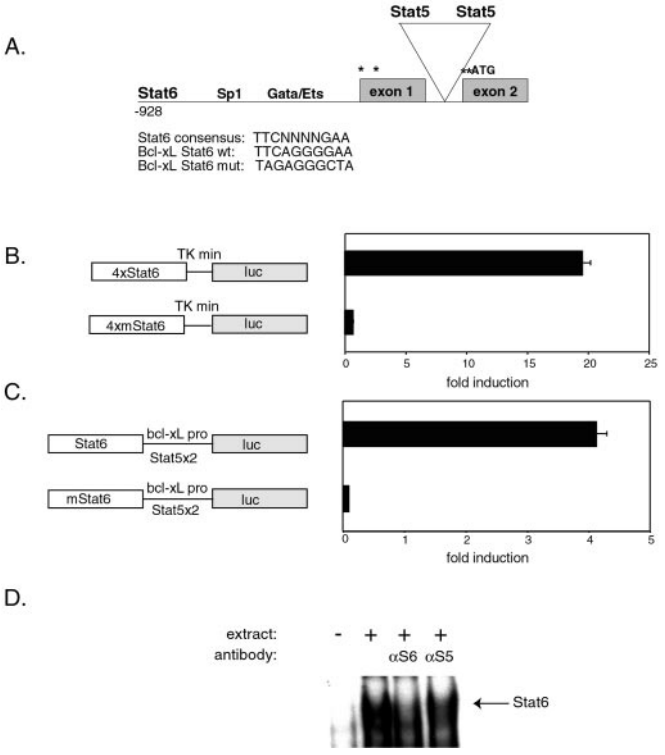

FIG. 4. **Stat6 directly activates a Bcl-xL promoter element.** *A*, schematic of the *bcl-xL* promoter. The previously identified Stat5 binding sites are shown in the first intron. The *asterisks* represent the transcriptional start sites reported previously (37). The Stat6 site described in this study is shown 928 base pairs upstream of the first exon. The sequence of the wild type Stat6 site identified in the *bcl-xL* promoter and the mutated site used in this study are also indicated. *B*, the genomic sequence surrounding and including the wild type Stat6 binding site from the Bcl-xL promoter and the genomic sequence including a mutant Stat6 binding site were each multimerized four times and cloned upstream of the minimal thymidine kinase promoter driving the expression of a luciferase reporter gene. The reporter constructs were transfected along with a Stat6 expression construct into the B cell lymphoma M12 in the presence and absence of recombinant IL-4. The experiments were carried out in duplicate, and the data shown are representative of three independent experiments. *C*, a single copy of the *bcl-xL* genomic sequence including wild type and mutant Stat6 binding sites described in *B* were cloned upstream of the 576-bp *bcl-xL* promoter region driving the expression of a luciferase reporter gene. The reporter constructs were transfected into the B cell lymphoma BJAB as described in *B*. *D*, nuclear extracts from untreated (−) and IL-4-treated (+) BJAB cells were incubated with a radiolabeled, double-stranded oligo including the wild type *bcl-xL* Stat6 site and analyzed by electrophoretic mobility shift assay. Extracts were preincubated with antibodies to Stat5 (*αS5*) and Stat6 (*αS6*) where indicated.

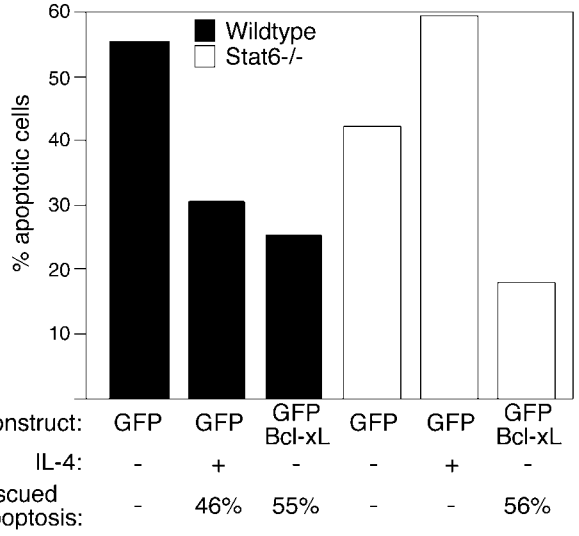

FIG. 5. **Ectopic expression of Bcl-xL rescues Stat6-deficient B cells from Fas-induced cell death.** Purified splenic B cells were stimulated as described under "Experimental Procedures" and transduced with packaging cell supernatants containing virus expressing GFP or GFP/Bcl-xL. The resulting transduced cells were sorted for GFP expression, stimulated with anti-CD40 for 48 h, and induced to die by exposure to anti-Fas for 6 h. Apoptosis was assessed by propidium iodide staining. Where indicated, IL-4 was included for the last 24 h of culture. The data represent the percentage of GFP-only expressing cells protected from apoptosis when either exposed to IL-4 or compared with cells transduced with GFP/Bcl-xL. The results are representative of four independent experiments.

their resistance to Fas-induced apoptosis (39). To determine whether reconstitution of Bcl-xL expression could protect Stat6-deficient B cells from undergoing apoptosis, we used a retroviral gene expression system to overexpress Bcl-xL. We cloned Bcl-xL cDNA into a bicistronic retroviral expression construct, allowing for the coexpression of Bcl-xL and GFP within the same cell. Purified wild type and Stat6-deficient B cells were activated *in vitro* by lipopolysaccharide for 24 h and then transduced with retroviral supernatants containing the empty GFP vector or the vector including GFP and Bcl-xL (GFP BclxL). The transduced cells were activated with anti-CD40 for 48 h to up-regulate Fas expression and subsequently treated with antibodies to Fas to trigger cell death. The extent of apoptosis was examined by comparing propidium iodide staining of GFP only to GFP/Bcl-xL-expressing transduced cells by fluorescence-activated cell sorter analysis. Similar to the data in Fig. 1*B*, a large percentage of wild type and Stat6-deficient cells transduced with the control GFP vector were sensitive to Fas-mediated apoptosis, and the addition of IL-4 to the wild type culture was able to rescue 46% of those cells from death (Fig. 5). IL-4 was unable to rescue any of the anti-Fas treated Stat6-deficient B cells from apoptosis and, if anything, enhanced cell death (Fig. 5). However, ectopic expression of Bcl-xL in either wild type or Stat6-deficient B cells led to a marked increase in the resistance of both wild type and Stat6-deificient cells to Fas-induced apoptosis, comparable with that observed for IL-4 and wild type B cells. Thus, Bcl-xL expression is sufficient to confer Fas-resistance in Stat6-deficient B cells.

DISCUSSION

In this report we have demonstrated that IL-4 fails to efficiently rescue Stat6-deficient B cells from apoptosis produced by growth factor withdrawal or Fas engagement. We find that the anti-apoptotic Bcl-2 family member, Bcl-xL, is one likely candidate target gene directly activated by Stat6 that can modulate the response of B cells to apoptotic stimuli.

In contrast to what we observed in B cells, Stat6 is not a critical regulator of the anti-apoptotic activity of IL-4 in T cells (Fig. 1*A* and Refs. 5, 13, and 14). This difference is reminiscent of the recent observation that IL-6 is a potent anti-apoptotic factor for resting T cells but not for activated T cells (40). In this situation, T cell activation appears to alter the cellular environment so that IL-6-induced Stat signaling is less potent. Although robust Stat6 activation is observed in both T cells and B cells after IL-4 exposure, the cellular context of this signal is clearly important in determining the anti-apoptotic outcome of IL-4 signaling.

A number of cytokines are capable of suppressing apoptosis, and Bcl-xL expression has been demonstrated to be cytokine-inducible in a variety of systems. For example, IL-3 is required as a survival factor for a number of cytokine-dependent cell lines and is capable of inducing Bcl-xL expression in both myeloid and pro-B cell lines in a Jak kinase-dependent manner (30). Additionally, IL-3 and granulocyte-macrophage colony-

stimulating factor are both capable of inducing Bcl-xL expression in wild type mouse bone marrow cells but not in Stat5a/b-deficient bone marrow cells, and the deficient cells are characterized by increased apoptosis (31). Stat5 and Stat3 have also been recently demonstrated to activate the *bcl-xL* expression directly although through a different Stat response element than the one we have described here for Stat6 (32, 41). The Stat5-responsive element is composed of two tandem GAS sites in the first intron of *bcl-xL* (Fig. 4*A*). Not surprisingly we found that Stat6 was incapable of activating transcription through the Stat5 sites in our reporter assays since these are GAS elements with only a three-base pair spacer within the palindromes (Fig. 4*C* and data not shown). These findings suggest that *bcl-xL* transcription can be regulated by a number of different cytokines through different Stat transcription factors, but this regulation is not necessarily mediated by the same Stat response elements in the *bcl-xL* gene. Interestingly, a recent report suggests that IL-15 is able to activate Stat6 specifically in mast cells and that in this cell type Stat6 is able to direct IL-15-induced *bcl-xL* transcription through the Stat5 sites (42). This result implies that the context of the cytokine-Stat signal can also strongly influence downstream changes in gene regulation.

We have demonstrated here that both IL-4 and IgM stimulation induce Fas-resistance in activated B cells in a manner that correlates with increased *bcl-xL* mRNA levels. It has been previously shown that IL-4- and anti-IgM-induced Fas resistance are mediated by signaling pathways that differ both in their time course and dependence on protein kinase C activation (8). Both IL-4 and anti-IgM stimulation ultimately result in increased *bcl-xL* transcription, although the mechanism of promoter activation is likely to be different for the two stimuli. Here we show that Stat6 is an IL-4-induced transcription factor that directly activates a *bcl-xL* regulatory element. Additionally we observed that the synergistic induction of Bcl-xL expression after IL-4 and antigen receptor stimulation is especially compromised in the absence of Stat6 (Fig. 3). Interestingly, anti-IgM has been shown to induce Stat6 phosphorylation, although this apparently plays no role in Ig-mediated Fas resistance or Bcl-xL expression (Figs. 2 and 3) (46). Anti-IgM stimulation has also been shown to induce the activation of NFκB and NFAT transcription factors, and a number of binding sites for these factors are present in the *bcl-xL* promoter region (37, 43–45). However, we have yet been able to identify an anti-IgM responsive element in the *bcl-xL* promoter region (data not shown), suggesting that regulatory elements outside of the proximal promoter element may contribute to anti-IgM-induced *bcl-xL* expression. It will be interesting to further define the regulatory elements that are involved in anti-IgM-induced *bcl-xL* transcription and how these elements potentially cooperate with Stat6 to activate *bcl-xL* transcription synergistically.

Even in the absence of Stat6, however, we observed that IL-4 was capable of partially rescuing B cells from passive cell death (Fig. 1*B*) and inducing low levels of Bcl-xL expression (Fig. 3). Clearly other signal transduction pathways can be activated in response to IL-4 in addition to Stat6, and these may also contribute to the anti-apoptotic effects of IL-4. IRS-2 is an additional signaling protein that associates with the IL-4 receptor and is rapidly phosphorylated in lymphocytes after IL-4 treatment (23). IRS-2 activation results in the recruitment and activation of phosphatidylinositol 3-kinase and ultimately Akt activation. This cascade has been suggested to lead to protection from apoptosis, and furthermore, B cells lacking the regulatory subunit of phosphatidylinositol 3-kinase have been shown to be deficient in their response to the anti-apoptotic effects of IL-4 (25, 47). However, we do not find that IRS-2 activation or Akt activation is absolutely required for IL-4-mediated rescue from growth factor withdrawal in that B cells from IRS-2-deficient mice are competent in their anti-apoptotic response to IL-4. (Fig. 1*C*). An additional pathway from the IL-4 receptor has also been implicated in rescue from apoptosis in myeloid cell lines (15). This pathway emanates from a different phosphorylated tyrosine residue on the IL-4 receptor that is not recognized by Stat6 or IRS-2. The nature of this pathway has not yet been defined, and its role in opposing apoptosis in primary B cells has not been reported.

Although our results suggest that Bcl-xL is an important regulator of B cell apoptosis, we do not observe a direct correlation between Bcl-xL expression and protection from apoptosis. Both IL-4 and anti-IgM stimulation induce similar amounts of Bcl-xL protein in Stat6-deficient B cells, but only anti-IgM stimulation is protective of Fas-mediated apoptosis (Fig. 3*A*). Additionally, overexpression of Bcl-xL in our retroviral gene expression experiments as well as in other reports resulted in only partial protection from Fas-induced apoptosis. These results suggest that other important factors are also induced by IL-4 or antigen receptor stimulation that modulate apoptosis. One likely candidate is a recently described anti-apoptotic factor, FAIM, which is induced specifically by anti-IgM stimulation in primary B cells and is capable of blocking Fas-induced cell death when overexpressed in B cell lines (48). Also, antigen receptor stimulation has been demonstrated to block the association of Fas with its direct downstream effector, FAS-associated death domain protein (FADD), in a manner that is independent of new protein synthesis (49). Additionally, we have found that BAG-1, a Bcl-2-associating protein with anti-apoptotic activities, is induced in primary B cells by IL-4 in a Stat6-dependent manner (50) (data not shown). Clearly, the coordinated expression of a number of pro- and anti-apoptotic factors that are regulated by multiple signals is critical for determining the appropriate cellular response to a death signal.

Because Bcl-2 family members are important regulators of cell survival, there has been a great deal of interest in understanding the role of these proteins in the generation of an oncogenic state. Bcl-xL in particular was shown to be specifically activated by retroviral insertion in a number of murine myeloid and T cell leukemias (30). Because Stat proteins play important roles in cellular proliferation and apoptosis, they have also been implicated in playing a causative role in oncogenesis. Indeed, a mutant form of Stat3 was recently demonstrated to directly mediate cellular transformation (51). Interestingly, a link between constitutive Stat3 activation and disregulated Bcl-xL expression was reported in squamous cell carcinomas (52). Constitutive Stat6 activation has also been associated with specific tumor cells (10). In the future, it will be interesting to determine whether disregulated Stat6 can affect cellular transformation through the induction of Bcl-xL expression.

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