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IL-6 Contributes to the Expression of RAGs in Human Mature B Cells

Sophie Hillion, Maryvonne Dueymes, Pierre Youinou, and Christophe Jamin

Mature B cells acquire the capacity to revise rearranged Ig V region genes in secondary lymphoid organs. In previous studies, we demonstrated that cross-linking the BCR and the CD40 induces the expression of the RAG1 and RAG2 enzymes and, thereby, secondary rearrangements. We examine herein the mechanism that underpins RAG1 and RAG2 expression in peripheral and tonsil B cells. Coordinated engagement of the BCR and CD40 promoted the synthesis of IL-6 and, thereby, up-regulation of its receptor on activated B lymphocytes. Furthermore, we provide evidence that IL-6 initiates the expression of RAGs in circulating B cells, and extends those in tonsil B cells. Thus, neutralization of IL-6 or blocking of its receptor inhibits RAG expression. Moreover, we demonstrate that IL-6 impedes BCR-mediated termination of RAG gene expression in both population of B cells. The recovered inhibition of RAG gene transcription by IL-6 receptor blockade supports the notion that once recombination is launched, its termination is also regulated by IL-6. Taken together, these studies provide new insight into the dual role of IL-6 in inducing and terminating expression of the recombinase machinery for secondary rearrangements in mature human B cells. The Journal of Immunology, 2007, 179: 6790–6798.

The B cell repertoire is shaped during early ontogeny through random rearrangement, replacement, and selection of Ig V region genes. These events are regulated by RAG1 and RAG2 products which help assemble V(D)J Ig genes in the bone marrow (BM). The expression of these enzymes is modulated by environmental factors. Thus, the early stages of B cell differentiation require IL-7, which influences Ig gene rearrangements and regulates repertoire diversification. In contrast, IL-3 and IL-6 activate RAG gene transcription following interactions between stromal cells and lymphoid progenitor cells. For RAG gene expression to occur in the latter setting, two signals are necessary: the first is mediated by contact through the cell membrane with stromal cells, while the second comes from soluble cytokines.

Because V(D)J genes are randomly selected to participate in rearrangements, self-reactive B cells may emerge in the BM. At least three mechanisms prevent the immune system from retaining such autoreactivity. These include deletion of autoreactive B cell clones by apoptosis, anergy (6), and receptor editing to shift the BCR. The latter mechanism, which is triggered as soon as immature B cells encounter their autoantigens, requires the assistance of newly synthesized RAG enzymes. With the benefit of successful secondary rearrangements, B cells purge autoreactivity, make up a nonself-reactive BCR repertoire, switch off their V(D)J recombination machinery, and migrate to a secondary lymphoid organ (SLO).

Based on the association of mature B cells with a decrease in the RAG proteins and an increase in the density level of the BCR (10), secondary RAG induction was originally thought to be restricted to immature B cells. RAG products have since been detected in mature B cells stimulated in culture (11), and in germinal centers (GCs), where peripheral V(D)J recombination may consume somatic hypermutations in centrocytes (12). Replacing the variable region of a rearranged Ig with another would generate BCRs with higher affinity for the Ag (13). RAG proteins were thus detected in B cells within and outside of the GCs in mice (12, 14, 15) and humans (16–18).

Recent evidence suggests that significant differences exist between mechanisms of RAG regulation in mice and humans. In mice, stimulation of mature splenic B cells with CD40L in the presence of IL-7 (19), or with LPS in the presence of IL-4 (11), induces the expression of RAG genes, whereas anti-IgM fails to do so in the presence of IL-1 (15). In humans, stimulation of blood B cells with Staphylococcus aureus Cowan I and IL-2 induces the expression of RAGs (20), whereas stimulation with CD40L plus IL-2, IL-4, or IL-10 exerts no effects on RAG up-regulation in GC B cells (21). The stage of B cell maturation also appears influential in the RAG response of B cells to a given stimulus.

Although external signals seem to be essential for the expression of RAGs in mature B cells, their precise nature remains elusive. Our previously reported model revealed that, following 5 days of culture, mature human pre-GC B cells expressed RAG proteins in response to CD40L and anti-IgM stimulation (18). In contrast to previous observations in immature and mature B lymphocytes, there was no need for further introduction of cytokine into the culture medium. Prompted by this observation, we addressed the issue of whether cytokines, derived from B cells, could enable RAG products to be synthesized in mature B cells following stimulation with CD40 and BCR cross-linking. We found that IL-6 is produced by costimulated B cells, contributes to the initiation and the extension of RAG expression, and modulates the subsequent BCR-mediated down-regulation.
Materials and Methods

Isolation of B cells

Normal blood was donated by volunteers from laboratory staff. Tonsils were obtained from children undergoing routine tonsillectomy. The tissues were minced up, suspended in PBS and filtered to remove tissue fragments and clumps. Tonsil cell suspensions and blood were layered onto Ficoll-Hypaque, and centrifuged for 30 min at 450 g. Mononuclear cells were incubated with neuraminidase-treated sheep RBC for 1 h at 4°C, and T cells depleted by a second 25-min round of centrifugation.

All mAbs were purchased from Beckman-Coulter, unless otherwise specified. B cells were stained with PE-conjugated anti-CD5 and FITC- or PE-linked to cyanin 5-conjugated anti-CD19 mAbs, and CD5-negative/CD19-positive B cells sorted on an Epics Elite (Beckman Coulter) FACS. All preparations were >98% pure CD19+ as determined by FACS.

For the murine National Institutes of Health-3T3 fibroblasts transfected or not with human CD40L gene were a gift from John Gordon (Department of Immunology, University of Birmingham Medical School, Birmingham, U.K.).

Flow cytometry

We used PE-anti-CD40L (clone TRAP1) from BD Biosciences, FITC-anti-CD23 (clone 9P25) PE-anti-Î· (Î·) and FITC-anti-Î· (Î·) from Dako-Cytomation, and FITC-anti-CD19 (clone J4.119), -anti-CD10 (clone ALB1), -anti-CD21 (clone J173), -anti-CD23 (clone BL13), FITC- and PE-anti-CD5 (clone BL1a), PE-anti-CD126 (clone M91), -anti-CD24 (clone ALB1), -anti-CD62L (clone DREG56), FITC-anti-Î·Ig, PE-linked to cyanin 5-labeled and PE-linked to cyanin 7-conjugated-anti-CD19. Intra-cellular staining of BCL-6 and IRF4 transcription factors were performed after permeabilization of the cells with 70% methanol. Anti-BCL-6 and anti-IRF4 (Abcam) Abs were revealed with FITC-conjugated anti-rabbit Abs (Jackson ImmunoResearch Laboratories).

Cell culture

All the cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine (Invitrogen Life Technologies), 200U/ml penicillin, and 100 Î¼g/ml streptomycin. To stimulate B cells (22), we used 105 NIH-3T3 fibroblasts transfected or not, and incubated with mitomycin C (Sigma-Aldrich). Isolated B cells were seeded at 2×107 cells/ml and cultured with or without, 1 Î¼g/ml anti-IgM Ab-coated beads for 24 h. Cells were also stimulated with 10 Î¼g/ml anti-IgM Ab-coated beads alone and, in some experiments, cultured in the presence of rIL-6 (ImmunoTools), anti-IL-6 Ab (Dako-Cytomation), or 40 ng/ml anti-Î·Ig Ab (R&D Systems) at a concentration known to block IL-6 activity.

RNA isolation and RT-PCR

RNA was extracted by the RNAble kit (Eurbio), and reverse-transcribed with Superscript II RNase H-RT according to the manufacturer’s instructions (Invitrogen Life Technologies). RAG1 and RAG2 were amplified by nested RT-PCR using 1 Î¼l of cDNA using TaqDNA polymerase (Invitrogen Life Technologies) and the primer pairs listed in Table 1. The PCRs were conducted as follows: in the first round, cDNA was amplified for 25 cycles of 30 s at 94°C, 1 min at 56°C, and 1 min at 72°C, with a 10-min final extension at 72°C. The second round consisted of 35 cycles of 30 s at 94°C, 1 min at 56°C, and 1 min at 72°C, with a 10-min final extension at 72°C. There was only 1 round of 40-cycle PCR for the GAPDH RT-PCR amplification. The final products were analyzed on 2% agarose gels stained with ethidium bromide (18). The specificity of all the fragments was verified by cycle threshold evaluation with the 2-Î·Ct method using 18S as internal control.

Ligation-mediated PCR (LM-PCR)

DNA was extracted from 105 cells using the DNAzol kit (Invitrogen Life Technologies) and 500 ng ligated to the LM-PCR linker at 20 pmol with T4 DNA ligase (Promega) as previously described (18). Following two rounds of PCR using primers upstream the 5’ end of either JÎ±3 to JÎ±7 or JÎµ2 to JÎµ5 genes paired with primer encompassing the LM-PCR linker and the recombination signal sequence, PCR products were analyzed on 2% agarose gel stained with ethidium bromide (18). The specificity of all the amplified products were verified by sequence analysis.

Indirect immunofluorescence

Cultured blood B cells were washed in PBS, centrifuged onto glass slides, fixed in 4% paraformaldehyde, and prepared as described (18). First, cells were incubated in PBS containing 0.1% Nonidet P-40 (Sigma-Aldrich), 2% nonfat dry milk, 5% FBS, and 0.02% sodium azide, followed by incubation with Cohn fragment (Sigma-Aldrich) to avoid nonspecific fixation. Cells were incubated overnight with goat anti-Rag2 Ab (Santa Cruz Biotechnology), followed by FITC-anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and visualized with tetramethylrhodamine isothiocyanate-streptavidin. Cells were then left overnight with rabbit anti-RAG1 Ab (Santa Cruz Biotechnology) followed by FITC-anti-rabbit IgG (Jackson Immunoresearch Laboratories). Apliques of cells were stained without anti-RAG Abs but in the presence of goat and rabbit Igs at the same concentrations and used as controls. Double stainings of either RAG protein with propidium iodide were also performed at the same time. Cells were examined by fluorescence microscopy using an oil immersion lens (Zeiss). Preliminary experiments were performed on 697 pre-B and Hep-2 epithelial cells to ensure the lymphoid specificity of the detected proteins.

ELISA

IL-6 was titrated in the supernatants of cultured cells by a commercial ELISA using paired Abs (Beckman Coulter). Supernatant of CD40L-transfected fibroblasts was used as negative control.

Results

Induction and termination of RAG gene transcription in tonsil and blood B cells

With reference to our previous demonstration that costimulation with anti-IgM and CD40L induces the transcription of RAGs in tonsil B cells (18), we analyzed the response of blood B cells to the same stimuli. Transitional CD10high B cells, that have the capacity to express RAG mRNA in secondary lymphoid organs of mice (23, 24), can be detected in the circulation of humans (25). In contrast to mouse B cells, however, circulating human transitional type 1 B lymphocytes also express CD5 (25),

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<th>Table 1. Sequences of oligonucleotides used as primers for the amplification of cDNA in RT-PCR and quantitative RT-PCR</th>
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B cells expressed both RAG1 and RAG2 mRNA following CD21 to be CD10 transitional type 1 B cells. Thus, sorted cells from tonsils were found to express RAGs, and tonsil B cells sustained to express RAGs, by nested RT-PCR using tonsillar and peripheral blood B cells, before (B) and after (C) 5 days of culture on CD40L-transfected fibroblasts in the presence of 1 μg/ml anti-IgM-coated beads, after 24 h of stimulation with 10 μg/ml anti-IgM alone (B'), or after 5 days of costimulation followed by 6 h of culture with 10 μg/ml anti-IgM alone (C'). GAPDH mRNA was analyzed by RT-PCR. Negative and positive controls with HEp-2 and 6792 IL-6 AND RAGs in mature B cells.

Therefore, to ensure accurate determination of RAG expression in circulating mature B cells, we sorted CD19+CD5− blood lymphocytes and excluded CD19+CD5+ that include transitional type 1 B cells. Thus, sorted cells from tonsils were found to be CD10−, CD21−, CD23−, CD24−, CD62L−, BCL-6low+IRF4+, and those from blood CD10−, CD44+, CD21−, CD23−, CD24+, CD62Llow, BCL-6low, and IRF4++ (Fig. 1A), confirming their mature status. B cells from tonsils contained mRNA for RAG1 and RAG2, whereas those from blood did not (Fig. 1B). We previously observed that 13.9 ± 4.2% of the CD19+CD5− tonsil B cells expressed RAG1 and RAG2 transcripts (18). Tonsil and blood B cells were then cultured on CD40L-transfected fibroblasts in the presence of anti-IgM Ab. After a 5-day culture (Fig. 1C), RAG1 and RAG2 mRNA were maintained in B cells from tonsils, and induced in those from blood. As shown in Fig. 2A, stimulated tonsil B cells were found CD10−, CD44++, CD21+, CD23++, CD24low+, CD62L−, BCL-6low, and IRF4++, and those from blood B cells were found CD10−, CD44++, CD21+, CD23++, CD24+, CD62Llow++, BCL-6low, and IRF4++. This is consistent with an activated mature status, and further indicates the lack of transitional or immature B cells. Using single-cell RT-PCR, 15.5 ± 3.3% of the tonsil B cells and 8.1 ± 1.9% of the blood B cells expressed both RAG1 and RAG2 mRNA following stimulation (Fig. 2B).

This raises the issues regarding the behavior of blood B cells following stimulation with anti-IgM Ab alone. Cross-linking the BCR (21, 26) on tonsil B cells down-regulated the RAG gene transcription (Fig. 1B). Similarly, blood B cells induced to express RAGs, and tonsil B cells sustained to express RAGs, by CD40L plus anti-IgM stimulation were impelled to down-regulate RAG gene transcription by a 6-h treatment with anti-IgM alone (Fig. 1C). To determine whether RAG mRNA expression was associated with effective proteins at initiating rearrangement, variation in the frequency of κ- and λ-positive B cells were used as indicator of gene recombination process (27, 28). Induction of RAG expression following CD40L plus anti-IgM stimulation was thus associated with modifications to the κ- and λ-positive B cells (Fig. 2C). The κ:λ ratio decreased from 1.86 ± 0.17 to 1.34 ± 0.19 in tonsil B cells, and from 1.6 ± 0.08 to 1.2 ± 0.07 in blood B cells, suggesting that the Ig recombinase process was efficiently induced. The presence of active Ig recombinase enzyme was further demonstrated by LM-PCR on DNA isolated from CD40L and anti-IgM costimulated cells. DNA breaks or recombination signal sequences due to recombinase activity were determined using primers upstream the 5′ end of each Jκ3 to Jκ7 and Jκ2 to Jκ5 genes paired with primer encompassing the LM-PCR linker and the recombination signal sequences. Specific PCR products corresponding to a recombinaction of the Jκ6, Jκ7, and Jκ5 genes were found in tonsil B cells. Recombination of the Jκ4, JA7, Jκ3, and Jκ4 genes were also found in blood B cells (Fig. 2D). All these products were sequenced, and alignments with the 3′ end upstream each recombinated gene of the genomic DNA underscored the specificity of the products (data not shown). These data confirm that Ig recombinase activity is ongoing in this costimulation model.

To gain insight into how costimulation triggers RAG up-regulation, blood B cells were stimulated with CD40L-transfected fibroblasts. This resulted in the up-regulation of CD40L on mature Ig-positive cells (Fig. 3A) but not in the RAG expression (left panels in Fig. 3A). Thus, preactivation with CD40L was not sufficient to induce RAG. After subsequent addition of anti-IgM Ab to cell culture, no RAG mRNA could be seen either (right panels in Fig. 3A). Moreover, RAG1 and RAG2 proteins were also induced in blood B cells costimulated with CD40L-transfected fibroblasts and anti-IgM Ab (Fig. 3B), as previously observed in tonsil B cells (18), but not in cells sequentially

**FIGURE 1.** RAG1 and RAG2 expression in human mature B cells. Tonsillar and peripheral blood B cells were analyzed by FACS before stimulation to evaluate their maturation status using FITC-anti-CD10, -anti-CD44, -anti-CD21, -anti-CD23, -anti-BCL-6, -anti-IRF4, or PE-anti-CD24, -anti-CD62L, or –anti-CD5 Abs. A representative example of three analyses is shown (A). RAG1 and RAG2 mRNA were amplified by nested RT-PCR using tonsillar and peripheral blood B cells, before (B) and after (C) 5 days of culture on CD40L-transfected fibroblasts in the presence of 1 μg/ml anti-IgM-coated beads, after 24 h of stimulation with 10 μg/ml anti-IgM alone (B'), or after 5 days of costimulation followed by 6 h of culture with 10 μg/ml anti-IgM alone (C'). GAPDH mRNA was analyzed by RT-PCR. Negative and positive controls with HEp-2 and 6792.
stimulated (Fig. 3B’). Overall, these data suggest that anti-IgM should be added simultaneously with CD40L to induce RAG expression.

Increased synthesis of IL-6 in mature activated B cells

IL-2, IL-4, and IL-10 are not required for RAG induction in blood B cells (21). However, there is evidence that IL-6 contributes to the regulation of RAG genes in immature B lymphocytes (4). This data raises the possibility that IL-6 might also induce RAG expression in mature B lymphocytes. To address this question, first, the amount of IL-6 mRNA was measured by quantitative RT-PCR in tonsil and peripheral blood B cells (Fig. 4, A and A’). The transcription of IL-6 was increased in B cells cultured with CD40L-transfected fibroblasts in the presence of anti-IgM Ab after a 4-day incubation. Interestingly, the elevated transcription of IL-6 mRNA was concomitant with the sustained RAG expression in tonsil B cells and the induction of RAG transcription in blood B cells, as shown in Fig. 1C. In addition, IL-6 protein was detected by ELISA in the supernatant of these cultured B cells (Fig. 4, B and B’). However, for IL-6 to be functional its receptor must be present on the surface of mature B cells. CD126 was absent before stimulation, or after a 24-h stimulation with anti-IgM Ab, but was expressed on 47.8 ± 3.3% of tonsil B cells stimulated with CD40L, and 53.3 ± 2.5% of those stimulated with CD40L plus anti-IgM Ab (Fig. 4C’). This frequency reached 38.5 ± 1.5% of the peripheral blood B cells (Fig. 4C’). Following CD40L and anti-IgM costimulation, we observed that RAG-expressing cells were restricted to those turned positive for CD5 (18). Interestingly, a CD5-positive subpopulation, appeared expressing CD126 and represented 17.9 ± 2.5% and 16.9 ± 1.4% of CD40L- and CD40L plus anti-IgM Ab-stimulated tonsillar B cells, respectively. Within the peripheral blood B cells, this subpopulation represented 18.4 ± 1.5%. These data suggest that IL-6 is a candidate that may provide the third signal for RAG gene transcription in B cells induced to up-regulate CD126.

To further decipher the role of IL-6, circulating blood B cells were activated for 24 h with increasing amount of rIL-6 and for several days with 1000 pg/ml rIL-6. RAG expression could not be induced in such conditions (Fig. 5A). When rIL-6 was added to CD40L-stimulated B cells (Fig. 5B) or to anti-IgM Ab-stimulated B cells (Fig. 5C), no RAG induction was observed either even with elevated level of rIL-6. These data indicate that exogenous IL-6 alone is unable to promote RAG expression, even in CD40L-stimulated cells. Furthermore, they also highlight that BCR signaling is absolutely essential together with CD40 signals.

IL-6 regulates RAG mRNA expression in mature B cells

To verify the requirement for IL-6 in RAG expression of CD40 and BCR costimulated B lymphocytes, tonsil and blood B cells...
were stimulated with anti-IgM in the presence of CD40L-transfected fibroblasts for 5 days, with and without anti-IL-6R mAb (Fig. 6A). The transcription of RAG genes was confirmed to depend on IL-6, because the extent of its reduction correlated with the quantity of anti-IL-6R mAb, with a peak inhibitory effect at 40 ng/ml. Under these conditions (Fig. 6B), blocking IL-6 signaling to its receptor prevent the induction of RAG expression in blood B cells, and inhibit its persistence in tonsil B cells. The role of IL-6 was further strengthened using anti-IL-6 mAb (Fig. 6C). Thus, neutralization of IL-6 also dampens the induction of RAG transcription.

To confirm that IL-6 directly maintains RAG expression and is not only important for the activation process, we washed out IL-6 and

FIGURE 3. CD40L and BCR requirement for RAG expression in human mature B cells. Peripheral blood B cells were activated for 5 days on CD40L-transfected fibroblasts. Expression of CD40L was analyzed before (Day 0) and after (Day 5) 5 days of CD40L-stimulation, and that of surface Ig analyzed at day 5, by FACS (A). Cells were then stimulated or not with 1 μg/ml anti-IgM for 24 h. RAG1 and RAG2 mRNA were amplified by nested RT-PCR and GAPDH mRNA by RT-PCR (A'). RAG1 and RAG2 proteins were detected by immunofluorescence staining of peripheral blood B cells after 5 days of costimulation (B) and after activation for 5 days on CD40L-transfected fibroblasts followed by stimulation for 24 h with 1 μg/ml anti-IgM alone (B'). Cells stained with isotypic control Abs and propidium iodide are shown. Positive and negative controls with 697 pre-B and HEp-2 cell lines, respectively, are shown (C). A representative experiment of at least six is shown.

FIGURE 4. IL-6 and IL-6R CD126 expression on stimulated mature human B cells. Tonsillar (A–C) and peripheral blood (A'–C') B cells were stimulated for 5 days on CD40L-transfected fibroblasts in the presence or absence of 1 μg/ml anti-IgM. A and A', IL-6 mRNA was amplified by quantitative RT-PCR and its level established following normalization relative to that of 18S mRNA. B and B', The concentration of IL-6 in supernatants was determined by ELISA using those of CD40L-transfected fibroblasts without B cells as negative control. C and C', Expression of IL-6αR, CD126, and of CD5 were analyzed by FACS using PE-anti-CD126 and FITC-anti-CD5 Abs on B cells stimulated for 5 days, and on cells stimulated for 24 h with 10 μg/ml anti-IgM alone. A representative experiment of at least three is shown.
BCR cross-linking one day before the endpoint cultures of tonsil B cells (day 4), and then added back IL-6 on these cells further cultured on CD40L-transfected fibroblasts or in medium alone. With the persistence of CD40L signals, RAG transcripts were maintained independently on the addition of rIL-6 (Fig. 7A), likely due to the endogenous secretion of IL-6 by these cells (Fig. 4B). However, when they were further cultured in medium alone, RAG transcription was sustained only in the presence of rIL-6 (Fig. 7B). These data indicate that, following their induction, RAG expression is maintained due to the contribution of IL-6.

FIGURE 5. Effect of rIL-6 on RAG expression in mature human B cells. RAG1 and RAG2 mRNA were amplified by nested RT-PCR in peripheral blood B cells following 24 h of stimulation with a range of rIL-6 concentrations (0, 10, 100, and 1000 pg/ml), and in blood B cells cultured with 1000 pg/ml rIL-6 for 5 days (A). Tonsillar and peripheral blood B cells were stimulated for 5 days on CD40L-transfected fibroblasts (B), or for 24 h with 10 μg/ml anti-IgM (C), with a range of rIL-6 concentrations (0, 10, 100, and 1000 pg/ml). RAG1 and RAG2 mRNA were amplified by nested RT-PCR and that of GAPDH by RT-PCR. A representative example of three experiments is shown.

FIGURE 6. IL-6-dependent induction of RAG in mature human B cells. RAG1 and RAG2 mRNA were amplified by nested RT-PCR in tonsillar and peripheral B cells following 5 days of culture on CD40L-transfected fibroblasts in the presence of 1 μg/ml anti-IgM Abs, with a range of anti-IL-6R Ab concentrations, (0, 4, 40, or 400 ng/ml) (A), before and after 5 days of stimulation on CD40L-transfected fibroblasts with 1 μg/ml anti-IgM Abs in the presence or absence of 40 ng/ml anti-IL-6R Abs (B), or with a range of anti-IL-6 Ab concentrations (0, 1, 10, and 100 ng/ml) (C). A representative example of four experiments is shown.
To determine whether IL-6 could also have an effect on the repression of RAG transcription, we measured RAG transcripts in anti-IgM-stimulated B cells cultured on CD40L-transfected fibroblasts. Both RAG1 and RAG2 mRNA were expressed in tonsil and blood B cells after a 5-day culture. The conditioned supernatants were collected, and the cells washed in RPMI 1640. Tonsil as well as blood B cells were then seeded into 3 flasks. All cells were cultured with anti-IgM Ab for 6 h. The first aliquots of tonsil B cells and blood B cells were placed into fresh medium (left column in Fig. 8), the second aliquots were cultured in conditioned supernatants (medium column in Fig. 8), and the third cultured in conditioned supernatants with anti-IL-6R mAb (right column in Fig. 8). Repression of RAG transcription was achieved by anti-IgM in the first aliquots, impeded in the second aliquots by the presence of IL-6 in conditioned supernatants, and recovered in the third aliquots by anti-IL-6R-mAb-induced blockade of IL-6 in the conditioned supernatants. These results demonstrate that IL-6 has the capacity to modulate the RAG gene transcription by inhibiting its repression.

**FIGURE 8.** Influence of IL-6 on RAG repression in mature human B cells. Tonsillar and peripheral blood B cells were cultured for 5 days on CD40L-transfected fibroblasts in the presence of 1 μg/ml anti-IgM to induce RAG1 and RAG2 expression. Cells and culture supernatants were collected. The cells were then stimulated for 6 h with 10 μg/ml anti-IgM alone, with the culture supernatant only, and in the presence of 40 ng/ml anti-IL-6R Abs. RAG1 and RAG2 mRNA were amplified by nested RT-PCR, and that of GAPDH by RT-PCR. A representative example of three experiments is shown.

**Discussion**

The expression of RAG is tightly regulated in immature, and mature B cells (2–4, 11, 12). We previously noted that, although cytokines are required for RAG up-regulation, the provision of cytokines is dispensable in our model of RAG induction in human mature B cells, in which the cells are costimulated by CD40L and anti-IgM Ab for up to 5 days (18). We hypothesized that, following 5 days of costimulation, some cytokines produced from the activated B cells acted in an autocrine manner to induce RAG expression. To test this hypothesis, we assessed the presence of cytokines in the culture medium, and searched for their receptors on activated B cells. The results revealed that, following stimulation, tonsil and blood B cells behaved similarly by secreting a cytokine required to induce the expression of RAGs, and to subsequently control their down-regulation.

Several investigations have demonstrated that, in SLOs, RAG-positive B cells could be immature transitional type 2 B cells (15, 23, 24). Given that transitional type 1 B cells can be detected in the circulation of human (25), circulating RAG-positive B cells could be precursor cells recently emigrated from the BM. However, we believe this is unlikely in our study. Indeed, human circulating transitional type 1 B cells belong to the CD5-positive subpopulation (25) that have been excluded from the analysis. Moreover, stimulation of immature B cells with anti-IgM Abs induced RAG expression (29), which is not the case in our sensitive RT-PCR assay (Fig. 1B). Furthermore, stimulation of splenic mouse B cells with anti-CD40 plus IL-7 (19) induces IL-7R, which ultimately leads to the transcription of RAG genes. Despite the presence of mRNA for CD127 in resting B cells, we were unable to detect CD127-positive B cells by FACS, and activation of B cells with CD40L and/or anti-IgM Ab, in the presence of IL-7 did not lead to IL-7R up-regulation on the membrane (data not shown). Because IL-7R is expressed on human pre-B lymphocytes but not on mature lymphocytes (30, 31), the lack of IL-7R expression is a further argument indicating that immature B cells are absent from the purified CD19+CD5– blood B cells in our study and that induction of RAG cannot be ascribed to the presence of these cells either in blood, or in tonsil B cells (18), following CD40L and anti-IgM costimulation. On top of that, these data indicate that human mature B cells do not require IL-7 to transcribe RAG genes, in contrast to murine B cells (19), and that secondary V(D)J rearrangements are, thus, likely to be regulated by other cytokines.

Given its high level in SLOs (32) and its involvement in RAG expression in BM progenitor cells (4), IL-6 appears to be a credible candidate for RAG regulation in peripheral B cells. The experiments describe in the current study show that activation of mature B cells by CD40L and anti-IgM increases the level of mRNA for IL-6 and the amount of IL-6 protein. For IL-6 to be functionally effective, its receptor must be expressed at detectable levels on the cell surface. Consistent with previous reports (33), we noted that activated, but not resting, B cells harbor CD126 on their surface. How IL-6 and IL-6R expression are regulated following CD40 and IgM coligation is still an open question. Because engagement of the BCR alone takes 30 h to induce apoptosis (34, 35), there is little time to evaluate IL-6 synthesis and IL-6R up-regulation. However, it appears that a 24-h stimulation of B cells had no effects on the expression of IL-6Rs. In contrast, engagement of CD40 alone was able to induce detectable, albeit a modest increase in IL-6 production. In accord with previous reports (36), this is associated with efficient up-regulation of CD126. Coligated CD40 and BCR act synergistically to increase the production of IL-6 and expression of its receptor. To demonstrate the direct role of IL-6 in the induction of RAGs, rIL-6-6 was added to cultured B cells. RAG1...
and RAG2 expression was not detected in IL-6-activated cells, and not up-regulated either in CD40L- or anti-IgM- and IL-6-stimulated B cells. These findings establish that IL-6 is unable to directly regulate RAG induction even in CD40L-activated B cells or anti-IgM-stimulated B cells. However, when IL-6 was neutralized by anti-IL-6 mAb, or IL-6 binding to its receptor inhibited by anti-IL-6R mAb, up-regulation of RAGs induced by CD40L plus anti-IgM could be inhibited. This indicates that RAG expression depends on IL-6 signaling in CD40L-activated mature B cells provided that BCR signaling pathways are also stimulated. Interestingly, following removal of inducing signals, IL-6 was also able to maintain RAG expression of stimulated B cells when cultured in medium without activating signals. This suggests that IL-6 participates to the induction of RAG expressions, and also contributes to their sustained up-regulation. However, it remains unclear how does IL-6 up-regulate RAG expression: IL-6 may possibly activate transcription factors involved in RAG gene transcription (37). Alternatively, IL-6 may activate transcription factors that induce a cis-element to counteract a distant silencer that, normally, represses the transcription of RAGs (38). The physiological setting that triggers such RAG gene transcription might be direct cognate interaction between B and T cells via CD40 after BCR engagement. This interaction could permit the Ag-specific B cells to secrete and respond to IL-6 in an autocrine manner. Furthermore, following IL-6 synthesis by follicular dendritic cells, T cells or stromal cells, IL-6 may also operate in a paracrine fashion (32). Secondary V(D)J recombination may thus modify specificity of the BCR and allow the tuning of a more efficient immune response. These events may affect a subpopulation of B cells, because only part of the cells was induced to express CD126. In this context, it is interesting that expression of the RAG products is associated with the expression of the CD5 marker (18). The phenotypic analysis confirmed that CD5-positive B cells are among those induced to express CD126. It is, therefore, likely that RAG expression might be confined to the CD126-positive subpopulation of activated B cells expressing the CD5 marker. The CD5-positive B cells are mainly located in the follicular mantle zone of the GCs in SLOs (39). They may thus recombine their Ig genes before they enter the GCs. CD5 has a negative function in the BCR signaling (40). Therefore, one may hypothesized that B cells with BCR affinity not strong enough to allow migration in the GCs and the ensuing maturation, would receive BCR signals triggering CD5 expression which will elevate the BCR threshold. Together with CD40 ligation, BCR engagement will induces IL-6 secretion. This third signal would then promote V(DJ) rearrangements. The relevance of RAG induction may be to raise the BCR affinity up to the level required for maturation (41).

The promotion of the recombinase machinery for secondary IgV gene rearrangements by IL-6 as a result of RAG expression prompted us to determine whether IL-6 was also involved in the termination of the receptor revision. Following a 5-day costimulation with CD40L and anti-IgM, human B cells transcribed RAG1 and RAG2, and up-regulated CD126 on their surface. The pheno-
type changes indicate that RAG-expressing mature B cells should respond to IL-6 stimulation. We first confirmed that a strong BCR-mediated signal alone restrains RAG expression (21, 26). This was reversed by keeping the cells in culture medium. Surprisingly, when IL-6 binding was blocked with anti-IL-6R, the BCR-induced repression of RAG expression was recovered. These experiments further demonstrate that IL-6 controls BCR-mediated down-regulation of RAGs. IL-6 is, thus, not only necessary for the induction of RAG genes but is also influential in its suppression. The remaining bands for RAG1 observed when IL-6 is absent or blocked (Fig. 8) suggest that IL-6 mainly affect RAG2 mRNA expression. IL-6 is able to induce G1 arrest (42). The possibility therefore exists that this effect may participate in the variation of RAG2 mRNA regulation. Finally, the current study indicates that IL-6 is key in the regulation of RAG expression in human mature B cells. This is intriguing, considering that IL-6 is overexpressed in autoimmune disease (43–45). High levels of IL-6 in patients with autoimmune diseases could impede BCR engagement to switch off RAG expression, and may thus participate in uncontrolled BCR revision (46–48) leading to the production of high-affinity auto-Abs (49, 50).

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Disclosures

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References

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