Co-ligation of CD44 on naive human tonsillar B cells induces progression towards a germinal center phenotype

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Abstract

The precise signaling pathways to induce a germinal center (GC) phenotype and somatic mutations in human B cells are presently not understood. Major phenotypical hallmarks of a human GC B cell are up-regulated expression of CD10 and CD95 together with a heterogeneous expression of CD77. Activation of resting human tonsillar B cells using anti-CD40 and anti-IgM antibodies normally only induces up-regulation of CD38 and CD71 but has no effect on the typical GC markers. However, we show here that an additional co-ligation of the glycoprotein CD44 on such tonsillar B cells up-regulated the typical human GC markers CD10, CD38, CD77 and CD95, and down-regulated CD24 and CD39 as well as induced progression towards apoptosis in these cells; all characteristics of GC B cells. These data indicate a functional role of CD44 during activation of human naive B lymphocytes and in the generation of GC B cells.

Introduction

During a T cell-dependent antigen response, B cells are activated extrafollicularly by T cells in association with inter-digitating dendritic cells. Activated B cells subsequently enter the primary B cell follicles and give rise to germinal centers (GC). In the dark zone of a GC the B cells, termed centroblasts, undergo rapid proliferation during which accumulation of V-region-localized somatic mutations becomes evident. The progeny of a centroblast is the centrocyte, populating the GC light zone, where selection of B cells takes place with respect to their affinity for the antigen (1–3). During the differentiation of naive B cells into GC B cells certain phenotypical changes occur due to cell–cell interactions, as well as to soluble mediators including cytokines. Different membrane-bound receptor–ligand pairs have been demonstrated to be crucial for the formation of functional GC and memory B cells, where in particular CD40–CD40 ligand (CD40L) (4) and CD80–CD28 (5) interactions play a major role. This unique T cell-dependent B cell differentiation results in a GC B cell phenotype in humans characterized by an up-regulation of CD10, CD38, CD77 and CD95, but a down-regulation of CD23, CD24, CD39, CD44 and surface Ig (sIg) in comparison to naive, resting B cells (6).

The involvement of cell–matrix interactions in GC and memory B cell formation is still elusive, but might constitute a way to regulate and direct cellular responses. The involvement of different adhesion molecules and homing receptors, i.e. integrins, selectins and members of the Ig super family, is, however, well established during cellular responses (for review, see 7). The CD44 molecule is a highly polymorphic glycoprotein, shown to have the ability to act as an organ-specific homing receptor for lymphocytes in cell–matrix inter-actions (8–11). It has also been described as a cell–cell adhesion molecule leading to homeotypic or heterotypic cell aggregation (12). CD44 is widely expressed on various cell types in man, such as T and B cells, monocytes, granulocytes, fibroblasts, epithelial cells, and most erythrocytes (for review, see 8,13). Hyaluronate has been described as the major ligand for CD44 (11), although other ligands have also been characterized, including fibronectin (14) and collagen (15). A further element of complexity in the ligand specificity of CD44 is its glycosylation pattern, which markedly influences the ligand-binding ability intrinsic to CD44 (16). The functional roles of CD44 in human B cells are still largely unknown, although the molecule has been implicated from several

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studies to be associated with B cell differentiation and activation (17–21). Furthermore, interaction via CD40–CD40L was shown to rapidly induce expression of the hematopoetic form of CD44 (CD44H) on B cells, subsequently exhibiting a co-stimulatory activity for clonal expansion of T cells (22).

The present study focuses on CD44 as a potential mediator of co-stimulatory signals during T cell-dependent B cell activation, in an effort to elucidate the signaling pathways causing antigen-primed B cells to enter primary follicles, acquire GC phenotype and to start hypermutation. The first step in trying to mimic such events *in vitro* is to generate a B cell phenotype similar to what is found in GC. We demonstrate that after stimulating resting tonsillar B cells with signals through CD40 and the B cell receptor, the additional ligation of the CD44 molecule induces a phenotype that in several aspects is similar to that of a GC B cells.

Methods

Reagents and antibodies

R-Phycoerythrin (RPE)-conjugated anti-CD23 and anti-CD38 antibodies were obtained from Becton Dickinson (San Jose, CA). FITC-conjugated anti-CD20 and RPE-conjugated anti-CD24 antibodies were purchased from Immunotech (Marseilles, France). RPE-conjugated anti-CD10, anti-CD20 and FITC-conjugated anti-CD71 together with streptavidin–RPE were all obtained from Dako (Glostrup, Denmark). The anti-CD95–RPE antibody and anti-CD138–RPE antibody were purchased from PharMingen (San Diego, CA) and Serotec (Oxford, UK) respectively. Mouse anti-human CD39–biotin (AC2), mouse anti-human IgM (AF6) and mouse anti-human CD44 (BU52) antibodies were kindly provided by I. C. M. MacLennan (University of Birmingham, UK) and the rat anti-human CD77 (38.13) antibody was a generous gift from J. Wiels (Institute Gustave Roussy, Villejuif, France). Goat anti-rat Ig was purchased from Zymed (San Francisco, CA). Mouse anti-human CD40 (S2C6) antibody was a generous gift from S. Pauli (Stockholm University, Sweden).

Cells

Human tonsils were obtained from patients undergoing routine tonsillectomy at the Lund University Hospital (Lund, Sweden) or Malmo Academic Hospital (Malmo, Sweden). Briefly, tonsils were minced and mononuclear cells isolated by Ficoll-Isopaque (Pharmacia) density centrifugation. T cells were removed by rosetting with neuraminidase-treated sheep red blood cells and the T cell-depleted fraction was separated, using 60% Percoll, into high–density resting cells and low-density GC B cells as previously described (23–25). The high density fraction, containing mainly resting B cells, was washed in PBS/BSA (1%) and stained with anti-CD38–RPE and anti-IgD–FITC antibodies. Positive selection, using flow cytometric cell sorting of IgD$^+$/CD38$^-$ B cells, was performed on a FACStar Plus cell sorter (Becton Dickinson, San Jose, CA) achieving >99.8% purity.

Cell culture condition

IgD$^+$/CD38$^-$ B cells (3×10^5) were cultured on 2.5×10^4 CD32-transfected fibroblasts together with 0.5 µg/ml anti-CD40 and 0.1 µg/ml anti-IgM antibodies in flat-bottomed 96-well plates (Costar, Cambridge, MA) for FACS analysis. The cells were cultured for 4 days in complete medium, i.e. RPMI 1640 supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 10% FCS (Gibco Grand Island, NY) and 50 µg/ml gentamycin (Biological Industries, Haemek, Israel). For analysis of DNA synthesis and annexin studies, 10^5 cells were cultured under the same conditions as described above for 2–7 days.

To analyze the proliferative response during the culture period cultured B cells were analyzed for [^{3}H]thymidine incorporation. After 2, 3, 5 or 7 days of culture cells were pulsed with 1 µCi [^{3}H]thymidine and harvested after 16 h. Radioactivity incorporated by the cells was then counted in a Wallac 1450 MicroBeta liquid scintillation counter.

Flow cytometry

All flow cytometry analysis were performed on a FACScan flow cytometer (Becton Dickinson). Live lymphocytes were gated, to separate them from dead cells and fibroblasts, using forward and side scatter, and 7000 events were collected using the Lysys II program (Becton Dickinson). All antibodies used were checked against positive and negative cells, and isotype controls relevant for each antibody were used for background staining. A mixture of resting and GC B cells was stained immediately after isolation with anti-CD20–FITC and RPE-conjugated antibodies for the different cell surface markers. CD20 expression on resting and GC B cells was measured and resting B cells were defined as CD20^{low} and GC B cells were defined as CD20^{high}. After 4 days of culture, cells to be analyzed were removed from the cultures and stained with anti-CD20–FITC antibody and the same panel of RPE-conjugated antibodies as used for the day 0 stainings. The only exception was CD71 where a FITC-conjugated antibody was used in combination with CD20–RPE.

Detection of apoptotic cells was done by staining the cells with Annexin V–FITC and propidium iodide (PI) to determine the proportion of apoptotic (Annexin V$^+$/PI$^-$) versus necrotic cells (Annexin V$^+$/PI$^+$) (26). Cells from these cultures were also analyzed for co-expression of Annexin V–FITC and CD10–RPE.

Results

Phenotype of human resting and GC B cells

In order to study phenotypical changes of naive B cells during *in vitro* stimulation and compare these to *in vivo* B cell subsets, we selected surface markers that have been reported to be characteristic for GC B cells (27,28). Resting and GC B cells isolated from human tonsils were therefore analyzed for the expression of CD10, CD20, CD23, CD24, CD38, CD39, CD71, CD77 and CD95 to define their phenotype. CD20 expression was used to distinguish between two B cell populations, where CD20^{low} defined the resting B cell population and CD20^{high} the GC cells, as previously described (24). As can be seen on CD20^{high} cells, the GC phenotype is characterized by high expression of CD38, intermediate expression of CD10, CD71, CD77 and CD95, and no expression of CD23, CD24

Table 1. MFI[a] values from tonsillar and *in vitro* cultured B cells

Molecule	GC B cells	Resting B cells	Stimulated B cells[b]	
			− Anti-CD44	+ Anti-CD44
CD10	280	10	15–90	110–270
CD23	5	90	30–60	90–180
CD24	5	75	5	5
CD38	1500	120	200–450	250–650
CD39	5	85	90	15
CD71	170	10	130	185
CD77	310–610[c]	5	20–90	150–880[c]
CD95	275	10	65–90	65–190

[a]Mean fluorescent intensity (channel number) of the specific staining.
[b]IgD$^+$/CD38$^-$ tonsillar B cells (3×10^5) were cultured on CD32-transfected fibroblasts together with anti-CD40 and anti-IgM with or without anti-CD44 for 3 days. The data represents nine experiments and the range in MFI is due to donor variation.
[c]Heterogeneous expression.

and CD39. The resting B cell population expressed intermediate levels of CD23, CD24, CD38 as well as CD39, and was negative for CD10, CD71, CD77 and CD95 (Table 1), confirming the previously published phenotype of these populations (27,28).

CD44 ligation on resting B cells induces progression towards a GC B cell phenotype

Resting B cells from human tonsils were cultured together with CD32-transfected fibroblasts and co-stimulated with anti-CD40 and anti-IgM antibodies in the presence or absence of an anti-CD44 antibody. After 4 days, the B cell cultures were analyzed for surface marker expression with flow cytometry. The presence of anti-CD40 was absolutely necessary for survival of resting B cells, as observed in cultures containing only anti-IgM and anti-CD44 antibodies. There was no up-regulation of any of the activation markers and no proliferation was observed in these cultures (data not shown). The B cells activated by CD40 and Ig stimulation, with or without CD44 ligation, were phenotypically compared to resting and GC B cells after a 4 day culture (Table 1). The B cell phenotype induced during the culture period clearly illustrated that stimulation with anti-CD44 up-regulated both CD10 and CD95, to similar levels as found on GC B cells (Fig. 1). Co-ligation through the CD44 molecule also induced a heterogeneous CD77 expression as well as CD38 up-regulation, both hallmarks of the GC B cell phenotype. Furthermore, the naive tonsillar B cells down-regulated CD24 and CD39 expression during the 4 days of culture, which again is characteristic of a GC phenotype, although the down-regulation of CD24 was not dependent on the CD44 ligation. The stimulated B cells also exhibited an increase in CD71 expression, which was slightly higher on CD44 activated B cells (Fig. 1).

CD23 was also slightly up-regulated by CD44 ligation, which is in contrast to human GC B cells, since these have been described as negative for CD23 expression (Table 1).

CD44 ligation together with sIg and CD40 cross-linking induces proliferation and progression towards apoptosis of naive B cells

Naive B cells were cultured *in vitro* together with anti-CD40 and anti-CD44 antibodies in the presence or absence of anti-IgM antibodies in order to study if the changes towards a GC B cell phenotype also induced the expected functional changes. Proliferation of the *in vitro* cultured cells was measured after 2, 3, 5 and 7 days by thymidine incorporation. Cross-linking of sIg resulted in a substantially increased proliferation, which peaked after 3 days (Fig. 2).

Naive B cells were cultured for 4 days with anti-CD40 and anti-IgM antibodies with or without anti-CD44 antibodies. The cells were then stained with Annexin V–FITC and propidium iodide or CD10–RPE. Figure 3 shows that in the presence of anti-CD44 antibodies increased binding of Annexin V was observed in the CD10$^+$ population. The cells were also stained with PI to look for necrotic cells, but <1% of the cells incorporated PI (data not shown).

Discussion

The process of GC formation is dependent on the CD40–CD40L interaction (4) and mice lacking this molecular interplay fail to form functional secondary B cell follicles. In *in vivo* blocking experiments, the role of CD40–CD40L interaction has been shown crucial both in the formation of GC and in further differentiation to a memory B cell pool (4). It has also been shown that patients suffering from X-linked hyper-IgM syndrome exhibit a genetic defect in the CD40L gene resulting in an accumulation of B cells capable of producing only IgM (29). It is a well established fact that different adhesion molecules participate in and facilitate cell–cell interactions as well as modulate signal transduction (7). During B cell activation and differentiation the expression of the CD44 molecule is variable and it has been postulated in earlier studies that these changes reflect different requirements of the B cell for this receptor (20,21). CD44 is highly expressed on resting B cells but is down-regulated on GC B cells followed by re-expression during the following post-GC B cell differentiation (20,24). These changes in expression of the CD44 molecule during B cell activation and differentiation might possibly reflect its involvement both in homing and signaling processes during humoral immune responses. Knowing that CD44 participates in cellular adhesion processes, signal transduction events, as well as being present in a variety of isoforms on many cell types has focused our interest on the functional aspects of this molecule, particularly in relation to GC. To simulate some of the earliest signaling events during a T cell-dependent B cell activation in secondary lymphoid organs, resting B cells from human tonsils were cultured together with CD32-transfected fibroblasts and co-stimulated with different antibodies. Anti-CD40 antibody was used as a T cell signal, since stimulation via this molecule can partially substitute T cells in *in vitro* culture systems (28,30) and the CD40–CD40L interaction has been shown to be crucial for the formation of GC. Earlier reports have shown that a partial GC phenotype can be obtained by signaling through the CD40 molecule and the B cell receptor. However, ligation of these molecules
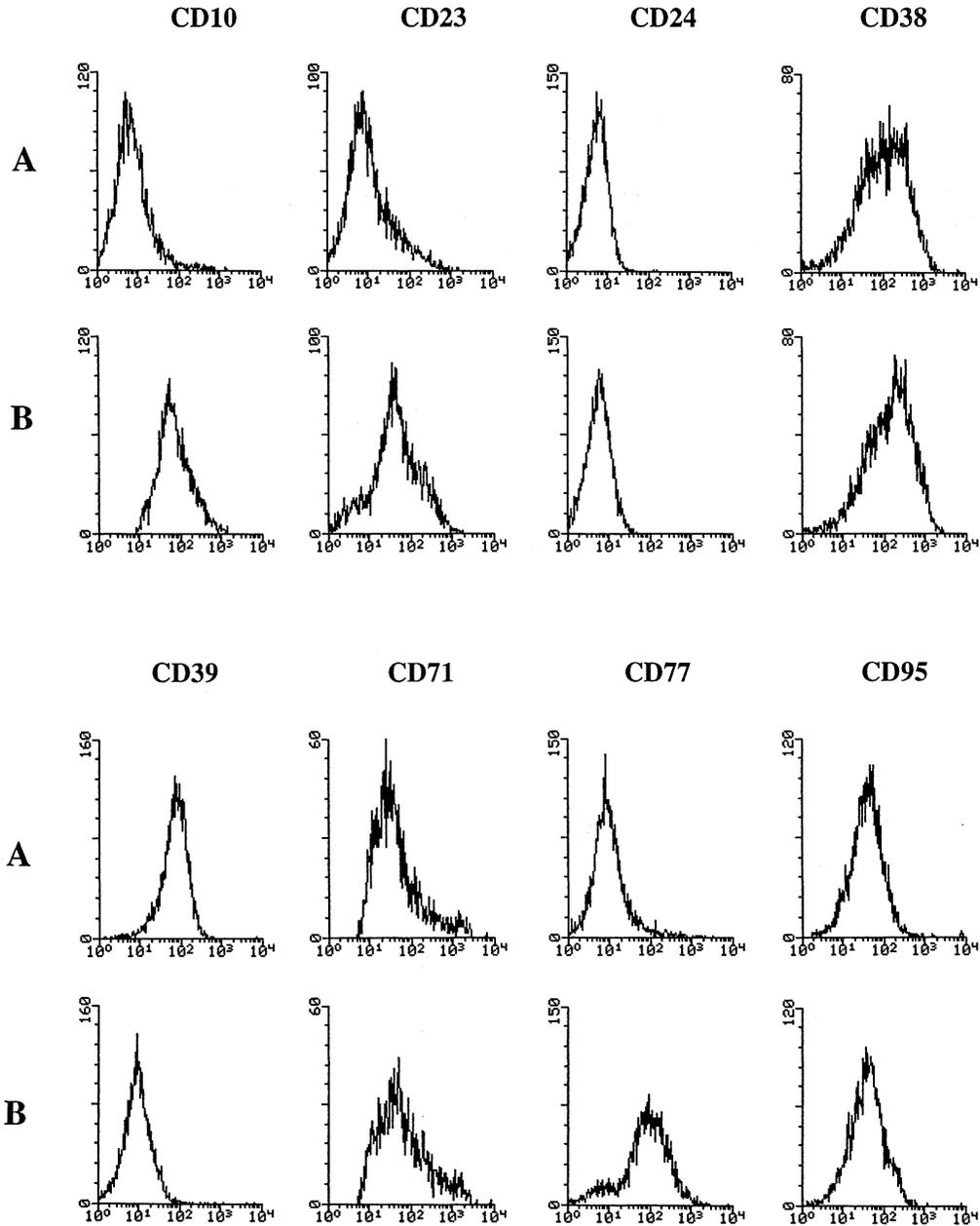
Fig. 1. A GC phenotype is induced by stimulating resting B cells with anti-CD40, anti-CD44 and anti-IgM. IgD$^+$/CD38$^-$ tonsillar B cells (3×10^5) were cultured on CD32-transfected fibroblasts together with (A) anti-CD40 and anti-IgM or (B) anti-CD40, anti-CD44 and anti-IgM. After 4 days of culture, the cells were stained with anti-CD20–FITC and CD20^{high} cells gated for further analyzed with anti-CD10, CD23, CD24, CD38, CD39, CD71, CD77 and CD95–RPE respectively. Data represents nine experiments and is presented as histograms for each marker.

failed to up-regulate CD10 and only slightly up-regulated CD77, both being characteristic GC markers, demonstrating that additional signal(s) was required to induce a complete GC phenotype (27,28).

In the present investigation we show that stimulation via CD44, in addition to CD40 and sIg ligation, up-regulated both CD10 and CD95, with CD10 to similar levels as found on GC B cells (Fig. 1). Co-ligation of the CD44 molecule also induced a heterogeneous CD77 expression as well as CD38 up-regulation, all being hallmarks of the GC B cell phenotype.

Compared to CD38 expression on GC B cells, the recorded CD38 expression in our cultures is lower. This might be explained by the fact that staining for CD38 is a step in the process of positive selection of naive B cells and CD38 might be down-regulated or blocked by the antibodies used in the isolation process.

The stimulated tonsillar B cells also exhibited an increase in CD71 expression, which was slightly higher on CD44-activated B cells (Table 1). Dynamical changes in the CD44 expression could not be assessed, since anti-CD44 was
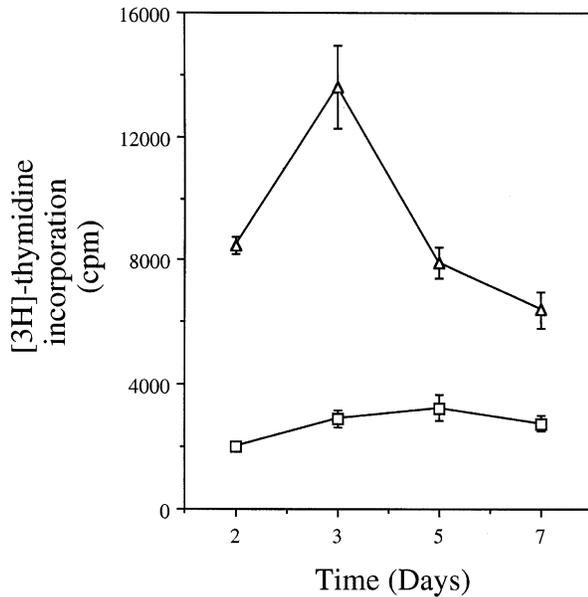
Fig. 2. Kinetics of cell proliferation during *in vitro* cultures of B cells with anti-CD40, anti-CD44 and anti-IgM antibodies. B cells (10^5) cells were cultured on CD32-transfected fibroblasts together with anti-CD40, anti-CD44 and anti-IgM antibodies (triangles) or anti-CD40 and anti-CD44 (squares). Data represents one of three experiments and the bars indicate mean value $\pm$ SD of triplicate cultures.
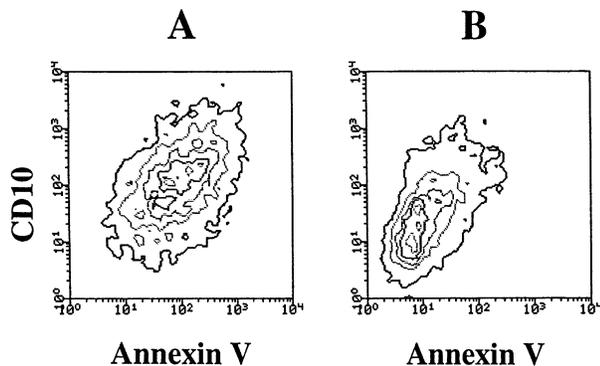
Fig. 3. *In vitro* generated GC B cells are destined for apoptosis. B cells (10^5) were cultured on CD32-transfected fibroblasts together with (A) anti-CD40, anti-CD44 and anti-IgM antibodies or (B) anti-CD40 and anti-IgM antibodies for 6 day. Cells were stained with Annexin–FITC and CD10–RPE, and apoptotic cells were detected by FACS analysis. Data represents one of three experiments and is presented as contour plots.

included as stimulator in our cultures and thus prevented further analysis. However, in cultures stimulated without anti-CD44 antibodies no decrease in CD44 expression could be detected (data not shown). For the same reason, we could not analyze sIg expression.

The only surface marker included in our analysis that did not follow the differentiation pattern towards a GC B cell phenotype was CD23 (Fig. 1). CD23 is down-regulated on GC B cells, but we see an increased heterogeneous expression of CD23 in our cultures (Table 1). The fact that we have constant CD40 stimulation in our cultures might, however, explain why

we do not see a down-regulation of CD23, since it has previously been reported that CD40 stimulation induced CD23 expression on human B cells (30,31). Cells from cultures with both CD40 and CD44 stimulation, but lacking anti-IgM antibodies, expressed CD10, CD77 and CD95, although there was no decrease in CD39 and CD24 expression (data not shown). Furthermore, CD38 was also expressed to a lower degree, when the surface Ig stimulation was lacking, indicating that all three signals had to be present to induce a phenotype similar to what is seen on GC B cells. These cells did, however, not express CD138, which is indicative of plasma cells (data not shown) (32).

We also analyzed the B cell proliferation in cultures providing signals via CD40, CD44 and sIg. The absence of anti-IgM antibodies resulted in >5 times decrease of proliferation, which indicates that signaling via the antigen receptor was necessary (Fig. 2). This also explained the low cell numbers that were obtained previously during phenotyping of cells lacking antigen stimulation. This indicates that proliferation of B cells in our culture system depends on a signal delivered via the antigen receptor.

It has been demonstrated that Burkitt lymphoma cell lines and GC B cells, cultured *in vitro*, loose membrane phospho-lipid asymmetry and that phosphatidylserine is exposed on their surface as they progress towards apoptosis. Annexin V binds to phosphatidylserine and has therefore been used to detect apoptotic cells (26). CD44 ligation, in combination with sIg and CD40 stimulation clearly induced the propensity of cells to bind annexin V after 3 days of culture, whereas the cells stimulated with only sIg and anti-CD40 did not (Fig. 1). As Fig. 3 shows, it is the CD10^{+} population that binds Annexin V, indicating that cells with the GC phenotype are becoming apoptotic. The binding of Annexin V and CD77 up-regulation in our cultures is, furthermore, in congruence with data showing that CD77 is expressed on GC B cells entering apoptosis (31,33). The induced GC phenotype seems to be dependent on CD44 ligation and this signal could be provided by the natural ligand for CD44 expressed on endothelial cells, stroma cells or T cells upon entry into secondary lymphoid organs (34).

Since the CD44 molecule is expressed in many different isoforms, different V-exon usage has a potential impact on the different signaling capacity of the CD44 molecule (35). The BU52 anti-CD44 antibody used in this study has in previous T cell studies also been shown to act in strong synergy with anti-CD2 antibodies.

In summary, our results indicate that co-ligation of the CD44 molecule allows naive human tonsillar B cells to differentiate and acquire a GC-like phenotype, as was illustrated by the up-regulation of CD10, CD38, CD71, CD77 and CD95 as well as down-regulation of CD24 and CD39. The *in vitro* generated GC B cells were also destined for apoptosis, which is another important feature of GC B cells *in vivo*. Based on these data, we suggest the CD44 triggering might to be of importance in the initiation of GC reactions *in vivo*.

Abbreviations

CD40L   CD40 ligand
GC   germinal center
PI   propidium iodide
RPE   R-phycoerythrin

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