Centrocytes rapidly adopt a memory B cell phenotype on co-culture with autologous germinal centre T cell-enriched preparations

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Abstract

B cells, after mutating their Ig V-region genes in germinal centres (GC), undergo apoptosis, unless they receive antigen-dependent selection signals. The signals appear to be delivered by GC T cells, require CD40 ligand expression and may induce differentiation to memory cells. Cultured GC B cells are prevented from entering apoptosis by ligating their surface CD40, but the resulting phenotype is not that associated with B cells found in vivo. Conversely, GC B cells rapidly adopt a memory B cell phenotype on culture with autologous memory CD4+ T cells that have been induced to express CD40 ligand transiently. This effect is prevented by blocking CD40 ligand. Naive CD4+ T cells, induced to express CD40 ligand, do not prevent GC B cells undergoing apoptosis.

Introduction

Germinal centres (GC) are sites of massive clonal expansion of B cells during T cell-dependent antibody responses (1,2). The B cells in GC activate a hypermutation mechanism that operates selectively on their Ig V-region genes (3-6). Available evidence indicates that cells that have modified their Ig V-region genes are selected by interaction with antigen on follicular dendritic cells (7) followed by cognate interaction with local T cells (8). Cells that are selected leave the GC to become memory B cells (9,10) or plasma cells (11). It is also possible that some selected cells remain in the GC to proliferate further and undergo additional mutation in their V regions (12,13). Non-selected cells die in situ by apoptosis (7).

GC B cells can be isolated from tonsils by removing T cells and B cells that express CD39 or IgD or both (7). Provided they are separated in the cold their viability is good. When they are cultured at 37°C they soon show signs of apoptosis. This can be delayed by cross-linking the GC B cells’ surface receptors for antigen, but longer survival is secured by culture with CD40 ligand (14) or mAb against CD40 (7). This effect of CD40 ligand is reinforced by IL-4 (15). Prevention of apoptosis in this way results in up-regulation of CD44 and surface Ig expression, suggesting that they might be inducing memory formation. Some features of the phenotype acquired differ markedly from those found among tonsil memory cells (16); this conclusion is reinforced in the study reported here. Memory B cells can be isolated from tonsil B cells by removing cells expressing CD38 or IgD or both (6,15,17-19). Cells with a putative memory B cell phenotype have been generated in vitro from GC B cells by culturing these cells with CD40 ligand-expressing fibroblasts and the addition of IL-2 and IL-10 for 7 days (20). In vivo this process occurs in <24 h (21). By 4 days, surviving GC B cells that have been cultured with fibroblasts with bound CD40 mAb have down regulated their surface CD77, but they remain CD38+ and express high levels of CD23 (22). The expression of CD23 is not a feature of memory B cells (18,23). In this report we describe experiments where CD4+ memory (CD45RO+) T cells are co-cultured with autologous GC B cells. It is shown that these T cells prevent the GC B cells entering apoptosis and induce a memory B cell phenotype, effects that are prevented by blocking CD40 ligation. These results are similar to those achieved by Lagresle et al. who co-cultured GC B cells with phytohemagglutinin-activated mitomycin C-blocked allogeneic CD4+ T lymphocytes (16). The present report goes on to show that autologous naive (CD45RA+) T cells, induced to express CD40 ligand transiently, do not prevent GC B cells from entering apoptosis.
Methods

Antibodies

Purified IgG preparations of the following mouse IgG1 mAb were used for negative selection of GC B cells, memory B cells and T cells: CD39, AC2 (a gift from M. Rowe University of Cardiff, UK); CD19, BU12 (23); CD38, OKT10 (ATCC, Bethesda, MD); CD45RA, GC6 (The Binding Site, Birmingham, UK); CD45RO, UCH-L1 (a gift from P. Beverley, University College Hospital, London) (24); CD8, B941 (a gift from C. Mawas, Marseille, France). Sheep IgG anti-human IgD (The Binding Site) was also used for negative selection. The antibodies were coupled either to sheep red blood cells (SRBC) to isolate B cells or to ox red blood cells (RBC) to isolate T cells using the chromic chloride method (25).

The following antibodies were used for FACScan analysis: a mouse isotype control—FITC/phycocerythrin (PE), UCHT1—PE (CD3), Bly1—FITC (CD20), MHM6—FITC (CD23), ACT1—FITC (CD25), MT310—FITC (CD4), DK25—PE (CD8) and rabbit F(ab)2 anti-κ/λ Ig light chains—FITC/PE (Dako, High Wycombe, UK); Leu17—PE (CD38) and leu 45RO—PE (CD45RO) (Becton Dickinson, Oxford, UK), 38.13—FITC, CD77 (a gift from Dr. J. Wels, Institute Gustave-Roussy, Villejuif, France) (26), CD45RA—ITC (Serotec, Oxford, UK), BU12—FITC, CD19 (23) and BUS2—FITC, CD44 (27), M90-biotinylated CD40 ligand (a gift from Richard Armitage, Immunex, Seattle) (28). Streptavidin—PE (Becton Dickinson) was used to reveal the biotinylated M90. Purified G28-5 (CD40) was a gift from J. A. Ledbetter (Oncogen, Seattle, WA) (23).

The following mAb were used for immunofluorescence microscopy: UCHT-1 (CD3), BU12-biotinylated (CD19), B52-biotinylated (CD44), OKT10 (CD38), 38-13 (CD77), anti-Bcl-2 (Dako) and MIB1, analogous to Ki67 (a gift from Johannes Gerdes, Borstal, Germany) (29). UCHT-1 mAb was revealed with a sheep anti-mouse y2b Texas-Red labelled (The Binding Site); the biotinylated mAb with streptavidin—FITC (The Binding Site); CD77 with goat anti-rat IgM—FITC (Nordic), and CD38, anti-Bcl-2 and MIB1 mAb with sheep anti mouse y1—FITC (The Binding Site). Engagement of CD40 ligand with CD40 was blocked with the CD40 ligand mAb M91 (a gift from R. Armitage, Immunex, Seattle, WA) (28).

Tonsils and the preparation of tonsil cell subsets

The separation of B cell subsets is described in detail in Methods. The levels of Ig secreted into these relatively short-term cultures were carefully resuspended in medium at 20°C, layered onto Ficoll-Isopaque and centrifuged at 450 g for 20 min. The interface (enriched for B cells) was then subjected to another cycle of T cell depletion using AET-treated SRBC, the resulting interface cells were washed in medium and layered onto a discontinuous Percoll gradient with bands of 60 and 65% Percoll (Pharmacia). Cells that enter the 65% Percoll gradient are highly enriched for follicular mantle B cells (18). Between 1 and 3 x 10⁸ cells not penetrating the 65% gradient (medium and light cells) were suspended in 7 ml medium containing 1.5 x 10⁹ CD39-coated SRBC and 10⁹ SRBC coated with sheep anti-human IgD. The suspension was then centrifuged for 3 min at 130 g and left undisturbed for 20 min at 4°C. Following careful resuspension in medium at 20°C, the cells were layered onto Ficoll-Isopaque and centrifuged as before. The interface, enriched for GC B cells, yields cells that are >95% CD19⁺, CD38low and CD44low. The memory B cell enriched fraction (memory B cells) was separated from the total B cell suspension in a similar way, but CD38-coated SRBC with anti-IgD-coated SRBC were used instead of CD39-coated SRBC. This separation yields CD38low, CD44high cells that have the features of memory B cells (6, 15, 17, 18). All the separation procedures were performed at 4°C except the density gradient centrifugations, which were carried out at 20°C.

To separate tonsil T cell subsets an aliquot of the tonsil mononuclear cell suspension, described above, was incubated with ox RBC coated with CD19 mAb for 20 min. This was then separated on a Ficoll-Isopaque gradient and the non-rosetting cells were harvested from the interface. These cells were then either incubated with ox RBC cells coated with CD45RA mAb and ox RBC cells coated with CD8 mAb to isolate memory CD4⁺ T cells, or with the CD45RO and CD8 mAb-coated ox RBC to isolate naive CD4⁺ T cells. Again the non-rosetting cells were separated by density centrifugation.

Cell culture and Ig production

All cell cultures were performed in 200 μl in 96-flat-bottom-well plates in medium supplemented with 10% FCS serum at a final cell density of 0.5 x 10⁶ cells/ml in a humidified incubator with 5% CO₂ at 37°C. The following additives were used in different experiments: (i) CD40 mAb at 1 μg/ml, (ii) phorbol dibutyrate (PDB) 1 nM (Sigma) with ionomycin (0.7 μg/ml, Calbiochem Novabiochem, Beeston, UK) and (iii) 15 μg/ml M91, a CD40 ligand mAb. PDB was used in preference to phorbol myristate acetate as the former can be removed from cells by washing.

In all experiments supernatants from cultured cells with the different additives were collected after 48 h culture and were screened for Ig production by haemaggulatination using SRBC coated with sheep anti-human IgG, A and M antibodies (The Binding Site) by the chromium chloride method (25). A standard human Ig preparation containing 1 μg/l IgG, 0.2 μg/l IgM and 0.1 μg/l IgA was used as a positive control. As total antibody production was minimal in the conditions used in the studies, Ig class and subclass levels were not assessed. The levels of Ig secreted into these relatively short-term cultures were at most only slightly higher than background levels; consequently these are not reported in detail.
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**Phenotypic analysis**

The expression of cell surface molecules was assessed by direct and indirect immunofluorescence staining using a FACScan (Becton Dickinson) and the Lysys FACScan software (Becton Dickinson). Cells were washed twice in PBS supplemented with 5% normal goat serum (Gibco) plus 0.1% sodium azide. The working dilution of the first antibody (or two antibodies in the case of double direct immunofluorescence staining) was added to 2 x 10^5 cells for 30 min on ice. The cells were then washed twice and the cells labelled with direct conjugates were fixed with 1% formaldehyde (Sigma). The cells stained with biotinylated M90 or BU52 were incubated with streptavidin–PE and the second FITC-labelled antibody for a further 30 min before the cells were washed and fixed. Cells stained with UCHT-1 were incubated with a Texas Red-labelled sheep anti-mouse IgG2b. The number of non-apoptotic cells was determined by counting the number of events in the viable lymphocyte-blast gate detected on FACScan in a fixed time. This gate established by forward and 90° light scatter plots includes viable lymphocytes and lymphoblasts but excludes cells undergoing apoptosis (31).

**Immunofluorescence microscopy**

Cells were stained as for FACScan analysis, but were not fixed, then transferred onto adhesion slides (BioRad, Munich, Germany), and left undisturbed for 15 min at room temperature. Slides were washed to remove unattached cells and mounted in glycerol containing DABCO to retard fading. When Bcl-2 or Ki67 staining was required for cells stained and mounted in this way, the slides were air dried and then fixed for 5 min with acetone at room temperature to permeabilize the cells. The cells were allowed to dry and were then incubated for 45 min with anti-Bcl-2 (Dako) or MIB1 mAb. Slides were washed in PBS followed by incubation with a FITC-labelled sheep anti-mouse IgG1 for 30 min. The nuclei were counterstained with Hoechst 33258. After washing, the slides were mounted in glycerol containing DABCO.

**Results and discussion**

GC B cells cultured with CD40 mAb or recombinant CD40 ligand are prevented from undergoing apoptosis but acquire a phenotype not associated with cells differentiating from GC B cells in vivo

Isolated GC B cells have been shown to undergo apoptosis spontaneously on culture at 37°C; this can be inhibited in many of the cells if mAb against CD40 or recombinant CD40 ligand is added to the cultures (7,14). This effect of cross-linking the surface CD40 of the GC B cells results in the generation of cells that have a phenotype not associated with any tonsil B cell subset in vivo—the cells co-express high levels of CD77, CD23, CD38, surface Ig and CD44 (Fig. 1). CD77 is a molecule associated with GC B cells (26,32) and particularly centroblasts (6,33). CD44 is expressed at high levels by both memory and virgin extra-follicular B cells. It is present at low levels on centrocytes—the non-proliferating B cells of the light zone of GC, while it is not detectable on most centroblasts—the proliferating B cells in the GC dark zone (17,18,23). CD23 is found on follicular mantle B cells.
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CD45RO\(^+\), CD8\(^-\) T cells

0 h

4 h PDB + Ionomycin

Preactivated T cells

+ autologous GC B cells

3 h

24 h

CD45RA\(^+\), CD8\(^-\) T cells

0 h

4 h PDB + Ionomycin

Preactivated T cells

+ autologous GC B cells

Fig. 2. The transient nature of CD40 ligand expression by naive and memory CD4\(^+\) T cells after 4 h of treatment with PDB and ionomycin when cultured with autologous GC B cells. CD40 ligand expression by activated naive (CD45RO\(^+\), CD8\(^-\) T cells) or memory (CD45RA\(^+\), CD8\(^-\) T cells) was assessed (top row) before (left) or at the end of activation (right) or (middle and bottom rows) after 3 and 24 h culture alone (left) or with autologous GC B cells (right). The surface expression of CD40 ligand was detected by one-color flow cytometry using biotinylated M90 (a CD40 ligand mAb). T cells were activated by culture for 4 h with PDB and ionomycin and were washed three times before culture with GC B cells.

and is up-regulated when these cells are activated (34–36) but is not a molecule characteristically associated with memory B cells or GC B cells (18,23). CD38 is expressed by tonsil GC B cells and plasma cells but not by most B cells outside GC (23). GC B cells express relatively low levels of slg compared with that on follicular mantle or memory B cells. These phenotypic studies indicate that it is unlikely that sustained signalling through the CD40 on the surface of GC B cells by itself is responsible for inducing their generation to memory B cells in vivo. In a search for possible signals that are responsible for this selection and differentiation stage further studies were carried out assessing the effects of coculturing GC B cells with different autologous tonsil T cell preparations.

Memory but not naive helper T cells with induced CD40 ligand expression prevent autologous GC B cells from undergoing apoptosis

Previous studies have shown that T cells with a memory or naive helper phenotype can be isolated from tonsil by negative selection and that the cells from both subsets can be induced to express CD40 ligand on their surface on culture with phorbol esters plus ionomycin (28,37,38). About half the memory cells, but no naive cells, contain preformed CD40 ligand that can be expressed on their surface within 5 min of cross-linking the TCR complex (28). The memory cells are prepared by removing cells expressing one or more of the following surface molecules: CD45RA, CD8 or CD19. Naive cells are isolated by removing cells with CD45RO, CD8 or CD19. None of the cells from either of the preparations express surface CD40 ligand constitutively. After culture with PDB and ionomycin for 4 h most cells from both preparations express CD40 ligand on their surface (Fig. 2). If the cells are then washed to remove these activating agents, the surface CD40 ligand is substantially down-regulated within 3 h and largely lost after 24 h culture. The addition of equal numbers of autologous GC B cells to either preparation results in the loss of all detectable CD40 ligand from the T cells within 3 h (Fig. 2). This rapid down regulation of CD40 ligand after cross-linking has been analysed in detail by Yellin et al. (39).

The survival of GC B cells was analysed when cultured with freshly-isolated memory or naive autologous helper T cells, or T cells in which CD40 ligand expression had been induced (Fig. 3b and c). As a positive and negative control, GC B cells were cultured with CD40 mAb or in medium only (Fig. 3a). As expected most of the GC B cells cultured in medium only are dead by 24 h while ~50% the cells cultured with CD40 mAb are still alive after 48 h. The GC B cells cultured with activated memory helper T cells show similar survival kinetics to those cultured with CD40 mAb. Those cultured with naive activated T cells show only marginal delay in entering apoptosis compared with the GC B cells cultured in medium alone. Consistently a small proportion of the GC B cells cultured with untreated memory helper T cells survive—
perhaps due to cognate interaction involving centrocytes that had taken up antigen from follicular dendritic cells in vivo and are presenting the processed peptides on their surface (40). The survival of GC B cells is not affected by co-culture with untreated naive T cells. To test if the rescue of GC B cells from entering apoptosis is dependent on signalling through CD40 ligand further cultures were set up in which the mAb M91 was added to cultures of activated memory helper T cells and GC B cells. This mAb has previously been shown to block binding of soluble recombinant CD40 to CD40 ligand (28). These experiments show that M91 inhibits the ability of the T cells to prevent apoptosis of the GC B cells (Fig. 3d). It seems that the rescue of GC B cells from apoptosis by memory T cells is dependent upon the expression of CD40 ligand, but that other signals have to be delivered by these cells to secure centrocyte survival. These other signals are not furnished by naive T cells that are expressing CD40 ligand. This finding indicates that transient expression of CD40 ligand as opposed to the sustained effect of recombinant CD40 ligand or CD40 mAb added to cultures is unable to prevent apoptosis of GC B cells. If selection of GC B cells, dependent on cognate interaction of T cells with B cells, is to be effective there should be no non-specific selection of bystander cells. For this to be achieved (i) molecules delivering rescue signals must be exposed on the T cell surface at the site of specific cell contact and (ii) the expression of these molecules must not linger on the surface after the selected cell has passed out of the GC. Hanna as long ago as 1964 (41) recognized that centroblasts are continually giving rise to centrocytes and that the transit time of centrocytes through the light zone is in the order of 7 h. This is confirmed by the findings that memory B cells in the marginal zone of the spleen are not in cell cycle (42) but can be derived from proliferating progenitors—presumably centroblasts—within 12 h (21). It follows that cognate interactions between centrocytes and T cells in the GC are transient. The rapid down-regulation of CD40 ligand expression observed in the present experiments and analysed in detail by Yellin et al. (39) fits with the concept of transient CD40 ligand expression at the site of cognate interaction in vivo. It is probable that this can only be achieved if the T cell has pre-formed CD40 ligand (28). It follows that experiments where GC B cells are stimulated chronically through their CD40 are not reflecting fully the events occurring within GC.

Most GC B cells cultured with activated autologous memory helper T cells rapidly change to a phenotype associated with memory B cells

The phenotype of GC B cells co-cultured with activated autologous memory T cells was assessed both by FACSScan analysis and visual immunofluorescence together with phase contrast microscopy. The co-cultured cells form into elongated aggregations of 10 cells or more across. After gentle pipetting and staining with CD20 plus CD2, or CD19 plus CD3, persistent small T cell–B cell conjugates are still seen (Fig. 4a). Using confocal microscopy, it is seen that co-expression of T and B cell antigens is due to the formation of T cell–B cell conjugates and not to the transfer of surface molecules. The conjugates form preferentially between CD77h (centrocytes) rather than CD77wil (centroblasts) GC B cells and T cells (Fig. 4b). Critical experiments involved assessing the relative levels of expression of CD77 and CD44 (Fig. 5). These show, as expected, that most cells rescued from apoptosis with CD40 mAb acquire the non-physiological CD44, CD77, CD23wil phenotype (Fig. 1 and 5b). Most cells cultured with autologous activated memory T cells by contrast become CD44wil, CD77wil (Fig. 5a) and remain CD23wil (Fig. 6). These rescued cells, like memory B cells, are CD23wil and have higher levels of surface Ig than GC B cells. Fluorescence microscopy showed that GC B cells cultured with activated memory T cells started to express Bcl-2 after the first 24 h culture with autologous memory T cells when most of these cells had stopped expressing Ki67 (Table 1). The latter finding is in accord with the non-proliferating state of the B cells with memory characteristic in tonsils (18). There do not appear to be any published data describing the rate of acquisition of Bcl-2 expression by GC B cells as they differentiate to memory cells. The only feature of the rescued cells that is not associated with memory B cells (6,18) is their continued expression of CD38. This seems to be explained by the results of a further series of experiments in which memory B cells were co-cultured with autologous activated memory CD4wil T cells. In these cultures CD38 expression was induced in the memory B cells (Fig. 6).

Persistence of a minority of cells with a centroblast phenotype in cultures of GC B cells and activated memory T cells

It will be seen from Fig. 5a, middle panel), that after 24 h culture with memory CD4wil T cells, ~1.5 of the original GC B
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A Memory T cells  GC B cells

\[ \uparrow \]

GC B cells with memory T cells

\[ 3 \text{ h} \quad 24 \text{ h} \]

CD2  CD20

B Memory T cells  GC B cells

\[ \uparrow \]

GC B cells with memory T cells

\[ 3 \text{ h} \quad 24 \text{ h} \]

CD2  CD77

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**Fig. 4.** CD77\text{low/intermediate} GC B cells preferentially form conjugates with autologous memory T cells. Conjugate formation between GC B cells and activated autologous memory CD4\text{+} T cells was assessed by flow cytometry. In (A) the formation of CD2\text{+}, CD20\text{+} T cell-B cell conjugates is shown; (B) shows the preferential formation of conjugates with CD77\text{low/intermediate} GC B cells, by 24 h all the cells in the conjugates are CD77\text{+}. The possibility that the non-conjugated CD77\text{high} cells seen at 24 h are derived from cells differentiating in conjugates is discussed in the text.

Cell preparation is still CD77\text{high}, CD44\text{low}, the centroblast phenotype. After an initial fall in the number of cells with a centroblast phenotype their numbers remain more or less the same between 6 and 24 h culture. Cells with this phenotype have almost all disappeared during this period in cultures of GC B cells in medium only (18). Two possible explanations for the relative persistence of centroblasts in the presence of activated memory T cells have been considered. The first is that T cells may be preventing centroblasts from dying. This is not favoured by the finding reported above that CD77\text{high} cells do not readily enter GC B cell-T cell conjugates. This leads to the second possible explanation—a proportion of the centrocytes that form conjugates with memory T cells

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**Fig. 5.** Co-culture of GC B cells with activated memory T cells induces B cells that are CD77\text{low}, CD44\text{high}, while soluble CD40 mAb induces CD77\text{high}, CD44\text{high} B cells. The percentage of cells with the phenotypes indicated was assessed by double immunofluorescence staining of the cells for CD44 and CD77 except for the B cells that are CD44\text{high}, CD77\text{low}; these cannot be distinguished by this staining from T cells which are also CD44\text{high}, CD77\text{low}. The number of CD44\text{high}, CD77\text{low} B cells was calculated by subtracting the number of surviving T cells (CD2\text{+} cells) from the number of CD44\text{high}, CD77\text{low} cells. Three of the phenotypes characterize physiological B cell phenotypes: CC centrocytes, CB centroblasts and MB memory B cells. CD77\text{high}, CD44\text{high} cells are generated from GC B cells on culture with CD40 mAb but this is not a phenotype associated with any tonsil B cell subset.
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Fig. 6. Comparison of the phenotype of GC B cells with that of memory B cells after both have been cultured with autologous pre-activated memory T cells. The phenotypes of both B cell subsets are compared after they have been co-cultured for 24 h with autologous memory CD4$^+$ T cells that had been pre-activated for 4 h with PDB and ionomycin.

Table 1. Percent GC B cells and activated memory T cells that express Bcl-2 and the proliferation-associated antigen Ki67 before and after co-culture

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<th>Co-culture (h)</th>
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Percent of T cells were identified as UCHT-1$^+$ cells and GC B cells as UCHT-1$^-$; apoptotic cells were distinguished from non-apoptotic cells by the presence of condensed and fragmented chromatin in the former using the Hoechst DNA-binding dye. Histologically GC T cells are typically Bcl-2$^-$; these data, therefore indicate that ~30% of the CD45RA$^+$, CD8$^+$ T cell preparation are GC T cells.

differentiate to a centroblast phenotype and these cells then dissociate from the conjugates and die over the next few hours by apoptosis. If centrocytes differentiate physiologically to the centroblast phenotype it would be expected that they would return to the dark zone. Some CD77$^{high}$ cells are found in the T cell-rich outer zone of tonsil GC and these might represent such cells in transit (43). Presumably the dark zone has a micro-environment that provides signals that prevent centroblasts undergoing apoptosis. Data in favour of the reversion of some centrocytes to centroblasts have been suggested by mathematical consideration of GC reactions (12). Experimental evidence is provided from experiments where CD40 ligand blockade in vivo was found to cause the rapid dissolution of GC (13). If centroblasts were a self-renewing population as opposed to one that continually produces centrocytes, blocking CD40 ligand might not be expected to affect these cells, unless they are maintained by homotypic signalling through CD40 and CD40 ligand; an explanation that is suggested by the finding by Lipsky's group that CD40 ligand expression can be induced on B cells (44). Further experiments are required to test these possibilities further.

Abbreviations

- AET: 2-aminoethylisothiouronium bromide
- GC: germinal centre
- PDB: phorbol dibutyrate
- PE: phycoerythrin

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