Activation of the Janus Kinase 3-STAT5a Pathway After CD40 Triggering of Human Monocytes But Not of Resting B Cells

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CD40/CD40 ligand interactions play a key role in the immune responses of B lymphocytes, monocytes, and dendritic cells. The signal transduction events triggered by cross-linking of the CD40 receptor have been widely studied in B cell lines, but little is known about signaling following CD40 stimulation of monocytes and resting tonsillar B cells. Therefore, we studied the CD40 pathway in highly purified human monocytes and resting B cells. After CD40 triggering, a similar activation of the NF-κB (but not of the AP-1) transcription factor complex occurred in both cell preparations. However, the components of the NF-κB complexes were different in monocytes and B cells, because p50 is part of the NF-κB complex induced by CD40 triggering in both monocytes and B cells, whereas p65 was only induced in B cells. In contrast, although the Janus kinase 3 tyrosine kinase was associated with CD40 molecules in both monocytes and resting B cells, Janus kinase 3 phosphorylation induction was observed only in CD40-activated monocytes, with subsequent induction of STAT5a DNA binding activity in the nucleus. These results suggest that the activation signals in human B cells and monocytes differ following CD40 stimulation. This observation is consistent with the detection of normal CD40-induced monocyte activation in patients with CD40 ligand+ hyper IgM syndrome in whom a defect in CD40-induced B cell activation has been reported. 


CD40 is a 50-kDa molecule that is present on the surface of B cells, monocyctic/dendritic cells, some carcinoma cell lines, and human thymus epithelium (1–4). The CD40-CD40 ligand (CD40L) interactions between B cells and activated Th cells play a key role in immune responses and have therefore been widely studied. Cross-linking of CD40 molecules on B cells by their ligand (CD40L/CD154) causes Ig class switching (5–10), cell proliferation, and rescue from apoptosis (11–14). The activation of monocytes by CD40 molecules leads to the production of proinflammatory cytokines (IL-1, IL-6, IL-8, and TNF-α), up-regulation of surface molecules (CD54, CD80, CD86, and HLA class II), tumoricidal activity (15–17), and rescue from apoptosis (18). The CD40 stimulation of dendritic cells plays a major role in the defense against pathogens, because large amounts of IL-12 are produced (19) and specific T cell cytotoxicity is induced (20–22). Studies of CD40- and CD40L-deficient mice (23–27) and human patients with CD40L mutations (X-linked hyper IgM syndrome) (28–32) have demonstrated that CD40-CD40L interactions are essential for cellular immune responses against intracellular pathogens. Another form of hyper IgM syndrome secondary to a defect in the CD40 activation pathway in B cells has also been reported (33–35) (CD40L+ hyper IgM). Patients with this syndrome have a defect in Ig switching but are not susceptible to infection by intracellular pathogens; these patients exhibit normal in vitro CD40-stimulated monocyte and dendritic cell activities (36). This may be due to T cell activation via the CD40L or to differences in the CD40 activation pathways of B lymphocytes and monocytes/dendritic cells, respectively. We investigated the latter hypothesis by comparing the biochemical events induced by the CD40 stimulation of highly purified human resting B cells and elutriated monocytes. Little is known about the biochemical events induced by the CD40 activation of monocytes and resting B cells, because most studies have focused on preactivated B cell lines. Several proteins are involved in the CD40 activation of B cell lines. They include protein kinases such as phosphatidylinositol-3-kinase, phospholipase C-γ2, and lyn, which are phosphorylated on tyrosine residues (37), and the transcription factor complexes NF-κB, AP-1, and NF-AT, which are induced (33, 38, 39). Proteins of the TNF receptor-associated factor (TRAF) family (TRAF2, TRAF3, TRAF5, and TRAF6) and others (TRAF family member associated NF-κB activator (TANK), NF-κB-inducing kinase (NIK), and c inhibitor of apoptosis protein (cIAP)) interact directly or indirectly with the intracytoplasmic tail of CD40 and are involved in NF-κB activation. The tyrosine kinase Janus kinase 3 (Jak3) has been found in B cell lines, in which it interacts with CD40. It is tyrosine-phosphorylated after CD40 triggering and induces STAT3 translocation (46). However, no phosphorylation of Jak3 was detected in CD40-activated murine spleen B cells, whereas STAT6 induction was reported (47). In a more recent study, Jabara et al. showed that CD40 signaling in Jak3-deficient human B cells, including up-regulation of membrane marker expression, proliferation, and Ig switching, is functional (48). This observation demonstrates that Jak3 is not required for CD40 phs cytokine-induced B cell activation.

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We investigated the induction of transcription factors and the activation of the tyrosine kinase protein Jak3 to better delineate CD40-mediated activation pathways in human monocytes and resting B cells. We obtained evidence for the induction of NF-κB (but not AP-1) transcription factors in monocytes and resting B cells after CD40 triggering. Jak3 was similarly associated with CD40 molecules in both cell populations, but tyrosine phosphorylation of Jak3 was detected only in monocytes after CD40 cross-linking. STAT5a was induced in activated monocytes, but no STAT DNA binding activity was detected after the CD40 stimulation of resting B cells. This finding suggests that there are differences in the CD40 activation pathways of resting B lymphocytes and monocytes.

Materials and Methods

Cell preparations

PBMCs that had been obtained from healthy donors by leukapheresis were subjected to Ficoll-Hypaque density centrifugation. The mononuclear cell interface was further separated into a monocyte-enriched fraction by countercurrent centrifugal elutriation using a Beckman J6 M/E centrifuge with a JE-5.6 elutriator rotor (Beckman Instruments, Palo Alto, CA). We used the technique described by Faradji et al. (49), slightly modified in terms of the elutriation medium, with Hank’s medium replaced by a phosphate buffer. The monocyte preparation was >90% pure as assessed by CD14 staining, with <2% B lymphocytes.

Tonsillar B cells were obtained by passing tonsil specimens through mesh and rosetting with 2-aminoethyl-isothiouronium bromide-treated SRBCs to remove T cells. Cells were subjected to Ficoll-Hypaque centrifugation and subsequently centrifuged through a discontinuous Percoll gradient to isolate small resting B cells. The cell population (recovered from the 50–55% interface) was analyzed by immunofluorescence. More than 90% of the cells were CD19+, μ+, δ−. Contaminating monocytes and T lymphocytes accounted for <5% of the cells.

Abs and reagents

For immunofluorescence studies, FITC-labeled anti-CD40 mAb was purchased from Dianclone (Besançon, France), PE-labeled anti-CD14 Ab and PE-labeled anti-CD19 Ab were obtained from Becton Dickinson (Mountain View, CA), and FITC-labeled polyclonal Abs directed against IgM and IgD were purchased from Caltag (San Francisco, CA).

For activation studies, the anti-CD40 mAb BB20 and an irrelevant mouse IgG1 control were obtained from Diaclon and used at a concentration of 10 μg/ml. The soluble CD40L (sCD40L), which was prepared as described previously (50), was used at a final concentration of 10 μg/ml. Ionomycin was purchased from Calbiochem (La Jolla, CA); PMA was obtained from Sigma (St. Louis, MO). IL-4 was obtained from R&D Systems (Minneapolis, MN).

Rabbit polyclonal Abs against Jak3, p65 (Rel-A), c-Rel, STAT1 (p84/p91), STAT2, STAT4, the C-terminal part of STAT5a (sc-1081 X), and STAT6 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), as was the anti-CD40 Ab (sc-974) used for immunoprecipitation. The Ab directed to the p50 component of NF-κB was kindly donated by R. Weil (Institut Pasteur, Paris, France). The antiphosphotyrosine Ab 4G10 and the anti-STAT3 Ab were purchased from Upstate Biotechnology (Lake Placid, NY). The Ab directed against the N-terminal part of STAT5a was kindly provided by Dr. B. Gröner (Institute for Experimental Cancer Research, Tumor Biology Center, Freiburg, Germany) (51).

Electrophoretic mobility shift assay (EMSA)

Monocytes or resting B cells (20 × 10^5) were activated for 30 min in RPMI 1640 in the presence of the anti-CD40 mAb BB20 (20 μg/ml), sCD40L (10 μg/ml), IL-4 (100 U/ml), or a combination of PMA (50 ng/ml) and ionomycin (10 μM). Cells were then washed once in cold PBS, and nuclear extracts were prepared as described elsewhere (52), with minor changes in procedure. Briefly, cells were allowed to swell on ice for 10 min in buffer A (10 mM HEPES [pH 7.9], 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 2 mM PMSF) containing the following protease inhibitors: leupeptin, aprotinin, pepstatin, and antipain, each at a concentration of 4 μg/ml for monocytes and 2 μg/ml for B cells. Samples were then centrifuged, and the pellet was suspended in 25 μl of buffer C (20 mM HEPES [pH 7.8], 420 mM NaCl, 1.5 mM MgCl₂, 0.5 mM DTT, and 2 mM PMSF) containing the protease inhibitors. The mixture was left for 20 min on ice. Cell debris was removed by centrifugation, and the resulting nuclear extracts were stored at −70°C. Protein concentration was measured with the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany).

Nuclear extracts (5–10 μg protein) were assayed for DNA-binding activity in a total volume of 20 μl of binding buffer (20 mM Tris-HCl [pH 8),
Monocytes or small resting B cells were incubated with the anti-CD40 mAb BB20 (10 μg/ml) or the irrelevant IgG1 control (10 μg/ml) for various times (from 1 to 30 min). Cells (20 x 10⁶) were lysed in 200 μl of lysis buffer (1% Nonidet P-40 or 0.5% for coinmunoprecipitation), 0.14 M NaCl, 50 mM NaF, 20 mM Tris (pH 7.4), 2 mM EDTA, 2 mM orthovanadate, 2 mM PMFS, 2% aprotinin, and 2-4 μg/ml testatin, anti-pain, and leupeptin). The cells were incubated for 20 min on ice and centrifuged for 20 min; the postnuclear extract was cleared by incubation with rabbit IgG (2 μg, Sigma) for 1 h at 4°C. Protein A-Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden) was added to precipitate nonspecific immune complexes. The cleared postnuclear extract was incubated overnight at 4°C with specific Abs. Protein A-Sepharose was added to precipitate the immune complexes. The precipitate was washed several times in lysis buffer, suspended in Laemmli sample buffer, and analyzed by SDS-PAGE followed by electrophoretic transfer to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA). Membranes were probed with an anti-rabbit IgG Ab or with the Ab of interest, with detection with enhanced chemiluminescence (Amersham, Buckinghamshire, U.K.). The probe was stripped from polyvinylidene difluoride membranes by incubating for 30 min at 50°C in 62.5 mM Tris-HCl (pH 6.8), 100 mM 2-ME, and 2% SDS. Membranes were washed several times in PBS containing 0.05% Tween 20 and subsequently reblotted.

### Results

**CD40-mediated stimulation of monocytes and resting B cells activated NF-κB but not AP-1**

CD40 triggering has been reported to induce the activation of the transcription factor complexes NF-κB and AP-1 in B cell lines (33, 38, 39). Therefore, we studied the activation of these transcription factors in CD40-stimulated monocytes and resting B cell preparations by EMSAs with specific labeled oligonucleotides. NF-κB DNA-binding activity was induced in monocyte nuclei after 30 min of stimulation with sCD40L or the anti-CD40 mAb (but not by the control IgG1) (Fig. 1A). The activation of AP-1 was not detected in the same nuclear extract (Fig. 1B). A similar pattern was obtained for the nuclei of resting B cells after stimulation with sCD40L or anti-CD40 mAb, with activation of NF-κB but not AP-1 transcription factors (Fig. 1, C and D). Similar results were obtained if B cells and monocytes were stimulated for ≤3 h (data not shown). As expected, PMA plus ionomycin, the positive control, activated both transcription factors in the nuclei of monocytes and resting B cells.

We investigated whether the same components were present in NF-κB complexes induced by CD40 stimulation in nuclei from monocytes and resting B cells by performing supershift assays. In both cell populations, anti-p50 Abs caused a complete supershift, whereas anti-c-Rel Abs had no effect. Anti-p65 Abs caused only a partial supershift in nuclei from B cells, and no supershift in nuclei from monocytes (Fig. 2, A and B). This observation indicates that the NF-κB complexes resulting from CD40 activation in nuclei from monocytes and B cells contain p50; however, p65 is present only in the NF-κB complexes induced in B cells.

**Jak3 is associated with CD40 in monocytes and resting B cells**

Hanissian and Geha showed that CD40 interacts with the tyrosine kinase Jak3 in B cells (46). Therefore, we investigated whether it could be also detected in monocytes and resting B cells by coinmunoprecipitation with an anti-CD40 Ab followed by Western blotting with an anti-Jak3 Ab. A band was detected on the Western blot at ~115 kDa; this size corresponds to that of the Jak3 protein in lysates from monocytes and resting B cells, indicating a Jak3 interaction with CD40 in both cell types (Fig. 3). In monocyte...
preparations, a slightly smaller (~110-kDa) protein was also detected with the anti-Jak3 Ab. This band may correspond to a degraded form of Jak3 because monocyte protein extracts are rich in proteases. It may also correspond to a shorter isoform of Jak3 that is present only in monocytes.

CD40-mediated stimulation of monocytes but not of resting B cells induced tyrosine phosphorylation of Jak3

As Jak3 has been shown to interact with CD40 molecules, we investigated the tyrosine phosphorylation of Jak3 in CD40-stimulated monocytes and resting B cells. Cell preparations were incubated with either anti-CD40 mAb (or a control IgG1) or IL-4 for various periods of time; next, the Jak3 protein was immunoprecipitated, and the extent of its tyrosine phosphorylation was assessed. In monocytes, anti-CD40 mAb activation (but not activation by the IgG1 control) induced Jak3 phosphorylation after 1–30 min of stimulation (Fig. 4A). Only the 115-kDa band was phosphorylated; the second band (110 kDa) was not. A higher molecular mass phosphorylated band (120 kDa, not found in IL-4-stimulated monocytes), precipitated with Jak3, was also detected. This band was not detected on the anti-Jak3 Ab immunoblot (Fig. 4A, lower panel); therefore, it may correspond to a Jak3-associated, tyrosine-phosphorylated protein.

In sharp contrast, the activation of resting B cells by either the sCD40L or anti-CD40 mAb resulted in no detectable phosphorylation of Jak3 (Fig. 4B), although significant amounts of Jak3 were precipitated (Fig. 4B, lower panel) and coprecipitated with CD40 (Fig. 3).

As expected, incubation with IL-4, the positive control, consistently induced Jak3 tyrosine phosphorylation in monocytes and B cells (Fig. 4, A and B).

The difference in CD40-mediated Jak3 activation did not result from the differential expression of CD40 molecules, because a similar level of CD40 receptors was present on monocytes and resting B cells (Fig. 5).

CD40-mediated stimulation of monocytes but not of resting B cells induced STAT5a activation

Jak tyrosine kinases transduce signals via the phosphorylation of STAT transcription factors, leading to STAT dimerization, translocation to the nucleus, and binding to DNA sequences (55). Therefore, we investigated the induction of STAT molecules following the CD40 stimulation of monocytes and resting B cells by EMSA. A 30-min incubation of monocytes with the anti-CD40 mAb BB20 (but not with the control IgG1) activated STAT proteins because the labeled GRR probe was retained on the gel. Moreover, in competitive assays, the addition of a 50-fold excess of unlabeled IRF-1 or β-CAS probes (both containing STAT consensus binding sites) led to the complete disappearance of the signal, confirming induction of the DNA-binding activity of STAT factors (Fig. 6A). STAT1, STAT2, STAT3, STAT4, and STAT6

![FIGURE 4. Tyrosine phosphorylation of Jak3 in response to CD40 ligation in monocytes but not in resting B cells. Monocytes (A) or resting B cells (B) (20 × 10⁶) were stimulated for the times indicated with anti-CD40 mAb (10 μg/ml), control IgG1 (10 μg/ml), or IL-4 (100 U/ml). Lysates were immunoprecipitated with anti-Jak3 Ab or purified rabbit IgG (nonspecific signal (NSP)) and immunoblotted with antiphosphotyrosine Ab (upper panel). The blots were then stripped and reprobed with anti-Jak3 Ab (lower panel). *, Jak3-associated phosphorylated protein. **, 110-kDa Jak3 form. sCD40L (10 μg/ml) activation of resting B cells gave similar results. Three independent experiments were conducted, and the results shown are typical.](http://www.jimmunol.org/)

![FIGURE 5. Expression of CD40 molecules on elutriated monocytes and tonsillar resting B cells. Double immunofluorescence staining was performed with FITC anti-CD40 mAb and PE-labeled anti-CD14 or anti-CD19 mAb.](http://www.jimmunol.org/)
proteins were not detected in supershift assays. In contrast, an Ab directed against the N-terminal part of the STAT5a protein consistently caused a complete supershift of the signal in CD40-activated monocytes (Fig. 7B). However, an Ab directed against the C-terminal part of STAT5a did not cause a significant supershift in CD40-activated monocytes (Fig. 7, A and B), but did induce a dramatic supershift in the IL-2-activated B cell line (Fig. 7C). This observation suggests that an actual truncated form of the STAT5a molecule, deleted of the C-terminus part, is translocated and activated in the nuclei of CD40-activated monocytes.

In contrast, no STAT activation was detected in resting B cells after CD40 stimulation (Fig. 6B), regardless of the length of the stimulation period (30–180 min). However, NF-κB was activated in these nuclear extracts (Fig. 6C), and IL-4 induced STAT binding activity, as expected (Fig. 6B). STAT5a was present in B cells as detected by Western blotting with the Ab directed against the C-terminal part of the STAT5a molecule (data not shown).

Discussion

In this study, we compared the biochemical events induced by CD40 ligation in peripheral blood-elutriated monocytes and tonsillar resting B cells isolated from normal subjects. The means of isolation used gave preparations of highly purified cells, with ≤5% contaminating cells. We obtained evidence that CD40 cross-linking activated the NF-κB transcription factor complex, but not AP-1, in both monocytes and B lymphocytes. To our knowledge, nothing has been published previously about the induction of transcription factors in CD40-stimulated monocytes, whereas conflicting reports have been published for B cells. The source of B lymphocytes used probably accounts for the observed differences. Berberich et al. (39) reported that CD40 activation leads to the induction of NF-κB and AP-1 in B cell lines, but that NF-κB is induced only in tonsillar B cells, consistent with our own observations. However, in another report, no NF-κB binding activity

FIGURE 6. Activation of STAT molecules in response to CD40 ligation in monocytes but not in B cells. Monocytes (20 × 10⁶) were stimulated by incubation for 30 min with anti-CD40 mAb (10 μg/ml). Nuclear extracts were used for EMSAs with labeled GRR probes. A, Competitive assays of STAT binding activity were done using a 50-fold excess of unlabeled STAT consensus binding probe, either IRF-1 or β-CAS. B, B cells (20 × 10⁶) were stimulated for 30 min with anti-CD40 mAb (10 μg/ml), IgG1 (10 μg/ml), or IL-4 (100 U/ml). Nuclear extracts were used for EMSAs with labeled GRR probes. C, Band shift analysis of the same nuclear extracts as for B, with labeled probe specific for NF-κB DNA-binding activity. Two independent experiments were conducted, and the results shown are typical.

FIGURE 7. Activation of a truncated form of STAT5a in response to CD40 ligation in monocytes but not in B cells. A, Supershift assay with specific Abs directed against STAT1, STAT2, STAT3, STAT4, STAT6, and the C-terminal part of STAT5a added to nuclear extracts from CD40-activated monocytes during the binding reaction. The different effects of the Abs directed against the C-terminal and the N-terminal parts of STAT5a are shown in B. As a control, the supershift assay was performed using the Ab against the C-terminal part of STAT5a with nuclear extracts of an IL-2-stimulated B cell line (C).
was detected in CD40-activated B cells from peripheral blood (56). We provide evidence herein that the CD40-induced NF-κB complexes were not identical in CD40-activated monocytes and B cells, because the p65 component was present only in the nuclei of B cells, whereas p50 was present in the NF-κB complexes of nuclei from both B cells and monocytes. Further work is required to fully characterize the NF-κB complexes detected in the nuclei of CD40-activated monocytes and B cells.

We also investigated the involvement of the Jak3 tyrosine kinase in CD40 activation. We used an immunoprecipitation study to demonstrate an association between CD40 molecules and Jak3 in both monocytes and resting B cells. Such an association was also observed in B cell lines and resting B cells by Hanissian and Gaha (46). However, our results clearly show that Jak3 was phosphorylated after CD40 triggering in monocytes but not in resting B cells. As Jak3 activates transcription factors of the STAT family, we performed EMSAs with a consensus probe for STAT proteins. Using a labeled GRR probe, STAT DNA-binding activity was consistently detected in CD40-stimulated monocyte nuclear extracts but not in CD40-activated resting B cell nuclear extracts. Super-shift assays with specific Abs showed that only STAT5α was induced by CD40 activation in monocytes. CD40-triggered Jak3 phosphorylation and STAT translocation, not studied previously in monocytes, have been studied in B cell lines. Hanissian and Gaha described the induction of Jak3 phosphorylation and STAT3 translocation (but no DNA-binding activity analysis of STATs was performed) following CD40 activation in human B cell lines (46). However, Karras et al. showed that CD40 triggering of unseparated murine spleen B cells led to the induction of STAT6, but not STAT3, and that Jak3 tyrosine kinase was not phosphorylated (47). These differences strongly suggest the existence of various CD40-induced biochemical pathways in B cells, according to source and activation status.

In this study, we also found that CD40 activation induced a peculiar form of STAT5α in monocyte nuclei. In the supershift assay, the Ab directed against the C-terminal part of the STAT5α protein did not recognize any STAT5α molecule, whereas the Ab directed against the N-terminal part of the protein did. This strongly suggests that CD40 ligation results in the induction of a truncated form of STAT5α. This truncated form of STAT5α may be a degradation product despite the use of protease inhibitors or it may be a monocyte-specific isoform. Similar monocyte truncated isoforms of STAT5α have been described in primary monocytes (57) and in monocyte lines (58).

The most striking data from our study are the differences in the patterns of CD40 activation in monocytes and resting B cells. CD40 triggering activates Jak3 and induces STAT5α activation in monocytes but not in resting B cells. The underlying mechanism is unknown. The amounts of CD40 present on the membranes of B cells and monocytes are similar. Jak3 appears to be associated with CD40 molecules in a similar way and to a similar extent in both cell populations. In addition, CD40 activation is known to increase the intracellular concentration of Jak3 in B cells (59). Cell activation status may affect the pattern of responses to CD40 stimulation. CD40 activation of B cell lines results in a pattern of Jak3 phosphorylation (46) similar to the one we describe here in monocytes. However, STAT5α is induced in monocytes, rather than STAT3, the translocation of which has been reported in B cell lines (46). The lack of Jak3 phosphorylation in CD40-activated B cells may be balanced by the need for a second signal. This second signal involves cytokines (IL-4 or IL-13), which are known to activate Jak kinases and induce STAT proteins (54, 60, 61). This suggests that regulatory molecules may be involved in CD40 activation in a cell-specific manner. Factors, especially those of the TRAF family, have been reported to be associated with CD40 molecules (40–44). Large amounts of TRAF3 are present in monocytes and B cell lines, whereas this factor is hardly detectable in resting B cells (62, 63). Therefore, TRAF3 could be a good candidate for the positive regulation of CD40 activation in monocytes; CD40 triggering alone is sufficient to induce the activation of Jak3 kinase and STAT protein in TRAF3+ cells (monocytes or activated B cells), but not in TRAF3− resting B cells. Our observation that the NF-κB transcription factor was induced in resting B cells activated by CD40 alone is not inconsistent with this hypothesis, because B cells do not require TRAF3 to induce NF-κB after CD40 stimulation (63). However, data from preliminary experiments indicate that a 24-h preincubation of resting B cells induces TRAF3 expression, but that activation of the Jak3/STAT pathway by CD40 agonists does not occur. Thus, other regulatory molecules, belonging to the TRAF family or interacting with TRAF factors, are probably involved.

The different patterns of CD40 activation of resting B lymphocytes and monocytes observed in this study are consistent with the clinical features of patients suffering from CD40L− hyper IgM syndrome (33–35). These patients suffer from Ig switch defect but are not susceptible to intracellular pathogens. In these patients, the normal CD40 activation of monocyte/dendritic cells contrasting with the complete lack of CD40 response in B lymphocytes (36) may be due to the existence of different CD40 activation pathways in the two cell types.

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