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Human germinal center B cells differ from naive and memory B cells by their aggregated MHC class II-rich compartments lacking HLA-DO

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Abstract

To generate memory B cells bearing high-affinity antibodies, naive B cells first encounter antigen in the T cell-rich areas of secondary lymphoid organs. There, they are activated by antigen-specific T cells and become germinal center (GC) founder B cells. GC founders enter the GC to become centroblasts that proliferate and mutate their BCR. Centroblasts differentiate into centrocytes that undergo selection, which requires both the recognition/capture of antigen on follicular dendritic cells and the presentation of processed antigen to GC T cells. Because at each stage of differentiation B cells act as antigen-presenting cells, we analyzed their content of HLA-DR$^+$-rich compartments (MIIC), as well as their expression of HLA-DM, which catalyzes peptide loading of class II molecules, and HLA-DO, which interacts with HLA-DM and focuses MHC class II peptide loading on antigens internalized by the BCR. Naive and memory B cells concentrate HLA-DR, -DM and -DO into compartments dispersed under the cell surface, which are identified by their expression of lysosome-associated membrane protein (Lamp)-1 as late endosomes/lysosomes. GC founders and GC B cells express larger Lamp-1$^+$DR$^+$ compartments that are concentrated in the juxta-nuclear region. These compartments express lower levels of HLA-DM and virtually no HLA-DO. Upon induction of a GC founder phenotype through the prolonged (days) co-ligation of BCR and CD40, the naive B cell's peripheral DR$^+$DM$^+$Lamp-1$^+$ compartments aggregate in a polar fashion close to the nucleus. Furthermore, HLA-DO expression virtually disappears, whereas low levels of HLA-DM remain co-localized with HLA-DR. Anti-κ/λ antibodies, used as surrogate antigens, are promptly (minutes) endocytosed in naive, memory and GC B cells. Then, naive and memory B cells target the surrogate antigen to their peripheral HLA-DO$^+$ MIIC, while GC B cells target it to their HLA-DO$^-$ MIIC aggregates. Taken together, our results show that human GC B cells differ from naive and memory B cells by their aggregated MIIC that lack HLA-DO.

Introduction

The production of high-affinity antibodies is dependent on memory B cells, whose generation requires a complex set of events that mostly occur within germinal centers (GC) (1–3). Upon capture of the antigen through the BCR and interaction with T_h cells, IgD$^+$CD38^{lo} naive B cells (4) give rise to CD38$^+$IgD$^+$ GC founder B cells (5). Within GC, these cells differentiate into centroblasts that proliferate and introduce somatic mutations within their variable region genes.

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Centroblasts then differentiate into centrocytes that are selected based on their ability to (i) recognize and capture through their BCR antigen presented as immune complexes on follicular dendritic cells (FDC) (6), and (ii) process and present the antigen-derived peptides through MHC class II complexes to memory GC T cells that, in return, drive the B cells to switch isotype (7–9) and differentiate into either memory or plasma cells (8). When a B cell encounters its specific antigen, the BCR is endocytosed and transported into the endosomal/lysosomal compartments. There the antigen is degraded into peptides that form complexes with MHC class II molecules. These MHC class II compartments have been extensively studied in B cell lines, either of human origin (4,10–13) or mouse origin (14–18). Studies with B cell lines also suggested that MHC II-rich compartments (MIIC) are altered following BCR engagement (15,16,19–21). These studies were extended to murine splenocytes expressing transgenic anti-egg lysozyme BCR (22). However, no information is available concerning the expression of these compartments in distinct B cell subsets.

MHC class II peptide loading is controlled by the invariant chain (Ii/CD74), and the non-classical MHC molecules HLA-DM and -DO (23–25). Newly synthesized MHC class II molecules associate in the endoplasmic reticulum with Ii/CD74, which facilitates MHC class II transport from the endoplasmic reticulum into MIIC. In this location, Ii/CD74 is cleaved by proteases that leave the class II-associated invariant chain peptide (CLIP) in the peptide-binding groove (26). Release of CLIP, necessary for peptide binding to MHC class II molecules, requires the action of HLA-DM (27–31). HLA-DM molecules are expressed in all antigen-presenting cells including B cells, dendritic cells and epithelial cells (32). In mutant cell lines or mice lacking functional H2-M, CLIP remains associated to class II, which results in a qualitatively altered $CD4^+$ T cell repertoire (33). In B cells, a considerable number of HLA-DM molecules are not present as free heterodimers, but are associated with HLA-DO in heterotetrameric complexes (19,30). HLA-DO is a non-polymorphic heterodimer closely related to classical MHC class II molecules. It is commonly accepted that HLA-DO molecules are restricted to B cells and to some cortical thymic epithelial cells (34), and play a role in regulating the activity of HLA-DM by focusing antigen presentation to antigens internalized through the BCR (33). HLA-DO was described as a negative regulator of HLA-DM activity (35,36) because its over-expression in transfected cell lines increases the amount of CLIP-containing MHC class II molecules at the cell surface. HLA-DO was also shown to restrict DM activity essentially at mildly acidic pH and to influence the repertoire of MHC class II-associated peptides (37,38).

The expression of MHC class II molecules is relevant to the different functional consequences of antigen presentation by the different types of B cells, as naive B cells induce tolerance (39), memory B cells induce immunity and GC B cells probe themselves for survival. To this end, we have analyzed the expression of the different components of the MHC class II antigen presentation machinery, HLA-DM, -DO, CD74 and CLIP, in naive B cells, GC founder B cells, and GC B cells (centroblasts and centrocytes) and memory B cells from tonsils and blood. Naive and memory B cells concentrate HLA-DR, -DM and -DO into compartments dispersed under the cell surface. In contrast, GC B cells aggregate in their juxta-nuclear region their MIIC that express virtually no HLA-DO. Accordingly, co-ligation of BCR and CD40 in naive B cells promptly down-regulates HLA-DO expression and induces re-organization of their MIIC. Finally, antigens in the form of anti-λ/κ are rapidly internalized in the various B cell subsets, and reach both the peripheral and deep MIIC.

Methods

Antibodies

Cell separation and staining was performed using the following antibodies: purified mouse anti-CD2, -CD3, -CD4, -CD16, -CD14, -CD56, -CerCLIP (clone CerCLIP), -DM (clone Map.DM1), -lysosome-associated membrane protein (Lamp)-1 and -mouse IgG1 (all purchased from PharMingen, San Diego, CA); mouse anti-HLA-DR (clone L243) and biotinylated anti-HLA-DR (Becton Dickinson, San Jose, CA); pure and FITC-conjugated mouse anti-CD38, -CD27, FITC–goat anti-IgA, FITC–goat anti-IgG, mouse IgG2a and mouse IgG2b (Caltag, Burlingame, CA); anti-CD39 and -HLA-DR (clone L243) (Immunotech, Marseilles, France); FITC- and phycoerythrin (PE)-conjugated goat anti-IgD (Southern Biotechnology Associates, Birmingham, AL); mouse anti-CD74 (Biosource, Camarillo, CA); mouse anti-DO (clone DOB.L1) (gift from Dr G. Moldenhauer, DKFZ, Germany); FITC-conjugated rabbit anti-human-λ/κ (Dako, Carpinteria, CA); secondary antibodies Texas Red- and Cy5-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA); and streptavidin–Cy5 (Caltag).

Fluorescent-labeled cells were analyzed on a flow cytometer (FACSCalibur; Becton Dickinson) or mounted in fluoromount mounting medium (Southern Biotechnology Associates) for confocal microscopy. All micrographs were recorded using a confocal microscopy system equipped with three Ar488, Kr568 and HeNe633 lasers (TCS-SP; Leica, Mannheim, Germany) as well as spectrophotometers.

B cell purification

Unseparated B cells were purified from mononuclear cells, isolated after Ficoll-Hypaque gradient centrifugation (Cellgro), using B cell-enrichment kits (Miltenyi, Auburn, CA) and magnetic columns (MACS). This procedure routinely provided >95% pure B cells. For naive B cells, tonsil mononuclear cells were enriched by the standard Ficoll-Hypaque gradient method and subjected to E-rosetting with sheep red blood cells to eliminate T cells. Non-rosetting cells were labeled with anti-CD2, -CD3, -CD4, -CD16, -CD14 and -CD56 to remove T cells and monocytes, anti-CD38 mAb to remove GC B cells, and anti-CD27mAb to remove memory B cells. Cells were subsequently incubated with goat anti-mouse IgG magnetic microbeads (Miltenyi) and labeled cells were eliminated using a magnetic column, leading to 80–95% pure naive B cells after two runs. For immunofluorescence of GC B cells, cells were purified and negatively selected as mentioned above, but using mouse anti-CD39 and FITC-conjugated goat anti-IgD to

label naive B cells. Cells were then incubated with goat anti-mouse IgG and anti-FITC–magnetic microbeads to eliminate labeled cells with the MACS system, leading to 85–95% pure GC B cells after two runs. For Western blotting, cells were sorted by FACS (FACS Vantage; Becton Dickinson) after labeling with FITC-conjugated anti-CD38 and PE-conjugated anti-IgD, leading to >95% purity. For immunohistochemistry on isolated tonsil plasma cells, plasma cells were sorted by FACS (FACS Vantage) after labeling of enriched B cells with anti-CD38.

Flow cytometry

To detect cell-surface markers, enriched B cells were stained with non-conjugated mAb, anti-HLA-DR, -DO, -DM, -CD74 and -CerCLIP, revealed by a Cy5-conjugated donkey anti-mouse IgG. Cells were then incubated with mouse serum 10%, and stained with PE-conjugated goat anti-IgD and FITC-conjugated mouse anti-CD38. For intracellular detection, cells were first labeled with anti-IgD–PE (Southern Biotechnology Associates) and anti-CD38–FITC, then incubated with goat Fab fragments anti-mouse IgG and permeabilized using a permeabilization kit (PharMingen) prior to indirect staining with anti-HLA-DR, -DO and -DM revealed by Cy5-conjugated donkey anti-mouse IgG. Controls were performed using non-conjugated isotype controls.

Immunohistochemistry

Tonsil sections were fixed for 15 min in acetone at 4°C and washed twice with PBS. After quenching with PBS/BSA 0.5% and fish gelatin 0.1%, staining was performed by incubating first one of the following indirect mouse mAb, anti-HLA-DR -DM, -DO, anti-CD74 or anti-CerCLIP, revealed by Cy5–donkey anti-mouse antibodies in PBS/BSA 0.5%/saponin 0.1%. The directly coupled FITC–anti-IgD antibody was then added following a quenching step with 10% mouse serum in PBS/BSA 0.5%. Preparations were mounted with fluoromount and micrographs were captured using the objective ×20, HC PL APO.

For cell suspensions, cells were allowed to adhere for 1 h on slides previously coated with 0.1 mg/ml poly-L-lysine (Sigma, St Louis, MO) for 1 h at room temperature and fixed with 4% paraformaldehyde. After quenching with PBS/glycine (50 mM), cells were permeabilized with Triton X-100 (0.1%) for 10 min. They were subsequently washed with PBS/saponin (0.2%) and quenched with PBS/BSA/fish gelatin before staining with one indirect antibody alone. These antibodies (anti-HLA-DR, -DO and -DM) were revealed by a Texas Red–anti-mouse IgG. Cells were then incubated with mouse serum 10% and stained using FITC-labeled antibody (anti-IgD, -IgA, -IgG and -λ/κ). For triple staining, streptavidin–Cy5 (Caltag) was used to detect biotinylated anti-HLA-DR. Preparations were mounted with fluoromount and micrographs were recorded using the objectives ×63 or ×100 PL APO with zoom 2.

Quantitative Western blot analysis

Cells (10^6) were lysed in 50 μl lysis buffer (20 mM Tris, pH 7.5, 5 mM MgCl2 and 1% Triton X-100) containing protease inhibitors (PMSF, pepstatin, chymostatin and leupeptin) for 1 h at 4°C. After removal of nuclei and cell debris, samples were supplemented with Laemmli sample buffer, boiled and loaded on a 12% SDS gel. After transfer onto an Immobilon PVDF membrane (Millipore, Bedford, MA), the membrane was blocked with Blocking reagent (Roche, Nutley, NJ) and stained with the respective primary antibodies (1B5 recognizing DRα, DM.K8 recognizing DMβ and DOB.L1 recognizing DOβ) followed by incubation with horseradish peroxidase-conjugated, isotype-specific secondary antibodies (Southern Biotechnology Associates). Staining was visualized with Super-Signal Dura West (Pierce, Rockford, IL). Quantitative analysis was performed on a LumiImager F1 (Roche) using LumiAnalyst software (Roche).

B cell cultures

Naive B cells were cultured in RPMI 1640 (Gibco, Grand Island, NY) with 10% FCS (Sigma) and antibiotics at 10^6/ml in 24-well plates, in the presence of 10^5/ml irradiated L cells, either transfected with the human Fc receptor CD32 only or double-transfected with CD40 ligand (CD40L) and CD32. Mouse anti-human λ/κ antibodies were added at 50 ng/ml to cross-link the BCR. Cells were harvested at day 6 for intracellular examination of HLA-DR, -DM and -DO expression by confocal immunofluorescence microscopy.

BCR internalization

Naive and memory B cells from blood and tonsil GC B cells were purified by negative selection. After staining at 4°C with anti-κ and anti-λ antibodies, these were cross-linked by a Texas Red-conjugated secondary antibody. Cells were warmed up at 37°C by addition of warm medium, and after 30 min cells were washed with cold PBS and placed to adhere on a poly-L-lysine-coated slide at 4°C. Staining with FITC–anti-HLA-DR was performed after permeabilization with PBS/Triton X-100, and a quenching step with PBS/BSA/saponin and mouse serum 10%.

Results

Distinct MIIC in distinct human B cell subsets

As different human B cell subsets display remarkably different functional properties, we questioned whether they would differ in their antigen-presenting machinery. As described earlier (4,40), purified tonsil B cells can be separated into four different B cell subsets using FITC-labeled anti-CD38 and PE-labeled anti-IgD antibodies (Fig. 1A). Naive (IgD^+CD38^-) and GC (IgD^-CD38^+) B cells were sorted and deposited on slides for confocal fluorescence microscopy. As shown in Fig. 1(B, a and b), most intracellular HLA-DR molecules of naive B cells reside within peripheral compartments dispersed beneath the cell surface. These compartments co-express the lysosome-associated membrane protein, Lamp-1 (Fig. 1B-a). HLA-DM (Fig. 1C, a, b, g and h) and -DO (Fig. 1C, d and e) molecules are also found within intracellular compartments, and dual staining indicates that these molecules are co-localized within the HLA-DR-rich compartments (Fig. 1C, a, d and g).

Purified GC B cells display a strikingly different organization of their MIIC, as none of these cells have the peripheral MIIC of naive B cells. Rather, GC B cells display aggregates of many small $HLA\text{-}DR^+$ compartments located next to the nucleus,

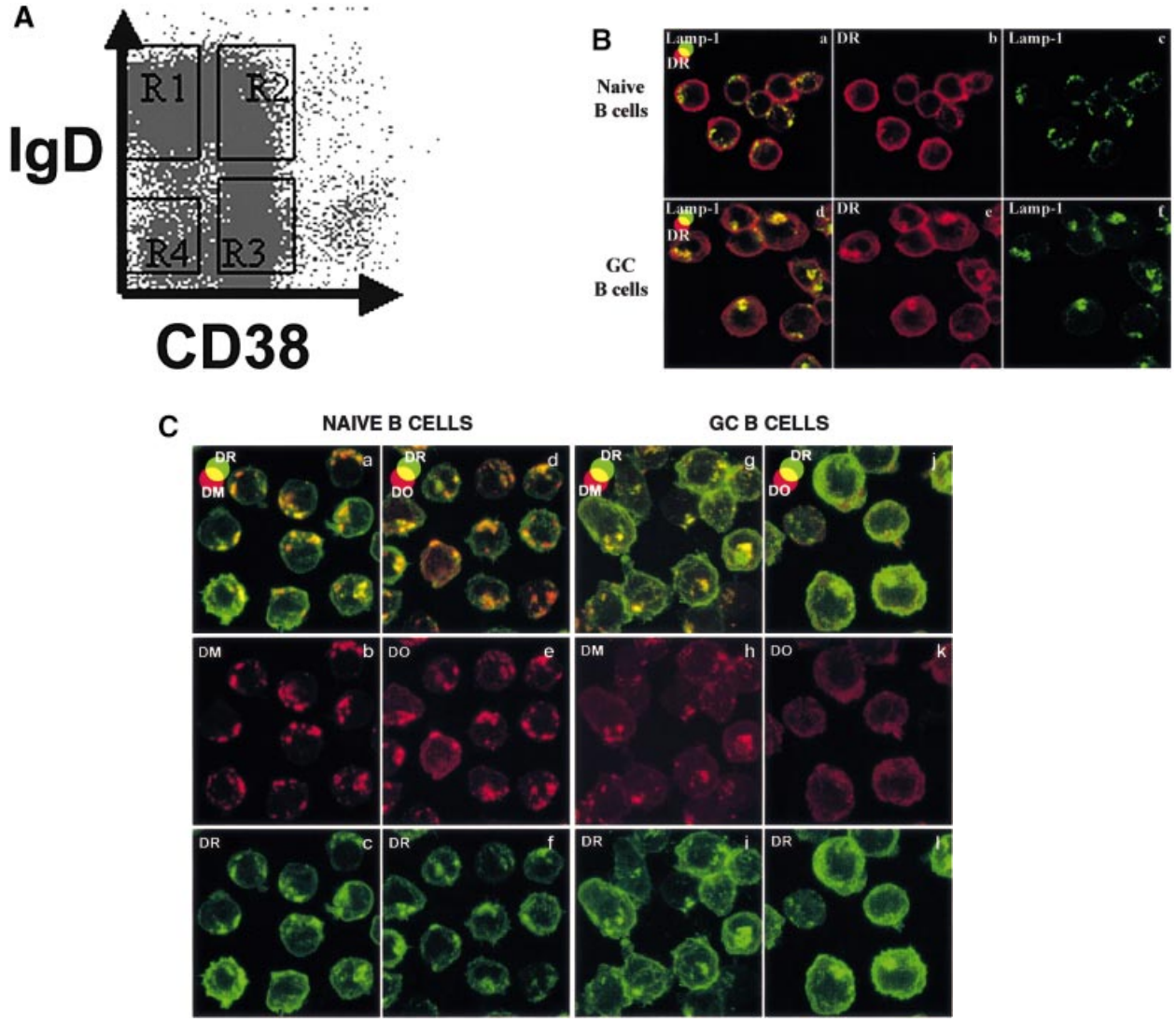


Fig. 1. Distinct MHC class II-rich compartments in naive and GC B cells. (A) Flow cytometry analysis of tonsillar B cell subsets. B cells were purified by negative selection using magnetic beads, and labeled with PE-labeled anti-IgD and FITC-labeled anti-CD38: IgD^+CD38^- naive B cells (R1); IgD^+CD38^+ pre-GC B cells (R2); IgD^-CD38^+ GC B cells (R3); IgD^-CD38^- memory B cells (R4). (B) Dispersed peripheral MIIC in naive B cells and deep aggregated MIIC in GC B cells. Sorted naive (upper panel) and GC (lower panel) B cells were deposited on slides, and analyzed by confocal microscopy using Texas Red-labeled anti-HLA-DR and FITC-labeled anti-Lamp. Left panel (a and d): merge; middle panel (b and e): anti-HLA-DR; right panel (c and f): anti-Lamp-1. Confocal microscopy, objective ×63, zoom 2. (C) HLA-DM/DO are co-localized with HLA-DR in naive B cells and down-regulated in GC B cells. B cell subsets were sorted by FACS to yield naive B cells (a–f) and GC B cells (g–l) for confocal immunofluorescence analysis of HLA-DR, -DM and -DO expression. The micrographs recorded with identical settings reveal that intracellular HLA-DR is co-localized with HLA-DM and -DO in naive B cells (yellow and orange dots in a–d). In GC B cells, HLA-DO expression is markedly down-regulated (j–k), whereas HLA-DM is only slightly reduced (g–h). Confocal microscopy, objective ×100, zoom 2.

likely within the peri-centriolar region of the cells (Fig. 1B, e, and C, i and l). These compartments co-express Lamp-1 (Fig. 1B, d), show reduced levels of HLA-DM (Fig. 1C, g and h) and virtually no HLA-DO (Fig. 1C, j and k). These compartments are likely equivalent to the MIIC identified earlier in B cell lines (4,10,14,41).

Thus all naive B cells express a collection of small peripheral MIIC, while GC B cells express larger MIIC aggregates in the juxta-nuclear region of the cells.

HLA-DM and -DO are down-regulated in GC B cells

The alterations of HLA-DM and -DO expression observed in GC B cells were further analyzed by immunohistochemical staining of tonsil sections. As opposed to anti-IgD staining that easily distinguishes follicular mantles from GC (Fig. 2A, a, c and e), anti-HLA-DR distinguishes T cell-rich areas from B cell-rich areas, but does not discriminate, within follicles, GC from mantle zones (Fig. 2A, b). In contrast, anti-HLA-DM reveals an

intense labeling of naive B cell-rich follicular mantles, contrasting with the GC areas that stain faintly (Fig. 2A, d). A monoclonal anti-HLA-DO antibody also revealed intense staining of follicular mantles, while GC were negative (Fig. 2A, f). Thus, consistent with previous information (42), naive B cells express DM/DO, but the present data indicate that GC B cells express DM/DO at much lower levels.

In situ staining with anti-HLA-DM does not permit us to appreciate: (i) the stage at which B cells loose DM/DO expression, i.e. GC founders or centroblasts, or (ii) the expression of DM/DO in memory B cells. To this end, the expression pattern of DM/DO was further analyzed by flow cytometry on suspensions of purified B cells stained with FITC-labeled anti-CD38 and PE-labeled anti-IgD. A third color, Cy5, can be used to study expression of MHC class II molecules. This analysis first showed that none of the subsets expresses DM/DO on their surface, unlike previous reports on human B cell lines (38). As shown in Fig. 2(B), IgD^+CD38^{lo} naive B cells express intracellular DM/DO. However, $CD38^{hi}IgD^-$ GC B cells express only very low levels of DM/DO, a result consistent with the *in situ* data reported above. GC founder B cells, which express both IgD and high levels of CD38, also display low levels of DM/DO, indicating a prompt down-regulation of these molecules following cognate activation of naive B cells. Quantitative Western blots analysis (25) of HLA-DR, -DM and -DO on purified B cell subsets (Fig. 2C) further shows that the HLA-DM content is slightly reduced in GC B cells compared to naive B cells, while HLA-DO is drastically reduced in GC B cells. The DO/DM ratio (calculated based on an absolute amount of purified HLA-DR used as standard) in naive B cells is 2.13 (mean of two independent experiments), while it is 0.30 in GC B cells.

Taken together, these data demonstrate that upon entry into a GC reaction, B cells promptly down-regulate their expression of HLA-DO.

Dual ligation of CD40 and BCR in naive B cells down-regulates HLA-DO and -DM

At least two signals are required for the formation of GC: antigen encounter and CD40 ligation (1,2). To assess their eventual role in the down-regulation of HLA-DM/DO within GC B cells, naive B cells were cultured over L cells expressing CD40L and CD32 (Ig Fc receptor) in the presence of anti-BCR antibodies, as surrogates for T cells and antigen respectively (43). Under these conditions, naive B cells differentiate into large blasts (Fig. 3d and i) and proliferate [(33) and not shown]. These B cell blasts display large aggregates of intracellular $HLA\text{-}DR^+$ compartments comparable to those of GC B cells. These compartments contain intermediate levels of HLA-DM (Fig. 3g and h). The *in vitro* generated GC-like B cells barely express HLA-DO, while they retain high levels of HLA-DR (Fig. 3d, f, g and i). Conversely, naive B cells cultured for 6 days on irradiated untransfected L cells preserve their naive phenotype, i.e. they remain small, express sIgD and maintain their HLA-DO molecules co-localized with HLA-DR in intracellular compartments (Fig. 3a and c). Single triggering of naive B cells cultured for 6 days with L cells expressing CD32 with anti-BCR led to significant cell death in the absence of CD40 ligation (not shown); yet the remaining viable cells still express HLA-DO. In contrast, CD40L signaling alone results in partial down-regulation of HLA-DO (data not shown).

These results indicate that *in vitro* signals that trigger a partial GC phenotype on naive B cells induce a reorganization of their MIIC and a down-regulation of HLA-DO, both typical of GC B cells.

Memory B cells, but not plasma cells, re-express HLA-DO and -DM

Once selected, high-affinity GC B cells develop into memory B cells or plasma cells. Flow cytometry analysis of purified tonsillar B cells identifies memory B cells as $CD38^{lo}IgD^-$ cells (gate R4 in Fig. 1A), and demonstrates that they express both HLA-DM and -DO at levels comparable to those observed in naive B cells (Fig. 2B, last row). Blood B cells are composed mainly of naive IgD^+ and memory IgA^+ or IgG^+ cells. Confocal analysis of purified blood B cells indicates that HLA-DO and -DR are co-expressed within intracellular compartments in memory IgA^+ B cells, as found above with naive B cells (Fig. 4A, a and e). Thus, GC B cells re-express HLA-DM/DO when they differentiate into memory B cells. Furthermore, these data indicate that naive and memory B cells of both blood and tonsils display a comparable HLA-DM/DO pattern.

While tonsillar plasma cells express high levels of surface HLA-DR (44), these cells do not express intracellular $HLA\text{-}DR^+$ compartments. Dual staining with either anti-HLA-DO or -DM further shows that they do not express these non-classical MHC molecules (Fig. 4B, a and d). Thus, upon differentiation into the plasma cell pathway, GC B cells further down-regulate their HLA-DM/DO, while they re-express these molecules while differentiating into the memory cell pathway. The lack of HLA-DM/DO expression in plasma cells is not unexpected, as these cells are not considered to act as antigen-presenting cells despite the presence of surface HLA-DR.

Memory and GC B cells express less CLIP within their HLA-DR molecules than naive B cells

The release of CLIP, the Ii/CD74-derived peptide, is necessary for exogenous peptide binding to the MHC class II molecules (12,45). Thus, we analyzed the expression of Ii/CD74 and the levels of CLIP peptides associated to HLA-DR molecules in the different B cell subsets. On tonsil sections, Ii/CD74 expression appears higher in GC B cells than in naive B cells from follicular mantles, while CerCLIP labeling is higher in follicular mantles than in GC (data not shown). Flow cytometry shows that Ii/CD74 is most expressed on GC founder B cells, and at comparable intensity on naive and memory B cells (Fig. 5, second column). The surface expression of CerCLIP is highest on naive B cells, decreases on GC founder B cells, and is lowest on GC and memory B cells (Fig. 5, third column). Thus, a large proportion of naive B cell HLA-DR surface molecules are engaged with CLIP peptide, leaving few molecules for self- and non-self-peptide presentation. In contrast, a higher proportion of memory B cell HLA-DR surface molecules is devoid of CLIP and thus presumably engaged with other peptides.

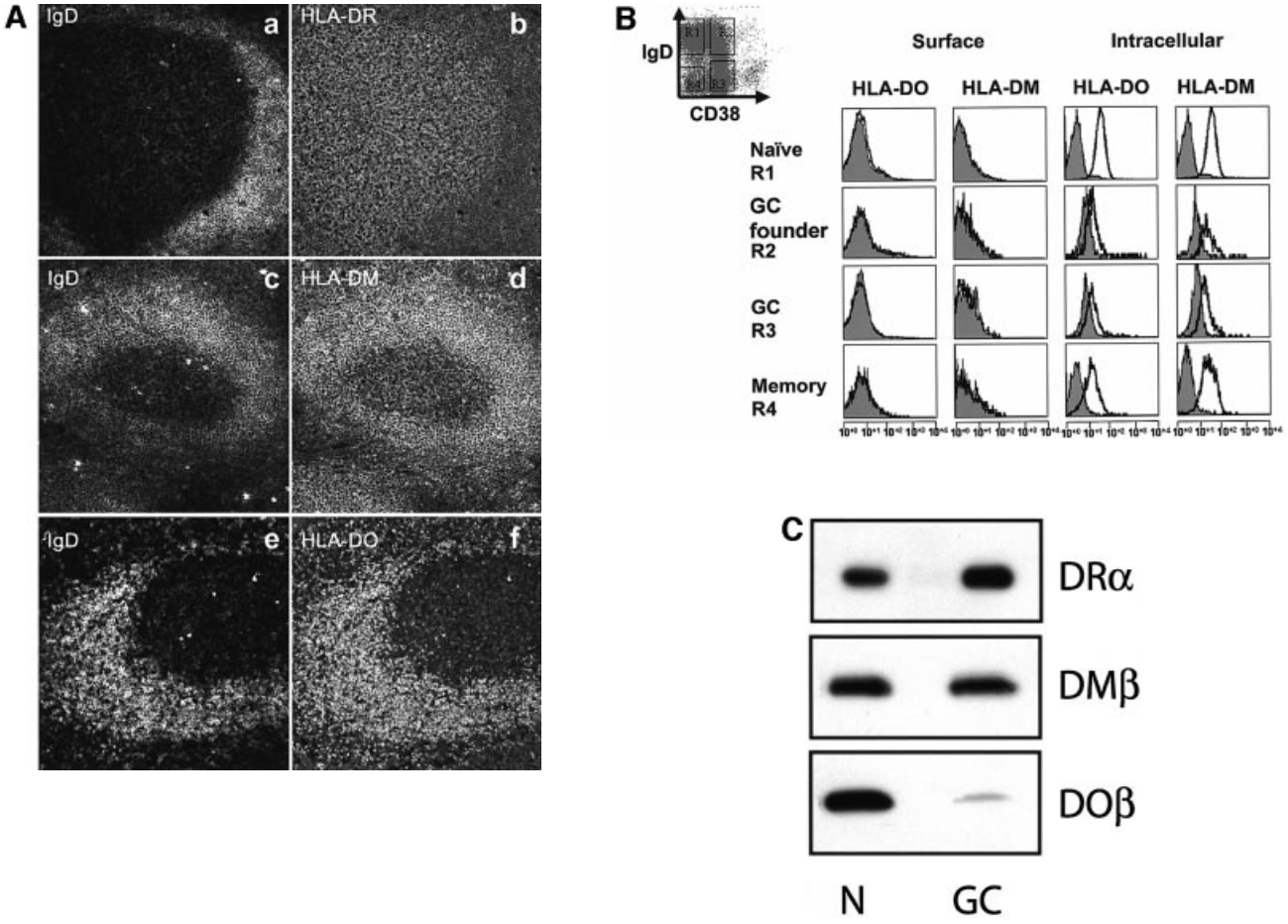


Fig. 2. Down-regulation of HLA-DM and -DO in GC B cells. (A) Confocal immunofluorescence microscopy. Acetone-fixed tonsil tissue sections labeled with anti-IgD (a, c and e) and with either anti-HLA-DR (n = 4), -DM (n = 3) or -DO (n = 5) antibodies (b, d and f). HLA-DR molecules are expressed at similar levels in the follicular mantle that contains naive B cells and in GC containing GC B cells. HLA-DM and -DO expression is reduced in GC B cells compared to the IgD^+ naive B cells of the follicular mantle zone. Confocal microscopy, objective ×20. (B) Down-regulation of intracellular HLA-DM and -DO in GC B cells. Purified B cells were labeled with PE-labeled anti-IgD, FITC-labeled anti-CD38: IgD^+CD38^- naive B cells (R1); IgD^+CD38^+ pre-GC B cells (R2); IgD^-CD38^+ GC B cells (R3); IgD^-CD38^- memory B cells (R4) and labeled with a third marker, HLA-DM/DO (thick line) and isotype control (shaded graph). Note the lack of HLA-DM/DO on the surface of all tonsil B cell subsets, and the down-regulation of intracellular HLA-DM/DO in pre-GC B cells (R2) and GC B cells (R3). (C) Western blot analysis of HLA-DR, -DM and -DO in sorted naive and GC B cell populations.

All human GC B cell subsets capture and target antigen–BCR complexes into HLA-DR compartments

As we have shown that different human B cell subsets express unique MIIC, we next analyzed whether they target antigen differently. The BCR is the main vehicle for B cells to capture and internalize antigen (46). Resting B cells express high levels of BCR on their cell surface, whereas GC B cells have lower amounts of BCR on their cell surface [(1) and data not shown]. Purified GC B cells from tonsils as well as naive and memory B cells from blood were incubated with anti-BCR, and then cross-linked with a secondary antibody to trigger clustering and internalization of the surrogate antigen. Naive, memory and GC B cells incubated at 4°C bear the cross-linked BCR molecules on their cell surface (Fig. 6a and d). After 30 min incubation at 37°C, naive and memory B cells internalize the surrogate antigen in peripheral MIIC (Fig. 6b), while GC B cells internalize it into their juxta-nuclear MIIC (Fig. 6d).

Thus, despite their apparent structural differences, the MIIC within normal human B cell subsets represent the targets of endocytosed BCR-captured antigens.

Discussion

The present study on normal human mature B cell subsets isolated from blood and tonsils, a major secondary lymphoid organ, reveals several important new features related to antigen presentation: (i) the presence in naive/memory B cells of multiple small peripheral MIIC distinct from the large juxta-nuclear aggregates of GC B cells, (ii) the lack of HLA-DO in GC B cells, (iii) the down-regulation of HLA-DO and

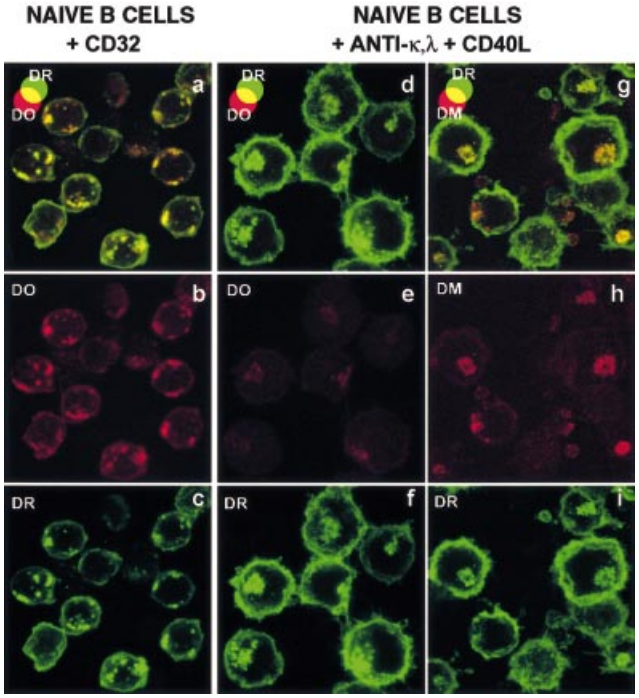
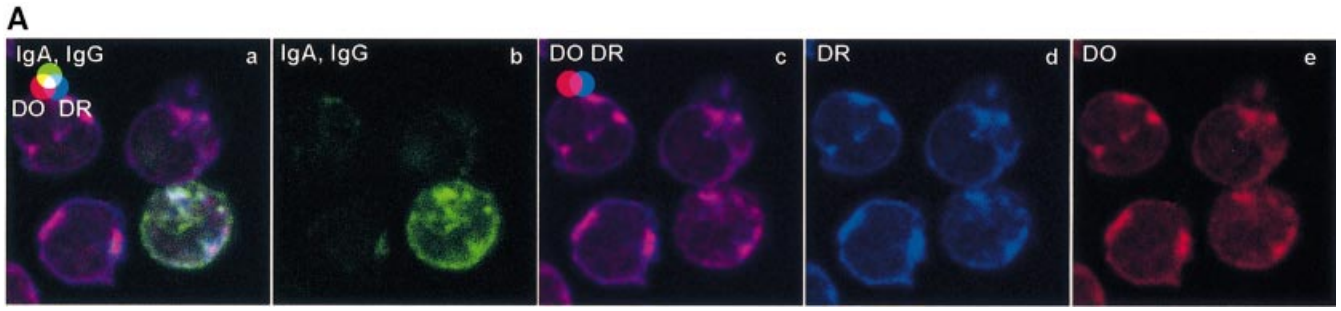


Fig. 3. Dual ligation of CD40 and BCR in naive B cells down-regulates HLA-DO. Purified naive B cells were cultured for 6 days with irradiated L cells either transfected with CD32 alone, to maintain the naive phenotype, or double-transfected with CD40L and CD32 supplemented with anti-λ/κ to generate a partial GC phenotype. Confocal micrographs with identical settings show co-localized intracellular HLA-DR and -DO molecules in naive B cells (a). The intracellular HLA-DO labeling is lost once naive B cells are converted into large B cell blasts after BCR engagement and CD40 ligation in culture (d and e), while HLA-DM expression is maintained at a low level (g and h). Confocal microscopy, objective ×100, zoom 2.



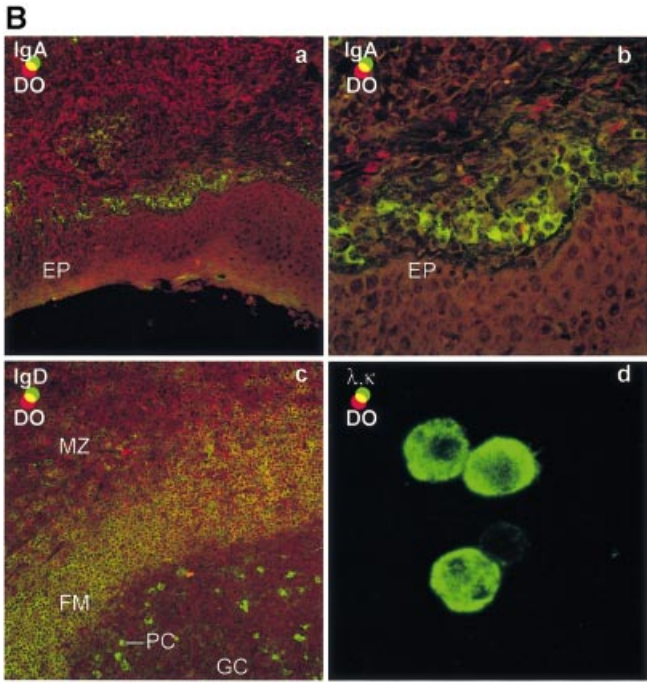


Fig. 4. Memory B cells re-express HLA-DO in peripheral MIIC. (A) HLA-DO and -DR are co-localized in purified blood naive and memory B cells. Purified blood B cells were labeled with anti-HLA-DO (red) (e), anti-DR (blue) (d), and anti-IgA and -IgG (b). In memory IgA^+/IgG^+ B cells, HLA-DO and -DR are co-localized in intracellular compartments as in naive B cells (c). Confocal microscopy, objective ×63, zoom 2. (B) Lack of HLA-DM/DO in tonsillar plasma cells. Tonsil tissue specimens (a–c) were labeled with FITC-labeled anti-IgA (a and b) and FITC-labeled anti-IgD (c), and purified tonsil plasma cells were labeled with FITC–anti-κ/λ (d), and counter-stained with Texas Red-coupled anti-HLA-DO antibody (a–d). The subepithelial IgA^+ plasma B cells (a and b), the IgD^+ plasma B cells (C) and the purified IgD^+ B cells (d) are devoid of HLA-DO. Confocal microscopy, objective ×10 (a), ×40 (b), ×20 (c), ×100, zoom 2 (d).

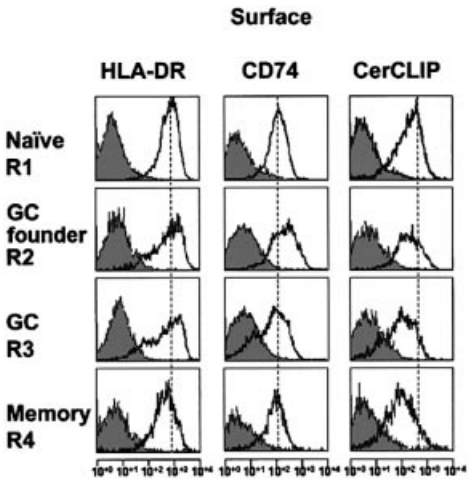


Fig. 5. Low levels of CLIP on GC and memory B cells. Purified tonsil B cells were triple labeled with PE-labeled anti-IgD, FITC-labeled anti-CD38 and Cy5-labeled anti-HLA-DR, CD74 or CerCLIP as the third marker for flow cytometry analysis of their surface expression. CerCLIP labeling is reduced on the surface of pre-GC, GC and memory B cells.

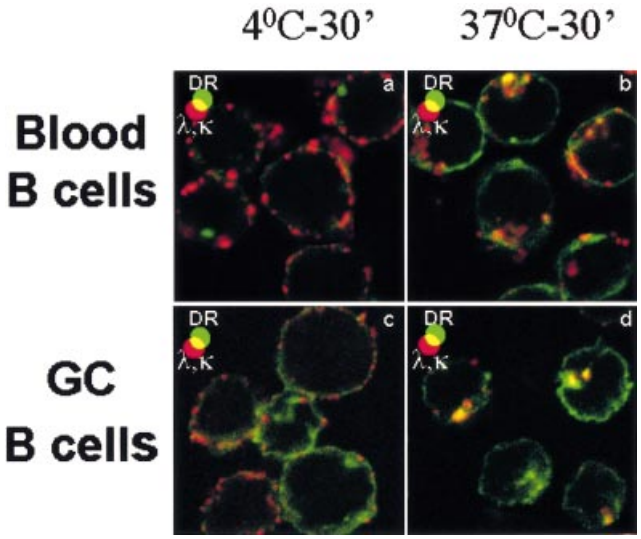


Fig. 6. Naive and GC B cells internalize BCR–anti-BCR complexes within MIIC. Purified naive (upper panel) and GC (lower panel) B cells were incubated at 4°C with anti-κ and anti-λ antibodies, and then with Texas Red-conjugated secondary antibody as surrogate BCR ligand. Cells were warmed up at 37°C by addition of warm medium, and after 0 and 30 min cells were washed with cold PBS, allowed to adhere on poly-L-lysine-coated slides at 4°C, and analyzed by confocal immunofluorescence microscopy for HLA-DR (green) and internalized surrogate BCR ligands (red). Confocal microscopy, objective ×63, zoom 2.

the merging of MIIC in naive B cells provided with GC-triggering signals, i.e. co-ligation of CD40 and BCR, and (iv) the internalization and targeting of the antigen–BCR complexes by naive and GC B cells into their MIIC.

We demonstrate that human B cells remodel their MHC class II compartments when they enter into a GC reaction. Naive and memory B cells concentrate HLA-DR molecules into numerous peripheral compartments located immediately under the cell surface, which also contain HLA-DM and -DO. As they also co-express Lamp-1, they qualify as late endosomes/lysosomes. This is at variance with studies on established murine B cell lines that share some characteristics of normal resting B cells (14,21). There, electron microscopy shows that, under a resting state, the murine B cell lymphoma line expresses MHC class II molecules in compartments distinct from Lamp-1$^+$ compartments. Two possibilities may explain the discrepancy between these studies: (i) the lack of sensitivity, in our study, of the confocal analysis which would not discriminate the two independent compartments and (ii) the use, in the other study, of proliferating B cell lines that may not be comparable to normal non-proliferating naive B cells.

Herein we further show that upon induction of a GC founder phenotype, through co-ligation of BCR and CD40, these DR$^+$DM$^+$Lamp-1$^+$ compartments aggregate deeper inside the cells. This finding extends to normal human B cells the remodeling, aggregation and fusion of the Lamp-1$^+$ compartments described, in response to BCR ligation, in murine B cell lines (15,16,19–21)**.** The studies with the B cell lines concluded that these newly generated compartments are more acidic, favoring the activity of HLA-DM and inhibiting the activity of HLA-DO (16). Our results on normal B cells yield a different conclusion, i.e. the disappearance of HLA-DO. Yet, the functional consequences are equivalent as the remaining HLA-DM is now able to fully exert its activity of peptide loading onto MHC II molecules in GC B cells.

The tonsillar GC founder cells (IgD$^+$CD38$^+$) express, on their cell surface, higher levels of HLA-DR and Ii/CD74 than naive and memory B cells, indicating synthesis of new MHC II molecules that are sent to the cell surface before integrating intracellular compartments. Studies on B cell lines, which display a constitutively activated CD40 signal transduction pathway (47), indicated that the loading of antigen through the BCR occurs onto newly synthesized MHC II molecules (48). Therefore, when naive B cells encounter their specific antigens and receive a T cell signal (most likely CD40L), new class II molecules are synthesized, possibly to provide the novel pool needed by the centroblasts for their selection. Our previous studies on murine splenic B cells showed that BCR ligands are uniquely presented by newly synthesized MHC class II molecules (49), and that the linked roles of antigen internalization and B cell activation of the BCR leads to potent Ii/CD74-dependent antigen presentation (49). In summary, the prolonged co-ligation of naive B cells, BCR and CD40 brings numerous features of GC B cells including morphology (size and merging of MIIC), phenotype with the exception of CD10 (43) and functions such as intense proliferation as well as a basic level of somatic mutations (50).

Our study further demonstrates that GC B cell MIIC, at variance with cell lines, do not express HLA-DO. The lack of HLA-DO apparently loosens the stringency of antigen presentation by GC B cells. This is likely to be needed in GC centroblasts, as fast probing of BCR quality is needed in response to their considerable proliferation that permits introduction of multiple somatic mutations to create variants with a higher affinity for the eliciting antigen. There, the cells which cycle every 6–7 min (1) need to be efficiently tested for

reactivity with the antigen that is presented by the FDC network. Indeed, our preliminary data suggest that GC B cells endocytose the antigen into MIIC within 5 min, while naive B cells do not show internalization after 5 min, but after 30 min. Thus faster antigen endocytosis and a less strict peptide selection, as implied by the lack of HLA-DO, may contribute to a faster selection of desired high-affinity GC mutants. The overall low stringency of antigen processing in GC B cells when compared to naive and memory cells is possible because: (i) GC B cells have already been antigen-selected outside the GC and (ii) GC are a 'hard nut to crack', penetrated by very few exogenous antigens and thus avoiding selection of undesired specificities that may arise as a consequence of somatic mutation (51).

A significant observation of this study is the higher density of CLIP on the surface of naive B cells when compared to GC and memory B cells. This indicates that naive B cells have a limited presentation of MHC class II–foreign peptide complexes. Naive cells may thus be compared to HLA-DO transfectants, which are deficient in antigen presentation (35,36). Upon differentiation into a GC founder, following antigen and specific T cell encounter, remodeling of the MIIC and loss of HLA-DO accelerate peptide-loading activity leading to the decrease of CLIP on the cell surface. These findings therefore extend to normal B cells earlier observations made with Epstein–Barr virus-transformed B cell lines and HLA-DM transfectants where free HLA-DM molecules catalyze CLIP removal from MHC class II molecules (33).

Once selected, GC B cells differentiate into memory B cells or plasma cells. The memory B cells re-express HLA-DO as well as MIIC dispersed in peripheral locations, suggesting the intriguing possibility that HLA-DO may actually be involved in the formation of these peripheral compartments. Memory B cells, at variance with naive B cells, have low CLIP on their surface. This likely reflects the presence of other self or exogenous peptides derived from antigens processed during GC reactions. Plasma cells, the antibody-producing cells, loose their system of antigen-processing capacity (lack of MIIC, and HLA-DO and -DM), even though they still express surface HLA-DR at high density (44), the function of which remains to be elucidated.

Down-regulation of HLA-DM and -DO in human GC B cells has been recently described by two different groups (52,53). These studies, however, did not address the redistribution of MIIC in purified GC B cell suspensions nor did they analyze the signals that contribute to the down-regulation of these molecules upon activation of naive B cells. Roucard *et al*. have reported the down-modulation of HLA-DM and -DO *in vitro* upon culture of cell lines and blood naive B cells with phorbol myristate acetate or soluble anti-IgM (54). We activated naive B cells with cross-linked anti-BCR and CD40L, the combination of signals that best reproduces the GC phenotype *in vitro*. These combined signals led to the down-regulation of HLA-DM and -DO, while isolated anti-BCR triggering led to B cell death.

In conclusion, human B cell subsets appear to have distinct mechanisms of antigen processing. The naive and memory B cells display small superficial MIIC that contain HLA-DM and -DO as described earlier with B cell lines. In contrast, GC founder B cells reorganize MIIC into large clusters that do not contain HLA-DO.

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Abbreviations

CD40L	CD40 ligand
CLIP	class II-associated invariant chain peptide
GC	germinal center
FDC	follicular dendritic cells
Lamp-1	lysosome-associated membrane protein-1
MIIC	MHC class II-rich compartment
PE	phycoerythrin

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