

Transgenic Expression of Insulin Receptor Substrate 2 in Murine B Cells Alters the Cell Density-Dependence of IgE Production In Vitro and Enhances IgE Production In Vivo¹

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Previous studies have shown that insulin receptor substrate (IRS)1 and IRS2 mediate proliferative and antiapoptotic signaling through the IL-4R in 32D cells; however their role in regulating normal B cell responses is not clear. To investigate the role of IRS2 in normal B cell function, we developed IRS2 transgenic (Tg) mice on the C57BL/6 background. Western blot analysis revealed a 2-fold elevation in IRS2 protein levels in Tg⁺ mice compared with littermate controls and a 3-fold increase in basal tyrosine phosphorylated IRS2 in the absence of IL-4 stimulation. IL-4-induced tyrosine phosphorylation of IRS2 was elevated in Tg⁺ B cells, whereas IL-4-induced phosphorylation of STAT6 was similar between Tg⁺ and Tg⁻ B cells. Tg expression of IRS2 had little effect on IL-4-mediated proliferation and no effect on protection from apoptosis. However, production of IgE and IgG1 by Tg⁺ B cells using standard in vitro conditions was diminished 50–60%. Because Ig production in vitro is known to be highly cell concentration-dependent, we performed experiments at different cell concentrations. Interestingly, at very low B cell concentrations (1000–5000 B cells/well), IgE and IgG1 production by Tg⁺ B cells was greater than that of controls, whereas at higher cell concentrations (10,000–20,000 cells/well) Ig production by Tg⁺ B cells was less than controls. Furthermore, in vivo immunization with OVA-alum or goat anti-IgD resulted in elevated serum IgE levels in the Tg⁺ mice. These results indicate that overexpression of IRS2 alters the B cell intrinsic density-dependence of IgE and IgG1 production in vitro and enhances IgE responses in vivo. *The Journal of Immunology*, 2004, 172: 2803–2810.

Interleukin-4 is a cytokine produced by T cells, mast cells, and basophils that stimulates profound effects on the growth and differentiation of B and T lymphocytes (1). These effects include the ability to regulate IgE and IgG1 production by B cells and the ability to regulate the lymphokine-producing phenotype of CD4⁺ Th cells. Much effort has been spent to gain an understanding of the molecular mechanism by which IL-4 mediates these effects with the future goal of developing rational strategies for manipulating immune responses.

Over the last 10 years, many groups have focused on understanding the structure of the receptor for IL-4 and the signal transduction pathways activated in cell lines by the binding of IL-4 to its receptor (2). The IL-4 receptor complex consists of a 140 kDa, high-affinity binding chain (IL-4R α) and the common γ -chain; this complex has been called the type I IL-4R. In addition, the low-affinity binding chain for IL-13 (IL-13R α 1), a cytokine that elicits many of the same biological responses as IL-4, is also able to complex with the IL-4R α (3). The IL-4R containing the IL-13R α 1

instead of γ -chain has been termed type II. The current data suggest that resting murine B lymphocytes only express the type I IL-4R because they do not respond to IL-13 (4).

One of the major signaling pathways activated by IL-4 is a latent cytoplasmic transcription factor, termed STAT6, which is a member of the signal transducers and activators of transcription family. STAT6 is recruited to the IL-4R α by binding to the second, third, or fourth cytoplasmic tyrosine residues via its Src homology 2 domain after they become phosphorylated (5). According to the general Janus kinase/STAT paradigm, STAT6 dimerizes in the cytoplasm after it becomes tyrosine phosphorylated, translocates to the nucleus, and binds to consensus sequences or gamma-activated sequences found within the promoter regions of IL-4-regulated genes. Signaling by the IL-4R complex results in the regulation of a number of genes. Many are dependent upon the activation of STAT6 including CD23, MHC class II, IL-4R α , G γ 1, or G ϵ (6, 7).

A second major signaling pathway activated by the IL-4R is the insulin receptor substrate (IRS)³ family (IRS1, IRS2, IRS3, and IRS4) pathway. These proteins are large cytoplasmic docking proteins that contain a protein tyrosine binding domain and many sites for serine/threonine and tyrosine phosphorylation (2). The IRS proteins are recruited to the IL-4R α by the first cytoplasmic tyrosine residue (Y1) that lies within a consensus motif also found in receptors for insulin and the insulin-like growth factor type I called the I4R-motif (8). The protein tyrosine binding domain of the IRS protein interacts directly with the I4R-motif of the IL-4R α and contributes to a number of signaling pathways (2). Tyrosine phosphorylated sites within the IRS proteins associate with cytoplasmic signaling molecules that contain Src homology 2 domains. One

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³ Abbreviations used in this paper: IRS, insulin receptor substrate; Tg, transgenic; HMG, high-mobility group; PI-3K, phosphatidylinositol 3'-kinase.

signaling molecule with which all IRS family members interact is the p85 subunit of phosphatidylinositol 3'-kinase (PI-3K). Numerous studies using long-term cell lines have shown that the interaction of IRS proteins with the p85 subunit results in the activation of the p110 catalytic subunit of the PI-3K enzyme. PI-3K activity is important for growth, survival, and regulation of gene expression in response to IL-4. Several signaling molecules, in which activities are downstream of PI-3K activity have been implicated in IL-4 responses, including p70^{S6} kinase, the Akt kinase, and the non-histone high-mobility group (HMG) DNA-binding protein HMG-I(Y) (9–12).

Treatment of B cells with IL-4 alone can induce or enhance the expression of CD23, MHC class II, and IL-4R α . In the presence of another stimulus, such as LPS or the ligand for CD40, IL-4 induces/enhances expression of the germline transcripts for the H chain of IgG1 (G γ 1) and IgE (G ϵ) leading to Ig class switch recombination. Although the IL-4-induced activation of STAT6 is necessary for expression of germline transcripts, there are a number of other cofactors involved in their expression, including C/EBP, NF- κ B, activating transcription factor 2, and HMG-I(Y) (13, 14). The IL-4-induced phosphorylation of HMG-I(Y), as well as other nuclear proteins, has been shown to be mediated through the IRS pathway (12). The phosphorylation of HMG-I(Y) participates in regulating G ϵ expression in B cells (15).

The role that the IRS pathway plays in normal immune responses is unclear. By RT-PCR, murine splenic B cells express IRS2 and IRS3, but not IRS1 (16). There have been limited reports of B cell defects in the IRS1 or the IRS2-deficient mouse (17–20). However, one IRS family member may compensate for the function of another family member during development. To address the role of IRS family members in regulating B cell responses, we used a transgenic (Tg) approach to drive ectopic expression of IRS2. We found that B cells derived from Tg mice expressing IRS2 levels elevated by 2- to 3-fold demonstrated alterations in the B cell intrinsic density-dependence of IgE and IgG1 production *in vitro*. Furthermore, we found an elevated serum IgE response in the Tg mice after *in vivo* challenge. These results suggest that, in addition to STAT6, IRS2 participates in the signaling pathway leading to IL-4-induced Ig production.

Materials and Methods

Mice

FVB/N and C57BL/6 mice were purchased from Taconic Farms (German town, NY). Animal care was provided in accordance with Institutional Animal Care and Use Committee procedures approved at the American Red Cross. FVB/N mice expressing IRS2 as a transgene were developed by standard techniques. The cDNA of IRS2 was cloned into the *Bam*HI site of the polylinker region in the p μ SV40 Tg vector obtained from Dr. S. Cory (Walter and Eliza Hall Institute, Melbourne, Australia). This vector uses the IgH enhancer (E μ) and the SV40 early region promoter (Psv). In addition, the vector contains the RNA splice signals of the SV40 small T Ag intron and the termination and polyadenylation sequence for the SV40 early transcripts. The Tg cassette is bound by *Not*I restriction sites. The IRS2-containing 6-kb *Not*I fragment was injected into FVB/N blastocysts before implantation into pseudopregnant females as previously described (21, 22). The animals delivered by these females were tested for transgene expression after they reached 3 wk of age by Southern blot analysis of tail DNA using the full *Not*I fragment as the probe. Genomic DNA was digested with either *Bam*HI or *Eco*RI to determine both copy number and differences in integration sites in the Tg mice by standard techniques. Three founder lines were identified. One founder line (no. 7) did not transmit the transgene to its progeny. The other two lines (nos. 3 and 2) did transmit the transgene to the F₁ progeny with a transmission rate of 50%.

Tg males from both founders were backcrossed more than six times onto the C57BL/6 strain. Experiments were performed using mice at least 6 wk of age. For line 2 (referred to as Tg^{low}), heterozygote transgene-positive mice were interbred to generate transgene homozygotes. Southern blot

analysis, as earlier described, was used to identify the IRS2-Tg homozygous mice.

B cell purification and reagents

Mouse spleen cells were obtained by mechanical disruption, followed by lysis of RBC in lysis buffer (0.15 M NH₄Cl, 1.0 mM KHCO₃, 0.1 M EDTA, pH 7.2). Small, resting splenic murine B cells were purified by Percoll gradient fractionation after treatment of total spleen cells with anti-Thy1 and complement. Purified B cells from the 70–66% Percoll layer were cultured at 37°C in RPMI 1640 culture medium (BioWhittaker, Walkersville, MD) supplemented with 10% ME FBS, 100 U/ml penicillin, 2 mM glutamine, and 1×10^{-7} M 2-ME. Purified B cells were incubated at various cell densities as described in the figures with the indicated cytokines and stimulants. Standard conditions included 10 ng/ml mouse IL-4 (R&D Systems, Minneapolis, MN), 30 μ g/ml LPS (Sigma-Aldrich, St. Louis, MO), and/or 1 μ g/ml anti-CD40 ligand (BD PharMingen, San Diego, CA).

RT-PCR and PCR analysis

RNA was isolated from purified B cells using RNazol B (Tel-Test, Friendswood, TX). RT-PCR was performed on RNA using oligo(dT) to generate cDNA. The following primers were used to detect endogenous IRS2, TGG TGA GGC AGG TAC CCG TCT (sense) and TCT GCA CGG ATG ACC TTA GCA (antisense); the IRS2 transgene, GAG ACC TCC GAG GGT TTC CAG (sense) and GAA TTC GAG CTC GCC CGG GGA TCC (antisense); and the housekeeping gene β -actin, AGA AGA GGT ATC CTG ACC CTG (sense) and ATC CAC ATC TGC TGG AAG GTG (antisense). Product was analyzed on agarose gels. Once the founder lines were clearly characterized by Southern blot analysis, new litters were screened for transgene expression by PCR analysis using the listed Tg primers on DNA extracted from ear punches.

For detection of germline ϵ transcripts and the I μ -C ϵ postswitch hybrid transcripts, purified B cells were stimulated with IL-4 (10 ng/ml) and LPS (30 μ g/ml) for 4 days. RNA was isolated and cDNA prepared as previously described. Various dilutions of cDNA were amplified using the previously described primer sets for I ϵ (23), I μ -C ϵ (ImF and CeR, Ref. 24), or β -actin for 28 cycles. To test for genomic DNA contamination in the total RNA preparation, RNA was incubated in the presence or absence of reverse transcriptase. The sample was then amplified with the primer sets for 40 cycles.

Immunoprecipitation and immunoblot

Analysis of IRS2 and STAT6 tyrosine phosphorylation and total protein expression was performed as previously described (25). Total splenocytes, or resting B cells, were deprived of serum for 2 h at 37°C. After washing, cells were incubated in RPMI 1640 in the presence or absence of IL-4 for 10 min at room temperature. The reaction was terminated by 10-fold dilution in ice-cold PBS. Cell pellets were lysed in HEPES lysis buffer (50 mM HEPES, pH 7.5, 0.5% Nonidet P-40, 5 mM NaCl, 5 mM EDTA, 10 mM napyrophosphate, 50 mM NaF, and protease inhibitor mixture) and clarified. The soluble fraction was immunoprecipitated with a polyclonal rabbit anti-IRS2 (Upstate Biotechnology, Lake Placid, NY), or anti-STAT6 (Santa Cruz Biotechnology, Santa Cruz, CA) and protein G beads (Life Technologies, Grand Island, NY). The precipitates were washed in lysis buffer and solubilized in SDS sample buffer. The samples were separated on 7.5% SDS-polyacrylamide gels before transfer to a polyvinylidene difluoride membrane. Membranes were probed with a monoclonal anti-phosphotyrosine Ab, RC-20H (BD Transduction Laboratories, Lexington, KY) to detect phosphorylated IRS2 or STAT6. The bound Ab was detected using ECL (Kirkegard & Perry Laboratories, Gaithersburg, MD). Blots were stripped using Re-blot (Chemicon International, Temecula, CA) and reprobed with anti-IRS2 or anti-STAT6. With certain lots of anti-IRS2 Ab, reprobing for IRS2 after blots were stripped resulted in high background. To avoid this problem, duplicate gels were run and probed directly with anti-IRS2 or RC-20H.

CD23 expression

Resting spleen cells were isolated as previously described and stimulated for 48 h with various concentrations of IL-4. After 48 h, cells were harvested and analyzed for induction of CD23 by staining with FITC anti-B220 and PE anti-CD23 (BD PharMingen, Mountain View, CA). The B220⁺ cells were analyzed for the intensity of staining with anti-CD23 using flow cytometry (FACScan; BD Biosciences, Mountain View, CA). The results are expressed as mean fluorescence intensity.

Cellular proliferation and Ig production

Cellular proliferation of stimulated resting B cells was performed by plating cells at increasing cell concentrations (1,000–50,000 cells/well) in a final volume of 0.2 ml/well. After 48 h, cells were pulsed with 1 μ Ci/well [^{3} H]thymidine (DuPont NEN, Wilmington, DE) for the final 4 h of culture before harvesting using a Packard harvester (Packard Bioscience, Meriden, CT) and the Matrix 9600 direct Beta count system.

Production of IgE and IgG1 was analyzed by ELISA after 10 days of culture. ELISA was performed as previously described (26). Briefly, 96-well plates were coated with 2 μ g/ml each of two monoclonal anti-IgE Abs (BD PharMingen and BioSource International, Camarillo, CA). Plates were blocked with PBS containing 2% FBS and 0.02% Tween 20. After blocking and overnight incubation with supernatant samples or IgE standard (a generous gift from Dr. D. H. Conrad, Virginia Commonwealth University, Richmond, VA), plates were developed with HRP-conjugated goat anti-IgE Abs (Southern Biotechnology Associates, Birmingham, AL) followed by Peroxidase Substrate (Bio-Rad, Hercules, CA). The plates were read at 415 nm using a Thermomax plate reader (Molecular Devices, Sunnyvale, CA). Standard curves were run and four-parameter analysis was performed using Molecular Devices software.

Total IgG1 production was measured using the Ab pair recommended by BD PharMingen. A 1/400 dilution of streptavidin-AP (Southern Biotechnology Associates) was used following incubation with the biotinylated anti-IgG1. OVA-specific IgG1 was measured by coating with 30 μ g/ml OVA to capture and detected using the biotinylated anti-IgG1. The plates were read at dual wavelength, 405–650 nm, and analyzed as previously described.

Analysis of *in vivo* Ig production

To test the effect of the IRS2 transgene on production of IgE *in vivo*, mice were immunized with OVA or goat anti-mouse IgD (27). For OVA responses, mice were immunized i.p. with 500 μ g/mouse OVA complexed with alum or alum alone on day 1 and boosted on day 14. On days 19, 22, and 27 all mice were challenged with OVA by inhalation (1% in PBS) for 20 min. Serum was collected 24 h after the last treatment. Total serum IgE and OVA-specific IgG1 levels were analyzed as previously described. To analyze the response to goat anti-mouse IgD, mice were injected i.v. on day 1 with 800 μ g of goat anti-mouse IgD. On day 5, mice were injected i.v. with 10 μ g BVD4-biotin, anti-IL-4 and bled 2 h later. Serum IL-4 levels were determined using the Cincinnati Cytokine Capture Assay as described (28). Mice were bled on days 5, 7, 9, and 11 to analyze serum IgE levels.

Results

Development of IRS2 Tg lines

To determine the contribution of IRS2 to normal B cell function, we undertook a transgenic approach to drive elevated expression of IRS2. This approach was based on previous studies in transgenic cell lines showing that overexpression of IRS proteins by ~3-fold was sufficient to alter the cellular responses and frequently resulted in constitutive activation of the IRS pathway (25, 29–33). The 4-kb cDNA encoding IRS2 was cloned into the *Bam*HI site of the polylinker in the pE μ SV40 Tg vector (Fig. 1A). This vector uses the IgH enhancer (E μ) and the SV40 early region promoter (Psv) and drives expression only in lymphocytes (34). In addition, the vector contains the RNA splice signals of the SV40 small T Ag intron and the termination and polyadenylation sequence for the SV40 early transcripts. The Tg cassette is bound by *Not*I restriction sites. The IRS2-containing 6-kb *Not*I fragment was injected into FVB/N blastocysts before implantation into pseudopregnant females. The animals delivered by these females were tested for transgene expression by Southern blot analysis of tail DNA using the full *Not*I fragment as the probe (Fig. 1B). Digesting the DNA isolated from two founder animals (nos. 2 and 3) with *Bam*HI demonstrated the 4-kb fragment encoding the IRS2 coding sequence and a 2-kb fragment derived from the Tg vector sequence, whereas other founders (represented by no. 4) lacked these fragments. DNA digests from all the founder animals demonstrated the endogenous 14-kb IRS2 fragment. The transgene-positive founder animals transmitted the transgene to the F₁ progeny with a transmission rate of 50%. The progeny were healthy with no obvious

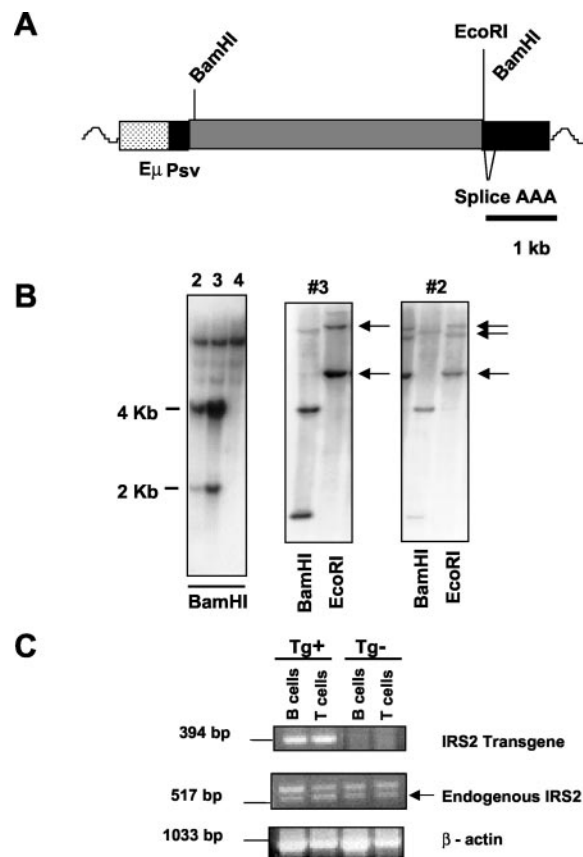


FIGURE 1. Construction and analysis of IRS2 Tg mice. **A**, Schematic representation of the IRS2 pE μ SV40 Tg vector constructed as described in *Materials and Methods*. The cDNA is flanked by *Bam*HI and *Eco*RI sites that were used to digest genomic DNA for Southern blot analysis. **B**, Southern blot analysis of founder mice. The full *Not*I fragment of IRS2 was used to probe genomic DNA isolated from the tails of founder mice nos. 2, 3, and 4 digested with *Bam*HI. Positive founders nos. 3 and 2 were further characterized. Copy number in the two founders, nos. 3 and 2, was estimated by digesting genomic DNA with *Bam*HI and comparing the signal intensities of the fragments to the intensity of endogenous IRS2 and to a control amount of purified fragment representing approximately one copy (data not shown). The high line (no. 3) contains approximately four copies of the transgene whereas the low line (no. 4) contains approximately two copies. Digestion with *Eco*RI was also performed to discriminate sites of integration; arrows indicate unique bands flanking the transgene. **C**, RT-PCR analysis was performed on RNA isolated from purified resting B cells and T cells.

abnormalities or alterations in lymphocyte subsets as determined by FACS analysis on spleen, thymus, and lymph nodes (data not shown) using Abs to the following cell surface markers: B220, CD19, CD5, κ -chain, CD4, CD8, CD44, CD69, CD25, TCR $\alpha\beta$, and MAC1.

To further characterize the founder animals, genomic DNA was digested with either *Bam*HI or *Eco*RI to estimate copy number and to compare integration sites. Comparison of the signal intensities of the fragments produced by *Bam*HI digestion with endogenous IRS2 (Fig. 1B) and a control amount of purified fragment representing approximately one copy (data not shown) allowed us to estimate approximately two copies of the transgene in founder no. 2, approximately four copies of the transgene in founder no. 3, and no copies in founder no. 4. Digestion with *Eco*RI was used to analyze integration because it would cut only once within the transgene. *Eco*RI digests of DNA isolated from founder no. 2 and no. 3 detected the 6-kb full Tg fragment and the 15-kb endogenous

IRS2 fragment. In addition, both samples demonstrated an additional band with a size unique to the founder. These results indicate the two founders have different sites of integration of the transgene and that the Tg DNA integrated in multiple copies. Progeny derived from founder no. 2 are henceforth referred to as Tg^{low} whereas progeny from founder no. 3 are called Tg^{high} . Both lines were backcrossed onto the C57BL/6 mouse strain. All experiments were performed using transgene-positive animals backcrossed six generations or more and their appropriate transgene littermate controls.

Characterization of transgene expression

Expression of mRNA derived from the IRS2 transgene was tested using RT-PCR (Fig. 1C). Both B cells and T cells purified from Tg^{high} mice, but not from transgene littermate control animals, were positive for transgene-specific mRNA. Other tissues from the Tg^{+} mice including liver and kidney were negative for Tg mRNA (data not shown). All samples tested positive for endogenous IRS2 and β -actin. Similar results were obtained with the Tg^{low} line (data not shown). Western blot analysis was performed to determine the level of IRS2 protein expression and its state of phosphorylation. Purified, resting B cells were isolated from IRS2 Tg or littermate controls and stimulated with IL-4 for 10 min. Lysates were immunoprecipitated with anti-IRS2 and separated by SDS-PAGE, and Western blot analysis was performed by probing with anti-phosphotyrosine (Fig. 2). In the absence of IL-4, B cells from IRS2 Tg^{high} mice showed an increased level of basal IRS2 phosphorylation compared with littermate controls. After IL-4 treatment we found ~2-fold more tyrosine phosphorylated IRS2 present in the Tg^{+} B cells compared with B cells from littermate controls. With respect to protein expression, B cells from Tg^{high} mice expressed ~2-fold more IRS2 than did littermate controls. However, B cells from the Tg^{low} line demonstrated similar levels of IRS2 phosphorylation after IL-4 treatment and similar levels of IRS2 protein as controls. Because the Tg^{low} line contained two copies of the transgene, whereas the Tg^{high} line contained four copies, we sought to increase the transgene dosage in the low line by breeding Tg^{low} -positive heterozygotes to generate homozygote mice. B cells isolated from the $Tg^{low+/+}$ animals demonstrated enhanced levels of tyrosine phosphorylated IRS2 after IL-4 stimulation to levels on par with those seen in B cells isolated from Tg^{high} mice (Fig. 2).

Previous experiments performed in tissue culture cell lines indicated that the IRS and STAT6 pathways are independent from one another (16). Therefore, the effect of the IRS2 transgene on the IL-4-induced activation of the STAT6 pathway in purified B cells was analyzed (Fig. 3). We first analyzed the ability of IL-4 to stimulate the tyrosine phosphorylation of STAT6 (Fig. 3A). IL-4 stimulation induced the tyrosine phosphorylation of STAT6 in B cells isolated from both Tg^{high} mice and littermate controls. We

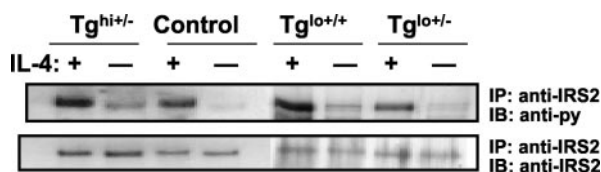


FIGURE 2. Increased levels of IRS2 in heterozygous Tg^{high} and homozygous $Tg^{low+/+}$ lines. Purified resting B cells from IRS2 Tg mice and littermate controls were stimulated $\pm$ IL-4 for 10 min. Lysates were immunoprecipitated with anti-IRS2 and separated by SDS-PAGE. The Western blots were probed with anti-phosphotyrosine or anti-IRS2 as indicated. Densitometric analysis of the film was performed using NIH Image software.

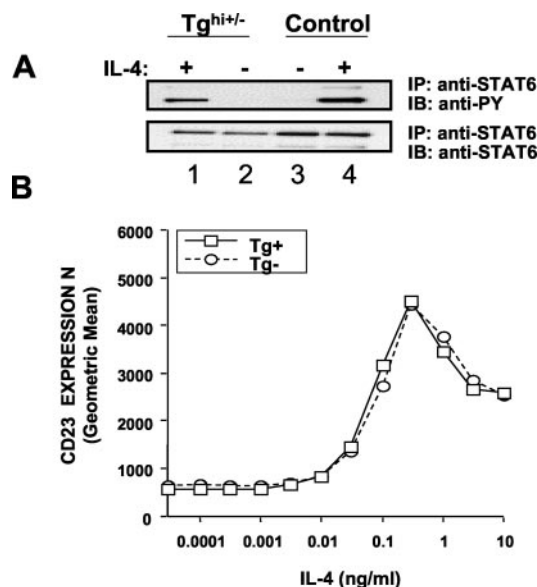


FIGURE 3. STAT6 and CD23 are not affected by overexpression of IRS2. A, Total splenic B cells were purified and stimulated in the presence or absence of IL-4 as indicated for 10 min. Phosphorylated STAT6 was detected by immunoblotting with anti-phosphotyrosine after immunoprecipitation with anti-STAT6 Ab. Blots were stripped and reprobed with anti-STAT6. IRS2 Tg^{high} (lanes 1 and 2) mice and littermate controls (lanes 3 and 4) are represented. B, Spleen cells isolated from control or Tg^{high} mice were stimulated for 48 h with various concentrations of IL-4. Cells were analyzed for surface expression of B220 and CD23. The mean intensity of CD23 staining on B220⁺ cells is shown.

found that the ability of IL-4 to induce the tyrosine phosphorylation of STAT6 was the same indicating that the STAT6 pathway is intact in the transgene-positive cells. We further tested the STAT6 pathway by analyzing the induction of CD23 by IL-4, a response highly dependent upon STAT6 (Fig. 4B). We consistently observed that the levels of IL-4-induced CD23 expression on resting B cells isolated from Tg^{high} or control mice were similar. Similar results were obtained when the B cells were stimulated with LPS and IL-4 (data not shown).

Functional characterization

In the IL-3-dependent 32D cell line, expression of IRS2 controls the proliferative and antiapoptotic responses to IL-4 (25). To determine whether this is also the case for primary B cells, we compared these responses using B cells purified from Tg^{high} mice and control mice (Fig. 4). We found that Tg expression of IRS2 had little effect on the proliferation of splenic B cells in response to LPS plus IL-4 (Fig. 4A), to IL-4 alone, or anti-CD40 plus IL-4 (data not shown). We also found that the IRS2 transgene had no effect on protection from apoptosis by IL-4. Treatment of B cells isolated from Tg^{high} or littermate controls with IL-4 was able to protect from anti-IgM-induced apoptosis at similar levels (Fig. 4B). Contrary to results obtained in factor dependent-cell lines (25, 35), these results indicate that the IRS pathway does not play a major role in IL-4-mediated proliferative or antiapoptotic responses in normal B cells. They are consistent with a recent report suggesting that STAT6 is more important for the regulation of lymphocyte growth and survival than is IRS2 (20, 36).

We next examined the effect of the IRS2 transgene on the ability of resting B cells to differentiate into IgE producers. Using standard in vitro culture conditions for IgE production (Fig. 5), we found that B cells isolated from Tg^{high} heterozygotes and Tg^{low}

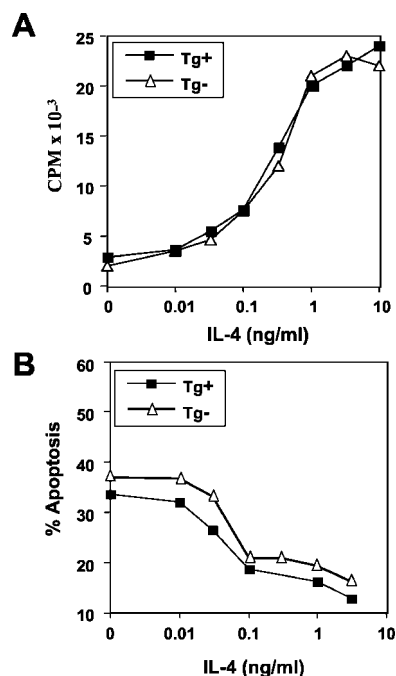


FIGURE 4. Effect of the transgene on proliferation and apoptosis. *A*, Resting, splenic B cells from IRS2 Tg^{high} (■) or littermate controls (△) were plated at 10,000 cells/well and stimulated with various concentrations of IL-4 in the presence of LPS (10 μ g/ml). *B*, Resting, splenic B cells from IRS2 Tg^{high} (■) or littermate controls (△) were stimulated with various concentrations of IL-4 in the presence of anti-IgM (30 μ g/ml) for 24 h. The levels of apoptosis were determined by FACScan analysis of DNA content. The percentage of cells expressing <2 N DNA content is shown. The level of apoptosis in the absence of anti-IgM was 10%. These results are representative of five independent experiments.

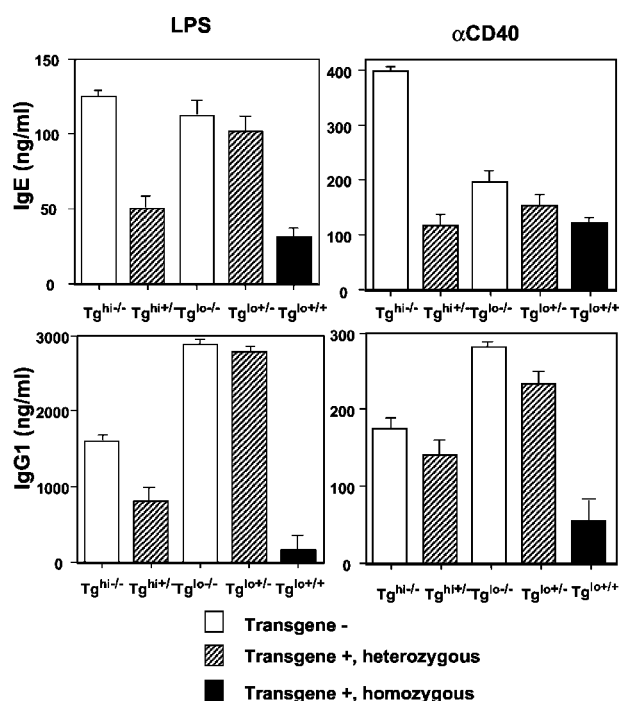


FIGURE 5. Using standard culture conditions, IgE and IgG1 levels are suppressed in vitro in heterozygous IRS2 Tg^{high} line and homozygous IRS2 Tg^{low+/+} line. Resting, purified B cells from homozygous IRS2 Tg (■), heterozygous IRS2 Tg (▨) and littermate controls (□) were cultured at 20,000 cells/well and stimulated in the presence of IL-4 and LPS or IL-4 and anti-CD40. Cell-free supernatants were analyzed for total IgE and IgG1 by ELISA.

homozygotes produced less IgE and IgG1 than did the littermate controls in response to LPS plus IL-4 and anti-CD40 plus IL-4. This reduction was observed in pooled B cells and in B cells purified from individual mice. Recently, it was found that the in vitro production of IgE is dependent on B cell density (37). Therefore, we also performed the stimulations at various B cell densities (Fig. 6A). Interestingly, we found that the dose-response curve for IgE, and to a lesser extent, for IgG1 was shifted to the left using B cells from Tg^{high} mice vs littermate controls and that there was a relatively steep drop in IgE production with increasing cell density in the Tg^{high} samples. These results suggest that IRS2 regulates the cell density-dependent production of IgE by primary B cells in vitro. The mechanism by which IRS2 might regulate this is not clear because we found no effect of the IRS2 Tg on B cell apoptosis (Fig. 4) or the number of B cell divisions stimulated by the

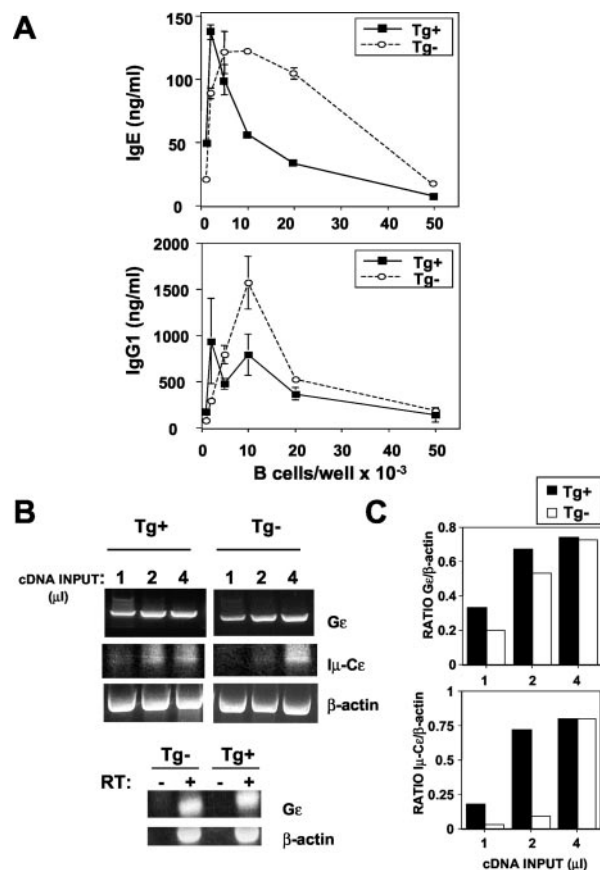


FIGURE 6. The IRS2 transgene alters the cell density-dependence of in vitro IgE and IgG1 production. *A*, Resting, purified B cells from IRS2 Tg^{high} (■) or littermate controls (○) were cultured at various cell densities and stimulated with IL-4 and 30 μ g/ml LPS. IgE and IgG1 production were analyzed by ELISA. *B*, Resting, purified B cells from IRS2 Tg^{high} or littermate controls were cultured as described in *Materials and Methods* for 4 days. Total RNA was prepared and cDNA generated. Amplification of the cDNA was performed on 2-fold serial dilutions of product as indicated using primer pairs specific for germline ϵ transcripts, postswitch transcripts, or β -actin. The PCR products were analyzed on agarose gels and stained with ethidium bromide. Photographs of the gels were scanned (*top*). To test for genomic DNA contamination in the RNA preparation, total RNA was incubated in the presence (+) or absence (-) of reverse transcriptase as indicated (*bottom*). The sample was then amplified with the primer sets for 40 cycles. *C*, Densitometry of the negative image of the bands in *B* (*top*) was performed using NIH Image software. The integrated density of the G ϵ or I μ -C ϵ band was divided by the integrated density of β -actin for each input. The relative amounts of G ϵ product and I μ -C ϵ product for Tg⁺ and Tg⁻ are shown.

in vitro conditions (data not shown). However, we did find that B cells isolated from IRS2 Tg^{high} or IRS2 Tg^{low+/+} proliferated less well in response to IL-4 at high cell densities (Fig. 7).

The ability of a B cell to switch to an IgE-producing cell has been clearly dissected (38). The initial step requires transcription of the H chain constant regions in their germline configuration. This is followed by the targeting of the active genomic region for switch recombination, producing a mature, productive transcript. Subsequently, the cell completes differentiation to become an IgE producing plasma cell. Once class switching has occurred, transcription is initiated at the I μ promoter and terminates at the 3' end of the switched ϵ constant region; the resulting mature ϵ mRNA transcript would be composed of an I μ exon spliced to the C ϵ exon. Therefore, to determine whether IRS2 was acting at the level of germline transcription and/or class switch recombination, we performed RT-PCR analyses (Fig. 6, B and C). When B cells were cultured under conditions of elevated IgE production, we found an enhancement of G ϵ production and a marked enhancement of I μ -C ϵ postswitch transcripts in Tg^{high} samples as compared with littermate controls (Fig. 6, B and C). As expected (23, 24), using RNA without reverse transcription as input, we did not detect any product for all primer pairs (Fig. 6B and data not shown).

Although the in vitro culture system has been very informative, it does not completely recapitulate activation of Ig production in vivo. Therefore, we tested the effect of the IRS2 Tg on IgE and IgG1 production in response to OVA or anti-IgD treatment of mice (Figs. 8 and 9). We found that the Tg^{high} animals produced 7 times the amount of IgE and 2.5 times the amount of OVA-specific IgG1 as their littermate controls in response to an OVA priming and challenge (Fig. 8). Furthermore, there was substantially more IgE in the serum of Tg^{high} animals 7 and 9 days after injection with goat anti-IgD (Fig. 9). Production of IgE Abs to protein Ags in vivo is dependent on IL-4. Therefore, during the course of this experiment the levels of serum IL-4 were also monitored using the

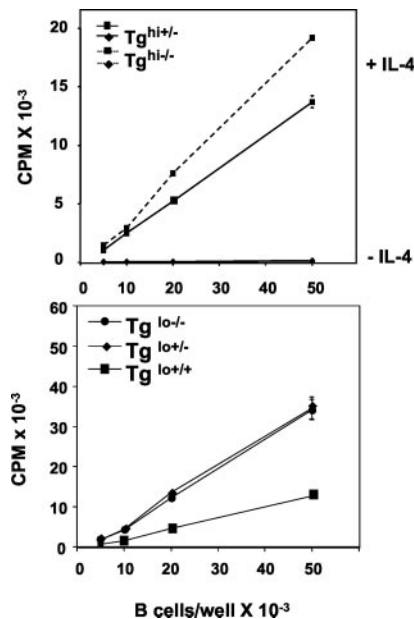


FIGURE 7. A, Resting, splenic B cells from IRS2 Tg^{high} (solid lines) or littermate controls (dotted lines) were plated at increasing cell dosages and stimulated with (+) IL-4 (■) or without (–) IL-4 (◆) in the presence of LPS (10 μ g/ml) or anti-CD40 (1 μ g/ml). B, Resting, splenic B cells from homozygous IRS2 Tg^{low+/+} (■), heterozygous IRS2 Tg^{low+/-} (◆), or littermate controls (●) were plated at increasing cell dosages and stimulated $\pm$ IL-4 in the presence of LPS (10 μ g/ml).

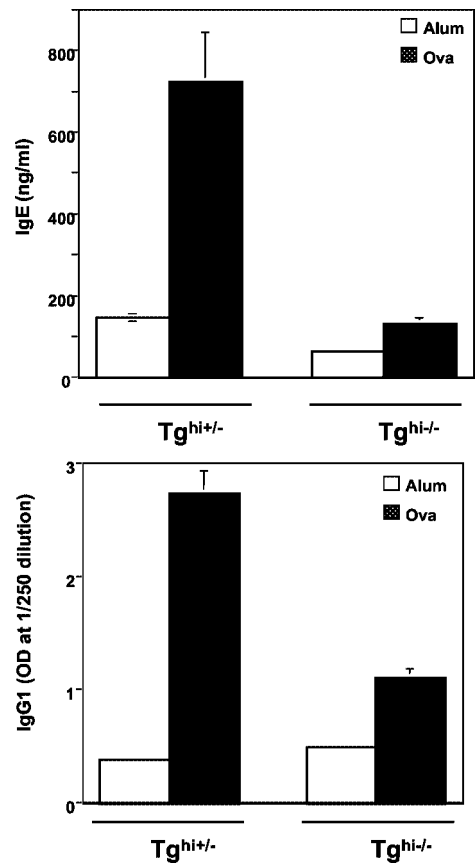


FIGURE 8. IRS2 Tg^{high} mice produce more IgE in response to OVA. IRS2 Tg^{high} or littermate controls were immunized and challenged with OVA as described in *Materials and Methods*. Serum samples were obtained and analyzed by ELISA for total serum IgE and OVA-specific IgG1 levels.

Cincinnati cytokine capture assay (28). Although there was a slight trend for increased serum IL-4 in the Tg^{high} group the increase was not significant.

Discussion

Previous studies demonstrated that overexpression of IRS proteins by ~3- to 5-fold was able to protect 32D cells from death induced

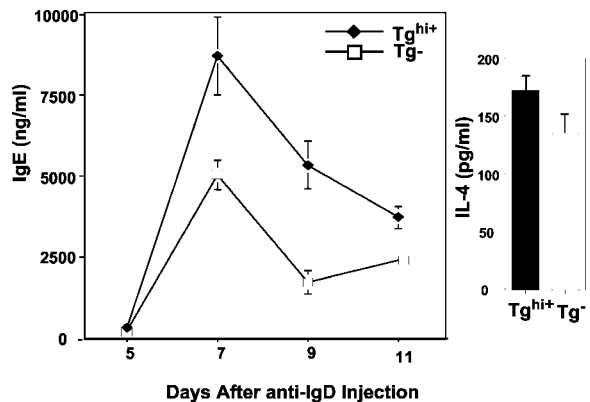


FIGURE 9. IgE and IgG1 production by IRS2 Tg^{high} mice is enhanced in vivo in response to goat anti-mouse IgD. IRS2 Tg^{high} (◆) or littermate controls (□) were immunized with goat anti-mouse IgD as described in *Materials and Methods*. Serum samples were obtained on days 5, 7, 9, and 11 after immunization. Serum IgE levels were determined by ELISA. Inset, On day 5, serum levels of IL-4 were analyzed using the Cincinnati Cytokine Capture Assay as described in *Materials and Methods*.

by IL-3 deprivation and to greatly enhance the IL-4-mediated response (25, 35). Overexpression of IRS1 by ~3-fold in human hepatocellular carcinoma cells resulted in constitutive tyrosine phosphorylation of IRS1, activation of PI-3K, and prevention of TGF β -induced apoptosis (25, 31–33). In these reports and others it was observed that the IRS/PI-3K pathway could be constitutively activated when IRS is overexpressed in long-term cell lines. These results lead to the hypothesis that the IL-4-induced activation of the IRS pathway would be responsible for protection of normal lymphocytes from apoptosis.

Recent efforts, including those reported in this study, specifically focused on the role of signaling pathways in regulating normal lymphocyte responses to IL-4. Surprisingly, it was shown that the ability of IL-4 to stimulate B cell proliferation in cells lacking IRS2 expression was slightly impaired, but that the ability of IL-4 to protect B cells from Fas-induced apoptosis was unaffected (20). Taking the opposite approach, we found that elevation of IRS2 had no effect on IL-4-induced protection of B cells from spontaneous or anti-IgM-induced death. However, B cells from IRS2 Tg mice showed a decrease in proliferation at high cell densities compared with littermate controls when stimulated with either LPS or anti-CD40 in the presence of IL-4. Because IRS2 and several other known signaling proteins dock to phosphorylated Y1 in the IL-4R α chain (2), it is possible that elevated IRS2 can block available Y1 docking sites thereby inhibiting the recruitment of another signaling molecule involved in B cell growth such as Shc (39).

In contrast to IL-3-dependent cell lines, these results indicate that IRS2 does not play a critical role in the antiapoptotic effect of IL-4 in normal B cells. Interestingly, we found that the IL-4-induced activation of IRS2 may be more important for B cell differentiation because IRS2 overexpression altered the cell density-dependence of IgE production in vitro. B cells from IRS2 Tg mice showed a decrease in both IgE and IgG1 production at cell concentrations ranging from 10,000 to 20,000 cells/well. However, at lower cell numbers (2,000 cells/well) the IRS2 Tg B cells produced more IgE and IgG1 compared with littermate controls.

Furthermore, we found that elevation of IRS2 enhanced the IgE response in vivo after priming and challenge with OVA or treatment with goat anti-IgD. We did not detect a significant difference in serum IL-4 levels in anti-IgD-treated Tg and control mice suggesting the possibility that the IRS2 effect in vivo is also B cell intrinsic. These results are consistent with a recent study reporting that the ability of TH2 cells derived from IRS2 knockout mice to produce IL-4 was not greatly altered (36).

At this time it is not clear what molecular mechanisms are affected by elevated IRS2 levels resulting in altered Ig production by B cells. Other investigators have shown that production of IgE and IgG1 in vitro is highly dependent upon cell density, the number of cell cycles that occur before Ig class switching takes place, and expression of other cell surface molecules (CD86, β 2-adrenergic receptor) (37, 40, 41). However, we have not detected any change in these parameters in the Tg B cells (data not shown). Although it has been reported that IFN- γ can use the IRS pathway for signal transduction (42), we did not detect IRS2 phosphorylation in response to IFN- γ in control or Tg B cells or any evidence for alteration in production of other Ig isotypes (data not shown). However, we did find that IRS2 could act at the molecular level because more G ϵ and I μ -C ϵ postswitch transcripts were detected in Tg^{high} B cells compared with littermate controls.

The IL-4-induced activation of STAT6 is critical for activation of the promoter for G ϵ and G γ 1 and precedes switching and Ig production (6, 7). However, some reports suggest it is not sufficient for maximal Ig production (43). The effect of elevated IRS2 expression on IgE production is most likely not due to a direct effect

on STAT6 activation per se because we did not observe significant differences in the levels of tyrosine phosphorylated STAT6 between control and IRS2 Tg B cells. Moreover, the ability of IL-4 to stimulate CD23 induction in controls and Tg B cells was similar. Thus, it appears more likely that the IL-4-activated IRS2 pathway is specifically regulating the STAT6-dependent differentiation of normal B cells to IgE or IgG1 producers, perhaps by influencing other transcription factors. Potential targets for influence by the IRS2 pathway are nuclear proteins involved in the regulation of G ϵ expression and formation of postswitch hybrids. IL-4 is known to induce the phosphorylation of a number of basic nuclear proteins, including HMG-I(Y) (15, 44), and their IL-4-induced phosphorylation is mediated through IRS (12). Further studies will be required to evaluate the effect of IRS2 expression on these factors in normal B cells.

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