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CD40 Ligand Exerts Differential Effects on the Expression of Ig Transcripts in Subclones of an IgM+ Human B Cell Lymphoma Line

Gregory S. Ford, Chun Hui Yin, Bryan Barnhart, Kevin Sztam, and Lori R. Covey

The CD40:CD40 ligand (CD40L) interaction plays a critical role in T cell-dependent isotype switching. To elucidate the role of CD40 signaling in the activation of γ germline transcription and as an extension, in targeting Cγ regions for isotype switching, an IgM+ Burkitt lymphoma cell line (Ramos 2G6) was assayed for the up-regulation of germline γ transcripts after CD40L stimulation. Independent Ramos 2G6 subclones that either expressed (Iγ+) or did not express (Iγ−) basal levels of Iγ transcripts were assessed for their transcriptional response to CD40L signaling by contact with either a Jurkat T cell line (D1.1) or a transfected CD40L-expressing epithelial cell line (293/CD40L) in the presence or absence of IL-4. Both Iγ− and Iγ+ Ramos 2G6 subclones cultured with IL-4 and CD40L markedly up-regulated germline transcription predominantly from the γ1, γ2, and γ3 subclasses over levels obtained with IL-4 alone. In addition, these two signals were required to obtain de novo switch recombination. However, incubation with CD40L alone resulted in a substantial increase in germline transcription only in the Iγ+ and not the Iγ− subclones. Observed basal transcription at the γ1 locus also correlated with the ability of not only the γ1 locus, but also the γ2 and γ3 loci, to up-regulate germline transcripts in response to CD40 signaling. These data are consistent with CD40:CD40L contact up-regulating germline transcription only after the B cell has received a signal that alters the transcriptional state of the heavy chain locus. The Journal of Immunology, 1998, 160: 595–605.

The differentiation of B cells in response to T cell-dependent (TD)3 Ags requires cognate interactions between CD40 expressed on B cells and CD40 ligand (CD40L) expressed on activated CD4+ T cells (reviewed in Ref. 1). The consequences of this interaction are that Ag-activated B cells undergo proliferation, homotypic adhesion, and class switch recombination, or isotype switching (2–6), to produce new clonotypic Abs that differ with respect to their C region effector functions, but still retain their unique specificity with regard to Ag binding.

In addition to the requirement for CD40L in isotype switching, cytokines are known to be involved in regulating the isootype profile in response to an antigenic challenge (for review, see Ref. 7). It is proposed that cytokines function at one level by regulating the accessibility of the different CH regions to switch factors, and that this process is correlated with chromatin changes that include increased DNase I hypersensitivity (8, 9) and hypomethylation (10) of specific CH regions, as well as the activation of transcription from sites upstream of the CH-associated switch (S) regions (11–16). The appearance of small CH-specific RNA transcripts, termed germline or sterile transcripts, is widely seen as an obligatory event preceding isotype switching in both mice (11–15, 17–19) and humans (16). Although germline transcription can be directly regulated by cytokines, soluble factors alone are clearly insufficient to induce significant TD class switching (20–24). This process requires an additional signal provided by contact through CD40L expressed on activated CD4+ T cells (reviewed in Refs. 25 and 26).

The critical importance of the CD40:CD40L interaction for T-dependent humoral immune responses has been demonstrated by an absence of switch recombination and deficient humoral immunity in animals lacking functional CD40 or CD40L (human X-linked hyper IgM syndrome (HIGMX-1) (27–32); and CD40 (33) and CD40L (34)-deficient mice). CD40 engagement mediates several cellular events, including the activation of multiple protein kinases and the specific phosphorylation of phospholipase C-γ2 and phosphoinositide-3’ kinases (35–38). In addition, nuclear factor-κB and other rel family members are induced in response to CD40 ligation (37, 39–41). However, the relationship between these initial signaling events and downstream processes, such as switch recombination, is poorly understood. With respect to iso- type switching, CD40 signaling appears to result both in the activation of the switch recombinase machinery and in chromatin changes (i.e., germline transcription) that may impart recombinational accessibility to the Cγ locus (2, 3, 5, 42–46).

It has been widely reported that CD40 signaling in the presence of IL-4 augments the level of germline transcription over the level obtained with IL-4 alone (43, 44, 47). However, it is unclear whether contact solely through CD40 can induce germline transcription. Studies conducted with murine B cells suggest that membrane from activated Th cells or CD40L expressed in S/J cells can induce germline transcription in the absence of cytokines (48, 49). However, other investigators found that activated Th membranes were insufficient to induce germline transcription (50). Studies using human B cells have also produced conflicting results. While specific studies revealed that multiple classes of germline

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3 Abbreviations used in this paper: TD, T cell-dependent; CD40L, CD40 ligand; PE, phycoerythrin; RT-PCR, reverse-transcriptase polymerase chain reaction; S, switch.
transcripts in purified peripheral B cells are induced in response to CD40 engagement (44, 51), others found that germline transcripts were not induced in response to signaling through CD40 (43, 46, 47). The results in many of these studies could be attributed to the different methods used to isolate B cells and also to the different sensitivities of the methods used for detecting germline transcripts (PCR vs RNase protection). Therefore, it remains unclear whether CD40 signaling mediates the induction of germline transcription or whether transcriptional targeting of specific Cg loci is primarily a consequence of cytokine signaling.

To examine this question more fully, we have conducted experiments using an IL-4-responsive Burkitt lymphoma cell line (Ramos 2G6) (52) that undergoes Cg transcriptional activation and limited class switching in response to TD signals (43, 53, 54). Isolated subclones that differed in their basal expression of Ig transcripts (Ig y and Ig y) were used in parallel to examine the effect CD40 stimulation has on the transcriptional activation of the different Cg genes.

We report in this work that CD40 signaling differentially regulates the expression of Ig transcripts in our defined model system. CD40L contact, in the absence of IL-4, was found to up-regulate Ig y transcription in the Ig y subclones. However, under identical conditions, marginal to no Ig y transcription was detected in the Ig y population. Finally, in confirmation of others’ findings (43, 44, 47, 49, 55, 56), measurable switch recombination was observed only after stimulation with both IL-4 and CD40L conditions that produced the highest level of Ig y expression in both Ig y and Ig y subclones. Our results support a role for CD40L in up-regulating Ig y expression only after the heavy chain locus has become transcriptionally active, and therefore argue against CD40L having a primary role in the selection of Cg genes involved in isotype switching.

Materials and Methods

Cell lines

The Jurkat clones D1.1 (CD40L positive) and B2.7 (CD40L negative) have been described previously (57, 58). The human B cell lymphoma clone Ramos 2G6.4C3F10 (Ramos 2G6) is an IL-4-responsive subclone of RA-1 (59) and has been previously described (52). B and T cell lines were maintained in RPMI 1640 supplemented with 10% FBS (Atlanta Biologics, Atlanta, GA). 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. 293 cells are derived from a primary human embryonic kidney cell transformed by adenovirus 5 and are available from American Type Culture Collection (Rockville, MD). The 293/CD40L line was constructed by the stable transfection of pCT-BAM into 293 cells, as previously described (54). Untransfected 293 and 293/CD40L cells were maintained in DMEM/F12 media with 10% FBS, 12 mM HEPES, pH 7.2, 0.2% NaHCO3, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine.

Abs and cytofluorographic analysis

For evaluation of cell surface Ig expression, FITC-labeled goat F(ab)’, anti-human IgM and anti-human IgG were purchased from Southern Biotechnology (Birmingham, AL). R-phycocerythrin (PE)-conjugated mouse anti-human CD20 mAb was purchased from PharMingen Corp. (San Diego, CA). Analysis of surface CD40L expression was conducted using the anti-CD40L mAb, 5C8, previously described by Lederman et al. (58). Approximately 2 × 106 cells were incubated with saturating concentrations of the indicated mAbs for 30 min at 4°C. After washing the cells once with 3.5 ml of FACS wash (MEM + 12 mM HEPES, pH 7.2, 0.2% NaHCO3), cells were resuspended in 150 μl FACS wash and fluorescence intensity measured using an Epics Profile II flow cytometer (Coulter Electronics, Hialeah, FL).

Mitomycin-c treatment

Jurkat D1.1 and B2.7, 293 cells, or 293/CD40L cells (107/ml) were incubated with 50 μg/ml of mitomycin-c (Sigma Chemical Co., St. Louis, MO) for 45 min at 37°C/5% CO2 to inhibit cell division. The treated cells were washed three times with PBS and allowed to incubate for 1 h at 37°C/5% CO2. Cells were again washed three times with PBS and resuspended in RPMI/10% FCS for use in T cell/B cell experiments.

T/B cocultures

To examine Cg y transcription, 2 × 105 Ramos 2G6 B cells were cultured for 6 days in 1 ml of RPMI/10% FBS, either alone or with mitomycin-c-treated Jurkat cells (D1.1 or B2.7) at a ratio of 2.5 to 5 T cells:1 B cell. Human rIL-4 (Life Technologies, Gaithersburg, MD) was added to replica wells at a concentration of 400 U/ml. Cocultures were established with 293 or 293/CD40L cells using the same conditions.

RNA isolation and PCR strategy

After the indicated incubation period, cells were harvested and RNA isolated using Trizol reagent (Life Technologies). cDNA was prepared by transcribing 2 μg of total RNA from the T/B cocultures in a 25-μl vol containing 200 μM of each dNTP, 10 pmol of random primer (Promega Corp., Madison, WI), 20 U RNasin (Promega Corp.), and 50 U AMV-RT (avian myeloblastosis virus reverse transcriptase) (Promega Corp.). After incubation at 37°C for 1 h, and heat inactivation for 10 min at 68°C, 5 μl was used in a 100-μl PCR reaction containing 200 μM of each dNTP, and 400 ng of 5’ and 3’ primers. Reactions were heated to 72°C for 3 min, after which 2.5 U of Taq polymerase (Promega Corp.) was added to each tube. The cycling conditions were as follows: 1.5 min at 94°C, 1 min at 55°C, and 1 min at 72°C for 30 cycles.

Oligonucleotides for RT-PCR

The PCR assay used to amplify total RNA from B/T cocultures was developed and described by Jumper et al. (44). The sequences of oligos used to amplify germine y transcripts are found in the I region (5’-Iy1, 5’-gctcctctctcagcagcagc-3’) and in the second exon of the Cg y region (3’-Cg2 Cy, 5’-ctctggggtttgggaggaa-3’). These oligos amplify Iy1 transcripts from all four subclones. Subclass-specific y probes used to identify specific Iy and VDJ-Cg y transcripts are as follows: hinge y1, 5’-aaatgttgtaaacaactca-3’, hinge y2, 5’-ggttgtagctgacccaac-3’, hinge y3, 5’-tcggctggcagcagcagc-3’, and hinge y4, 5’-tgctggaacatgctgacac-3’. The specific size of each amplified product was based on previously reported sequences for the different y subclasses (60–64). For synthesis of VDJ-Cg-Cy and VDJ-Cy PCR products, the following primers were used: VDJ-Cy (5’-Jmu) 5’-acccgttgcttcctctcacta-3’ and (3’-Cg2 Cy) 5’-tctctggggtttgggaggaa-3’; VDJ-Ca (5’-Vca.1.3) 5’-gacacgctcttgattta-3’ and (3’-Cg1 5’-gggaatcaagaggagcctgagc-3’). Semiquantitative PCR was conducted using conditions described above, but with 10-fold and 100-fold less cDNA. Primers specific for Iy1 and Cy were included in each reaction. Quantification of the Iy signal was determined by dividing the Iy signal at each point by the Cy signal. We did not detect a difference in the expression of Cy under the different conditions of our assay. This was established by determining the absolute numerical values of the Cy signal by phosphor image analysis and quantitation.

S1 nuclease protection assays

T cell/B cell cultures were established as indicated above and RNA isolated using the Trizol method. The probe used in these studies has been described previously (43), and is a uniformly labeled RNA probe produced by in vitro transcription of a 440-bp SacI fragment derived from the 15-bp-Cy3 cDNA. A quantity amounting to 200,000 cpm of labeled probe was incubated together with 15 μg of total RNA for 20 h in 80% formamide, 40 mM PIPES, 1 mM EDTA, and 400 mM NaCl at 60°C. The reaction mixture was then digested with 100 U of S1 nuclease (Promega Corp.) for 1 h at 37°C, followed by electrophoresis on a 5% acrylamide/urea gel to resolve protected bands.

Southern blotting and hinge region-specific hybridization

PCR products were separated on 1.5% agarose gels and transferred to nylon membranes for Southern blotting. In experiments in which Cg sub-class specificity was determined, four replicate gels were run. Blots were pre-hybridized for 1 h, followed by overnight hybridization at the appropriate temperature with 1 × 106 cpm/ml of kinased hinge region probe. The following temperatures were used for prehybridization and hybridization: y1, 52°C; y2 to y4, 56°C; and 5’ Jh, 56°C. Blots were washed once at room temperature and twice at the probe-specific temperature in 2 × SSC + 0.5% SDS. To quantitate signals in experiments conducted semiquantitatively, filters were exposed to a phosphor screen and analyzed using a Storm Imaging System (Molecular Dynamics, Sunnyvale, CA).
Isolation and identification of reciprocal switch circle products

Isolation of switched circle products: $2 \times 10^5$ Ramos B cells from the A/G8 (1y-) subclone were cultured either alone, or with $0.5 \times 10^5$ mitomycin-treated 293/CD40L cells with or without 400 U of human rIL-4. Mitomycin treatment and coculture conditions were identical to those described above. After 3 days, cultures were refed with RPMI complete and rIL-4, where appropriate.

After 6 days, supercoiled circular DNA molecules were isolated using an alkaline lysis protocol described by Zhang et al. (65). Briefly, cell pellets were resuspended in 400 μl alkaline lysis buffer (50 mM NaCl, 2 mM EDTA, and 1% SDS, pH 12.4), vortexed for 5 min, and incubated at 30°C for 1 h. After addition of 20 μl 1 M Tris-HCl, 7.4 and 40 μl 5 M NaCl, lysates were incubated with protease K (100 μg/ml final concentration) for 30 min at 37°C. After phenol/chloroform extractions and ethanol precipitation, DNA pellets were resuspended in 50 μl Tris-EDTA (pH 7.4) and digested for 1 h with EcoRI and RNase A before PCR amplification. As a positive control for amplification, we used 10 pg of the SμI plasmid described by Malisan et al. (66).

PCR amplification

Samples were subjected to two rounds of amplification using a modified protocol of Fujieda et al. (46) and Malisan et al. (66). Briefly, 0.5 μl of digested circular DNA was amplified in a 50-μl reaction containing 50 mM KCl, 10 mM Tris-HCl, pH 9, 1.5 mM MgCl2, 0.1% Triton X-100, 200 μl dNTP, 5% DMSO, 0.5 U Taq DNA polymerase (Promega Corp.), and 100 nM of each primer. Samples were incubated initially at 95°C for 10 min, 60°C for 10 min (at which point the enzyme was added), and 72°C for 10 min. These steps were followed by 40 cycles of: 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min. The second round of amplification was conducted with 5 μl of the first round products using the same conditions and cycling times as described above. The primers used in the first round amplification were as follows: M1, 5'-gtgaggtgtgaggtgaggtgaggtgaggtgaggtgaggtgaggtgaggtgaggtgaggtgaggtgaggtgaggtgaggtgaggtgaggtgaggtgaggtgagg-3', corresponding to nucleotides 3870–3844 of Sμ; and G1, 5'-ggtcactgttgcccccctgcctgtcctggc-3', corresponding to nucleotides 1859–1885 in the Sγ region (67). The primers used in the second round were: M2 (described above) and G3, 5'-aaggtcagcttgttgcccccctgcctgtcctggc-3', corresponding to nucleotides 1193–1222 in the Sγ region (68).

Ten microliters of the second round PCR products were separated on a 1% agarose gel and transferred to nylon membrane. Reciprocal switch products were identified using a random primed Sγ probe described by Malisan et al. (66). This probe includes the nucleotides between 1280 and 1546 of the Sγ region (266 bp) (68). The primers used for PCR amplification of the Sγ probe DNA were: 5'-Sγ-5'-cccagcaagagcttccaagccaa-3' and 3'-Sγ-5'-ggtcactgttgcccccctgcctgtcctggc-3'.

Results

Ramos 2G6 B cell line is a model for IgM+ B cell responses to T cell signals

To study the relationship between IL-4- and CD40-mediated signaling and heavy chain class switching, we used a model system in which discrete aspects of B cell differentiation could be analyzed within a clonal population. The Ramos 2G6 B cell line retains many of the signaling pathways thought to mediate physiologic class switch events, as well as the genetic recombination/deletion mechanism involved in class switching in vivo. This line can up-regulate expression of IgM transcripts in response to IL-4, and undergoes limited switch recombination in response to IL-4 and CD40L (43). The Ramos 2G6 line is surface IgM+/IgD− and secretes low levels of IgM Ab (52). Therefore, this B cell line appears to represent a B cell that has received an initial activation signal, but has not yet undergone switch recombination to express downstream isotypes.

Ramos 2G6 B cells remain IgM+ after growth in culture

To use the Ramos 2G6 cell line as a model system to study signals involved in switch recombination, we wished to confirm that this line was not undergoing isotype switching in culture. To establish the uniformity of the Ramos 2G6 B cell line with respect to Ig surface expression, we conducted two-color FACS analysis using either FITC-labeled α-IgG or FITC-labeled α-IgM in conjunction with PE-labeled α-CD20. We found that the vast majority of the cells were positive for surface IgM (Fig. 1A) and negative for surface IgG expression (Fig. 1B). Additionally, supernatants from actively growing, unstimulated, Ramos 2G6 B cells lack IgG protein, as determined by ELISA (data not shown).

Individual Ramos 2G6 subclones differ with respect to Ig transcription

An increase in steady state levels of germline transcripts by cytokines is tightly correlated with switch recombination to specific CH loci (11–19). Because we wished to determine whether the Ramos 2G6 B cell line was actively producing IgY transcripts, and therefore primed to undergo switch recombination to a particular CH region, we conducted S1 analysis on RNA isolated from unstimulated as well as IL-4- and T cell-stimulated Ramos 2G6 B cells. Previous analysis of Ramos 2G6 B cells by the S1 mapping technique revealed that germline IgY transcription was induced and augmented only after stimulation with either IL-4 or IL-4 together with an activated Jurkat cell line (D1.1), respectively. This previous study did not detect IgY transcription in unstimulated Ramos 2G6 B cells or in Ramos cells stimulated with D1.1 Jurkat T cells alone (43).

Using a probe that protected sequences in both the IgY and the Cγ regions (Fig. 2B), we were able to confirm these previous observations. Initially we only detected germline (IgY-Cγ) transcripts after stimulation with IL-4 either alone or with the D1.1 cell line plus...
IL-4 (Fig. 2A, lanes 5 and 7). Mature (Cy) transcripts were detected only with IL-4 plus D1.1 cells (lane 7). However, in assaying different populations of Ramos 2G6 B cells after repeated passage in culture, we frequently detected a protected band that corresponded in size to the IyCy transcript (Fig. 2A, lanes 15–18). This observation suggested that a basal level of Iy transcription was occurring in some cells before cytokine or T cell stimulation.

To isolate independent subclones that were expressing varying levels of germline transcripts, we conducted limiting dilution subcloning and identified several Ramos subclones (designated Iy- or Iy+) that were positive for Iy transcription (data not shown). We postulated that observed transcriptional differences in the Iy+ populations were a reflection of different numbers of cells becoming transcriptionally active upon growth in culture. Neither mature Cy message (Fig. 2A, lanes 15–18) nor surface IgG (data not shown) was detected in the Iy+ populations, indicating that these subclones have not undergone switch recombination to Cy.

**Subclass-specific germline transcript expression can be measured using RT-PCR**

Because the S1 probe used to analyze Ramos 2G6 RNA after different conditions of coculture is cross-reactive with transcripts from all four Cy subclasses, we were unable to establish subclass-specific Iy expression (Fig. 2). To extend our earlier observations, we assessed the subclass-specific transcriptional response of Iy+ and Iy- Ramos subclones in response to the CD40L+ Jurkat T cell line, D1.1. We used the RT-PCR protocol developed by Jumper et al. that is based on the use of 5' and 3' primers specific for homologous sequences within the four Iy and Cy genes (44). After amplification, Iy-specific PCR products are identified by hybridizing duplicate Southern blots with hinge region probes specific for each Cy subclass.

Because this PCR strategy depends on a particular hinge probe being specific for the indicated γ subclass, before performing experiments we wanted to confirm the specificity of each hinge region probe. We used isotype-specific primers to amplify and isolate the different Iy products from D1.1 plus IL-4-stimulated Ramos cells. After we confirmed the identity of each PCR product by sequencing, cloned fragments were transferred to duplicate nylon membranes, and probed with the different Cy hinge probes. We were particularly concerned with the possible cross-hybridization of the γ1, γ2, and γ4 hinge probes because we were unable to unambiguously resolve the subclass-specific PCR products by size (γ1 = 428 bp, γ2 = 416 bp, and γ4 = 419 bp). However, as shown in Figure 3, we detected virtually no cross-hybridization among the γ1, γ2, and γ4 probes. We did detect a low level of cross-hybridization between the γ3 hinge probe and the amplified

![Image of Figure 2](http://www.jimmunol.org/iyguestonOctober15,2017)
Iγ1 fragment, but because these PCR products are readily separable by size (γ1 = 428 bp vs γ3 = 569 bp), this level of crosshybridization did not affect our ability to detect Iγ1 and Iγ3 transcripts.

**Distinct subclones of Ramos 2G6 are transcribing basal levels of Iγ1, Iγ2, and Iγ3, but not Iγ4 transcripts**

To further characterize the basal transcription in an Iγ+ population, one line (C2) was subcloned and independent subclones were analyzed by RT-PCR for subclass-specific Iγ expression. We found that all but one subclone expressed Iγ1 transcripts. Subsets of Iγ1+ subclones also expressed either 1) Iγ2 or Iγ3 transcripts or 2) both Iγ2 and Iγ3 transcripts. We did not detect any Iγ4 expression in any of the subclones tested (Fig. 4). Further analysis of the Iγ+ subclone after growth in culture revealed that it eventually began to express detectable Iγ1 transcripts. This suggested that the Ramos 2G6 subclone was not stable with respect to Iγ transcription, but was becoming Iγ+ upon growth in culture.

**Iγ1+ subclones show a unique pattern of regulation in response to IL-4 and T cell contact**

To assess whether an Iγ+ subclone (D9) up-regulates germline transcripts in response to T cell contact, we established cocultures with Jurkat T cell lines that either express (D1.1) or do not express (B2.7) the CD40L. Using an anti-CD40L mAb, 5c8, we confirmed the CD40L expression pattern of the B2.7 and D1.1 Jurkat cell lines (Fig. 5A, top and middle panels). After 6 days, RNA was isolated and analyzed by RT-PCR and hybridization with hinge region-specific probes. This analysis revealed that expression of Iγ1, Iγ2, and Iγ3 transcripts was markedly increased in Ramos D9 cultures incubated with IL-4 (Fig. 5B, lane 2). In addition, Iγ transcription from these three subclasses was up-regulated in response to the CD40L+ Jurkat line, D1.1 (lane 5). With D1.1 T cells plus IL-4, we observed an augmentation of Iγ transcription from the γ1, γ2, and γ3 loci over what was seen with IL-4 alone (Fig. 5B, compare lane 6 with lane 2). We also observed after a long exposure a low level increase in Iγ4 transcripts with IL-4 and/or CD40L (data not shown).

To establish that the observed D1.1-specific augmentation of Iγ transcripts was due to the interaction between CD40 and CD40L, we cocultured the D9 B cell line with either 293 cells or 293 cells stably transfected with a plasmid expressing CD40L (293/CD40L) (54). The level of CD40L expression on the 293/CD40L cells was determined by staining with the 5c8 mAb and was found to be comparable with the level expressed on D1.1 Jurkat cells (Fig. 5A, lower panel). Coculturing the B2.7 cells with 293/CD40L alone resulted in a measurable increase in Iγ1 transcription compared with the level observed after culturing with the untransfected 293 cells (Fig. 5C, compare lane 3 vs 4). We also observed a further increase in germline transcript expression after incubation with CD40L plus IL-4.

Because it has been shown previously that specific B cell subsets and B cell lines can express a low level of CD40L upon activation (69–71), we wanted to rule out the possibility that CD40L expression by the Ramos 2G6 line was contributing to the response we observed. Using an Ab against CD40L on Ramos 2G6 cells either before or after IL-4 stimulation, we were unable to detect by FACS any surface expression of CD40L (data not shown).

**CD40L stimulation alone is a poor stimulator of Iγ transcription in the Iγ+ subclone**

We next assessed the transcriptional activation of the γ loci in a Ramos 2G6 subclone that showed no basal level of Iγ transcription. A representative experiment with one Iγ+ subclone, designated C8, is shown in Figure 6.

Cocultures of C8 cells were established with D1.1 and B2.7 Jurkat T cell lines in the presence or absence of IL-4. After performing RT-PCR and Southern analysis with the different γ hinge region probes, we found that germline transcripts from all four Cγ subclasses were induced in response to IL-4 (Fig. 6A, lane 2). In contrast, expression of germline transcripts from any Cγ subclass was negligible after stimulating the C8 cells with the CD40L+ Jurkat line, D1.1 (Fig. 6A, lane 3). Only after a very long exposure of the filter could we begin to detect a faint γ1 signal, but not γ2, γ3, or γ4 signal from C8 cells cocultured with D1.1 cells alone.
This lack of transcriptional activity in response to CD40L signaling was not due to a failure of the C8 (IgG2) cells to respond to CD40L, because when these cells were cocultured with D1.1 T cells plus IL-4, we did observe an increased level of IgG expression from all subclasses over the level seen with IL-4 alone (Fig. 6A, compare lanes 2 and 5). This enhanced response suggested the IgG2 B cells were fully capable of responding to CD40L-mediated signals in the presence of IL-4. We observed this result with three independent IgG2 subclones.

Together these experiments reveal that Ramos 2G6 B cells with an IgG2 phenotype generally do not up-regulate Ig germline transcripts in response to CD40L. This result is in direct contrast to what we observed in the IgG2 subclones incubated under the same conditions (Fig. 5B, lane 5). However, under the same conditions and in the presence of IL-4, both the IgG2 and IgG3 populations augment the expression of IgY transcripts to a level that is significantly greater than what is observed with IL-4 alone.

To establish that our results were the consequence of CD40 signaling and not a result of other lymphoid-specific molecule(s), we again repeated our experiments using 293/CD40L cells. In the presence of 293/CD40L cells plus IL-4, we observed increased induction of IgG1 transcripts relative to stimulation with 293 plus IL-4 (Fig. 6C, compare lanes 5 and 6). We also observed a modest induction of transcription in response to CD40L alone (lane 4). Since we could also detect a slight induction of IgY transcripts in the IgG2 population by D1.1 T cells after a long exposure (Fig. 6B),
one possibility is that there is a small number of \( \gamma^+ \) cells that can up-regulate germline transcripts in response to CD40L. To test this possibility, we subcloned an \( \gamma^+ \) subclone and analyzed 3 secondary subclones for basal 1y1 expression. We identified two subclones that were positive for low level basal \( \gamma^+ \) transcription (data not shown). Therefore, the low level of 1y1 germline transcription that is seen in response to CD40 signaling is consistent with the presence of a small number of \( \gamma^+ \) cells present within the \( \gamma^+ \) population.

**Quantitative measurement of 1y1 transcription after incubation of \( \gamma^- \) and \( \gamma^+ \) Ramos 2G6 B cells with IL-4 and Jurkat T cells**

To quantify the up-regulation of 1y1 transcripts under different conditions of stimulation, we conducted semiquantitative RT-PCR on RNA isolated from cocultures established with 1y- (C8) and 1y+ (D9) Ramos B cells. To control for B cell-specific RNA in the PCR reactions, we coamplified a fragment that corresponded to sequences in the VDJ-\( C\mu \) mRNA. The extent of augmentation was determined by quantifying both signals within a linear range of amplification and dividing the 1y1 signal by the \( C\mu \) signal (Fig. 7).

Analyzing the C8 samples semiquantitatively, we found that the conditions of B2.7 cells plus IL-4 and IL-4 alone induced 1y1 transcription to an approximately similar extent (Fig. 7A, lanes 1–3 and lanes 7–9). However, the up-regulation with D1.1 cells plus IL-4 was threefold greater than with B2.7 plus IL-4, suggesting that molecules expressed on D1.1 and not on B2.7 cells are inducing the expression of germline transcripts in the Ramos C8 B cells. Analysis of 1y expression in the \( \gamma^+ \) subclone D9 showed that IL-4 increased the basal 1y1 expression approximately fivefold over the signal obtained with unstimulated cells (Fig. 7B). After incubating the B cells with CD40L+ D1.1 T cells, we observed a similar induction of 1y1 transcripts over the unstimulated D9 B cells (approximately sixfold). In contrast, there was no discernible increase in 1y1 expression after incubating the D9 B cells with the B2.7 T cells (see lanes 7–9). In confirmation of others’ findings (43, 44, 47, 49, 55, 56), incubation of both the C8 and D9 B cell lines with IL-4 plus D1.1 T cells produced an increase in 1y1 expression that was greater than what was measured with either IL-4 or CD40L+ D1.1 T cells alone.

**Switch recombination in an \( \gamma^+ \) subclone requires both IL-4 and CD40L**

In an effort to relate the expression of I\( \gamma \) transcripts in the Ramos 2G6 line to isotype class switching, we assayed the expression of mature Cy transcripts in response to different conditions of coculture. In Figure 8A, a subclone, RA3, that displays a relatively high basal level of 1y1 expression and a very low level of 1y2 expression was analyzed for the induction of germline transcription in response to different conditions of coculture. Upon culturing this line with 293/CD40L cells, we observed the up-regulation of 1y1, and to a much lesser degree, 1y2 and 1y4 transcription compared with what is observed with 293 cells alone (compare lanes 4 and 8, respectively). After longer exposures, we could also observe the induction of 1y3 transcripts (data not shown). We repeated these experiments using semiquantitative PCR and assessed the expression of all four \( \gamma \) subclasses. We analyzed the expression of I\( \gamma \) and \( C\mu \) transcripts at a time when these signals were increasing linearly with respect to input cDNA. Upon quantification of the different \( \gamma \) signals, it was evident that there was a clear synergistic effect of CD40L and IL-4 on the different \( \gamma \) loci. However, the magnitude of expression was much greater at the \( \gamma^+ \) locus compared with the other three loci (Table I).

Upon analyzing the RA3 RNA samples for mature \( C\gamma \) transcripts, we found that substantial levels of \( C\gamma \) were detected only in cultures in which both IL-4 and CD40L were added (Fig. 8B, lane 6). In addition, we only detected expression from the \( C\gamma 1 \), and to a much lesser extent, the \( C\gamma 3 \) loci. Although the RA3 population up-regulates 1y1 expression in response to CD40L alone, this condition was insufficient to induce expression of mature transcripts from any \( \gamma \) subclass.

To confirm that we were inducing de novo switch recombination and not just selecting previously switched cells, we analyzed DNA from cocultures of Ramos 2G6 using an assay that detects the reciprocal recombination products of switch recombination events (46, 66, 68, 72). As shown in Figure 9, we only detected switch circle products in samples of Ramos 2G6 B cells that had been cocultured with 293/CD40L cells plus IL-4. In addition, the presence of multiple discrete bands is consistent with several Ramos B...
cells, with distinct S\(\mu\)-S\(\gamma\) breakpoints, having undergone switch recombination as a consequence of CD40L plus IL-4 signaling.

**Discussion**

In this study, we have examined the transcriptional response of an IgM\(^+\) B cell line, Ramos 2G6, to CD40L expressed either by a Jurkat T cell line, D1.1, or a transfected 293 cell line (293/CD40L). Using subclones of Ramos 2G6 that differed in their basal expression of IgY transcripts, we sought to establish whether 1) upon CD40/CD40L contact there are differential effects on the subclass-specific expression of IgY and mature C\(\gamma\)Y transcripts, and 2) whether basal IgY transcription changes the transcriptional response of the C\(\gamma\l) locus to CD40-mediated signaling pathways.

Using this model system, we found that D1.1 T cells efficiently induce or up-regulate IgY transcription in Ramos 2G6 subclones that display a basal level of IgY transcription. We found in IgY subclones that IgY expression was up-regulated at multiple loci, irrespective of the transcriptional state of a particular locus before coculturing. For example, in a subclone that only expressed detectable basal levels of IgY transcripts, after incubation with D1.1 T cells alone, we observed the induction of IgY2 and IgY3 as well as IgY4 transcripts. In contrast, we found that the CD40L failed to induce germline transcription at the \(\gamma_2\), \(\gamma_3\), and \(\gamma_4\) loci, and induced very weak transcription at the \(\gamma_1\) locus in the Ramos 2G6 IgY subclones.

This finding was reinforced when we analyzed the induction of IgY transcripts using semiquantitative PCR. Whereas the induction of IgY1 transcripts in the transcriptionally positive subclone was approximately the same with either IL-4 or CD40L, the IgY\(^+\) subclone was induced to a much greater extent with IL-4 compared with the minimal induction seen with CD40L contact alone.

Together these results suggest that only after the Ramos B cells have undergone a change that is reflected in the induction of basal C\(\gamma\l) transcription can IgY transcripts be effectively induced by CD40 signaling. To extend this model to normal B cell responses, the IgY\(^+\) phenotype appears to represent a stage of B cell activation in which there is a general increase in B cell responsiveness to CD40 signaling.

This model could explain differences obtained in analogous studies in humans and mouse B cells. Kitani and Strober studying IgY expression in IgG\(^+\) high density resting B cells found that IgY1 and IgY3 expression generally required a proliferative stimulus, whereas IgY2 and IgY4 transcripts could be induced with cytokines alone (IFN-\(\gamma\) and IL-4, respectively) (73). However, Fujieda et al. found that in IgD\(^+\) tonsilar B cells, IgY1, IgY3, and IgY4, but not IgY2 transcripts were induced with IL-4 alone (46). This same study found that CD40 triggering alone failed to induce the expression of any class of IgY transcripts. However, Jumper et al. (44) and Warren and Berton (49) found that multiple classes of germline transcripts were up-regulated in response to CD40 signaling. One possible explanation for these contrasting results is that the different methods used to isolate B cells resulted in populations or subpopulations at different stages of B cell activation that also differed in their ability to up-regulate germline transcripts in response to CD40 triggering.

Other evidence also supports the concept that up-regulation of germline transcripts by CD40 signaling may be contingent upon the activation state of the B cell. Berberich et al. found that transformed B cell lines, compared with human tonsilar B cells, differentially activated a number of kinases in response to signaling through CD40 (40). The capacity to activate specific signaling pathways correlated with a specific maturation stage of the responding B cells.

The up-regulation of IgY transcripts by stimulation with either IL-4 plus CD40L or CD40L alone suggests that CD40-responsive elements are associated with different C\(\gamma\)Y genes. Such response elements recently have been identified in the promoter region of the human Ie gene (51) and the murine IgY1 gene (41). It is possible...
IgD to establish conditions of stimulation that would induce mature \( \text{C}_{\gamma1} \) transcripts in an \( \text{I}_{\gamma}^+ \) (RA3) population and the subclass-specific expression of these transcripts, RA3 cells were incubated under different coculture conditions of stimulation. Shown in A is the amplification of cDNA with primers specific for \( \text{I}_{\gamma} \) sequences. In B is the result of PCR amplification of the same cDNAs using primers specific for mature \( \text{C}_{\gamma1} \) transcript. The different conditions of coculture are as follows: alone (lane 1); with IL-4 (lane 2); with mitomycin-treated 293 cells (lanes 3); with mitomycin-treated 293 cells plus IL-4 (lanes 4); with mitomycin-treated 293/CD40L cells (lanes 5); and with 293/CD40L cells plus IL-4 (lanes 6). All hinge region probes were labeled to the same approximate sp. act. The exposure of all blots within an experiment was for the same length of time. To control for RNA integrity and the efficacy of the PCR reaction, primers specific for regions in the \( \text{C}_{\mu} \) message were included in each PCR reaction. One \( \text{I}_{\gamma} \) blt was rerun probed with the \( 5'-\text{J}_{\gamma} \) oligo to identify the \( \text{C}_{\mu} \)-specific band.

### Table I. Quantitation of subclass \( \text{I}_{\gamma} \) transcripts in the RA3 subclone after stimulation with IL-4, CD40L, and CD40L plus IL-4

<table>
<thead>
<tr>
<th>RA3 Cells Cocultured With</th>
<th>Fold Induction of Germline Transcripts</th>
<th>( \text{I}_{\gamma}^1 )</th>
<th>( \text{I}_{\gamma}^2 )</th>
<th>( \text{I}_{\gamma}^3 )</th>
<th>( \text{I}_{\gamma}^4 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>293</td>
<td>1.0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>293 + IL-4</td>
<td>3.5</td>
<td>0.67</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>293/CD40L</td>
<td>2.3</td>
<td>1.07</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>293/CD40L + IL-4</td>
<td>13.8</td>
<td>3.8</td>
<td>2.8</td>
<td>1.3</td>
<td></td>
</tr>
</tbody>
</table>

RA3 RNA was extracted 6 days after coculture, reverse transcribed, amplified by PCR, and analyzed for \( \text{I}_{\gamma} \) expression by hybridization to the different \( \text{I}_{\gamma} \) hinge probes. In the same reaction, primers to amplify the \( \text{C}_{\mu} \) message were included to quantify and standardize the amount of B cell-specific RNA in each reaction. PCR products amplified from DNA isolated from Ramos 2G6 B cells cultured either alone (lane 2) with 293/CD40L (lane 3), or with 293/CD40L plus IL-4 (lane 4). Molecular weight markers (\( \lambda \) HindIII/EcoRI) are shown in lane 1.

FIGURE B. Ramos 2G6 B cells express mature \( \text{C}_{\gamma1} \) transcripts only under conditions of IL-4 and CD40L stimulation. To establish the conditions of stimulation that would induce mature \( \text{C}_{\gamma1} \) transcripts in an \( \text{I}_{\gamma}^+ \) (RA3) population and the subclass-specific expression of these transcripts, RA3 cells were incubated under different coculture conditions of stimulation. Shown in A is the amplification of cDNA with primers specific for \( \text{I}_{\gamma} \) sequences. In B is the result of PCR amplification of the same cDNAs using primers specific for mature \( \text{C}_{\gamma1} \) transcript. The different conditions of coculture are as follows: alone (lane 1); with IL-4 (lane 2); with mitomycin-treated 293 cells (lanes 3); with mitomycin-treated 293 cells plus IL-4 (lanes 4); with mitomycin-treated 293/CD40L cells (lanes 5); and with 293/CD40L cells plus IL-4 (lanes 6). All hinge region probes were labeled to the same approximate sp. act. The exposure of all blots within an experiment was for the same length of time. To control for RNA integrity and the efficacy of the PCR reaction, primers specific for regions in the \( \text{C}_{\mu} \) message were included in each PCR reaction. One \( \text{I}_{\gamma} \) blt was rerun probed with the \( 5'-\text{J}_{\gamma} \) oligo to identify the \( \text{C}_{\mu} \)-specific band.

Table I. Quantitation of subclass \( \text{I}_{\gamma} \) transcripts in the RA3 subclone after stimulation with IL-4, CD40L, and CD40L plus IL-4

that signaling through CD40 induces the activation of a unique set of transcription factors distinct from those induced with IL-4. Alternatively, because IL-4 and CD40 signaling are known to up-regulate a number of common transcription factors, including nuclear factor-κB, AP-1, and nuclear factor of activated T cells (NFAT) (39, 74–76), signals provided by coactivation could increase the number of occupied binding sites at each \( \text{I}_{\gamma} \) promoter, resulting in increased transcription.

Switch recombination, as measured by the appearance of both mature \( \text{C}_{\gamma1} \) transcripts and reciprocal products, was observed only after Ramos 2G6 B cells were stimulated with both IL-4 and CD40L. This result supports others’ findings that both signals are necessary for switch recombination (43, 44, 47, 49, 55, 56). However, contact through CD40, which results in increased germline transcription, is not sufficient to obtain switch recombination in our model system. This is evident in our \( \text{I}_{\gamma}^+ \) subclone, RA3. This subclone expresses an elevated basal level of \( \text{I}_{\gamma1} \) transcription and clearly up-regulates the expression of \( \text{I}_{\gamma1} \) transcripts in response to CD40L, but does not undergo switch recombination when stimulated through CD40 alone. This result contrasts with the findings of Malisan et al., who detected a low level of switch recombination in \( \text{I}_{\gamma1} \) B cells cocultured with CD40L-expressing L cells. Whether these rare events represented a small number of B cells that were already cytokine and/or Ag activated in the population is unknown. It is clear, however, that the majority of B cells undergoing switch recombination did require both IL-10 and CD40L (66).

We found in the RA3 line that transcription was up-regulated at the \( \text{I}_{\gamma1} \) locus in response to CD40L plus IL-4 approximately 14-fold over the basal level of transcription. This level of induction was not approached at the \( \text{I}_{\gamma1} \) locus as a consequence of other culturing conditions or at the other \( \gamma \) loci in response to any condition of activation (Table I). Since we only saw measurable levels of VDJ-\( \text{C}_{\gamma1} \) transcripts, it is possible, as suggested by Snapper et
al. (77), that recombination occurs only after a critical threshold of germine transcription is reached. Alternatively, IL-4 and CD40L signaling may induce factors actually involved in the switching mechanism.

In conclusion, we find a differential effect of CD40 signaling on the induction of Ig transcripts in phenotypically distinct subclones of Ramos 2G6. This difference in signaling may ultimately reflect chromatin changes at the Cig locus that facilitate or prevent the binding of CD40-induced transcription factors. Chromatin changes have been seen previously at the Cig locus in response to cytokine signaling (8, 9). We are currently examining whether analogous changes occur in our Ramos clones as they move from an Ig- to an Ig- phenotype.

Acknowledgments

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References


