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Engagement of CD153 (CD30 Ligand) by CD30+ T Cells Inhibits Class Switch DNA Recombination and Antibody Production in Human IgD+ IgM+ B Cells[1]

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Abstract

CD153 (CD30 ligand) is a member of the TNF ligand/cytokine family expressed on the surface of human B cells. Upon exposure to IL-4, a critical Ig class switch-inducing cytokine, Ag-activated T cells express CD30, the CD153 receptor. The observation that dysregulated IgG, IgA, and/or IgE production is often associated with up-regulation of T cell CD30 prompted us to test the hypothesis that engagement of B cell CD153 by T cell CD30 modulates Ig class switching. In this study, we show that IgD^+ IgM^+ B cells up-regulate CD153 in the presence of CD154 (CD40 ligand), IL-4, and B cell Ag receptor engagement. In these cells, CD153 engagement by an agonistic anti-CD153 mAb or T cell CD30 inhibits $S\mu \rightarrow S\gamma$, $S\mu \rightarrow S\alpha$, and $S\mu \rightarrow S\varepsilon$ class switch DNA recombination (CSR). This inhibition is associated with decreased TNFR-associated factor-2 binding to CD40, decreased NF-κB binding to the CD40-responsive element of the $C\gamma 3$ promoter, decreased $I\gamma 3$-$C\gamma 3$ germline gene transcription, and decreased expression of Ku70, Ku80, DNA protein kinase, switch-associated protein-70, and Msh2 CSR-associated transcripts. In addition, CD153 engagement inhibits IgG, IgA, and IgE production, and this effect is associated with reduced levels of B lymphocyte maturation protein-1 transcripts, and increased binding of B cell-specific activation protein to the Ig 3′ enhancer. These findings suggest that $CD30^+$ T cells modulate CSR as well as IgG, IgA, and IgE production by inducing reverse signaling through B cell CD153.

Human IgD^+ IgM^+ naive B cells undergo switching to IgG, IgA, or IgE upon exposure to IL-4 and CD154 (CD40 ligand, CD40L)[3] (1–3), a molecule expressed by $CD4^+$ T cells 4–6 h after TCR engagement by Ag exposed on APCs (4, 5). Engagement of CD40 by CD154

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induces rapid recruitment of TNFR-associated factors (TRAFs) to the CD40 cytoplasmic domain (6–9). This CD40:TRAF complex is thought to elicit the activation and nuclear translocation of NF-κB (10, 11), a transcription factor that, together with IL-4-induced STAT-6, plays a critical role in the germline transcription of downstream Ig C_H genes (12, 13). Within 2–3 days, I_H-C_H germline transcription of a targeted C_H gene induces class switch DNA recombination (CSR) to the same gene (12). Although the inducers of CSR are largely known, the mechanisms deputed to the negative modulation of this process remain elusive.

Ag-stimulated $CD4^+$ T cells initiate the Ab response by activating naive B cells in the T cell-rich extrafollicular areas of secondary lymphoid organs (4, 12). Upon recruitment to the lymphoid follicle, activated B cells undergo proliferation, germinal center (GC) phenotypic differentiation, Ig V(D)J gene somatic hypermutation, and Ig class switching (12, 14). Within the GC, Ag-activated CD95L $(FasL)^+$ T cells modulate Ig production by deleting autoreactive and low affinity B cells through CD95 (Fas), a TNFR family member expressed by B cells upon CD40 engagement by CD154 (15, 16). Among $CD95^+$ GC B cells, only high affinity (Ag-selected) B cells are rescued from CD95-mediated apoptosis and differentiate into Ig class-switched memory B cells or plasma cells (14–17).

Recent studies suggest that additional T cell-dependent mechanisms modulate B cell selection in the GC. In the presence of CD154, IgD^+ IgM^+ B cells express CD30, a death domain-less TNFR family member (18). In these cells, CD30 engagement by CD153 (CD30L) on $CD8^+$ T cells inhibits CD154 and IL-4-induced Ig class switching. Because B cell Ag receptor (BCR, surface Ig) engagement down-regulates CD30 expression (18), CD30 would represent a CD95-independent pathway limiting the CD40-mediated GC progression of low affinity B cells. In these cells, CD30 and CD95 might constitute complementary checkpoints at distinct B cell differentiation stages, as CD30 and CD95 are mainly expressed by perifollicular activated B cells and GC B cells, respectively (16, 17, 19).

The finding that CD95- and CD30-mediated signals are mainly effective in non-Ag-selected B cells suggests that additional mechanisms limit CSR and Ab production in B cells that have been positively selected by Ag. Few days after CD28 and IL-4R engagement, Ag-activated T cells express CD30 while undergoing clonal expansion as part of a specific immune response (20, 21). The expression of T cell CD30 is up-regulated in disorders associated with dysregulated IgG, IgA, and/or IgE production, including autoimmune and allergic diseases (22–24). This observation, together with the notion that human mature B cells express CD153 (CD30L) (25–27), prompted us to hypothesize that engagement of B cell CD153 by T cell CD30 modulates CSR.

CD153 is a member of the TNFL superfamily, a group of type II transmembrane glycoproteins that includes CD27L (CD70), FasL (CD95L), OX40L (CD134L), 4-1BB

[3]Abbreviations used in this paper: L, ligand; BCR, B cell Ag receptor; Blimp-l, B lymphocyte maturation protein-1; BSAP, B cell-specific activation protein (Pax5); CSR, Ig class switch DNA recombination; DNA-PK, DNA protein kinase; FM, follicular mantle; GC, germinal center; htCD154, soluble human trimeric CD154; PB, peripheral blood; RAG, recombination-activating gene; RE, responsive element; SA, streptavidin; SWAP-70, switch-associated protein-70; TRAF, TNFR-associated factor.

ligand (CD137L), and CD40L (CD154) (28). Several studies have shown that, while binding to their receptors, these molecules, including CD134L and CD154, elicit important biologic effects (29–41). The cytoplasmic domain of CD153 consists of 38 aa and is highly conserved across species (28, 42), suggesting that, like CD134L or CD154, whose cytoplasmic tails consists of 24 and 22 aa, respectively, CD153 can transmit intracellular signals. In agreement with this possibility, CD153 cross-linking by an immobilized agonistic mAb or a human CD30:Fc IgG1 chimeric protein has been shown to induce gene expression and metabolic activation in human T cells and neutrophils (43). The function of CD153 in human B cells remains unknown.

In this study, we show that, in the presence of CD154, IL-4, and BCR engagement, human IgD^+ IgM^+ B cells up-regulate surface CD153. In these cells, engagement of CD153 by T cell CD30 inhibits CSR as well as IgG, IgA, and IgE production. Our findings suggest that, by inducing reverse signaling through CD153, $CD30^+$ T cells modulate the CD154-dependent entry of IgD^+ and/or IgM^+ B cells, including Ag-selected $CD30^-$ B cells, into the pool of B cells producing IgG, IgA, or IgE Abs.

Materials and Methods

Cells

The CL-01 B cell line has been described (2, 3, 13, 18, 44, 45). PB or tonsil B cells were isolated, as reported (13, 44). IgD^+, IgD^-, IgG^+, and IgG^- B cell fractions were magnetically segregated upon B cell incubation with FITC-conjugated mouse or goat Abs to IgD or IgG (Southern Biotechnology Associates, Birmingham, AL) and anti-FITC MicroBeads (Miltenyi Biotec, Auburn, CA). Tonsil IgD^+ $CD38^-$ and IgD^+ $CD38^+$ B cells were selected upon incubation of purified IgD^+ B cells with MultiSort Release Reagent (Miltenyi Biotec), FITC-conjugated mouse mAb to CD38 (PharMingen, San Diego, CA), and anti-FITC MicroBeads. Tonsil IgD^- $CD38^+$ and IgD^- $CD38^-$ B cells were segregated in a similar fashion from purified IgD^- B cells. Plasmacytoid differentiation was induced by culturing B cells with 1 μg/ml of htCD154 (Immunex, Seattle, WA), 100 U/ml of IL-2 (Life Technologies, Grand Island, NY), 100 U/ml of IL-4, and 200 ng/ml of IL-10 (Schering-Plough, Kenilworth, NJ). Under these conditions, activated B cells produced large amounts of IL-6 (3). To obtain $CD4^+$ $CD30^+$ T cells, PBMCs were stimulated with 100 U/ml of IL-4 (Schering-Plough) and 1 μg/ml of anti-CD3 (from Dr. K. Smith, Weill Medical College of Cornell University, New York, NY), anti-CD28 (PharMingen), and anti-CD134 (anti-OX40; a gift from Dr. Y. Tozawa, Kitasato University, Kanagawa, Japan) mAbs immobilized on irradiated (7000 rad) mouse CD32 expressing L cells. Anti-CD134 mAb was added 24 h after the onset of the culture. Four days later, $CD4^+$ T cells were magnetically sorted by incubating monocyte-depleted PBMCs with FITC-conjugated mouse mAb to CD4 (Southern Biotechnology Associates) and anti-FITC mAb-conjugated MicroBeads. $CD4^+$ $CD30^+$ T cells were purified by incubating sorted $CD4^+$ T cells with MultiSort Release Reagent, a biotinylated mAb to CD30 (PharMingen), and SA MicroBeads. Upon expansion with 100 U/ml of IL-2, 1:100 (v/v) PHA (Life Technologies), and 5 μM of TNF-α protease inhibitor (Immunex), $CD30^-$ and $CD30^+$ T cells were fixed with 1% paraformaldehyde.

Cell cultures

Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Life Technologies), 2 mM L-glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. IgD^+ or IgD^- B cells and $CD4^+$ (total, $CD30^-$ or $CD30^+$) T cells were seeded in 96-microwell plates at 0.5×10^5 and 2×10^5 cells/well, respectively. Mouse M81 or M80 mAbs to CD153 (Immunex), human CD30:Fc IgG1 (Immunex), human CTLA-4 (CD152):Fc IgG1 (R&D Systems, Minneapolis, MN), mouse control MOPC-21 mAb (Sigma, St. Louis, MO), mouse BU52 mAb to CD44 (The Binding Site, San Diego, CA), and mouse TAG34 mAb to CD134L (OX40L) (from Dr. T. Hori, Kyoto University, Kyoto, Japan) were immobilized on irradiated CD32 expressing L cells or on plastic plates at 5 μg/ml. In some experiments, anti-CD153 or control mAbs were added to naive B cells 24 h after htCD154 and cytokines. Blocking mouse mAbs to CD27 (M86; Immunex), CD30 (Ber-H2; Dako, Carpenteria, CA), and CD154 (24–31; Ancell, Bayport, MN) were used at 30 μg/ml. htCD154, IL-4, IL-10 (Schering-Plough), and IL-6 (Genzyme, Cambridge, MA), and Sepharose-conjugated Ab to Ig H and L chain (anti-BCR) (Irvine Scientific, Santa Ana, CA) were used at 1 μg/ml, 250 U/ml, 200 ng/ml, 100 U/ml, and 4 μg/ml, respectively.

Flow cytometry

FITC- or PE-conjugated mAbs to the following Ags were used: CD154 (Ancell), CD23, CD77 (Coulter, Miami, FL), CD30 (Dako), CD40, CD38 (PharMingen), CD3, CD4, CD8, CD19 (Sigma), CD10, CD19 (Becton Dickinson, San Jose, CA), CD38, IgD, IgM, IgA, IgG (Southern Biotechnology Associates), and CD138 (Serotec, Oxford, U.K.). To detect CD153 in tonsil B cell subsets, enriched B cells were labeled with unconjugated mouse mAb to CD153 (Immunex), washed, and further incubated with PE-conjugated Ab to mouse Igs (PharMingen). After extensive washes, these cells were incubated with FITC-conjugated anti-CD38 and biotin-conjugated anti-IgD (Southern Biotechnology Associates), washed, and finally labeled with peridinin chlorophyl protein-conjugated SA (Becton Dickinson). To analyze CD30 and CD154 on PB T cells, PBMCs were stained with FITC anti-CD3, biotin-conjugated anti-CD30 (labeled with peridinin chlorophyl protein-conjugated SA), and PE-conjugated anti-CD154, or anti-CD4 mAbs. Cell cycle analysis and apoptosis assays were performed with propidium iodide (Sigma) and annexin-V (R&D Systems). Cells (10^4) were acquired using a FACSCalibur analyzer (Becton Dickinson), and data processed using a MacIntosh CELL-Quest software program (Becton Dickinson).

Immunohistochemistry

Immunohistochemistry was performed by indirect immunoperoxidase staining of Formalin-fixed, paraffin-embedded tissue sections from different human lymphoid organs, including lymph nodes, tonsils, and spleen. A TechMate500TM BioTek automated immunostainer (Ventana Medical Systems, Tucson, AZ) was used with Abs recognizing IgD (Dako) and CD153 (Immunex). The pressure cooker and trypsinization Ag-retrieval methods were used for IgD and CDl53 staining, respectively.

ELISAs and cell proliferation assays

Culture supernatants were tested for IgG, IgA, IgE, and IL-6 concentration using specific ELISAs (Biosource International, Camarillo, CA). For proliferation assays, B cells (0.5×10^5) were seeded in 96-well plates and pulsed with 1 μCi of [^{3}H]TdR at day 3 of culture. After 18 h, cells were harvested for the measurement of [^{3}H]TdR uptake (18).

PCRs and Southern blots

cDNAs were reversed transcribed from equal amounts of total RNA (3 μg) (18). PCR analysis was made semiquantitative by varying the number of amplification cycles and performing dilutional analysis so that there was a linear relationship between the amount of cDNA used and the intensity of the PCR product. PCR conditions consisted of 1-min denaturation at 94°C, 1-min annealing at 60°C, and 1-min extension at 72°C. β-actin, Iγ3-Cγ3, V_HDJ_H-Cγ3, TdT, and RAG-2 were PCR amplified, as reported (18, 26, 46). Extrachromosomal Sγ-Sμ, Sα-Sμ, and Sε-Sμ were PCR amplified from 500 ng of genomic DNA, blotted, and hybridized with probes specific for the recombined switch regions (2). The following sense and antisense primer pairs were also used: Ku70, 5′-GTGATGTCCAATTCAAGATGA GTC-3′ and 5′-GACCTCTTGGTATCGCTAGGCAG-3′; Ku80, 5′-GT ATGGACGTGGGCTTTACCATGAG-3′ and 5′-CCTAAGCGAAAGG GGCCATCTCCTC-3′; DNA-PK (cetelytic subunit) 5′-TACCAGAGAGCA TTCCAGCACC-3′ and 5′-TCTTGAATCACTCCTCCTTGATCC-3′; SWAP-70, 5′-GGAAGAAACTGGAGGAAGCAG-3′ and 5′-CCATGATG GGCCACTTTGTCCT-3′; Msh2, 5′-CGTTCATGGCTGAAATGTTGG-3′ and 5′-GCACTTCTTTGCGCTGGTTCC-3′; Blimp-1, 5′-AAGAGAAAA GCAACTGGATGCG-3′ and 5′-GGGTGAAATGTTAGAACGGTA GAGG-3′. The human SWAP-70 cDNA sequence was obtained from Gen-Bank (accession no. AB014540) through comparative analysis of mouse SWAP-70 cDNA (accession no. AF053974).

Immunoblotting and immunoprecipitation

Cytoplasmic proteins were extracted as described (13), fractionated on 10% SDS-PAGE gels, and transferred to nitrocellulose membranes. After blocking, membranes were incubated with a rabbit Ab to TRAF-2 (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA; 1:500), washed, and further incubated with a HRP-conjugated Ab to rabbit Igs (Santa Cruz Biotechnology; 1:2000). Bound Abs were detected with an enhanced chemiluminescence detection system (Amersham, Little Chalfont, U.K.). After stripping, the same membranes were reprobed with a rabbit mAb to actin (Santa Cruz Biotechnology; 1:500). CD40-bound TRAF-2 was detected in a similar fashion after immunoprecipitation of total cell lysates with mouse BE1 (IgG1) mAb to CD40 (Ancell) and protein G plus Agarose (Santa Cruz Biotechnology) (7).

Luciferase reporter assays and EMSAs

A total of 2×10^6 CL-01 cells in 500 μl was mixed with 40 μl of plasmid DNA-TRIS-EDTA solution containing 25 μg of ECS-Iγ3 (−449/+265) pGL3 reporter construct and 10 ng of pRL-CMV control vector. Electroporation was conducted at 525 V/cm and 950 μF using a

Gene Pulser II apparatus (Bio-Rad Laboratories, Hercules, CA). Transfected cells were cultured for 24 h, and luciferase activities were measured as reported (13). NF-κB and STAT-6 shift and supershift assays were performed as described (13). A double-stranded probe encompassing the BSAP site 1 of the mouse Ig H chain 3′ enhancer was prepared by annealing the 5′-CA GAATTGTGAAGCGTGACCATAG-3′ primer to the 5′-CTATGGT CACGCTTCACAATTCTG-3′ primer. Inhibition assays were performed using an Ab to BSAP/Pax5 (Santa Cruz Biotechnology).

Results

B cells up-regulate CD153 in the germinal center of secondary lymphoid organs

Tonsil B cells include IgD^+ $CD38^-$ naive B cells, IgD^+ $CD38^+$ GC founder B cells, IgD^- $CD38^+$ GC B cells (further divided in $CD77^+$ centroblasts and $CD77^-$ centrocytes), and IgD^- $CD38^-$ memory B cells (47, 48). These B cell subsets are localized in the follicular mantle (FM), FM-GC junction, GC, and marginal zone of the secondary lymphoid follicle, respectively (14, 17). Although naive B cells, GC founder, and GC centroblasts mostly consist of preswitched IgD^+ IgM^+ and IgD^- IgM^+ B cells, respectively, centrocytes and memory B cells are mostly switching or already class-switched IgD^- IgM^- (IgG^+ or IgA^+) B cells (14, 17). In this study, CD153 as well as the transcription status of the Ig H chain locus were analyzed in sorted tonsil B cell subsets. Naive B cells (B1) expressed low levels of CD153 and lacked germline Iγ3-Cγ3 and productive V_HDJ_H-Cγ3 transcripts (Fig. 1*A*). Founder GC B cells (B2) up-regulated CD153 and did not express Iγ3-Cγ3 or V_HDJ_H-Cγ3 transcripts. GC B cells (B3) coexpressed CD153 as well as Iγ3-Cγ3 or V_HDJ_H-Cγ3 transcripts. Among these cells, centroblasts expressed CD153 at higher density than centrocytes (not shown). Finally, switched memory B cells (B4) lacked CD153 and Iγ3-Cγ3, but expressed V_HDJ_H-Cγ3.

The immunohistochemistry analysis of IgD, CD79a (Igα coreceptor, a molecule expressed by all B cells), and CD153 on tonsil sections showed that CD153 is expressed by IgD^- $CD79a^+$ B cells within the GC of secondary lymphoid follicles (Fig. 1*B*). By contrast, IgD^+ $CD79a^+$ B cells within the FM surrounding the GC did not express significant CD153. This latter was up-regulated in tonsil IgD^+ IgM^+ naive B cells 1 day after exposure to GC differentiation and CSR-inducing stimuli, including htCD154, IL-4, and anti-BCR Abs, and it was down-regulated 1 day later (Fig. 1*C*). These findings indicate that IgD^+ IgM^+ naive B cells up-regulate CD153 in the GC of secondary lymphoid organs, and suggest that class-switched B cells, including memory B cells, express CD153 at lower density than preswitched B cells.

CD153 engagement inhibits CD154- and IL-4-induced Iγ3-Cγ3 germline transcription and Sμ→Sγ3 CSR

Previous studies have shown that mouse M81 anti-CD153 mAbs or human CD30:Fc IgG1 fusion protein specifically bind B cell CD153 (25–27). These reagents were immobilized on CD32 (FcγR)-expressing L cells, a condition that mimics CD30:CD153-mediated cell:cell interaction and elicits significant CD153-dependent signaling in T cells and neutrophils (43). Upon exposure to htCD154 and IL-4 for 2 days, PB IgD^+ IgM^+ B cells induced Iγ3-Cγ3

germline transcripts (Fig. 2). In these cells, immobilized anti-CD153 mAbs or human CD30:Fc IgG1 virtually abrogated the expression of Iγ3-Cγ3 transcripts, whereas MOPC-21 mAb, Abs to B cell CD44 or CD134L (17, 49), or human CD152 (CTLA-4, the CD80 and CD86 receptor expressed by activated T cells):Fc IgG1 chimeric protein did not. In addition to IgD^+ IgM^+ normal B cells, CD153 cross-linking inhibited Iε-Cε germline transcription in Ramos and BL-2, two $CD153^+$ neoplastic B cell lines expressing an IgD^- IgM^+ centroblastic-like phenotype (not shown). This inhibitory effect was not associated with CD40 down-regulation, and was not dependent on the secretion of endogenous cytokines, as a combination of neutralizing Abs to IL-6, IL-10, TNF-α, TGF-β, and/or IFN-γ did not reverse the inhibitory activity of anti-CD153 mAb (not shown).

In the presence of htCD154, IL-4, and MOPC-21 mAb, PB IgD^+ IgM^+ B cells expressed extrachromosomal Sγ3-Sμ reciprocal DNA recombination products (Fig. 3*B*), V_HDJ_H-Cγ3 productive transcripts (Fig. 3*C*), and surface IgG (Fig. 3*D*) 4 days after exposure to htCD154 and IL-4. In these cells, switching to Cγ3 was associated with increased expression of transcripts encoding proteins that are relevant to CSR (50–53), including Ku70, Ku80, DNA-PK, SWAP-70, and Msh2 (Fig. 3*C*). Similar results were obtained when htCD154- and IL-4-stimulated B cells were exposed to an anti-CD44 mAb (not shown). Compared with these cells, B cells exposed to htCD154, IL-4, and anti-CD153 mAb strongly inhibited Sμ→Sγ3 DNA recombination (Fig. 3*B*), V_HDJ_H-Cγ3 transcription, expression of CSR-associated transcripts (Fig. 3*C*), and surface IgG expression (Fig. 3*D*). In similar cells, anti-CD153 mAb did not down-regulate TdT, a gene involved in V(D)J gene diversification (46), and, more importantly, did not affect B cell survival or proliferation (Fig. 3*A*). These findings indicate that CD153 cross-linking inhibits Iγ3-Cγ3 germline transcription and subsequent Sμ →Sγ3 CSR in CD154- and IL-4-induced IgD^+ and/or IgM^+ B cells.

CD153 engagement inhibits the CD154- and IL-4-induced transcriptional activation of the Cγ3 germline gene promoter

The role of CD153 engagement in Cγ3 gene transcriptional activation was assessed in human IgD^+ IgM^+ CL-01 cells. Two days after exposure to htCD154 and IL-4, these B cells induced Iγ3-Cγ3 transcripts (Fig. 4*A*), and activated the Cγ3 germline gene promoter (Fig. 4*B*). In similar B cells, immobilized anti-CD153 but not MOPC-21 or anti-CD44 (not shown) mAbs down-regulated Iγ3-Cγ3, but not RAG-2 transcripts (46), and significantly hampered the transcriptional activation of the Cγ3 gene promoter (about 50% inhibition). This promoter is induced upon NF-κB and STAT-6 binding to specific CD40 and IL-4R DNA REs, respectively (13). In a 4-h culture, anti-CD153 but not MOPC-21 or anti-CD44 (not shown) mAbs significantly decreased the htCD154-induced binding of p50/p65, p50/c-Rel, and p50/p50 NF-κB/Rel complexes to the CD40 RE of the Cγ3 germline gene promoter, but did not affect the IL-4-induced binding of STAT-6 to the IL-4R RE of the same promoter (Fig. 4*C*). Similar findings were obtained in IgD^- IgM^+ Ramos and BL-2 B cells (not shown), suggesting that CD153 engagement inhibits CD154- and IL-4-induced transcriptional activation of the Cγ3 germline gene promoter by hampering NF-κB binding to the CD40 RE of this promoter.

CD153 engagement interferes with the CD154-induced binding of TRAF-2 to CD40

Because TRAF-2 has been suggested to play an important role in Ig germline transcription (10), we reasoned that CD153 cross-linking could interfere with the CD154-induced assembly of the CD40:TRAF-2 receptor complex. Cytoplasmic TRAF-2 was constitutively expressed in unstimulated IgD^+ IgM^+ CL-01 B cells (Fig. 4*D*). Exposure of these cells to htCD154 (with or without IL-4) but not IL-4 dramatically reduced the levels of cytoplasmic TRAF-2, which was instead bound to CD40, suggesting that CD154:CD40 engagement depletes cytosolic TRAF-2 at least in part by inducing TRAF-2 recruitment to the CD40 cytoplasmic tail. Immobilized anti-CD153 but not MOPC-21 or anti-CD44 (not shown) mAbs prevented htCD154-induced down-regulation of cytoplasmic TRAF-2 as well as htCD154-induced binding of TRAF-2 to CD40, indicating that CD153 cross-linking interferes with the assembly of the CD40:TRAF-2 receptor complex.

CD153 engagement inhibits CD154- and cytokine-induced IgG secretion and plasmacytoid phenotypic differentiation

The role of CD153 engagement in plasmacytoid differentiation was analyzed in purified tonsil IgD^+ (IgM^+ IgG^-) and IgG^+ (IgD^- IgM^-) B cells. Upon exposure to htCD154 and cytokines for 8 days, IgD^+ B cells secreted IgG and differentiated to $CD38^{++}$ $CD138^+$ cells (Fig. 5*A*), all traits of plasmacytoid differentiation (11). Compared with IgD^+ B cells, similarly induced IgG^+ B cells secreted higher amounts of IgG and gave rise to a higher proportion of $CD38^{++}$ $CD138^+$ B cells (Fig. 5*A*). Upon exposure to anti-CD153 but not MOPC-21 or anti-CD44 (not shown) mAbs, stimulated IgD^+ but not IgG^+ B cells secreted lower amounts of IgG and gave rise to a significantly lower proportion of $CD38^{++}$ $CD138^+$ B cells (Fig. 5*A*). These findings suggest that CD153 cross-linking inhibits plasmacytoid differentiation in CD154- and cytokine-induced IgD^+ B cells.

CD153 engagement modulates Blimp-1 transcripts and BSAP DNA-binding activity in a reciprocal fashion

Upon exposure to htCD154 and cytokines for 4 days, IgD^+ IgM^+ B cells secreted IL-6, an inducer of plasmacytoid differentiation, and expressed transcripts encoding Blimp-1 (Fig. 5*B*), a transcription factor involved in the transition of mature B cells into plasma cells (54, 55). Compared with B cells exposed to MOPC-21 mAb, B cells exposed to anti-CD153 mAb increased secretion of IL-6 (56), but virtually lacked Blimp-1 transcripts (Fig. 5*B*). These CD153-stimulated B cells also increased the binding of BSAP to the Ig 3′ enhancer (Fig. 5*B*), a DNA element that up-regulates the Ig H chain transcription in plasma cells and is repressed by BSAP (57). Thus, in CD154- and cytokine-induced IgD^+ B cells, CD153 engagement inhibits Blimp-1 transcription while up-regulating BSAP binding to DNA.

T cells express functionally active CD30 and CD154 4 days after TCR, CD28, and CD134 engagement

Ag-activated $CD4^+$ T cells express CD30 in the presence of CD28 engagement and IL-4 (21), a cytokine produced by T cells upon engagement of CD134 by CD134L on APCs (36, 58). PBMCs were incubated with IL-4 and immobilized agonistic mAbs to CD3, CD28, and CD134. After 8 h, 34 ± 8% of $CD4^+$ T cells expressed CD154, but not CD30 (Fig. 6*A*). Four

days later, 12 ± 5% of these T cells still expressed CD154, whereas 62 ± 14% of them expressed CD30 (Fig. 6*B*). These 4-day-stimulated $CD4^+$ T cells were sorted, fixed (to prevent cytokine secretion), washed, and cultured with IgD^+ IgM^+ B cells in the presence or absence of exogenous cytokines and anti-BCR Ab (the latter was added to minimize CD30 expression by B cells). Under these conditions, 4-day-stimulated $CD4^+$ T cells induced significant switching to IgG in IgD^+ B cells, and increased the IgG production in IgG^+ B cells (Fig. 6*C*). These effects were dependent on residual T cell CD154, as they were abolished by preincubating 4-day-activated $CD4^+$ T cells with saturating amounts of a blocking mAb to CD154. Preincubation of T cells with a blocking mAb to CD30 (59), but not to CD27 (42), enhanced IgG production in IgD^+, but not IgG^+ B cells, suggesting that T cell CD30 limits IgG synthesis in Ag-selected IgD^+ B cells.

$CD30^+$ T cells inhibit CD154- and cytokine-induced CSR to Cγ, Cα, and Cε through B cell CD153

To formally verify the inhibitory ability of $CD4^+$ $CD30^+$ T cells, $CD30^-$ and $CD30^+$ T cells were sorted from 4-day-stimulated $CD4^+$ T cells. Sorted cells were fixed, washed, incubated with a mAb-blocking residual surface CD154, washed, and then cultured with IgD^+ IgM^+ B cells in the presence or the absence of exogenous htCD154, cytokines, and anti-BCR Abs. Purified $CD30^+$ but not $CD30^-$ T cells significantly inhibited htCD154- and cytokine-induced Sγ-Sμ, Sα-Sμ, and Sε-Sμ CSR (Fig. 7*A*) as well as IgG, IgA, and IgE production (Fig. 7*B*). Preincubation of $CD30^+$ T cells with a saturating amount of blocking mAb to T cell CD30 restored CSR as well as IgG, IgA, and IgE secretion, while blocking mAb to T cell CD27 did not. These findings indicate that $CD4^+$ $CD30^+$ T cells negatively modulate CD154- and cytokine-induced CSR in Ag-selected IgD^+ B cells, and suggest that this inhibitory activity is largely dependent upon CD30:CD153 interaction.

Discussion

These studies define a novel mechanism that modulates Ig class switching in human B cells. IgD^+ IgM^+ B cells up-regulate CD153 expression upon exposure to CD154, IL-4, and BCR engagement. In these B cells, CD153 engagement by immobilized agonistic mAbs or CD30:Fc inhibits NF-κB activation, I_H-C_H germline transcription, CSR as well as expression of transcripts encoding Ku70, Ku80, DNA-PK, SWAP-70, and Msh2 CSR-related products. It also inhibits IgG secretion and plasmacytoid differentiation, and this effect is associated with increased BSAP binding to the Ig 3′ enhancer and down-regulation of Blimp-1 transcripts. Finally, purified $CD30^+$ T cells inhibit CSR as well as IgG, IgA, and IgE production in CD154- and cytokine-stimulated IgD^+ IgM^+ B cells. These findings indicate that reverse signaling through CD153 inhibits Ig class switching in B cells that are positively selected by Ag, and suggest that $CD30^+$ T cells play an important role in the modulation of the Ab response.

We have recently demonstrated that human IgD^+ IgM^+ B cells express CD30 upon CD40 engagement and in the absence of BCR coengagement (18). In these B cells, engagement of CD30 by $CD153^+$ $CD8^+$ T cells inhibits CD154- and IL-4-induced CSR as well as IgG, IgA, and IgE production. This CD30-dependent pathway would be critical to rapidly inhibit CSR

in non-Ag-selected naive B cells, and would complement the CD95-dependent deletion of non-Ag-selected GC B cells (14, 16, 17). The observation that Ag-specific T cells up-regulate CD30 in disorders associated with dysregulated IgG, IgA, or IgE production (22–24) prompted us to postulate that engagement of B cell CD153 by T cell CD30 modulates CSR.

We show in this study that $CD4^+$ $CD30^+$ T cells inhibit CD154-and IL-4-induced CSR in IgD^+ and/or IgM^+ B cells, including Ag-selected $CD30^-$ B cells. The CSR-inhibitory activity of $CD30^+$ T cells is dependent on B cell CD153, as emphasized by the finding that blockade of CD30:CD153 interaction restores CSR as well as IgG, IgA, and IgE secretion. Furthermore, CD153 cross-linking by immobilized CD30:Fc or specific agonistic anti-CD153 mAbs inhibits CD154- and cytokine-induced CSR. This CD153-mediated inhibitory activity seems to be CSR specific, as CD153 engagement does not affect CD40-mediated B cell proliferation or survival, nor does it affect CD23, CD54 (ICAM-1), CD80 (B7.1), or CD86 (B7.2) up-regulation. In addition to inhibiting CSR, CD153 engagement significantly enhances the production of endogenous IL-6 as well as DNA binding by BSAP, a transcription factor that critically modulates B cell proliferation and differentiation (57). Thus, CD153 transmits both positive and negative signals in CD40-activated B cells.

These findings are consistent with the reported signal-transducing properties of other members of the TNFL superfamily, including CD70 (CD27L), CD95L (FasL), CD134L (OX40L), CD137L (4-1BBL), and CD154 (CD40L) (29–41), and extend previous findings showing that CD153 cross-linking by agonistic anti-CD153 mAbs or CD30:Fc induces reverse signaling in T cells and neutrophils (43). In CD154- and IL-4-activated IgD^+ IgM^+ B cells, CD153 engagement inhibits the transcription of $I\gamma3$-$C\gamma3$ germline transcripts and turns off CSR to $C\gamma3$. This inhibitory effect is associated with decreased levels of transcripts encoding DNA-PK, Ku70, Ku80, SWAP-70, and Msh2. Because DNA-PK, Ku, and Msh2 proteins play a role in CSR, and SWAP-70 has been suggested to be an element of the switch recombinase machinery (50–53, 60), it is tempting to speculate that CD153 signaling modulates CSR not only by inhibiting the germline transcription of downstream C_H genes, but also by modulating the expression of critical CSR-related molecules. This inhibition appears to be specific, as CD153 engagement does not significantly affect the level of transcripts encoding TdT and RAG-2, two genes involved in Ig V(D)J gene diversification and recombination (46).

Several studies have shown that Ig germline transcription and CSR are critically dependent on the integrity of the CD40 signaling pathway (12). Upon CD154-induced oligomerization, B cell CD40 recruits multiple TNFR adaptor proteins, including TRAF-1, TRAF-2, TRAF-3, TRAF-5, and TRAF-6 (6–11). By activating downstream kinases, including NF-κB-inducing kinase, IκB kinase, and c-Jun N-terminal protein kinase, TRAFs up-regulate the binding of critical transcription factors, including NF-κB and AP-1, to B cell gene promoters (6, 7, 10, 11, 61, 62). In mouse B cells, the recruitment of TRAF-2, TRAF-3, TRAF-5, and/or TRAF-6 to the CD40 cytoplasmic tail is critical for CD154-dependent induction of $C\gamma$ gene germline transcription and IgG production (8, 9). In human IgD^+ IgM^+ B cells, CD153 cross-linking inhibits CD154-mediated TRAF-2 recruitment to CD40, and concomitantly reduces the binding of NF-κB to the CD40 RE of the $C\gamma3$ germline gene

promoter (Fig. 4). These findings, together with our preliminary experiments showing the CD153 cross-linking decreases the recruitment of TRAF-3 to CD40 (not shown), suggest that CD153 signaling may modulate the CD40-mediated transcriptional activation of downstream C_H genes by inducing an overall desensitization of the CD40 signaling complex.

It has been recently shown that $CD153^+$ B cells coexpress high levels of a truncated form of CD30, referred to as CD30 variant (63). This CD30 variant encompasses the cytoplasmic tail of trans-membrane CD30; recruits TRAF-2, TRAF-3, and TRAF-5; and induces NF-κB activation when overexpressed in a myeloid cell line (63, 64). Our preliminary experiments indicate that, in CD40-activated B cells, engagement of CD153 up-regulates the binding of TRAF-2 and TRAF-3 to CD30 variant (not shown). Thus, CD30:CDl53 interaction would interfere with the CD40:NF-κB-associated signaling pathway by recruiting multiple TRAFs to CD30 variant, and thereby hampering them from binding to the CD40 cytoplasmic tail. The presence of the same signaling element (i.e., the CD30 cytoplasmic tail) in both the CD153- and CD30-associated signaling pathways would be consistent with our observation that engagement of CD30 or CD153 elicits partially overlapping functional effects in CD154:CD40-activated IgD^+ IgM^+ B cells (18). It would also be consistent with the possibility that bidirectional B cell CD30:B cell CD153 signals synergistically inhibit the GC progression of perifollicular non-Ag-selected $CD30^+$ $CD153^+$ B cells (18).

In addition to hampering CSR, CD153 engagement significantly inhibits IgG, IgA, and IgE as well as plasmacytoid phenotypic differentiation in CD154- and cytokine-induced IgD^+ B cells. This inhibition is not associated with decreased production of B cell-derived IL-6 (56), but, rather, with decreased transcription of Blimp-1, a poorly defined molecule involved in the transition of mature B cells into plasma cells (54). It is also associated with up-regulation of BSAP, a transcription factor that, when overexpressed in GC-like B cells, represses the 3′ Ig enhancer (a critical regulator of Ig H chain production) (57, 65). These findings suggest that, in CD154- and cytokine-induced IgD^+ IgM^+ B cells, CD153 signaling turns off the plasmacytoid differentiation program by modulating Blimp-l and BSAP transcription factors in a reciprocal fashion.

By defining a specific role for B cell CD153 in the regulation of Ig CSR and plasmacytoid differentiation, our findings outline a novel CD95-independent mechanism in the homeostasis of B cell differentiation. They suggest that the process that initiates a specific Ab response and the rapid expansion of $CD4^+$ $CD154^+$ T cells with CSR-inducing activity later on gives rise to $CD4^+$ $CD30^+$ T cells with CSR-inhibitory activity (Fig. 8). As early as 8 h after optimal TCR, CD28, and CD134 activation by Ag and costimulatory molecules (i.e., CD80, CD86, and CD134L) on APCs (5, 36, 58), $CD4^+$ T cells express CD154 that engages CD40 on IgD^+ IgM^+ B cells. In the presence of BCR engagement, these naive B cells undergo clonal expansion, acquire GC phenotypic traits, and up-regulate CD153 expression. Within 4 days, a significant proportion of these GC B cells completes CSR to IgG, IgA, or IgE; initiates plasmacytoid or memory differentiation; and down-regulates CD153 expression. At this time and in the presence of IL-4, activated $CD4^+$ T cells induce CD30 (20, 21) while still expressing functionally significant levels of CD154 (66). These $CD4^+$ $CD30^+$ T cells would inhibit CSR and plasmacytoid differentiation in newly

CDl54:CD40-activated IgD^+ and/or IgM^+ $CD153^+$ B cells, while sparing ongoing IgG, IgA, or IgE production in already class-switched GC, plasmacytoid, or memory B cells. Thus, a few days after the initiation of the Ab response, $CD4^+$ $CD30^+$ T cells would modulate the magnitude of IgG, IgA, or IgE production by inhibiting the late CD154-dependent entry of $CD153^+$ B cells, including Ag-selected $CD30^-$ B cells, into the pool of B cells producing class-switched Abs.

Expression of cell surface CD30 is modulated by disintegrin metalloproteinases, a family of proteins produced by activated mononuclear cells, including dendritic cells (67, 68). The in vivo relevance of cell-bound CD30 cleavage is suggested by the presence of increased levels of circulating soluble CD30 in disorders characterized by abnormally high CSR and IgG, IgA, and/or IgE production, including systemic lupus erythematosus, rheumatoid arthritis, systemic sclerosis, and atopic dermatitis (22–24). Our preliminary experiments indicate that activated dendritic cells counteract the CSR-inhibitory activity of $CD4^+$ $CD30^+$ T cells by inducing metalloprotease-mediated cleavage of T cell surface CD30 (not shown). The cleaved soluble CD30 would further reduce B cell CD153 engagement by T cell CD30 (27, 68). Thus, extensive cleavage of T cell CD30, as found in autoimmune and allergic disorders (22–24), would increase the production of pathogenic IgG, IgA, and/or IgE by depleting the pool of $CD30^+$ suppressor T cells and by reducing the frequency of the inhibitory CD30:CD153-mediated T:B cell interaction.

By adding to previous reports indicating that CD30 signaling negatively regulates the cytotoxic activity of NK and $CD8^+$ T cells (69–71), our experiments suggest that the CD30:CD153 interaction functions as a biological brake that physiologically limits the immune response by transmitting bidirectional negative signals. Given the CD30 ability to inhibit the in vivo clonal expansion of $CD8^+$ cytotoxic T cells (71), human viruses, including HIV, measles, and hepatitis B and C viruses, might up-regulate CD30 expression on T cells to evade both Ab- and cell-mediated virus-specific immune responses (22, 23). In addition, expression of CD30 may confer immune privilege to a given tissue or organ. For instance, IL-4- and progesterone-induced $CD30^+$ placental T cells as well as $CD30^+$ decidual cells might contribute to inhibit the Ab response to fetal alloantigens by maternal $CD153^+$ B cells (72). In conclusion, B cell CD153 could represent a novel target for the immunotherapy of disorders associated with dysregulated Ab production.

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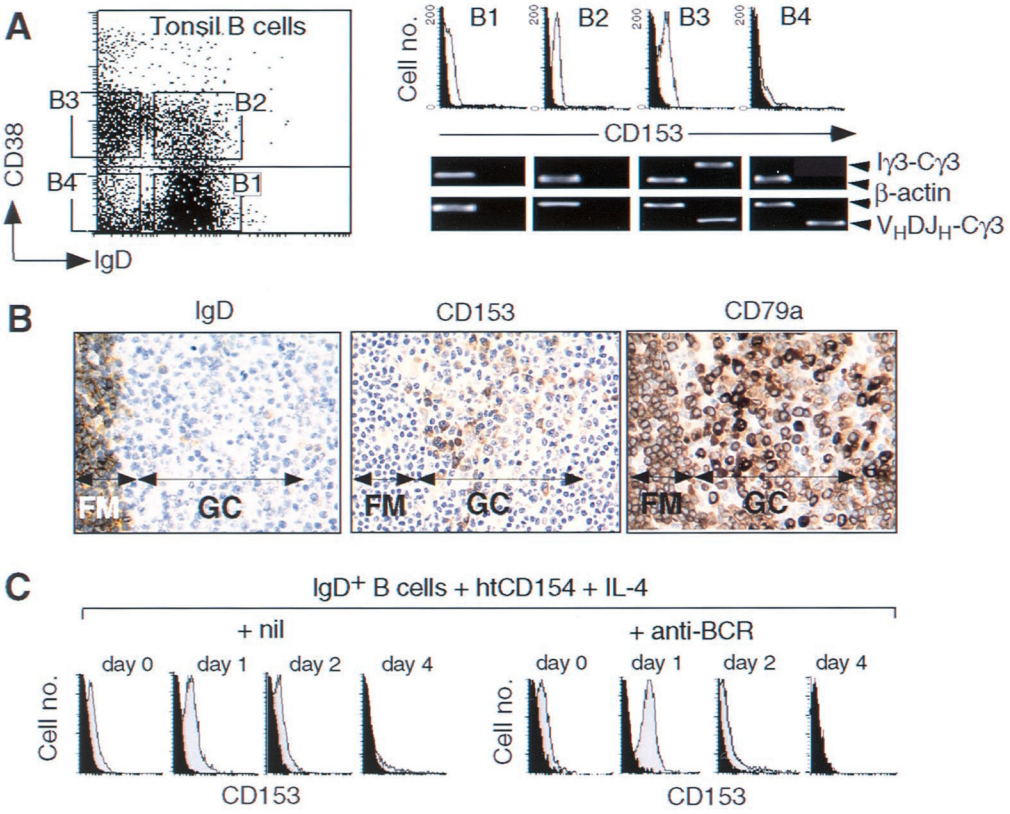

FIGURE 1.

Human B cells up-regulate CD153 in the GC of secondary lymphoid organs. *A*, CD153 expression was analyzed by flow cytometry on electronically gated IgD^+ $CD38^-$ (B1, naive), IgD^+ $CD38^+$ (B2, founder GC), IgD^- $CD38^+$ (B3, GC), and $CD38^-$ IgD^- (B4, memory) B cells. Filled and shaded histograms correspond to control and anti-CD153 mAbs, respectively. *β*-actin (593-bp), I*γ*3-C*γ*3 (670-bp), and VDJ-C*γ*3 (416-bp) transcripts were PCR amplified (35 cycles) from each B cell fraction. *B*, IgD, CD153, and CD79a (Ig*α*) were visualized (brown cells) in serial sections from the same lymphoid follicle by immunohistochemistry (×400). *C*, Expression of CD153 was analyzed on freshly isolated tonsil IgD^+ B cells (day 0) or on IgD^+ B cells exposed to htCD154 and IL-4 with or without anti-BCR Abs.

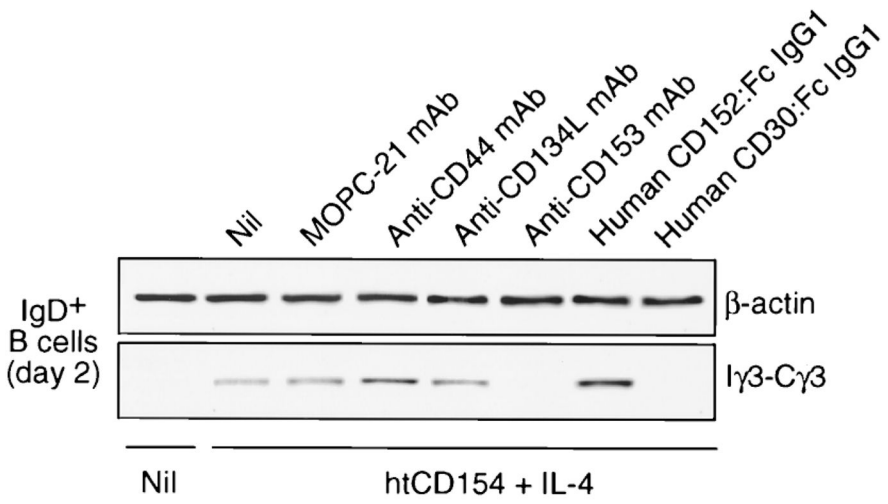

FIGURE 2.

CD153 cross-linking inhibits germline Iγ3-Cγ3 transcription in CD154- and IL-4-induced IgD^+ B cells. PB IgD^+ B cells were cultured for 2 days with or without htCD154 and IL-4, and in the presence or absence of MOPC-21 mAb, anti-CD44 mAb, anti-CD134L mAb, anti-CD153 mAb, human CD192:Fc IgG1, or human CD30:Fc IgG1 immobilized on irradiated CD32 L cells. β-actin (593-bp) and Iγ3-Cγ3 (670-bp) transcripts were PCR amplified (18 cycles) from equal amounts of cDNA in the presence of [α-^{32}P]dCTP. PCR products were then fractionated on a 6% polyacrylamide gel. These findings were derived from one of three experiments yielding comparable results.

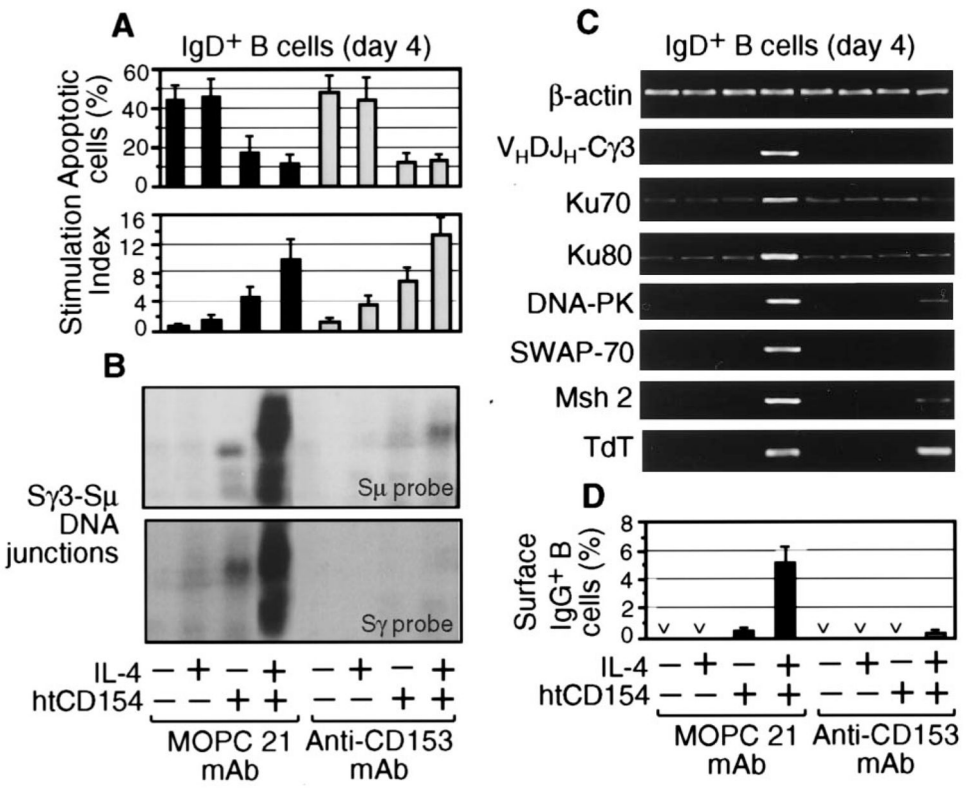

FIGURE 3.

CD153 cross-linking inhibits S$\mu \rightarrow$Sγ3 CSR in CD154-and IL-4-induced IgD$^+$ B cells. PB IgD$^+$ B cells were cultured for 4 days with or without htCD154 and/or IL-4, and in the presence or absence of MOPC-21 or anti-CD153 mAbs immobilized on CD32 L cells. *A*, B cell apoptosis and proliferation measured by propidium iodide plus annexin V staining and [^{3}H]TdR incorporation assays, respectively. *B*, Sγ3-Sμ DNAs (0.5–3 kb) PCR amplified (30 cycles) from 500 ng of genomic DNA, and hybridized with Sμ and Sγ probes. *C*, β-actin (593 bp), V_HDJ_H-Cγ3 (416 bp), Ku70 (518 bp), Ku80 (492 bp), DNA-PK (578 bp), SWAP-70 (548 bp), Msh2 (449 bp), and TdT (276 bp) PCR amplified (25 cycles; β-actin 20 cycles) from equal amounts of cDNAs. *D*, Percentage of CD19$^+$ IgD$^+$ B cells expressing surface IgG (arrows indicate <0.05). These findings were derived from one of five experiments yielding comparable results (error bars indicate ± SD).

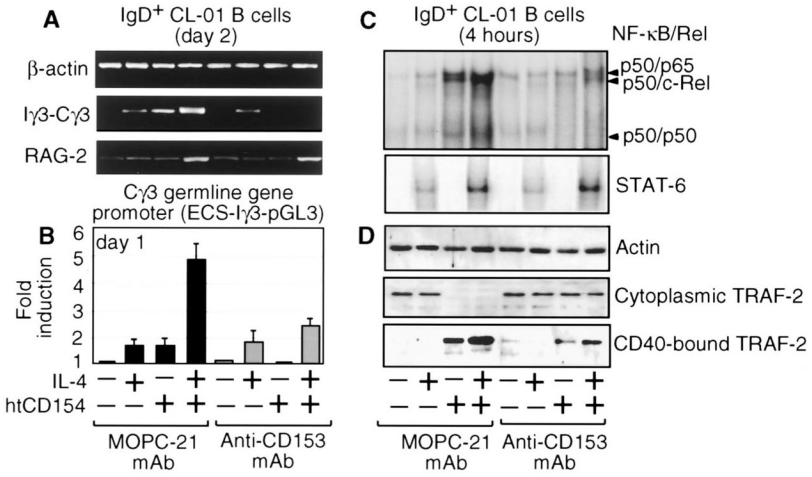

FIGURE 4.

CD153 cross-linking inhibits activation of the Cγ3 germline gene promoter, nuclear translocation of NF-κB, and CD40:TRAF-2 association in CD154- and IL-4-induced IgD$^+$ B cells. CL-01 B cells were cultured with or without htCD154 and/or IL-4, and in the presence of MOPC-21 or anti-CD153 mAbs immobilized on the plastic plate. *A*, β-actin (593-bp), RAG-2 (1,105 bp), and Iγ3-Cγ3 (670-bp) transcripts were PCR amplified (25 cycles) from equal amounts of cDNA. *B*, CL-01 cells were transfected with an ECS-Iγ3-pGL3 construct, and the luciferase activity was measured after 24 h. *C*, The binding of NF-κB and STAT-6 to the CD40 and IL-4R REs of the Cγ3 promoter was assessed by EMSA. *D*, Cytoplasmic proteins obtained from 4-h-stimulated B cells were transferred to nitrocellulose membranes, and immunoblotted for TRAF-2 and actin. In additional experiments, total proteins immunoprecipitated with an anti-CD40 mAb were immunoblotted for TRAF-2. These findings were derived from one of three experiments yielding comparable results (error bars indicate ± SD).

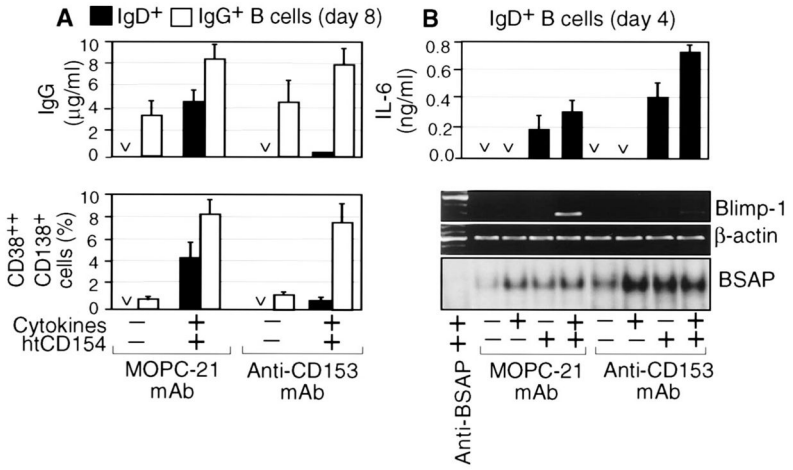

FIGURE 5.

CD153 cross-linking down-regulates IgG secretion and Blimp-1 transcripts while up-regulating BSAP nuclear activity in CD154- and cytokine-induced IgD^+ B cells. *A*, Tonsil IgD^+ (■) or IgG^+ (□) B cells were cultured with or without htCD154 and cytokines (IL-4 and IL-10), and in the presence of MOPC-21 or anti-CD153 mAbs immobilized on CD32 L cells. IgG concentration and $CD38^{++}$ $CD138^+$ plasmacytoid B cells were analyzed after 8 days (arrows indicate IgG and plasma cell values <100 ng/ml and <0.1%, respectively). *B*, Tonsil IgD^+ B cells were cultured as above. After 4 days, IL-6 was measured by ELISA; Blimp-1 (355-bp) and *β*-actin (593-bp) transcripts were PCR amplified (25 cycles) from equal amounts of cDNA; and BSAP binding to the BSAP site 1 of the mouse Ig 3′ enhancer was determined by EMSA. These findings were derived from one of three experiments yielding comparable results (error bars indicate ± SD).

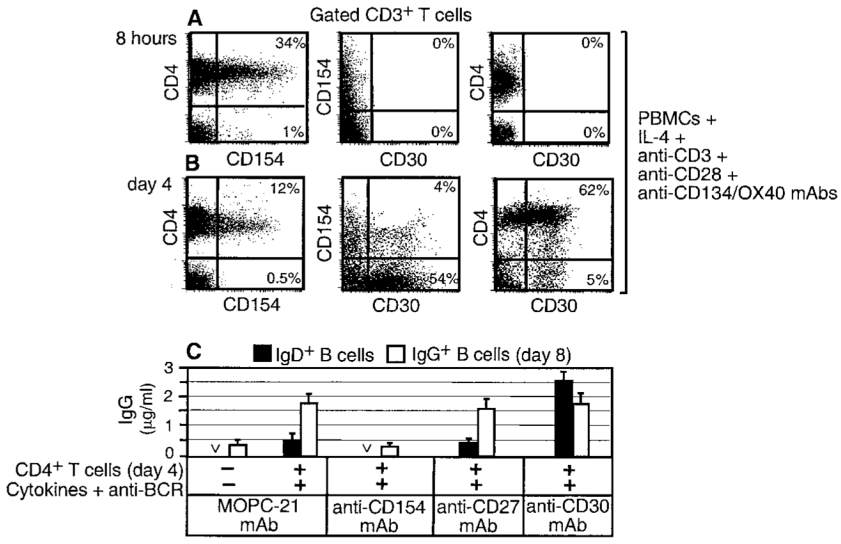

FIGURE 6.

$CD4^+$ T cells express CD154 and CD30 4 days after TCR, CD28, and CD134 stimulation. *A* and *B*, PBMCs were incubated with IL-4 and mAbs to CD3, CD28, and CD134 immobilized on CD32 L cells. After 8 h (*A*) or 4 days (*B*), CD4, CD154, and CD30 were analyzed on gated $CD3^+$ T cells. *C*, IgD^+ (■) or IgG^+ (□) B cells were cultured with or without cytokines (IL-4 and IL-10), anti-BCR Abs, and $CD4^+$ T cells isolated from 4-day-stimulated PBMCs. Before culture, $CD4^+$ T cells were fixed and preincubated with MOPC-21 or blocking mAbs to CD154, CD27, or CD30. IgG were measured after 8 days. These findings were derived from one of three experiments yielding comparable results (error bars indicate ± SD).

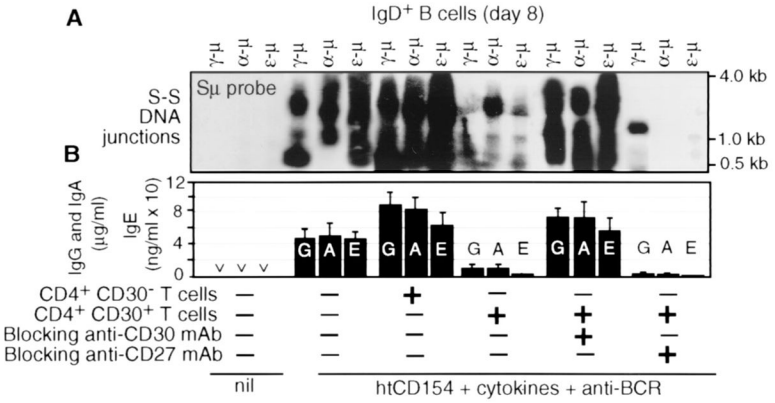

FIGURE 7.

CD30$^+$ T cells inhibit CSR in CD154- and cytokine-induced IgD$^+$ B cells. IgD$^+$ B cells were cultured with htCD154, cytokines (IL-4 and IL-10), and anti-BCR Abs in the presence or absence of CD4$^+$ CD30$^-$ or CD4$^+$ CD30$^+$ T cells sorted from 4-day-stimulated PBMCs. Before culture, all CD4$^+$ T cells were fixed, washed, incubated with a blocking anti-CD154 mAb, and washed. CD30$^+$ T cells were also preincubated with blocking anti-CD30 or anti-CD27 mAbs. Sγ-Sμ (γ), Sα-Sμ (α), and Sε-Sμ (ε) junction DNAs (*A*) as well as IgG, IgA, and IgE secretion (*B*) were assessed after 8 days. These findings were derived from one of three experiments yielding comparable results (error bars indicate ± SD).

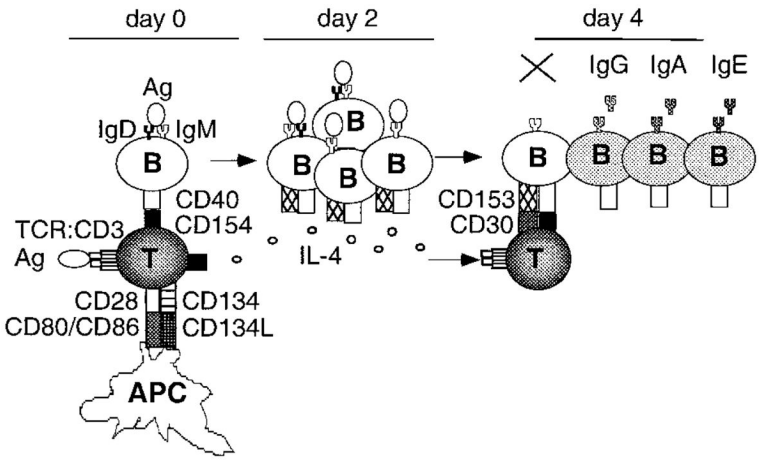

FIGURE 8.

Proposed role of $CD30^+$ T cells in the regulation of CSR and IgG, IgA, and IgE production. Upon TCR, CD28, and CD134 engagement by Ag and costimulatory molecules on APCs, $CD4^+$ Th cells express CD154 and secrete IL-4. In the presence of Ag, CD154, and IL-4, IgM^+ IgD^+ $CD30^-$ B cells undergo clonal expansion and up-regulate CD153. Within 4 days, Ag-selected B cells complete CSR to IgG, IgA, or IgE and down-regulate CD153. At this time, $CD4^+$ T cells induce CD30 while still expressing significant levels of CD154. These $CD30^+$ T cells inhibit CSR in newly CD154:CD40-activated IgD^+ and/or IgM^+ B cells by inducing reverse signaling through CD153.