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Expression of a Constitutively Active Stat6 In Vivo Alters Lymphocyte Homeostasis with Distinct Effects in T and B Cells

Heather A. Bruns,* Ulrike Schindler,‡ and Mark H. Kaplan2*

IL-4 is a critical cytokine in the regulation of immune responses and genesis of atopy. Engagement of the IL-4R activates multiple signaling pathways, including the transcription factor Stat6. Stat6-deficient mice demonstrate the importance of this factor in lymphocyte proliferation, gene expression, and Th cell differentiation. Recently, a mutant Stat6 (Stat6VT) was generated that is transcriptionally active independent of IL-4 stimulation. To determine the ability of a constitutively active Stat6 to mimic IL-4-stimulated responses, we have generated transgenic mice expressing Stat6VT under control of the CD2 locus control region, restricting expression to lymphoid populations. The phenotype of Stat6VT transgenic mice is similar, but not identical, to IL-4 transgenic mice, suggesting a critical role for Stat6-independent signaling pathways in the generation of some IL-4 responses in vivo. The expression of a constitutively active Stat6 in vivo increases surface expression of IL-4-induced genes and increases serum levels of IgG1 and IgE, compared with nontransgenic mice. Stat6VT expression increases Th2 differentiation in vivo and in vitro. Stat6VT expression also dramatically alters homeostasis of peripheral lymphocyte populations resulting in decreased CD3+ cells and increased B220+ cells, compared with nontransgenic littermates. Altered T and B cell populations correlate with an activated phenotype and increased cell death in transgenic T cell, but not B cell, populations. Together these results suggest that expression of a constitutively active Stat6 has distinct effects on B and T lymphocytes. The Journal of Immunology, 2003, 170: 3478–3487.

IL-4 specifically activates the transcription factor Stat6 via the Janus kinase (Jak)3–Stat pathway (23–25). After IL-4 stimulation, the Jak3 phosphorylates tyrosine residues within the IL-4Rα and γc receptor chains (26, 27). Stat6 is then recruited to the phosphorylated receptor through a Src homology (SH)2 domain and phosphorylated on Y-641 (28, 29). The phosphorylated monomers then dimerize, translocate to the nucleus, and bind DNA to activate transcription. The importance of Stat6 in mediating IL-4 signaling has been demonstrated using Stat6-deficient mice. Experiments using these mice have shown that most IL-4-mediated functions are lost in the absence of Stat6 (30–32). B cells are unable to switch Ig production to IgE. There is no induction of IL-4-induced cell surface markers, and lymphocytes have impaired proliferative responses to IL-4. Furthermore, T cells from Stat6-deficient mice are greatly reduced in their ability to differentiate into Th2 cells. Recently, a mutant Stat6, where valine and threonine in the SH2 domain are converted to alanines, was generated and designated Stat6VT (33). Stat6VT is constitutively phosphorylated and transcriptionally active in the absence of IL-4. Studies done to characterize Stat6VT revealed that it is constitutively phosphorylated on the critical tyrosine residue, Y-641, and that this phosphorylation is essential for the dimerization of Stat6VT and its ability to activate transcription. The mutation causes a conformational change in the protein that does not allow association of Stat6VT with the unphosphorylated IL-4R chains. Furthermore, the constitutive activity of Stat6VT is independent of phosphorylation by the IL-4-activated kinases Jak1 and Jak3.

To determine the ability of a constitutively active Stat6 to mimic IL-4-stimulated functions and alter in vivo immune function, we have generated transgenic mice expressing Stat6VT only in lymphoid tissues. In this report, we have found that Stat6VT is sufficient to alter lymphocyte homeostasis, increasing peripheral B cell populations and decreasing T cell populations. This is due, in part, to increased apoptosis by Stat6VT T lymphocytes. Expression of

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3 Abbreviations used in this paper: Jak, Janus kinase; SH, Src homology; LCR, locus control region; DN, double negative; MFI, mean fluorescence intensity.
Stat6VT in B lymphocytes leads to increased expression of IL-4-induced genes and increased production of IgG1 and IgE, but does not result in B cell activation. In T lymphocytes, Stat6VT expression skews T cell differentiation toward the Th2 phenotype, and strikingly, induces the activation of T cells, characterized by cell surface marker expression and increased proliferative response. Thus, a constitutively active Stat6 has distinct effects on B and T cell populations.

Materials and Methods

Generation of Stat6VT transgenic mice

Stat6, with a VT/AA mutation at amino acids 625 and 626, was previously described (33, 34) and was engineered with a C-terminal FLAG tag. The vector containing the Stat6VT cDNA was digested with EcoRI and the fragment was cloned into VACD2, a plasmid containing the CD2 locus control region (LCR) (35, 36). The CD2:Stat6VT vector was digested with KpnI and XhoI. The resulting 15.7-kb fragment was purified and used for the generation of transgenic mice. Mice were generated by the Indiana University Transgenic Facility (Indianapolis, IN) on a C3H genetic background. Transgene-positive founders were backcrossed to C57BL/6 mice (Harlan Bioproducts, Indianapolis, IN) were used between 6 and 12 wk of age.

Phenotyping of Stat6VT transgenic mice

Tail DNA was purified by phenol:chloroform extraction, digested with BamHI, separated on a 0.8% agarose gel, and transferred onto a nylon transfer membrane (Schleicher & Schuell, Keene, NH). The membrane was prehydrated for 4 h at 42°C, and hybridization was performed with a 32P-labeled Stat6 cDNA probe at 42°C for 18 h. The membrane was first washed in 2× SSC containing 0.1% SDS at 60°C for 30 min, then in 0.2× SSC containing 0.1% SDS for 30 min to 1 h. After washing, the membrane was exposed to x-ray film overnight at −80°C. The copy number of the transgene expressed in each founder relative to the signal from wild-type Stat6 was determined using densitometry. For analysis of protein expression, whole cell extracts (5 × 10⁷−1 × 10⁸ cells per sample) from various tissues were generated as previously described (37). Lysates were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membrane was blocked for 1 h with 1/1000 diluted PBS (Life Technologies, Rockville, MD) at pH 7.0 or pH 4.0 for 2 h and incubated with goat anti-mouse alkaline phosphatase-conjugated secondary Ab (Bio-Rad, Hercules, CA) for 30 min, then washed. The protein was detected using an ECL kit (Bio-Rad). The blots were stripped, then reprobed with a monoclonal anti-FLAG Ab (Bioss, Saco, ME) diluted 1/5000.

Affinity purification of DNA binding proteins

A 5′-biotinylated oligonucleotide sequence (TGTAATTCCGTGCGACTAATGC, TATG), isolated by binding site selection as described previously (28), was coupled to streptavidin-conjugated agarose beads. Per sample, 3 μg of biotinylated oligonucleotide was incubated with 40 μl of 50% streptavidin-conjugated bead slurry in a total volume of 100 μl of a lysis buffer comprising 50 mM Tris-HCl, pH 8.0, 0.5% Igepal, 15 mM NaCl, 0.1 mM EDTA, 10% glycerol, 10 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF, 1 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml chymostatin for 2 h at 4°C. Whole cell extract (400 μg) was incubated with 40 μl of DNA-coupled agarose beads in lysis buffer at a total volume of 500 μl for 3 h at 4°C. Complexes were washed once in lysis buffer before elution by boiling in miniblotting buffer. Proteins were separated on a 10% SDS-PAGE and transferred as described above. Western blot analysis was performed using an anti-Stat6 mAb (BD Transduction Laboratories, Lexington, KY) diluted 1/5000.

Cellular phenotypic analysis by flow cytometry

Cells (1 × 10⁴–3 × 10⁶ per sample) were washed and stained in PBS with 2% BSA and 0.1% NaN₃ (FACS buffer). Cells were first incubated with anti-Fc-μR Abs, clone 2.4G2 (BD PharMingen, San Diego, CA) for 10 min. All staining was done at 4°C followed by one wash with FACS buffer, then fixed in PBS with 2% BSA, 0.1% NaN₃, and 0.5% formaldehyde. Samples stained with Abs directly conjugated to FITC, PE, or CyChrom (BD PharMingen; IgM, IgE, CD23 from eBioscience, San Diego, CA) were incubated for 15 min. Samples stained with Abs not directly conjugated, IL-4R (R&D Systems, Minneapolis, MN) and anti-CD40 clone 3/23 (BD PharMingen) were incubated for 30 min with the primary Ab, washed, and then incubated with a goat anti-rat PE-conjugated secondary Ab (BD PharMingen) for 5 min then washed and fixed. For propidium iodide staining, cells were stained as described above and resuspended in buffer without fixative. Five minutes before analysis, 2.5 μg of propidium iodide was added.

Isolation of splenic B220⁺ cells

Spleens were obtained from mice and ground into a cell suspension that was treated with RBC lysis solution (Sigma-Aldrich) for 5 min. The remaining cells were washed in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated FBS (HyClone Laboratories, Logan, UT), penicillin-streptomycin, sodium pyruvate, nonessential amino acids, L-glutamine, HEPES (all from BioWhittaker) and 5 × 10⁻⁵ M 2-ME (Sigma-Aldrich). B220⁺ cells from splenocytes were purified with rat anti-mouse B220 microbeads (Miltenyi Biotec, Auburn, CA) according to manufacturer’s protocol. The purified cells were then collected and analyzed by flow cytometry (FACScan; BD Biosciences, Mountain View, CA) to determine the purity of the population (>95%).

In vitro T cell stimulation

Spleenic and lymph node CD4⁺ T cells were purified with rat anti-mouse CD4 microbeads (Miltenyi Biotec) according to manufacturer’s protocol. The purified cells were then stimulated in media at a 1 × 10⁶ cell/ml with 4 μg/ml anti-CD3 (clone 2C11) and 1 μg/ml anti-CD28 (clone 37.51; BD PharMingen). After 3 days, supernatants were harvested and analyzed for cytokine production by ELISA as previously described (38). For the in vitro Th cell differentiation assay, pooled splenocytes and lymph nodes were first enriched for CD4⁺ cells by negative selection. Abs to CD8 (clone 2.43), MHC class II (clone M5/114.15.2), CD16/CD32 (clone 2.4G2), and B220 (BD PharMingen) were incubated (1 μg/ml) with cells (5 × 10⁶/ml) at 4°C. After 30 min, cells were washed and incubated with goat anti-IgG magnetic beads (Polysciences, Warrington, PA) for 30 min at 4°C. Purified cells were plated at 4 × 10⁶ cells/ml and stimulated with 4 μg/ml anti-CD3 (2C11) and 1 μg/ml anti-CD28 (clone 37.51; BD PharMingen). After 6 days, cells were harvested, plated at 200,000 cells/0.5 ml and restimulated with 4 μg/ml anti-CD3. After 48 h, supernatants were harvested and cytokine production was analyzed by ELISA.

Acid stripping

Freshly isolated B lymphocytes (5 × 10⁶) from transgenic mice were incubated in PBS (Life Technologies, Rockville, MD) at pH 7.0 or pH 4.0 for 20 min on ice, then washed once in FACS buffer, and analyzed as described above.

Proliferation assay

Purified CD4⁺ or B220⁺ splenocytes were plated in increasing numbers (20,000–80,000/well) in a 96-well dish with or without 5 ng/ml IL-4 and immediately pulsed with 0.8 μCi [³H]thymidine. Cells were incubated at 37°C for 24 h then harvested and counted in a scintillation counter for [³H]thymidine incorporation.

RNase protection assay

RNA was isolated from CD4⁺ or B220⁺ splenocytes by TRIzol reagent (Life Technologies). RNA (5 μg) was used for a MultiProbe RNase protection assay (BD PharMingen) according to the manufacturer’s protocol.

Ab isotype ELISA

Serum levels of Ig isotypes were analyzed by ELISA using IgM, IgG2a, IgE (BD PharMingen), and IgG1 (Southern Biotechnology Associates, Birmingham, AL) Abs according to manufacturer’s recommendation.

Results

Generation of Stat6VT transgenic mice

Stat6VT transgenic mice were generated using the CD2 LCR, which restricted expression of the transgene primarily to B and T lymphocytes (35, 36). The Stat6VT cDNA, with a FLAG epitope at its C-terminal end, was inserted into the VACD2 plasmid between the CD2 promoter and CD2 LCR (Fig. 1A). Six founders positive for the Stat6VT transgene had similar phenotypes and two founder lines were analyzed in detail. Southern analysis of genomic DNA from the founder lines was used to determine the copy number of the integrated transgene relative to wild type (Fig. 3479The Journal of Immunology [3] downloaded from http://www.jimmunol.org by guest on July 27, 2017
1B). Cell lysates from the spleens, lymph nodes, and thymi of transgenic mice blotted with an anti-FLAG Ab demonstrated that both lines, CD2:Stat6VT(24) and (78), expressed Stat6VT in the spleen and lymph nodes and at a higher level in the thymus (Fig. 1C). Immunoblot analysis of cell lysates from various tissues confirmed that expression of Stat6VT was limited to lymphoid tissues in these animals (data not shown). To confirm that Stat6VT was activated, dimerized Stat6 molecules were precipitated from thymocytes and purified B220<sup>+</sup> cell lysates from the spleens of wild-type (nontransgenic littermates) and transgenic mice using an agarose-coupled high affinity Stat6 DNA binding site. The presence of Stat6 in the B cell and thymocyte cell lysates from Stat6VT transgenic mice and not from wild-type mice demonstrated that Stat6VT was constitutively activated in each of the founder lines (Fig. 1D).

CD2:Stat6VT mice have altered lymphoid homeostasis

The CD2:Stat6VT mice were indistinguishable from their control littermates except for consistently enlarged spleens and, less frequently, enlarged lymph nodes. Analysis of lymphocyte populations within the bone marrow of wild-type and transgenic mice by flow cytometry indicated that B cell development was normal (data not shown). Additionally, thymic development appeared grossly normal, and there was no significant change in the cellularity of the thymus in the CD2:Stat6VT mice compared with wild-type mice. However, as CD2:Stat6VT mice increased in age, there was an increase in the CD4<sup>-</sup>CD8<sup>-</sup> double-negative (DN) and CD4<sup>+</sup> single-positive thymic populations in comparison to controls (Fig. 2A). The increased DN thymocytes from older CD2:Stat6VT mice correlated with a reduced number of DN CD25<sup>+</sup>CD44<sup>low</sup> cells and an increased number of DN CD25<sup>+</sup>CD44<sup>low</sup> cells compared with wild-type thymocyte populations (data not shown). These results suggest that expression of Stat6VT has only minor effects on the maturation of thymocytes.

Despite the normal development of B and T lymphocytes within the bone marrow and thymus, analysis of peripheral lymphocyte populations by FACS revealed that in comparison to control mice, there was an expanded B220<sup>+</sup> cell population (wild type: 43 ± 7%, CD2:Stat6VT(24): 70 ± 15% (p < 0.001 vs wild type), CD2: Stat6VT(78): 65 ± 15% (p < 0.01 vs wild type)) and decreased CD3<sup>+</sup> population (wild type: 29 ± 11%, CD2:Stat6VT(24): 8 ± 2% (p < 0.002 vs wild-type), CD2:Stat6VT(78): 6 ± 2% (p < 0.003 vs wild-type)) in the spleens (Fig. 2B) and lymph nodes (data not shown) of CD2:Stat6VT(24) and (78) mice. To explore the altered peripheral lymphocyte populations, we examined the amount of cell death in B and T cells from wild-type and transgenic mice. Splenocytes from these mice were isolated and stained with propidium iodide and Abs to either B220 or CD3. Uptake of

1B). Cell lysates from the spleens, lymph nodes, and thymi of transgenic mice blotted with an anti-FLAG Ab demonstrated that both lines, CD2:Stat6VT(24) and (78), expressed Stat6VT in the spleen and lymph nodes and at a higher level in the thymus (Fig. 1C). Immunoblot analysis of cell lysates from various tissues confirmed that expression of Stat6VT was limited to lymphoid tissues in these animals (data not shown). To confirm that Stat6VT was activated, dimerized Stat6 molecules were precipitated from thymocytes and purified B220<sup>+</sup> cell lysates from the spleens of wild-type (nontransgenic littermates) and transgenic mice using an agarose-coupled high affinity Stat6 DNA binding site. The presence of Stat6 in the B cell and thymocyte cell lysates from Stat6VT transgenic mice and not from wild-type mice demonstrated that Stat6VT was constitutively activated in each of the founder lines (Fig. 1D).

FIGURE 1. Analysis of Stat6VT expression in transgenic mice. A, Schematic of the construct used to generate the Stat6VT transgenic mice and diagram of the Stat6VT protein, indicating the critical tyrosine residue and VT mutation. ID, interaction domain; CCD, coiled-coil domain; DBD, DNA binding domain; LD, linker domain; TAD, transactivation domain. B, Southern analysis of tail DNA from each founder line digested with BamHI, which yielded a 2.8-kb fragment that hybridized with the Stat6 cDNA probe. The numbers in each lane represent the copy number of the transgene relative to wild type. C, Total cell extracts (50 µg) from the indicated tissues from CD2:Stat6VT mice were immunoblotted with anti-FLAG Ab and anti-GAPDH as loading control. D, Western analysis of DNA-binding Stat6 molecules precipitated from 200 µg of whole cell extract generated from either thymocytes (upper panel) or purified B220<sup>+</sup> cells (lower panel).

FIGURE 2. FACS analysis of lymphocyte populations from CD2:Stat6VT mice. A, Thymocytes from wild-type and transgenic mice, either 9- or 12-wk-old, were analyzed by FACS following staining with the indicated Abs. Numbers indicate the percentages of cells in each quadrant. B, Splenocytes from wild-type and transgenic mice were analyzed by FACS following staining with the indicated Abs. The percentages of B220<sup>+</sup> and CD3<sup>-</sup> cells are indicated.
propidium iodide results from a breakdown in the cell membrane, an indicator of cell death. We found that while B lymphocytes from transgenic mice had comparable or decreased levels of cell death in comparison to wild-type B cells, CD2:Stat6VT T cells had a marked increase in cell death compared with controls (Fig. 3A). To examine a mechanism for these alterations, we determined the expression of several apoptosis-related genes. Using flow cytometry, we stained freshly isolated splenocytes from wild-type and transgenic mice for CD3 or B220 and Fas or Fas ligand expression and found no difference in the levels of expression between wild-type and CD2:Stat6VT B and T cells (data not shown). We also isolated RNA from wild-type and transgenic B and T lymphocytes and performed RNase protection assays. Using a multiprobe template set, we first examined the expression of caspase family members (Fig. 3B) and found no difference in the expression of any of the genes between the four cell types. We then looked at the expression of Bcl-2 family members (Fig. 3C). Although there appeared to be no significant difference in expression of many of the Bcl-2 family member genes among the cell types, Bcl-x was found to be absent or present at low levels in wild-type B cells but distinctly up-regulated in transgenic B cells. This is consistent with a recent report that demonstrates that the protective effects of IL-4 on B lymphocytes are due to the Stat6-dependent up-regulation of Bcl-x (17). In contrast, the decreased peripheral T cell population does not appear to be the result of the ability of a constitutively active Stat6 to regulate the expression of common apoptosis-related genes.

Stat6VT is sufficient to up-regulate expression of cell surface markers whose expression is normally increased after stimulation with IL-4

Transgenic mice overexpressing IL-4 under the control of the Ig promoter/enhancer, also have altered peripheral lymphocyte populations, similar to our findings with the CD2:Stat6VT mice (39). Furthermore, the phenotypes of B and T cells in the IL-4 transgenic mice were altered. To examine the ability of Stat6 to mediate these effects, we examined the phenotypes of B and T lymphocytes from Stat6VT transgenic mice. Cell surface markers, such as MHC class II and IL-4R, have been shown to be up-regulated by IL-4 stimulation, and this increased expression was shown to be dependent upon Stat6 (30–32). To examine the effects of a constitutively active Stat6 on the expression of these genes, we analyzed the expression of MHC class II and IL-4R on splenocytes from wild-type and CD2:Stat6VT mice incubated for 48 h in the presence or absence of IL-4 by flow cytometry. As demonstrated in Fig. 4, MHC class II and IL-4R were expressed at low levels on wild-type B220+ cells in the absence of IL-4. After stimulation with IL-4, the expression of these genes was increased. In contrast, MHC class II and IL-4R were expressed at higher levels on B220+ cells from transgenic mice even in the absence of IL-4, while treatment with IL-4 had little effect. The expression of other cell surface markers, such as CD80 and CD86, has also been shown to be increased on B lymphocytes after stimulation with IL-4 (12), and we have determined that this response is Stat6-dependent (data not shown).

FIGURE 3. Analysis of cell death and expression of apoptosis-related genes. A, Freshly isolated splenocytes were stained with either Abs to CD3 or B220 and propidium iodide, then analyzed by FACS; % cell death indicates the percent of cells that are propidium iodide-positive. B and C, RNA was isolated from wild-type and transgenic B or T cells and used for a MultiProbe RNase protection assay (BD PharMingen). Templates for caspase (B) and Bcl-2 (C) family members were used to determine expression levels. Densitometric analysis revealed no differences in the expression of genes in T cells.
To examine the effect of Stat6VT on the expression of these genes, splenocytes from wild-type and transgenic mice were treated and analyzed as above. There was a low level of CD86 expression on B220\(^{+}\) wild-type cells in the absence of IL-4, but after stimulation with IL-4, CD86 expression was significantly increased (Fig. 4). B220\(^{+}\) cells from transgenic mice had high levels of CD86 in the absence of IL-4, and incubation with IL-4 had minimal effects. Similar results were observed for CD80 (data not shown). Thus, expression of a constitutively active Stat6 up-regulates the expression of genes normally regulated by IL-4.

**Stat6VT does not induce an activated phenotype in B lymphocytes**

It is important to note that while MHC class II, CD80, and CD86 are IL-4-inducible, they can also be indicators of B cell activation (40–42). To determine whether CD2:Stat6VT B cells are activated or simply have increased expression of Stat6-inducible genes, we analyzed the expression of other cell surface markers that indicate activation or maturation of B lymphocytes and are not regulated by IL-4. FACS analysis of freshly isolated B220\(^{+}\) cells from wild-type and CD2:Stat6VT mice showed that CD40 expression, which is up-regulated on activated or mature B lymphocytes (43–45), was increased on transgenic B cells compared with wild type (Fig. 5A). The expression of CD40 on B cells from wild-type and CD2:Stat6VT mice is not induced by IL-4, and basal expression of CD40 on Stat6-deficient B220\(^{+}\) cells was indistinguishable from levels on wild-type B cells (data not shown). However, we also compared wild-type and CD2:Stat6VT splenic B cells for the expression of CD44 (Pgp-1/Ly-24) and CD95 (Fas), whose expression is increased upon B cell activation (46–48) and CD138 (syndecan-1), which is expressed on differentiated plasma cells (49), and found no difference (data not shown). Furthermore, increased cell size is an indicator of activation, and we found no difference in cell size between wild-type and transgenic B cells (data not shown). Lastly, because lymphocyte proliferation in response to cytokine is indicative of activation, we examined the ability of wild-type and CD2:Stat6VT B cells to proliferate in response to IL-4. Purified B cells from wild-type and CD2:Stat6VT mice were plated in the presence or absence of IL-4 then immediately pulsed with \(^{3}\)H]thymidine for 24 h, harvested, and analyzed for \(^{3}\)H]thymidine incorporation. B cells from control and transgenic mice proliferated similarly in the presence or absence of IL-4 (Fig. 5B). These results taken together demonstrate that Stat6VT can alter gene expression but does not induce an activated state in B lymphocytes.

**Increased Class Switching to IgG1 and IgE in Stat6VT transgenic mice**

IL-4 is known to promote class switching in activated B cells to IgG1 and IgE, and Stat6 is essential for this IL-4-induced switch in Ig production (13, 14, 50). To analyze the effects of a constitutively active Stat6 on Ig production in vivo, we obtained sera from wild-type and transgenic mice and analyzed Ig levels by ELISA. Serum levels of IgM and IgG2a were similar between control and Stat6VT transgenic mice (Fig. 6A). However, the serum levels of

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** Analysis of cell surface marker expression on wild-type and CD2:Stat6VT B lymphocytes. A, Splenocytes from wild-type and transgenic mice were incubated for 48 h in the absence or presence of 5 ng/ml IL-4 and stained with the indicated Abs before FACS analysis. Histograms represent the expression of the indicated cell surface markers on B220\(^{+}\) splenocytes. The numbers indicate the mean fluorescence intensity (MFI) of each sample in the presence (gray line) or absence (black line) of IL-4.

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** Analysis of B lymphocyte activation. A, Expression of CD40 on freshly isolated B220\(^{+}\) splenocytes from wild-type and Stat6VT transgenic mice. MFI is indicated in the upper right corner. Data shown are representative of at least three separate experiments. B, Purified B220\(^{+}\) cells from wild-type and transgenic mice were incubated in the presence or absence of 5 ng/ml IL-4, immediately pulsed with \(^{3}\)H]thymidine for 24 h, and assayed for incorporation.
IgG1 and IgE were elevated in the transgenic mice (Fig. 6A). To further analyze Ig production in the transgenic mice, we analyzed the surface Ig expression on B220+ splenocytes from wild-type and transgenic mice. Only a small percentage of cells were IgG2a+ in wild-type mice, and that percentage did not change with expression of Stat6VT (Fig. 6B). However, there was an increase in splenic IgG1+ cells from Stat6VT transgenic mice compared with control mice (Fig. 6B). The percentages of IgG1+ B cells among the founder lines corresponded with Ig production seen in vivo where CD2:Stat6VT(78) mice had higher serum levels of IgG1 than CD2:Stat6VT(24).

Because IgE levels were also increased in the serum of transgenic mice, we also wanted to examine the percentage of IgE+ B cells. Analysis of surface Ig expression revealed that a significant percentage of B cells from Stat6VT transgenic mice were found to be both IgM and IgE double-positive (Fig. 7A). This was surprising, because under normal circumstances, it is highly unlikely that

**FIGURE 6.** Analysis of serum Ig levels and surface Ig expression. A, Sera obtained from wild-type and transgenic mice were analyzed for Ig levels by ELISA. Each point represents an individual mouse. Bars indicate average amount of serum Ig levels ± SE. B, Splenocytes from wild-type and transgenic mice were analyzed by FACS following staining with the indicated Abs. The percentage of IgG2a+ or IgG1+ cells is noted in the upper right quadrant. FACS analysis is representative of at least three separate experiments.

**FIGURE 7.** IgE+ CD2:Stat6VT B lymphocytes are the result of IgE binding of FceRII (CD23). A, FACS analysis of freshly isolated B220+ splenocytes from wild-type and transgenic mice following staining with PE-labeled anti-IgE and FITC-labeled anti-IgM. B, Wild-type B cells were incubated for 24 h in the absence or presence of 5 ng/ml IL-4. Expression of CD23 on freshly isolated B220+ splenocytes from transgenic mice. MFI for each sample is indicated. C, B220+ splenocytes from Stat6VT transgenic mice were incubated in PBS at pH 7.0 or 4.0 for 20 min, washed, and then stained with PE-labeled anti-IgE and analyzed by FACS. Histograms represent the relative expression of IgE on the surface of wild-type B220+ splenocytes. Data shown are representative of at least three separate experiments.
analysis of any one B cell would express both IgM and IgE. FcεRII (CD23) has been shown to be up-regulated on lymphocytes in response to IL-4, and this inducible expression is dependent upon Stat6 (31, 32, 51). Increased levels of CD23 on the surface of transgenic B cells, combined with increased serum IgE levels may provide an explanation for the IgM/IgE double-positive B cell phenomenon. To determine whether Stat6VT is sufficient to increase expression of CD23 on B lymphocytes, we stained B220⁺ cells from the spleens of transgenic mice with an anti-CD23 Ab and analyzed them by FACS. Wild-type cells were incubated for 48 h in the presence or absence of IL-4 then stained for expression of CD23. On wild-type B cells, there was already a significant level of CD23 expression that was further increased after incubation with IL-4. However, B cells from Stat6VT transgenic mice constitutively expressed high levels of CD23 without IL-4 stimulation (Fig. 7B), similar to other surface proteins examined in Fig. 4. To confirm that the IgM/IgE double-positive B cell phenomenon was due to the binding of IgE to CD23, transgenic B220⁺ cells were washed in PBS at neutral pH or at acidic pH. The latter releases Abs from FcRs and prevents rebinding. Cells were then analyzed for surface expression of IgE by FACS. As Fig. 7C shows, transgenic B cells washed at neutral pH remain IgE⁺, while cells washed at acidic pH had surface IgE expression at or below the levels of control cells. Surface levels of IgM and CD23 were minimally affected (Fig. 7C). Thus, a constitutively active Stat6 induces class switching to IgG1 and IgE and increases CD23 expression on B220⁺ cells, leading to the increased binding of IgE to CD23 on the B cell surface.

Expression of Stat6VT promotes Th2 differentiation

Because Stat6VT expression alters B lymphocyte function and gene expression, we wanted to examine the effects of Stat6VT expression on T lymphocyte function. Stat6-deficient mice have demonstrated the importance of Stat6 in the differentiation of naive CD4⁺ T lymphocytes into the Th2 phenotype. To analyze the effects of Stat6VT expression on T lymphocyte function, purified CD4⁺ T cells pooled from the spleens and lymph nodes of wild-type and transgenic mice were activated with anti-CD3 for 3 days.

Analysis of supernatants for cytokine production revealed that T cells from CD2:Stat6VT transgenic mice secreted increased levels of the Th2 cytokines, IL-4, -5, and -13, compared with wild-type mice (Fig. 8A). To further examine the ability of a constitutively active Stat6 to regulate Th2 differentiation, we stimulated purified CD4⁺ T cells with anti-CD3 and anti-CD28 under unskewed conditions. After 6 days, cells were harvested and restimulated with anti-CD3 for 48 h. Analysis of cytokine production demonstrated that under unskewed conditions, T cells from CD2:Stat6VT mice secreted Th2 cytokines, while wild-type T cells secreted the Th1 cytokine, IFN-γ, characteristic of the C57BL/6 genetic background (Fig. 8B). Thus, expression of Stat6VT promotes T lymphocyte differentiation toward the Th2 phenotype.

Stat6VT induces an activated phenotype in T lymphocytes

Because expression of a constitutively active Stat6 can influence T cell function, we wanted to examine its ability to alter the phenotype of peripheral T cells. T lymphocytes in the periphery that have not encountered Ag are considered naive, characterized by the high level of CD62L expression and low expression of CD44. Splenocytes from wild-type and CD2:Stat6VT mice were isolated and stained with Abs to CD4, CD44, and CD62L. Compared with wild-type T cells, CD2:Stat6VT T cells had increased percentages of CD62Llow and CD44hi cells, indicative of an activated or memory phenotype (Fig. 9A). Although CD2:Stat6VT T cells did not have increased expression of the T cell activation marker IL-2Rα (data not shown), they were slightly increased in size compared with wild-type cells (Fig. 9B). To further test the activation state, purified T cells from the spleens of wild-type and transgenic mice were plated in the absence or presence of IL-4 then immediately pulsed with [³H]thymidine for 24 h, harvested, and analyzed for [³H]thymidine incorporation. Transgenic T cells stimulated with or without IL-4 had much greater proliferation than wild-type T cells stimulated with or without IL-4 (Fig. 9C), indicating that T cells from CD2:Stat6VT mice were activated. Thus, expression of a constitutively active Stat6 can induce an activated state in T lymphocytes.

![FIGURE 8](http://www.jimmunol.org/) Analysis of in vitro cytokine production by wild-type and CD2:Stat6VT CD4⁺ T lymphocytes. A, Purified CD4⁺ T cells from wild-type and CD2:Stat6VT mice were stimulated with anti-CD3 for 3 days. Supernatants were harvested and analyzed for cytokine production by ELISA. B, Purified CD4⁺ T cells were differentiated in vitro under unskewed conditions for 1 wk. Cells were restimulated, and cytokine production was analyzed by ELISA. Cytokine production was measured as nanograms/4 × 10⁶ CD4⁺ cells. Data shown are representative of at least three separate experiments.
The forward scatter (size) of the CD4/H11001 assayed for incorporation.

absence of 5 ng/ml IL-4, immediately pulsed with [3 H]thymidine and from wild-type and transgenic mice were incubated in the presence or IL-4 was originally identified as a B cell growth factor, promoting functions both in vitro and in vivo. Our results demonstrate that the expression of Stat6VT has a dramatic effect on B and T cell functions within the spleen. CD2:Stat6VT mice have an increased peripheral B cell population and decreased T cell population. Stat6VT B lymphocytes have enhanced production of IgG1 and IgE, and increased expression of IL-4-regulated genes. T lymphocytes from transgenic mice have an activated phenotype and increased Th2 differentiation similar to previous reports using a Stat6:ER fusion protein (52) and Stat6VT retroviruses (53, 54). However, in contrast to B cells, T cells from transgenic mice do have an activated phenotype, suggesting that the mechanisms by which a constitutively active Stat6 can alter lymphocyte homeostasis is distinct between B and T lymphocytes.

The ability of IL-4 to promote Th2 differentiation and increase IgE production in vivo has made it an important factor in the promotion of atopic diseases, such as asthma and allergies. Furthermore, mutations in the IL-4Rα chain and within the Stat6 gene have been linked to atopic disease (55–60), suggesting that alterations of IL-4 signaling may be associated with allergic disease. We have shown that Stat6VT transgenic mice have elevated serum IgE levels and B lymphocytes with increased expression of FcεRII (CD23) similar to the human atopic phenotypes (61–64) and contribute to the constitutive binding of IgE to CD23 on the surface of IgM+ B cells. The importance of this finding is that B cells with IgE bound to CD23, the only IgE receptor on B cells, have the increased ability to potentiate allergic responses via IgE/CD23-mediated Ag presentation (65–68). These results suggest that an activated IL-4 signaling pathway may promote and perpetuate allergic inflammation.

There are both similarities and differences between transgenic Stat6VT mice and transgenic mice overexpressing IL-4 (15, 39, 69). Transgenic IL-4 had dramatic effects on thymic cellularity and thymocyte maturation, suggesting that an active Stat6 does not mimic all effects of transgenic IL-4 expression. In contrast, there are similar reductions in peripheral T cells in IL-4 and Stat6VT transgenic mice. This may be due to IL-4/Stat6-mediated decreases in thymic emigration as previously proposed (69), but may also be due to the IL-4/Stat6-mediated increases in T cell activation and cell death in vivo. Expression of many IL-4-stimulated surface markers and the y1 isotypes are increased in both IL-4 and Stat6VT transgensics. Additionally, peripheral B cell numbers/percentages are increased in both transgensics, which correlates well with other reports that examine the protective effects of IL-4 on B cells. IL-4 promotes the survival of circulating B cells and their migration to the spleen (70). Furthermore, some antiapoptotic effects of IL-4 are due to a Stat6-dependent increase in Bcl-x expression (17). These findings provide a mechanism for the expansion of the peripheral B cell population seen in CD2:Stat6VT and IL-4 transgenic mice.

Because both B and T cells express the constitutively active Stat6 in CD2:Stat6VT mice, some aspects of the phenotype of these mice may result from interactions between these cells. The increased expression of MHC class II and costimulatory markers on B cells could contribute to the activated phenotypes of the T cells. IL-4 liberated from T cells could then enhance the Stat6-induced phenotype in B cells. Enhanced IL-4 secretion from T cells may contribute to other phenotypes including the splenomegaly that is shared with IL-4 transgenic as well as helminth-infected and anti-IgG1-treated mice and may be a common result of a “Th2” response.

In this report, using transgenic mice we have demonstrated the ability of a constitutively active Stat6 to alter lymphocyte homeostasis as well as mediate and enhance lymphocyte function and activation by mechanisms that are distinct between B and T lymphocytes. Further studies using the CD2:Stat6VT mice will allow us not only to better understand the ability of Stat6 to regulate transcription, but also to examine the differences in transcription regulation by Stat6 between T and B lymphocytes and how this regulation alters immune responses in vivo.

**Discussion**

IL-4 was originally identified as a B cell growth factor, promoting the survival and proliferation of B cells, and regulating gene expression. Studies with Stat6-deficient mice have shown that Stat6 is required for many IL-4-induced functions. In this report, using a constitutively active Stat6 mutant, we investigated the ability of this transcription factor to mediate IL-4-stimulated B and T cell functions. The results indicate that Stat6VT B lymphocytes have enhanced production of IgG1 and IgE, and increased expression of IL-4-regulated genes. T lymphocytes from transgenic mice have an activated phenotype and increased Th2 differentiation similar to previous reports using a Stat6:ER fusion protein (52) and Stat6VT retroviruses (53, 54). However, in contrast to B cells, T cells from transgenic mice do have an activated phenotype, suggesting that the mechanisms by which a constitutively active Stat6 can alter lymphocyte homeostasis is distinct between B and T lymphocytes.

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References


