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Stat6 Is Required for Mediating Responses to IL-4 and for the Development of Th2 Cells

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Summary

Interleukin-4 (IL-4) stimulation of cells leads to the activation of multiple signaling pathways, one of which involves Stat6. We have generated Stat6-deficient mice by gene targeting in embryonic stem cells to determine the role of this transcription factor in mediating the biologic functions of IL-4. IL-4-induced increases in the cell surface expression of both MHC class II antigens and IL-4 receptor are completely abrogated, and lymphocytes from Stat6-deficient animals fail to proliferate in response to IL-4. Stat6-deficient B cells do not produce IgE following in vivo immunization with anti-IgD. In addition, Stat6-deficient T lymphocytes fail to differentiate into Th2 cells in response to either IL-4 or IL-13. These results demonstrate that, despite the existence of multiple signaling pathways activated by IL-4, Stat6 is essential for mediating responses to IL-4 in lymphocytes.

Introduction

Signal transducers and activators of transcription (STAT) proteins are a recently identified class of transcription factors responsible for mediating many cytokine-induced responses. These proteins exist in a latent form in the cytoplasm and become phosphorylated by the Janus kinase (JAK) family of tyrosine kinases following cytokine–receptor interactions. Once phosphorylated, STAT proteins dimerize, translocate to the nucleus, and bind to specific DNA sequences to regulate gene transcription (Ihle, 1995; Schindler and Darnell, 1995). Of the presently known STAT proteins, only Stat6 is activated in response to the cytokine interleukin-4 (IL-4) (Kotanides and Reich, 1993; Hou et al., 1994; Schindler et al., 1994; Quelle et al., 1995).

IL-4 is secreted by several cell types including stimulated T lymphocytes, mast cells, and basophils (Howard et al., 1982; Lee et al., 1986; Paul and Ohara, 1987; Yoshimoto and Paul, 1994; Sad et al., 1995). While initially identified by its ability to support the growth and differentiation of B lymphocytes costimulated with submitogenic doses of anti-immunoglobulin (Howard et al., 1982), IL-4 is now known to have pleiotropic effects on the immune system. IL-4 is essential for the induction of immunoglobulin E (IgE) synthesis by activated B lymphocytes and influences class switching to IgG1 as well (Vitetta et al., 1985; Coffman et al., 1986). B cells stimulated with IL-4 increase their cell surface expression of major histocompatibility complex (MHC) class II molecules (Noelle et al., 1984), IL-4 receptor (IL-4R) (Ohara and Paul, 1988), and the low affinity IgE receptor, CD23 (Conrad et al., 1987). IL-4 also induces the proliferation of T lymphocytes and is important for the differentiation of T helper 2 (Th2) cells (Le Gros et al., 1990; Swain et al., 1990). Indeed, the analysis of IL-4-deficient mice generated by gene targeting in embryonic stem (ES) cells has confirmed the importance of this cytokine in mediating many of these responses (Kuhn et al., 1991; Kopf et al., 1993).

IL-4-induced responses result from the interaction of ligand with a cell surface receptor composed of a cytokine-specific α chain and the common γc chain also used by IL-2 (Takeshita et al., 1992), IL-7 (Noguchi et al., 1993; Kondo et al., 1994), IL-9 (Russell et al., 1994), and IL-15 (Giri et al., 1994) receptors. Recent studies have shown that the α chain of the IL-4R is also a component of the high affinity IL-13 receptor (Lin et al., 1995; Zurawski et al., 1995; Hilton et al., 1996). Engagement of the IL-4R leads to the activation of at least two distinct signaling pathways. One involves the activation of Stat6 through phosphorylation by Jak1 and Jak3 (Johnston et al., 1994; Witthuhn et al., 1994). The Stat pathway is thought to be important because the promoters of several genes known to be regulated by IL-4 contain the consensus Stat6-binding site $TTCN_4GAA$ (Schindler et al., 1995), and fragments of promoters of Iϵ and other genes containing this sequence bind Stat6 present in extracts from cells stimulated with IL-4 (Ichiki et al., 1993; Kotanides and Reich, 1993; Schindler et al., 1994). In addition to Stat6 activation, stimulation of the IL-4R has also been shown to induce the phosphorylation of an insulin receptor substrate (IRS) termed 4PS or IRS-2 (Keegan et al., 1994; Sun et al., 1995). Activated IRS-2 associates with phosphatidylinositol 3-kinase and may be responsible for certain IL-4-induced responses. For example, cell lines transfected with an IL-4R mutant that is incapable of inducing the tyrosine phosphorylation of IRS-2 have an impaired ability to proliferate in response to IL-4 (Keegan et al., 1994).

To determine the relative importance of the Stat6 signaling pathway in mediating the biologic functions of IL-4, we have produced mice deficient in Stat6. Our results demonstrate that lymphocytes from *Stat6*$^{-/-}$ mice are unable to respond to IL-4 as measured in a variety of in vitro and in vivo assays. Furthermore, *Stat6*$^{-/-}$ mice show a striking defect in the generation of Th2 cells. These results suggest that the Stat6 signaling pathway is critical for mediating IL-4-induced responses in lymphocytes.

Results and Discussion

Generation of Stat6-Deficient Mice

A gene-targeting construct was generated that replaces exons encoding amino acids 505–584 of Stat6 with a

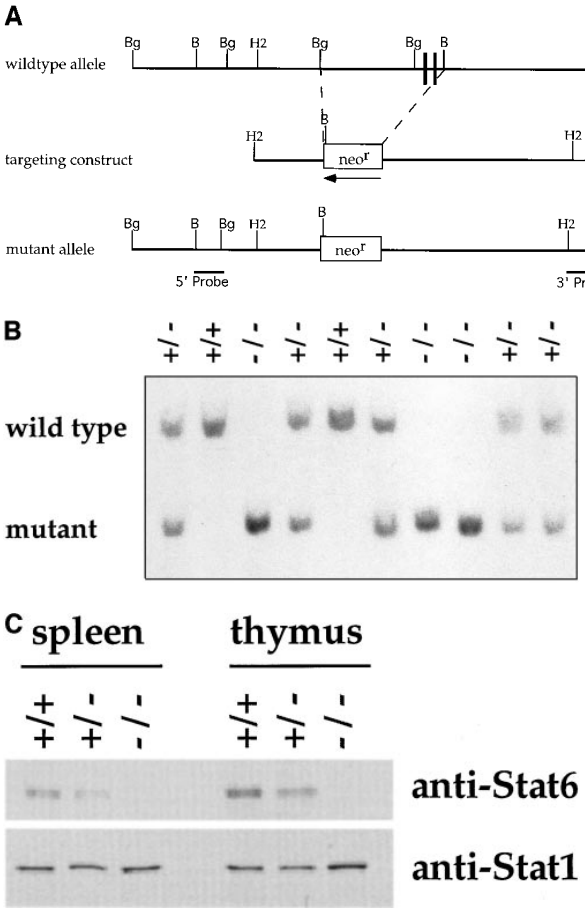

Figure 1. Generation and Analysis of *Stat6*$^{-/-}$ Mice

(A) Schematic of the targeting construct used to disrupt the *Stat6* gene. The two exons of the *Stat6* gene that were replaced with the neomycin resistance gene are indicated as closed bars and the positions of other exons are not shown. 5′ and 3′ probes used to verify correctly targeted clones are indicated under the mutant allele. Restriction enzymes are the following: B, BamHI; Bg, BglI; H2, HindII.

(B) Southern (DNA) analysis of tail DNA from an intercross litter digested with BamHI and hybridized with the 5′ probe indicated in (A). The approximate size of the wild-type band is 3.5 kb and the mutant band is 1.9 kb.

(C) Immunoblot analysis of total cell extracts from spleen and thymus of mice wild-type, heterozygous, or homozygous-deficient for the *Stat6* gene. As a control, filters were also probed with an antibody specific for Stat1.

cassette containing the neomycin resistance gene (Figure 1A). This region of the *Stat6* gene encodes the SH2 domain required for Stat dimerization, thereby insuring that any mutant protein that may be made from the targeted locus will not be functional. The targeting construct was electroporated into D3 ES cells, and Southern (DNA) analysis of G418-resistant clones revealed that 6 of 60 clones had undergone homologous recombination at the *Stat6* locus. Correctly targeted clones were injected into BALB/c blastocyts to generate chimeras. Once the disrupted *Stat6* allele was transmitted through the germline, heterozygotes were intercrossed to generate mice homozygous for the *Stat6* mutation (Figure 1B). Despite the widespread expression of *Stat6* transcripts in adult tissues (Hou et al., 1994), *Stat6*$^{-/-}$ mice are grossly indistinguishable from their control littermates. Immunoblot analysis of cell lysates from both spleen and thymus confirmed that *Stat6*$^{-/-}$ mice do not express Stat6 protein (Figure 1C). All lymphocyte subsets in the thymus and peripheral lymphoid organs of *Stat6*$^{-/-}$ mice appear to be represented normally when analyzed by flow cytometry for CD3, CD4, CD8, and B220 (data not shown).

Stat6 Is Required for IL-4-Induced Increases in the Expression of Cell Surface Markers

Several cell surface molecules, including MHC class II antigens and the IL-4R are known to be up-regulated on lymphocytes in response to IL-4 (Noelle et al., 1984; Ohara and Paul, 1988). To examine the role of Stat6 in the regulation of IL-4-inducible genes, lymph node cells from control or *Stat6*$^{-/-}$ mice were incubated for 18 hr in 1,000 U/ml IL-4 and then examined by flow cytometry. As shown in Figure 2, B cells from control mice show a striking increase in their cell surface expression of MHC

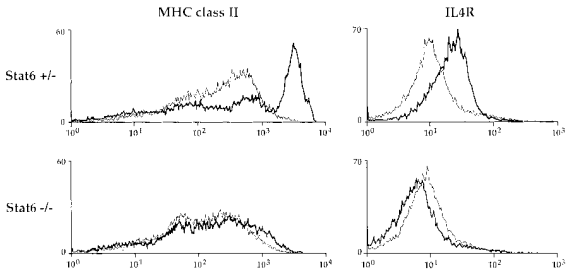

Figure 2. Flow Cytometric Analysis of Lymphocytes From Control and *Stat6*$^{-/-}$ Mice

Lymph node cells from heterozygous or Stat6 homozygous-deficient mice were incubated for 18 hr in either medium alone (fine lines) or medium plus 1,000 U/ml IL-4 (bold lines). Cells were stained with either a cocktail of antibodies specific for I-A^d, I-E^d, and I-A^b and analyzed for MHC class II expression on B220$^+$ cells (left) or with an antibody specific for the α chain of the IL-4R (right). Log fluorescence intensity is shown on the X axis and cell number on the Y axis.

class II molecules in response to stimulation with IL-4, while both B and T cells demonstrate an increase in the expression of IL-4R. In contrast, no induction of these genes is seen in lymphocytes from *Stat6*$^{-/-}$ mice in response to IL-4. The basal level of expression of these genes on lymphocytes from control and *Stat6*$^{-/-}$ mice is similar, and lipopolysaccharide stimulation of B lymphocytes from *Stat6*$^{-/-}$ mice and their control littermates leads to an equivalent up-regulation in the cell surface expression of MHC class II antigens (data not shown). These results demonstrate that Stat6 is required for the IL-4-induced increase in expression of genes regulated by this cytokine.

Lymphocytes From *Stat6*$^{-/-}$ Mice Have a Greatly Impaired Proliferative Response to IL-4

As mentioned above, cell lines transfected with an IL-4R mutant that is incapable of inducing the tyrosine phosphorylation of IRS-2 have an impaired ability to proliferate in response to IL-4 (Keegan et al., 1994). Similar transfectants containing a truncated IL-4R incapable of mediating the tyrosine phosphorylation of Stat6 retain their mitogenic response to IL-4 (Keegan et al., 1994; Quelle et al., 1995). These results suggested that the IRS-2 signaling pathway, rather than the Stat6 pathway, is important for the proliferative response to IL-4. To examine more definitively the role of Stat6 in mediating the proliferation of lymphocytes in response to IL-4, lymph node cells from *Stat6*$^{-/-}$ mice and control littermates were stimulated for 48 hr in the presence of graded doses of IL-4 and subsequently assayed for [^{3}H]thymidine incorporation. As shown in Figure 3A, the proliferative response of lymphocytes from *Stat6*$^{-/-}$ mice is almost completely abrogated. In contrast with lymphocytes from control mice, which show a 40-fold increase in proliferation in response to 1,000 U/ml IL-4, the proliferative response of lymphocytes from *Stat6*$^{-/-}$ mice is only 4- to 5-fold over background at this concentration of IL-4 and undetectable at 100 U/ml IL-4. No differences were observed in the proliferation of lymphocytes from *Stat6*$^{-/-}$ mice or control littermates to graded doses of IL-2, whose receptor uses the same γc chain as IL-4 (Figure 3B), or in their proliferation in response to either anti-CD3 or lipopolysaccharide (data not shown).

It is noteworthy that the ability of mutant forms of the IL-4R both to induce the phosphorylation of signaling molecules and to generate a proliferative response was assayed using transfectants of a myeloid progenitor cell line (Keegan et al., 1994; Quelle et al., 1995). Therefore, the apparently greater importance of IRS-2 in mediating proliferative signals in myeloid cells in response to IL-4 may reflect differences in the cell types examined. However, these results do not preclude a role for IRS-2 in mediating the proliferative signal in lymphocytes. Indeed, the small amount of proliferation of *Stat6*$^{-/-}$ lymphocytes to high concentrations of IL-4 may be due to signals generated through other pathways, such as that involving IRS-2. Additionally, we cannot rule out the possibility that the reduced proliferation of *Stat6*$^{-/-}$ lymphocytes in response to IL-4 results from the inability of IL-4 to induce the up-regulation of IL-4R and amplify the proliferative response. Additional studies will be required to address this issue. Nevertheless, our data clearly demonstrate that a normal IL-4-induced proliferative response in lymphocytes is dependent on Stat6. Furthermore, these results are consistent with previous observations suggesting that STAT proteins may be involved in transformation and cell growth (Danial et al., 1995; Migone et al., 1995; Yu et al., 1995).

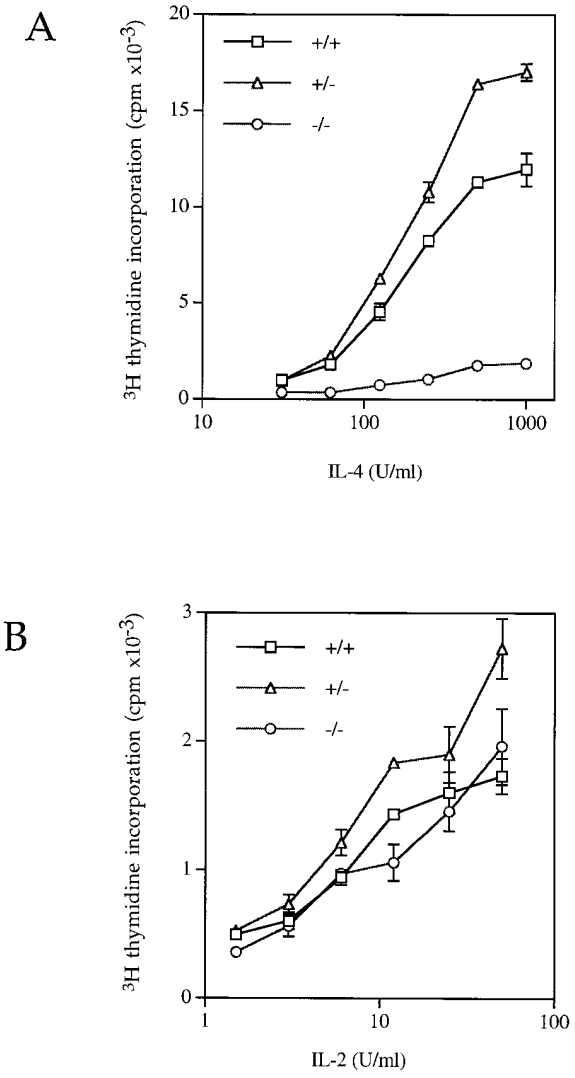

Figure 3. Proliferation of Lymphocytes from Control or *Stat6*$^{-/-}$ in Response to IL-4 or IL-2

Lymph node cells were stimulated with increasing doses of either (A) IL-4 or (B) IL-2 for 48 hr and pulsed for the last 18 hr of culture with [^{3}H]thymidine. Symbols indicate the mean of triplicate wells ± SEM.

Stat6 Is Required for the Generation of an IgE Response

IL-4 has been demonstrated to be essential for the generation of IgE in response to a number of antigenic challenges, including immunization with anti-IgD (Conrad et al., 1990; Finkelman et al., 1991). To analyze the role of Stat6 in this IL-4-mediated response, *Stat6*$^{-/-}$

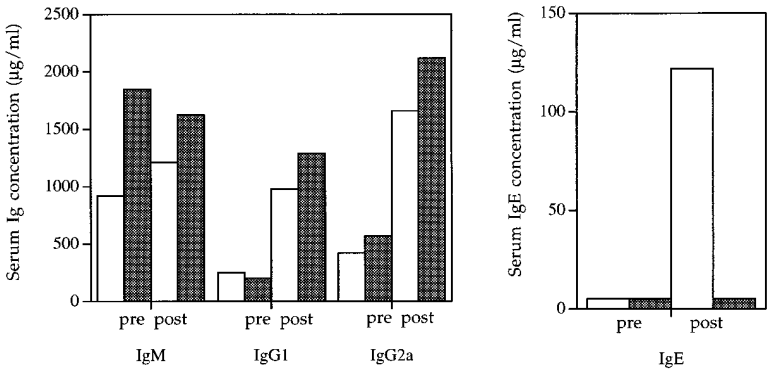

Figure 4. Analysis of Immunoglobulin Isotype Production by Control and *Stat6*$^{-/-}$ B Lymphocytes

Control (open bars) and *Stat6*$^{-/-}$ (stippled bars) mice were immunized with 200 μg anti-IgD and bled 9 days after injection. Immunoglobulin isotypes in pre- and post-bleeds were quantitated by ELISA. Concentrations of antibody are shown in micrograms/milliliter.

mice and control littermates were injected with IgD-specific monoclonal antibodies. All mice, regardless of their genotype, developed splenomegaly in response to immunization with anti-IgD (data not shown). Sera were analyzed by enzyme-linked immunosorbent assay (ELISA) for the presence of all immunoglobulin isotypes both before immunization and 9 days postinjection with anti-IgD. As shown in Figure 4, the basal levels of IgM, IgG1, and IgG2a were similar between *Stat6*$^{-/-}$ mice and control littermates. Following immunization with anti-IgD, control mice show an increase in serum levels of each of these immunoglobulin isotypes and, consistent with previous reports, a striking induction in the level of IgE is evident. While sera from *Stat6*$^{-/-}$ mice immunized with anti-IgD show an increase in the levels of IgG1 and IgG2a similar to that seen in the sera of immunized control mice, *Stat6*$^{-/-}$ mice did not have detectable levels of IgE. The induction of IgG1 in *Stat6*$^{-/-}$ mice is not surprising, since IL-4-deficient mice also have detectable levels of IgG1 (Kuhn et al., 1991) and, we have noted, an increase in serum IgG1 following anti-IgD injection is seen in IL-4-deficient mice and therefore is not dependent on IL-4 (M. H. K. and M. J. G., unpublished data).

T Lymphocytes From *Stat6*$^{-/-}$ Mice Do Not Differentiate Into Th2 Cells

To address directly the role of Stat6 in the differentiation of Th cell subsets, spleen cells from control and *Stat6*$^{-/-}$ mice were stimulated in vitro with anti-CD3 and cultured in the presence of either IL-12 and anti-IL-4 to generate Th1 cells, or IL-4 and anti-interferon-γ (IFNγ) to generate Th2 cells. After 1 week in culture, cells were washed, restimulated with anti-CD3, and culture supernatants were assayed for the presence of various cytokines by ELISA. Th1 cell differentiation is unimpaired in *Stat6*$^{-/-}$ mice as evidenced by their ability to produce similar amounts of IFNγ and granulocyte/macrophage colony-stimulating factor compared with that produced by control cells cultured under identical conditions (Figure 5A). As expected, barely detectable levels of IL-4 and IL-5 are produced by cells cultured under Th1-inducing conditions irrespective of their genotype (data not shown). When lymphocytes from control mice were cultured under conditions that favor the generation of Th2 cells and subsequently assayed for their ability to produce IL-4 and IL-5, they were found to secrete high levels of these cytokines (Figure 5B). In contrast, lymphocytes from *Stat6*$^{-/-}$ mice demonstrated an inability to produce IL-4 and IL-5 when cultured under identical conditions (Figure 5B). Thus, T lymphocytes from *Stat6*$^{-/-}$ mice are almost completely impaired in their ability to differentiate into Th2 cells, a result consistent with the notion that Stat6 is essential for mediating the differentiation signals induced by IL-4.

It is noteworthy that the impairment in Th2 development seen in *Stat6*$^{-/-}$ mice is more pronounced than that observed in IL-4-deficient mice. Despite the importance of IL-4 in mediating the differentiation of Th2 cells, the limited generation of these cells can be detected in IL-4-deficient mice in response to infection with Nippostrongylus brasilienis (Kopf et al., 1993) and following in vitro differentiation (M. H. K. and M. J. G., unpublished data). The more profound defect in Th2 development seen in *Stat6*$^{-/-}$ mice suggests that a cytokine other than IL-4 may be capable of inducing the differentiation of Th2 cells, but that, like IL-4, this cytokine generates differentiation signals through Stat6. IL-13 is one such candidate, since this cytokine shares many properties with IL-4. Recent studies have shown that the α chain of the IL-4R is also involved in the formation of the high affinity IL-13 receptor (Lin et al., 1995; Zurawski et al., 1995; Hilton et al., 1996). Furthermore, while IL-13 does not induce T cell proliferation, IL-13 stimulation does lead to the tyrosine phosphorylation of Stat6 (M. H. K., U. S., and M. J. G., unpublished data).

To determine whether IL-13 is capable of inducing the differentiation of Th2 cells and, if so, whether this process is mediated by Stat6, spleen cells from control and *Stat6*$^{-/-}$ mice were stimulated in vitro with anti-CD3 and cultured in the presence of IL-13 and anti-IFNγ. As shown in Figure 5C, lymphocytes from control mice stimulated in the presence of IL-13 developed into Th2 cells, as evidenced by their production of high levels of IL-4 and IL-5 following subsequent restimulation. In contrast, IL-13 was not capable of generating Th2 cells from *Stat6*$^{-/-}$ lymphocytes. These results demonstrate that IL-13 is indeed capable of inducing the differentiation of Th2 cells, and that Stat6 is essential for the differentiation signals generated by both IL-4 and IL-13 in T cells. Furthermore, the ability of IL-13 to drive the differentiation of Th2 cells may account for the presence of Th2 responses in IL-4-deficient mice.

Taken together, our results with *Stat6*$^{-/-}$ mice demonstrate a critical role for this transcription factor in mediating IL-4-induced responses in lymphocytes. Other signaling pathways, including that involving the tyrosine

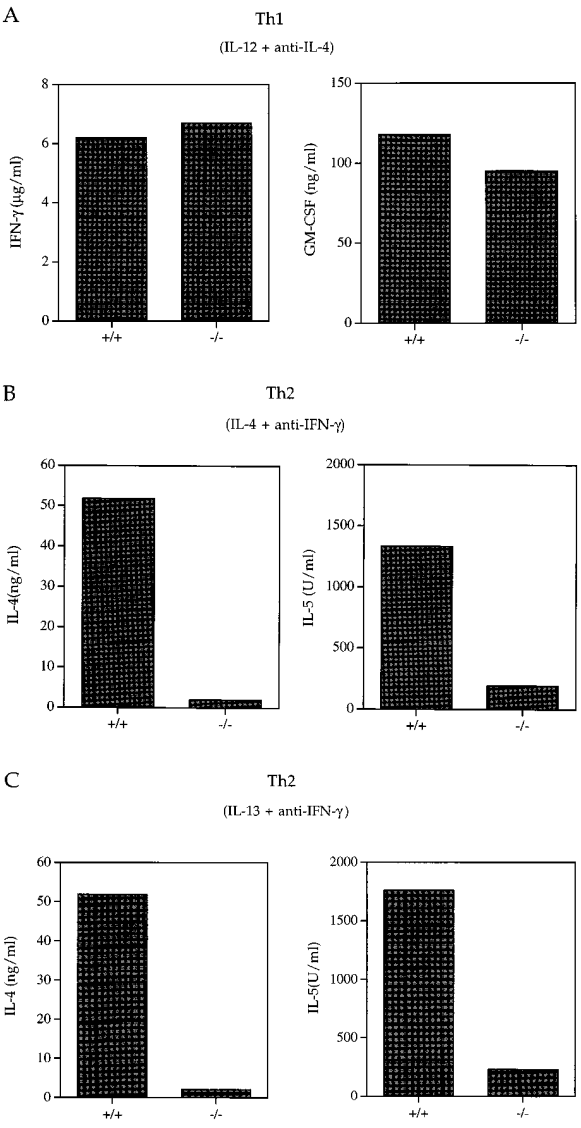

Figure 5. Cytokine Analysis of In Vitro Differentiated T Lymphocytes from Control and *Stat6*$^{-/-}$ Mice

Spleen cells from control and *Stat6*$^{-/-}$ mice were activated with anti-CD3 and cultured under conditions that skew their development towards either (A) Th1 cells (IL-12 plus anti-IL-4), (B) Th2 cells (IL-4 plus anti-IFNγ), or (C) Th2 cells (IL-13 + anti-IFNγ). After 7 days in culture, cells were restimulated with anti-CD3 and supernatants were collected 24 hr later and analyzed for the presence of cytokines by ELISA. The amount of IL-4 and IL-5 produced by Th2-differentiated cells from *Stat6*$^{-/-}$ mice was at or below the level of detection (2 ng/ml, IL-4; 50 U/ml, IL-5).

phosphorylation and activation of other STATs or IRS-2, clearly cannot compensate for Stat6. The role of these latter pathways in mediating cytokine responses in both lymphocytes and other hematopoietic cells remains to be determined.

Our results also suggest that Stat6 is required for mediating the differentiation signals leading to the development of Th2 cells in response to IL-4. Interestingly, the defect in Th2 development seen in *Stat6*$^{-/-}$ mice is more severe than that seen in IL-4-deficient mice. These results may be explained by the observation that, like IL-4, IL-13 is also capable of inducing the Stat6-dependent differentiation of Th2 cells. *Stat6*$^{-/-}$ mice will be useful in identifying genetic programs necessary for the development of Th2 cells.

Experimental Procedures

Stem Cells and Blastocyst Injections

A gene targeting construct containing approximately 1 kb of 5′ flanking sequence and 3 kb of 3′ flanking sequence of the *Stat6* gene was generated. The targeting construct (20 μg) was electroporated into D3 ES cells using a Bio-Rad gene pulser set at 25 μF/350 V. After 9 days of selection in 180 μg/ml G418 and 2 μM gancyclovir, drug-resistant clones were picked into 24-well plates and expanded in culture. Screening for correctly targeted clones was done by Southern (DNA) analysis and, once identified, they were injected into 3.5 day postcoital BALB/c blastocysts.

Immunoblot Analysis

Total cellular extracts were prepared as previously described (Quelle et al., 1995), except that following centrifugation, extracts were dialyzed against 1 XD (Dignam et al., 1983). Protein (20 μg) was separated on an 8% polyacrylamide gel, and electrophoretically transferred onto nitrocellulose. The filter was blocked in 10% dry milk in 1× TBST for 14 hr and subsequently probed with antisera directed against recombinant Stat6 (Schindler et al., 1995) at a 1:10,000 dilution for 1 hr. Stat1 anti-serum was produced against the C-terminal 33 aa (Schindler et al., 1995). Protein bands were detected by enhanced chemiluminescence (Amersham, Arlington Heights, Illinois).

Antibodies and Cytokines

Antibodies to CD3 (145-2C11) and fluorescein isothiocyanate– and phycoerythrin-conjugated antibodies to I-A^d, I-E^d, I-A^b, and B220 were purchased from Pharmingen (San Diego, California). Recombinant IL-4 and antibody specific for IL-4R were purchased from Genzyme (Cambridge, Massachusetts). A fluorescein isothiocyanate–conjugated goat anti-rat immunoglobulin was purchased from KPL (Gaithersburg, Maryland) and used as a secondary reagent for the IL-4R antibody. Mouse monoclonal antibodies specific for IgD^a (Finkelman et al., 1991) were the gift of Dr. F. Finkelman. Anti-IL4 (11B11) was the gift of Dr. W. Paul. Anti-IFNγ was purified from supernatant of the XMG-1 hybridoma. Recombinant IL-2 was obtained from Boehringer-Mannheim (Indianapolis, Indiana). Recombinant IL-12 was the gift of Dr. M. Gately. Recombinant IL-13 was purchased from R and D Systems (Minneapolis, Minnesota).

Flow Cytometry

Single cell suspensions were made from the indicated tissues. Cells (1×10^6) were incubated on ice with 1 μg monoclonal antibody for 1 hr and washed before analysis on a Becton-Dickenson FACScan.

Proliferation Assays

Lymph node cells were plated at 5×10^4/round-bottomed microwell with dilutions of the indicated lymphokine in RPMI 1640 (Mediatech, Herndon, Virginia) supplemented with 10% fetal calf serum (Hyclone, Logan, Utah), penicillin–streptomycin, sodium pyruvate, nonessential amino acids, L-glutamine, HEPES (all from Mediatech) and 5×10^{-5} M 2-mercaptoethanol. Cells were pulsed with 1 μCi [^{3}H]thymidine (New England Nuclear, Boston, Massachusetts) for the last 18 hr of a 48 hr culture period. Spleen cells were incubated at 10^5/well for lipopolysaccharide and anti-CD3 stimulations.

In Vivo IgE Production

Mice were injected with 100 μg of two anti-allotypic anti-IgD monoclonals in 200 μl phosphate-buffered saline. Mice were bled by the tail vein to obtain preimmunization serum. At 9 days postinjection, mice were sacrificed and bled by cardiac puncture. Immunoglobulin isotypes were analyzed by ELISA as previously described (Markowitz et al., 1993).

In Vitro T Cell Differentiation

For in vitro differentiation assays, 2×10^6 spleen cells/ml were cultured in medium as above and stimulated with 1 μg/ml plate-bound anti-CD3 and incubated with 10 μg/ml anti-IL-4 plus 100 U/ml IL-12 (Th1) or 10 μg/ml anti-IFNγ (Th2). After activation (24 hr), we added 50 U/ml of IL-2 to all cultures and 1,000 U/ml IL-4 or 25 pg/ml IL-13 to Th2 cultures. Cultures were supplemented with fresh medium after 4 days, washed, and restimulated after 7 days with plate-bound anti-CD3 in the absence of any additional reagents. Supernatants were collected 24 hr later. Cytokine ELISAs were performed as previously described (Kuchroo et al., 1995).

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Note Added in Proof

Similar observations have been made by Drs. Kishimoto, Akira, and colleagues on mice selectively deficient in Stat6. We thank Drs. Kishimoto and Akira for sharing such observations with us prior to publication.