Redirection of B cell responsiveness by transforming growth factor β receptor

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Communicated by N. Avrion Mitchison, University College London, London, United Kingdom, April 1, 2003 (received for review January 12, 2003)

The multifunctional transforming growth factor β receptor (TβR) ligand pair plays a central role in the regulation of lymphocyte homeostasis and prevention of autoimmunity. Although the mechanisms underlying the induction of transcriptional modulators by TβR have been studied in considerable detail, relatively little is known about the regulatory pathways targeted. To shed light on the mechanisms involved in negative regulation of B cell responses we identified TβR-dependent transcriptome changes by comparative gene expression profiling of normal and TβR-deficient primary B cells. The data reveal TβR-mediated induction of inhibitors of antigen receptor signaling (Ship-1, CD72) as well as inhibitors of the Jak/Stat pathway and signaling by means of Toll-like receptors (SOCS1,3). These inhibitory effects are complemented by induction of antiproliferative transcription factors. In contrast to this inhibition, G protein-coupled receptors such as CXCR4 and agonists mediating Ca^{2+} flux (inositol trisphosphate receptor subtype 2) are induced by TβR, indicating enhancement of the Ca^{2+} storage/release system and chemotactic responses. Suppression of proapoptotic genes suggests support of cell survival. Confirming the shift in B cell responsiveness, antigen-receptor-mediated activation of Syk and phospholipase C-γ2, as well as Stat6 phosphorylation, is inhibited, whereas chemotaxis, Ca^{2+} release, and cell survival are enhanced in transforming growth factor-β-sensitive B cells. The data provide a molecular basis for TβR-mediated inhibition of B cell responsiveness and indicate that TβR maintains homeostasis not only through inhibition of the cell cycle but also by delivering a coherent instructive signal that redirects responsiveness to microenvironmental cues.

While the central role of transforming growth factor β (TGF-β) in the negative regulation of immune responses is well established (reviewed in refs. 1 and 2), relatively little is known about the cellular mechanisms modulated in response to TGF-β receptor (TβR) signaling. Experimental approaches using a dominant-negative receptor transgene or Cre/*loxP*-mediated, cell-type-specific inactivation of the TβRII gene in mice showed that the homeostatic activity of TGF-β involves direct interaction with lymphocytes. T cells with impaired TβR signaling spontaneously differentiate into effector cells and cause autoimmunity (3). Mice lacking the ligand-binding chain of TβR in B cells (TβRII-B) through conditional mutagenesis (Cre/*loxP*) showed B cell hyperresponsiveness, enlargement of the B1 cell population, enhanced antibody production, and substantial responses to normally weak antigens, with increased autoreactive DNA-binding activity and a selective defect in IgA production (4). Thus, TβR signals regulate lymphocyte activation, differentiation and the maintenance of peripheral tolerance. The ability of TGF-β to inhibit B cell activation has been reported earlier (5, 6), but the mechanisms involved have remained unclear. Induction of cell cycle arrest through induction of cyclin-dependent kinase inhibitors and down-regulation of c-Myc (7) would in principle be sufficient to maintain homeostasis and prevent inappropriate lymphocyte responses. The fact that inactivation of c-Myc in primary B cells compromises their activation and proliferation (8) supports this possibility. However, expression profiling of transformed cells (9, 10) showed that TGF-β can affect gene expression more extensively. The multifunctional nature and the cell type-specific, context-dependent outcome of TGF-β signaling calls for a comprehensive approach to enable the development of coherent models for TβR function in primary cells.

To obtain a comprehensive picture of the effects of TGF-β on B cell responses, we identified TβR-induced transcriptome changes by comparative gene expression profiling. Using primary B cells that lack TβRII (4) as a negative control, we excluded tonic or autocrine signals through this receptor that could compromise detection TGF-β-response genes. Prior knowledge of the molecular mechanisms controlling B cell function (reviewed in refs. 11–15) facilitates the evaluation of TβR-mediated effects on the molecular program, and the phenotype of TβRII-B mice provides further help in relating TGF-β-mediated effects to regulation of B cell responses *in vivo*. Expression of about 100 of ≈6,500 known genes was modulated at least 2-fold by TβR signaling within 1 or 6 h. Approximately half of these were classified as "housekeeping" genes or genes with unknown function (Fig. 6, which is published as supporting information on the PNAS web site, www.pnas.org), The remaining genes encompassed regulators of transcription and of major signaling pathways, indicating a shift of B cell responsiveness from activation to homeostasis. This effect was confirmed by inhibition of B cell antigen receptor (BCR) and Jak/Stat-mediated signal transduction, accompanied by enhanced cell migration and survival *in vitro*, thus demonstrating the ability of TβR not only to inhibit but also to redirect B cell responsiveness to microenvironmental cues.

Methods

Animals. Experiments on animals were carried out according to the regulations under the Home Office (U.K.) Animals (Scientific Procedures) Act 1986. Mice carrying the floxed (i.e., flanked by *loxP* recognition sites for Cre-mediated gene inactivation *in vivo*) TβRII-fl locus (4) were intercrossed with mice expressing the Cre recombinase in B cells (16). B cells of the genotype $T\beta RII^{fl/fl}$; $CD19^{Cre/+}$ served as the test group and B cells from $T\beta RII^{+/+}$; $CD19^{Cre/+}$ mice were used as controls. Mice of the mixed 129×BALB/c genetic background (75%/25%) were used initially for gene expression profiling. Effects on B cell responsiveness *in vitro* were confirmed by using TβRII-B mice of 129 background.

B Cell Enrichment and Stimulation. Splenic B cells were isolated by magnetic anti-CD43 depletion (≥95% purity; Miltenyi Biotec, Auburn, CA) and used directly for stimulation with TGF-β1 to obtain gene expression profiles. The phosphorothioate-modified oligonucleotide 5′-TCCATGACGTTCCTGACGTT-3′ (CpG-ODN) was used at 0.1–1 μM for polyclonal B cell activation. TGF-β1 (R & D Systems) was used at 2–5 ng/ml. Similar

Abbreviations: TGF-β, transforming growth factor β; TβR, TGF-β receptor; BCR, B cell antigen receptor; CpG-ODN, CpG-oligodeoxynucleotide.

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numbers of live control and mutant cells were recovered from the stimulations by using a Histopaque-1083 cushion (Sigma).

RNA Purification and Expression Analysis. RNA was extracted from unstimulated or TGF-β1-treated, depletion-purified B cells and processed by following the recommendations of the microarray manufacturer (Affymetrix). Labeled fragmented antisense RNA was hybridized to MG-U74A v1 or v2 microarrays, washed, and stained as recommended. Microarrays were scanned and the image was checked visually for even hybridization. Comparative analysis of gene expression profiles was carried out by using the statistical expression algorithm of the Affymetrix Microarray Suite V5.0. To identify genes induced by TGF-β, expression profiles from mutants were used as a baseline in the comparative analysis. TGF-β-suppressed genes were identified, using the controls as a base line. The homogeneity of hybridization over a probe array representing a single gene is reflected in a "detection p value." Probe sets that matched or exceeded the following criteria were selected: signal intensity >200, detection p value <0.1, >2-fold difference in signal intensity (signal $\log_2$ ratio >1), increased or medium increased expression over baseline, and an expression change p value <0.1. The latter gives the significance of the change between the comparison files. By using Affymetrix software MICRODB and Data Mining Tool (DMT), probe sets that passed the above criteria in at least three of four comparisons (unstimulated cells) or four of five comparisons (stimulated cells) in one or more of the culture conditions were identified. Only ORFs with known or inferred function were considered for further analysis. MHC genes were excluded because of heterogeneous genetic background of the mutants. Detection p values for the qualifying ORFs were <0.067, with expression change p values <0.003. Fold change expression values in the analysis for TGF-β-suppressed genes (mutants vs. control baseline) were multiplied by -1 to indicate suppression and combined with the data for TGF-β-induced genes (control vs. mutant baseline). Values (fold differences) for genes with established function were then grouped according to known or inferred function and graphically presented by using TREEVIEW software (17) available at http://rana.lbl.gov/EisenSoftware.htm.

Signaling Studies, Ca^{2+} Flux, Apoptosis, and Migration Assays. Live prestimulated B cells were washed and incubated for 2–4 h in Hanks' balanced salt solution (HBSS)/0.5% BSA/20 mM Hepes before stimulation with anti-IgM (Jackson ImmunoResearch), or anti-IgH (Southern Biotechnology Associates) for 5 min or IL-4 and IFN-γ (Insight Biotechnology, Wembley, U.K.) for 20 min. Antibodies for Western blotting were from Cell Signaling Technology (Beverly, MA), for flow cytometry including anti-CXCR4 antibody (clone 2B11) and anti-CD72abd were from BD PharMingen. For Ca^{2+} flux, CpG-ODN-prestimulated B cells were washed and equilibrated in B cell medium (DMEM/10% FCS/sodium bicarbonate/nonessential amino acids/sodium pyruvate/2-mercaptoethanol/Hepes/antibiotics) for 3 h at 37°C, under a 5% CO_2/95% air atmosphere, washed in DMEM/0.5% BSA/25 mM Hepes, and labeled in this medium with 1 μM Indo-1 acetoxymethyl ester (Molecular Probes) in the presence of 4 mM probenecid (Sigma) for 45–60 min at 37°C. Analysis was carried out on a LSR flow cytometer (Becton Dickinson) at 37°C with live cells gated by using the Fl5 parameter. Baseline fluorescence was acquired for 30 s, before addition of the agonist. The change in fluorescence ratio between free (520 nm) and Ca^{2+}-bound Indo-1 (390 nm) was recorded as the ratio of Fl4/Fl5 fluorescence. For chemotaxis assays, unstimulated depletion-purified B cells were incubated in a humidified 5% CO_2 atmosphere at 37°C, for 45 min in RPMI medium 1640/10% FCS in the absence or presence of TGF-β1 (2 ng/ml). Cells (10^6) were placed in the top chamber of transwells (5-μm-pore filter, Costar), in RPMI medium 1640/0.2% BSA and incubated for 2–4 h with agonist in the bottom chamber before cells that had migrated into the bottom chamber were counted. Apoptotic cell death was assessed by flow cytometry using propidium iodide. To determine the extent of DNA fragmentation cells were fixed with 70% ethanol, treated with RNase A (200 μg/ml) in PBS/0.1% Triton X-100, which causes loss of fragmented DNA from the cell, and emergence of a sub-G_1 peak upon staining with propidium iodide. The effects of TβR on the B cell response have been confirmed in at least three independent experiments.

Results and Discussion

Identification of TGF-β Response Genes in Primary B Cells. To detect genes modulated by TβR, splenic depletion-purified B cells were stimulated with TGF-β alone or in conjunction with anti-IgM. The latter was included because costimulation of the BCR should reveal any negative effects of TGF-β on genes induced by BCR crosslinking. Whereas freshly purified B cells showed remarkably few differences, stimulation with TGF-β and/or anti-IgM led to differential expression of ≈100 of ≈6,500 genes with known or inferred function, within 1 or 6 h of stimulation (Fig. 1). The representation of known TGF-β response genes such as the negative-feedback mediator of TGF-β signaling, *Madh7/Smad7*, the Ski-related transcriptional corepressor (*Skir*, Fig. 1 *Top*), as well as the transcription factors *Myc*, and *Junb* (Fig. 1), confirmed that TGF-β response genes were readily detected by this experimental approach. Importantly, IgG3 (Cγ3) emerged as a TGF-β-suppressed gene, whereas IgA (Cα) transcripts were induced in TGF-β-responsive cells (Fig. 1 *Top*), reflecting the differential effects of TβR on IgG3 and IgA responses *in vivo* (4). The remaining TβR-modulated genes were classified by their known or inferred function and grouped tentatively as transcription factors, modulators of signal transduction through BCR, cytokine receptors, G protein-coupled receptors, and regulators of cell cycle and apoptosis. The main implications of the results and the confirmation of the implied TGF-β effects on B cell responses *in vitro* are discussed below.

Modulators of Transcription and Proliferation. Repressor functions or antiproliferative effects have been demonstrated for most of the transcriptional modulators induced by TβR (Fig. 1). Significantly, *Bhlhb2/Stra13*, which is induced by TβR, prevents spontaneous activation of lymphocytes and autoimmunity (18), indicating its essential role in the maintenance of lymphocyte homeostasis. The suppression of the agonists c-*Myc* and *Utf1* would support the overall inhibitory effect. Induction of the cyclin-dependent kinase inhibitor $p21^{waf}$ (*Cdkn1a*), suppression of calcyclin (*S100a6*) and of the ataxia telangiectasia gene (*Atm*) product, which are involved in cell cycle control, are in line with TβR-induced inhibition of cell proliferation.

Antigen Receptor Signaling. The BCR complex not only is necessary for the development and survival of B cells (11) but also regulates differentiation of B cell subpopulations according to signal strength (15). Accessory molecules that are recruited to the BCR signaling complex (12, 14) control the initiation of a signaling cascade that leads by means of phosphatidylinositol 3-kinase and tyrosine kinases to activation of phospholipase C-γ2, induction of Ca^{2+} flux, activation of mitogen-activated protein kinases and protein kinase Cs, and the mobilization of transcription factors (13). As shown in Fig. 1, TβR induces receptor-proximal and -distal regulators of the BCR signaling pathway. The inositol-polyphosphate phosphatase Ship-1 (*Inpp5d*) antagonizes phosphatidylinositol 3-kinase activity and inhibits BCR signaling by hydrolysis of phosphatidylinositol trisphosphate. Ship-1 can be induced directly via the Smad pathway (19). CD72 recruits the inhibitory phosphotyrosine phosphatase SHP-1, which antagonizes the activity of Src-family

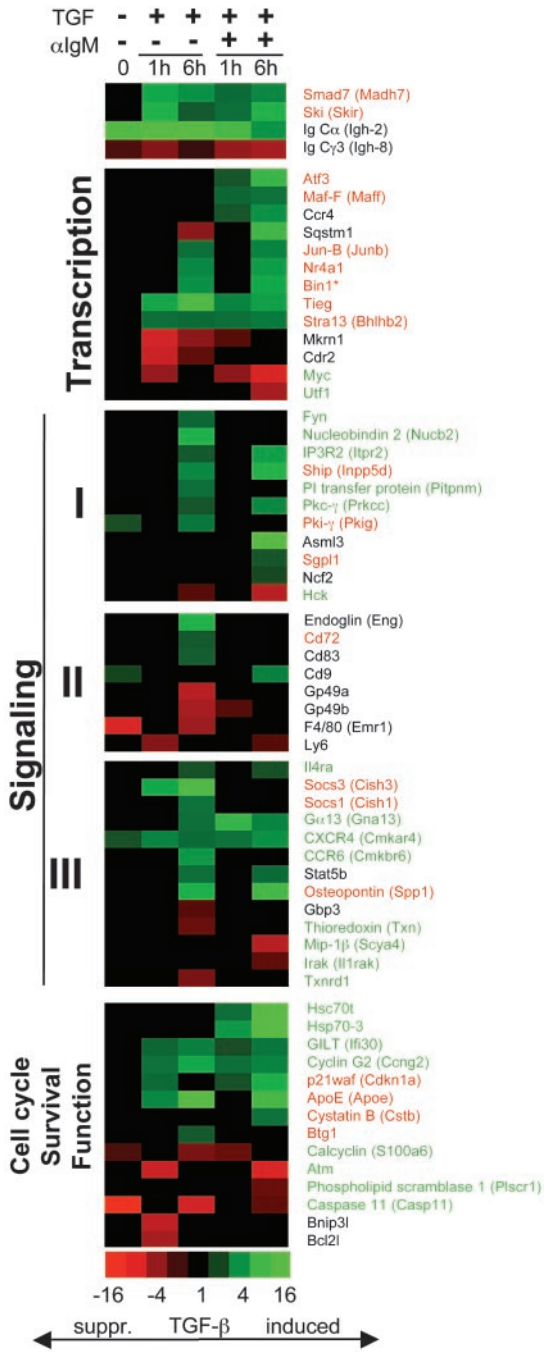

Fig. 1. TβR-dependent transcriptome modulation in primary B cells. Depletion purified B cells were left untreated or were stimulated for 1 or 6 h with TGF-β alone or in combination with anti-IgM as indicated. TβR-mediated effects on known TGF-β response genes and the Ig gene loci deregulated in TβRII-B mice are shown in *Top*. Genes with reproducibly ≥2-fold differences in three of four (unstimulated cells) or four of five (stimulated cells) comparisons are tentatively grouped by known or inferred function. Green in the clusters indicates up-regulation, and red indicates suppression in TGF-β-responsive controls relative to the mutants. Green text indicates ''agonistic'' function, and red text indicates inhibitory function. Where commonly used gene names are given, they are followed by the official gene symbol in parentheses. * indicates interim gene symbol.

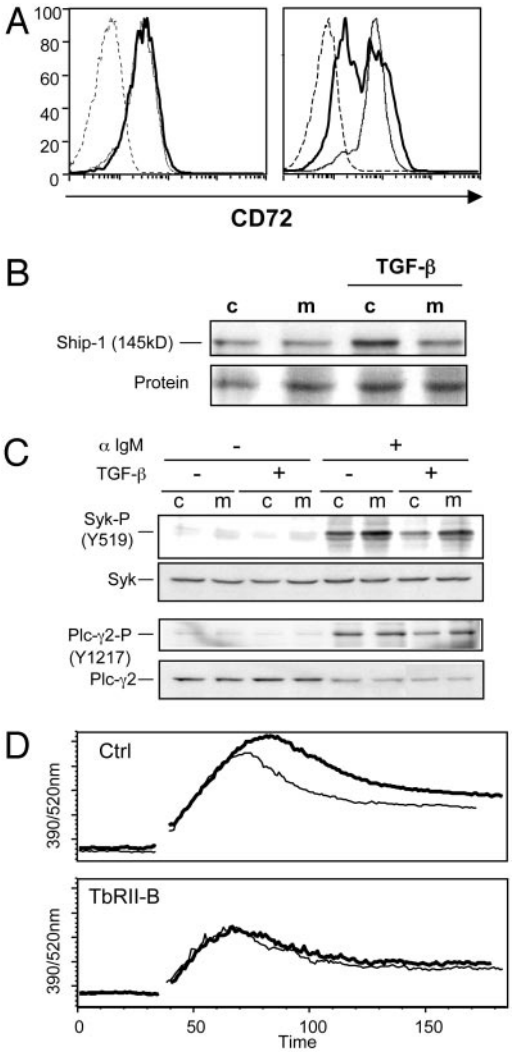

Fig. 2. Modulation of BCR signaling by TβR in CpG-ODN-activated B cells. (*A*) CD72 expression of control (thin line) or TβRII-B B cells (bold line) before (*Left*) or after (*Right*) stimulation with 1 μM CpG-ODN for 72 h and TGF-β for the last 16 h is shown. Negative staining control is indicated by the dashed line. (*B*) Expression levels of Ship-1 in control (c) and TβRII-B mutant B cells (m) are revealed by Western blot analysis of purified B cells stimulated as in *A*. Ponceau staining (protein) is shown to document equivalent loading. (*C*) Western blots of cell lysates from purified B cells prestimulated as in *A* for total and tyrosine-phosphorylated Syk and phospholipase C-γ2 with and without stimulation by 10 μg/ml anti-IgM. (*D*) Ca^{2+} response profiles of control (*Upper*) and mutant (*Lower*) B cells stimulated with 10 μg/ml anti-IgM, with (bold line) or without (thin line) TGF-β treatment. Data were confirmed in independent experiments.

kinases activated upon BCR engagement. Significantly, inactivation of Ship or CD72 in cell lines or mice causes B cell hyperresponsiveness, increased antibody production, and impaired B cell homeostasis (20–23), with similarities to the TβRII-B phenotype (4).

Despite the modulation of mRNA expression, treatment of resting B cells with TGF-β did not affect signaling via the BCR and other pathways significantly (data not shown). To establish whether the regulatory effects indicated by the TβR-dependent transcriptome change led to corresponding changes at the protein level, induction of metabolic activity was necessary to enhance protein turnover and facilitate *de novo* protein synthesis. We chose CpG-ODN, which mediate B cell activation via TLR9 (24), for polyclonal B cell activation. Whereas TGF-β inhibited CpG-ODN-induced B cell activation and proliferation in controls, mutants were refractory to this inhibition, confirming their impaired responsiveness to TGF-β (see below and data

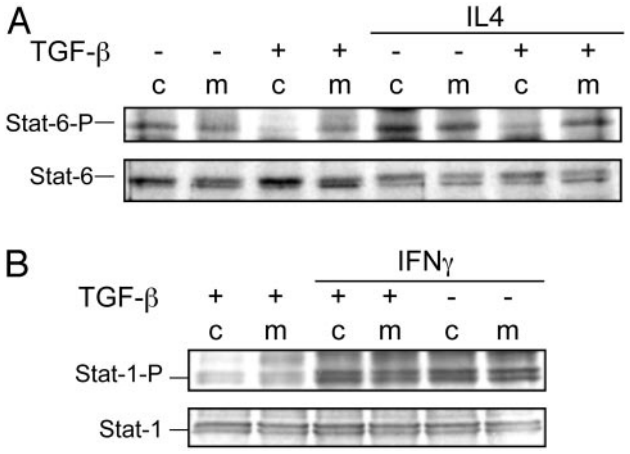

Fig. 3. Inhibition of Stat6 phosphorylation by TβR. Shown is Western blot analysis of control (c) or mutant (m) B cells stimulated for 3 days with CpG-ODN in the absence or presence of TGF-β during the last 16 h, probed with antibodies specific for Stat6 or Stat1 (*Lower*) or their phosphorylated forms (*Upper*). Cell recovery after density gradient purification was similar in controls and mutants (data not shown). (*A*) Activated B cells show constitutive Stat6 phosphorylation on Tyr-641, perhaps because of IL4 derived from small numbers of T cells in these primary cultures. Addition of IL4 leads to an increase. Stat6 phosphorylation is suppressed by TGF-β in the controls, whereas it is unaffected in the receptor mutants. (*B*) Stat1 phosphorylation on Tyr-701 by IFN-γ is not affected by TβR signaling. Data were confirmed in independent experiments.

not shown). As shown in Fig. 2*A*, CD72 was expressed at high levels on activated B cells. Unlike controls, a major fraction of mutant B cells showed reduced expression of this inhibitory receptor (Fig. 2). Notably, this did not require addition of exogenous TGF-β (data not shown) suggesting that "tonic" TβR signaling, and/or autocrine stimulation in activated B cells is sufficient to sustain this inhibitory mechanism. Also, the induction of Ship-1 indicated by the mRNA expression data above was confirmed by Western blotting (Fig. 2*B*).

Activation of the tyrosine kinase Syk is critical for phospholipase C-γ2 activation in response to BCR signaling. Because Src family kinases contribute to Syk activation (13, 15) TβR-mediated enhancement of the CD72/Shp-1 complex is implicated in the negative regulation of this process. As shown in Fig. 2*C*, BCR-induced phosphorylation of tyrosine kinase Syk at activating tyrosine-519 is indeed suppressed by TGF-β in the controls, but not in mutant B cells. Similarly, phospholipase C-γ2 activation is suppressed in TGF-β-responsive cells. Impaired TβR-mediated inhibition of BCR signaling may contribute to the B cell hyperresponsiveness of TβRII-B mice (4) and explain the increased numbers of B1 cells *in vivo*, as the size of this population is positively correlated with BCR signal strength (reviewed in ref. 15).

While inhibiting proximal BCR signaling, TβR had a positive effect on distal regulators of this pathway (*IP3R2*, *Nucb2*, *Pitpnm*, *Prkcc*). For example, the inositol trisphosphate receptor subtype 2 (*IP3R2*) modulates BCR-induced Ca^{2+} responses, whereas the tyrosine kinase Fyn can activate inositol trisphosphate-gated Ca^{2+} channels. By measuring BCR-induced Ca^{2+} flux directly we found that the enhancing effects of TβR on distal components of the Ca^{2+} storage/release system seemed to overcompensate the inhibitory effects on BCR signaling in this experimental system. As shown in Fig. 2*D*, Ca^{2+} flux in response to BCR crosslinking is enhanced by TGF-β in controls. Mutant B cells are not only refractory to the TGF-β-induced enhancement, but show on overall blunted Ca^{2+} response. The proximal inhibition of the BCR may serve to counterbalance the enhancement of the distal Ca^{2+} storage/release system on which distinct signaling pathways converge (see below).

Inhibition of Cytokine Receptor Signaling. IL-4 produced by type 2 helper T cells (Th2) enhances the proliferation and survival of B cells, while promoting Ig isotype switching to IgG1 and IgE via the Jak/Stat pathway, specifically Stat6 (25). As shown in Fig. 1, *Cish1* and *Cish3* are induced in TGF-β-responsive cells. They encode members of a small family of pleiotropic regulators (SOCS/JAB/SSI) known to inhibit signaling of a wide range of growth factor receptors linked to the Jak/Stat pathway, and also inhibit Toll-like receptors (26–28). In line with the induction of these inhibitors and earlier studies reporting TGF-β-mediated inhibition of IL4 signaling in transgenic T cells (29), activated mutant B cells showed increased phosphorylation of Stat6 (Fig. 3). Notably, this is observed without addition of IL4, possibly triggered by IL4 derived from small numbers of contaminating T cells in these primary cultures. Addition of IL4 caused a small increase in Stat6 phosphorylation. Importantly, TGF-β suppressed Stat6 phosphorylation in the controls, whereas phosphorylation levels in the mutants remained high. The inhibitory effect of TGF-β on Stat6 was selective, as Stat-1 phosphorylation upon stimulation with IFN-γ was not affected. Induction of pleiotropic negative regulators such as SOCS1 and SOCS3 would explain the wide-ranging regulatory potential of TβR. The negative effects on Stat6 phosphorylation indicate that TβR can inhibit T cell-dependent production of IgG1 and IgE, thus providing an explanation for the disproportional increase in IgG1 production seen in TβRII-B mice (4).

Enhancement of G Protein-Coupled Receptor Pathways, Chemokine Responsiveness, and Cell Survival. The chemokine receptor genes *Cmkar4* (CXCR4) and *Cmkbr6* (CCR6) are consistently induced

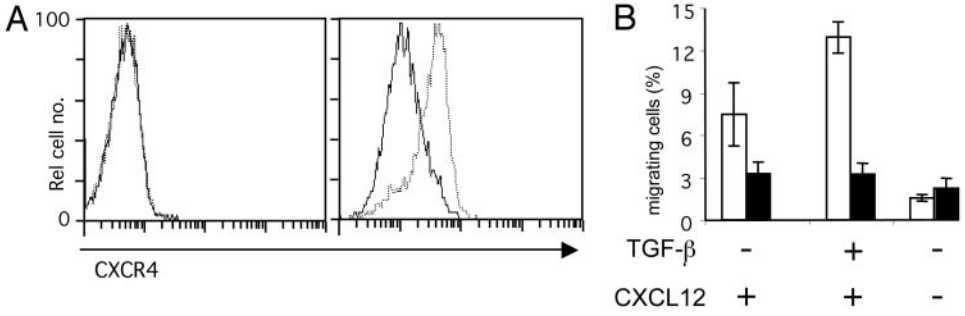

Fig. 4. TβR-mediated induction of CXCR4 and responsiveness to CXCL12 in B cells. (*A*) CXCR4 expression on B cells (CD45R/B220$^+$) from controls (dotted line) or TβRII-B mice (solid line) before (*Left*) or after (*Right*) overnight incubation in serum-containing medium. (*B*) Migratory response of depletion-purified control (open bars) or mutant B cells (filled bars) to the CXCR4 ligand CXCL12. Inclusion of ligand in the top chamber blocked migration to background levels (data not shown). Data were confirmed in independent experiments.

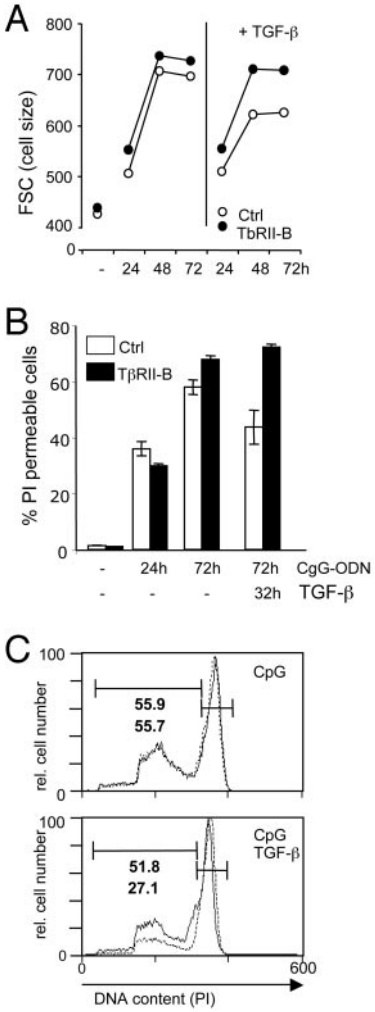

Fig. 5. Inhibition of activation and cell death in primary B cells by TβR. (*A*) Blastogenesis in response to stimulation with CpG-ODN was determined by flow cytometry. Depletion-purified B cells from control (○) and TβRII-B (●) mice were treated with 1 μM CpG-ODN and TGF-β (2 ng/ml) as indicated. Standard deviations of triplicate values were <5%. (*B*) The bar chart shows the accumulation of propidium iodide (PI)-permeable cells in CpG-ODN-stimulated B cell cultures. TGF-β suppresses the accumulation of dead cells in controls (open bars) but not mutants (filled bars). Cells were stimulated as above, and the number of cells with permeable membranes was determined by exclusion of propidium iodide, in triplicate. (*C*) Stimulation with CpG-ODN for 72 h is accompanied with apoptotic DNA fragmentation indicated by the sub-G_1 peak, which derives from loss of fragmented DNA. Control (dotted line) and mutant (solid line) B cells (*Upper*) showed similar levels of DNA fragmentation unless TGF-β1 was added, suppressing fragmentation in the controls (*Lower*). Numbers in the histograms indicate the frequency (%) of cells in the sub-G_1 peak. The upper number at the marker refers to mutant, and the lower number refers to control. Data were confirmed in independent experiments.

in a TβR-dependent manner (Fig. 1). The concomitant suppression of the chemotactic factor genes *Scya4* (Mip1β) and *Txn* (thioredoxin), which can attract costimulatory cells such as T cells, is in line with protection from stimulatory activity. Although support of CXCR4 levels by TβR was evident at the level of mRNA in unstimulated splenic B cells, protein levels were below the detection limit (Fig. 4). Upon incubation *in vitro*, however, CXCR4 levels increased ≈8-fold in the controls. Mutant B cells also showed a 2- to 3-fold increase in expression, indicating that CXCR4 induction is not absolutely dependent on TβR signaling. Reflecting the reduced levels of CXCR4 expression, mutant B cells (Fig. 4) showed markedly impaired chemotaxis toward the CXCR4 ligand CXCL12 (SDF1). Moreover, addition of TGF-β boosted chemotaxis in the controls, whereas migration of the mutant cells was unaffected. Our *in vivo* analysis of TβRII-B mice did not reveal major positive effects of TβR on B cell homing to lymph nodes or Peyer's patches, which may be partly controlled by CXCR4 (30). Functional overlap of CXCR4 and CCR7 and/or increased proliferation of hyperresponsive TβR-negative B cells could explain this apparent discrepancy. The enhancement of GPCR signaling by TβR is further supported by induction of *Gna13* (Fig. 1), which encodes the Gα13 subunit of heterotrimeric G proteins. Gα13 regulates p115RhoGEF/Lsc and Pyk-2, which both contribute to marginal-zone B cell homeostasis (31, 32), and couples with the phospholipid receptor G2a, inactivation of which leads to late-onset autoimmunity (33). Because chemokine receptor signaling triggers Ca^{2+} flux, the positive effects of TβR on the Ca^{2+} storage release system described above would further support the enhancement of this signaling pathway.

Suppression of genes encoding proapoptotic products including caspase 11 (*Casp11*) and phospholipid scramblase 1 (*Plscr1*) indicates TβR-mediated support of cell survival. As shown in Fig. 5, TGF-β inhibited CpG-ODN-induced B cell blastogenesis, in line with the induction of Toll-like receptor inhibitors discussed above. This inhibition was accompanied by protection from cell death as indicated by the reduced accumulation of cells with permeable membranes. The positive effect on survival of activated B cells was confirmed by reduced apoptotic DNA fragmentation in controls (Fig. 5*C*). The antiapoptotic effect of TGF-β described here contrasts with the widely reported direct proapoptotic effects on B cells and transformed cell lines (34–36). Our data show that in the activated mature primary B cell, TGF-β-mediated inhibition of activation is accompanied by support of cell survival, thus highlighting the context-dependent nature of this signaling mechanism and its ability to modulate complex apoptotic programs specifically. Constructive support of B cell function is further indicated by induction of IfI30/GILT, a lysosomal reductase, which enhances processing and MHC class II-restricted presentation of proteins containing disulfide bonds (37).

Effects on Other Signaling Pathways. Induction of a number of genes involved in responses to pleiotropic mediators is consistent with TβR-mediated inhibition of activation and support of migratory responses. These include *Pki*γ (Fig. 1), which encodes a potent inhibitor of PKA, *ApoE*, which has antiproliferative properties and is induced in tolerized lymphocytes (38, 39), and sphingosine-1-phosphate (S1-P) lyase (*Sgpl1*) and acid sphingomyelinase-like gene 3a (*Asml3*). Induction of S1-P lyase would favor degradation of S1-P, a pleiotropic agonist with activating and mitogenic properties, which has also been shown to inhibit lymphocyte recirculation by inducing sequestration in lymph nodes (40).

Concluding Remarks. The present study aims to provide a global picture of the TβR effects that modulate β cell responsiveness and homeostasis. Suppression of c-Myc and cell cycle progression could in principle account for TβR-mediated inhibition of B cell responses (8), by limiting clonal expansion of antigen-specific B cells. However, the regulatory mechanisms mobilized by TβR evidently reach far beyond modulation of transcription factors and cell cycle regulators. The data presented reveal the ability of TβR to modulate major signaling pathways controlling B cell activation, differentiation, and homeostasis. Apart from increased numbers of B1 cells, TβRII-B mice show both B cell hyperplasia and hyperproduction of IgG1 in the gut-associated Peyer's patches, indicating the key role of TGF-β in the negative regulation and direction of B cell responses in these anatomical sites of high immunogenic drive (4). The profound inhibitory

effects of TβR on B cell activation by means of BCR and Toll-like receptors would help maintain homeostasis, whereas inhibition of Stat6 activation would suppress differentiation of IgG1-expressing cells, in favor of TGF-β-mediated induction of IgA responses.

The extended connectivity reflected in the TβR-dependent transcriptome changes enables a coherent instructive signal, which not only mediates homeostatic control through reinforced suppression of activation, proliferation, and differentiation but also provides for concomitant support of B cell survival and enhancement of homeostatic pathways. It indicates that this ancient signaling mechanism, whose evolutionary origin seems to coincide with the appearance of the bilateral body plan about 10^9 years ago (41), has been progressively refined and integrated into the regulatory network of specialized mammalian cells. Further characterization of TβR target genes and their contribution to control of differentiation and homeostasis should help identify critical nodes in the complex regulatory network and facilitate prediction of effects arising from malfunction or therapeutic modulation of the TβR/Smad pathway. For example, TGF-β-induced chemokine receptors such as CXCR4 can direct tumor metastasis (42). Thus, modulation of TGF-β levels or Smad signaling, through genetic polymorphisms (43) or therapeutic intervention, may affect the metastatic behavior of tumor cells considerably. The comprehensive analysis of transcriptome changes in response to multifunctional growth and differentiation factors is instrumental in revealing the spectrum of coordinated transcriptome changes, which, taken together, ultimately (re)direct cellular responses in adaptation to microenvironmental cues. The integration of appropriate genetic tools can enhance the signal-to-noise ratio and the quality of the overall picture obtained.

We thank Mike Hubank at the Institute of Child Health for facilitating access to the Affymetrix microarray suite and Gemma Lee and Leigh Madden for technical support. This work was supported by the Wellcome Trust.

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