The Journal of Immunology

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J Immunol 2000; 165:5462-5471; ;
doi: 10.4049/jimmunol.165.10.5462
http://www.jimmunol.org/content/165/10/5462

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Commitment of B Lymphocytes to a Plasma Cell Fate Is Associated with Blimp-1 Expression In Vivo[1]

Cristina Angelin-Duclos,* Giorgio Cattoretti,† Kuo-I Lin,* and Kathryn Calame[2]*

B lymphocyte-induced maturation protein-1 (Blimp-1) is a transcriptional repressor that is sufficient to trigger terminal differentiation in the B cell lymphoma BCL-1. In this study, we have determined the expression pattern of Blimp-1 in vivo in primary and secondary lymphoid organs of humans and immunized mice. Blimp-1 is expressed in plasma cells derived from either a T-independent or T-dependent response in plasma cells that have undergone isotype switching and those resulting from secondary immunization. Blimp-1 is also present in long-lived plasma cells residing in the bone marrow. However, Blimp-1 was not detected in memory B cells. This expression pattern provides further evidence of a critical role for Blimp-1 in plasma cell development, supporting earlier studies in cultured lines. Significantly, Blimp-1 was also found in a fraction (4–15%) of germinal center B cells in murine spleen and human tonsils. Blimp-1 expression in the germinal center is associated with an interesting subset of cells with a phenotype intermediate between germinal center B cells and plasma cells. In the mouse, Blimp-1$^+$ germinal center B cells peak at day 12 postimmunization and disappear soon thereafter. They are not apoptotic, some are proliferating, they express germinal center markers peanut agglutinin or CD10 but not Bcl-6, and most express CD138 (syndecan-1), IRF4, and cytoplasmic Ig. Together, these data support a model in which B cell fate decisions occur within the germinal center and Blimp-1 expression is critical for commitment to a plasma cell, rather than a memory cell, fate. *The Journal of Immunology,* **2000, 165: 5462–5471.**

Naive B lymphocytes develop continuously from pluripotential progenitors in the bone marrow and migrate to peripheral lymphoid organs where they encounter Ags. Binding of cognate Ag initiates a complex set of events that ultimately results in formation of both Ag-specific memory B cells and Ab-secreting plasma cells (1–3). Plasma cells, which represent the terminal stage of B cell development, are effector cells that secrete large amounts of Ab, no longer express MHC class II or respond to T cell help and do not divide.

The decision to become a plasma cell occurs at varying times and in different sites during a humoral immune response (4). Exposure of B cells to T-independent (TI)[3] Ags activates proliferation and formation of foci of Ab-forming cells (AFC) in periarteriolar lymphoid sheaths (PALS) of secondary lymphoid organs. These plasma cells secrete Abs with relatively low affinity for Ag, usually of the IgM class, and are short-lived. Following exposure to T-dependent (TD) Ags, two distinct paths of B cell development are possible. The first is similar to the TI response and involves rapid clonal expansion of activated B cells which form foci of AFC in the T cell-rich areas of lymphoid organs, providing immediate Ag clearance. These early foci of AFC, which mainly produce low-affinity Abs, peak 8–10 days after immunization and then regress rapidly (5, 6). The alternate developmental choice for TD-activated B cells is entry into a lymphoid follicle to establish a germinal center (GC) (5, 7, 8). GC B cells, in association with follicular dendritic cells and T_H cells, undergo rapid proliferation, somatic hypermutation, and affinity maturation. B cells that exit the GC adopt one of two fates: they become either memory cells or plasma cells (9–11). Plasma cells, that develop in the GC, migrate to the bone marrow (12–14) where they secrete high-affinity Abs and where they may persist for many months in the absence of further proliferation (1, 15, 16). Upon secondary exposure to TD Ags, circulating or marginal zone memory cells proliferate both in T cell-rich regions and in GCs of spleen and lymph nodes. This secondary response leads rapidly to development of plasma cells secreting Abs with high affinity for Ag (17).

Little is known about the molecular mechanisms that determine the development of B cells following exposure to cognate Ag, particularly those involved in the choices between a short-lived plasma cell vs GC development or plasma vs memory fate following the GC reaction. We are studying the role of a transcriptional repressor called B lymphocyte maturation protein-1 (Blimp-1) in terminal B cell development. Although several transcription factors (NF-κB/Rel, BSAP, PU.1, Spi-B, and Aiolos) are important for early steps in B cell lymphopoiesis and others (Oct-2, OCA-B, IRF4, E2A, and Bcl-6) are required for activated or GC B cells (18, 19), Blimp-1 is the only transcription factor known to be expressed in B cell lines representing mature or plasma cells but not in lines representing earlier stages of B cell development (20, 21).

Blimp-1 is a 98-kDa protein containing five Kruppel-type zinc fingers that confer sequence specific DNA binding (22, 23). Based on studies in B cell lines, Blimp-1 has been postulated to be a master regulator of terminal B cell differentiation. In the BCL-1 lymphoma model of differentiation from a mature B cell to a plasma cell, ectopic expression of Blimp-1 is sufficient to cause terminal differentiation evidenced by loss of surface Ig, IgM secretion, expression of syndecan-1 on the cell surface, and cessation of cell division (20, 24, 25).

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Received for publication June 28, 2000. Accepted for publication August 28, 2000.

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[1] This work was supported by Grant RO1AI43576 (to K.C.).

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[3] Abbreviations used in this paper: TI, T independent; AFC, Ab-forming cell; PALS, periarteriolar lymphoid sheath; TD, T dependent; GC, germinal center; Blimp-1, B lymphocyte-induced maturation protein-1; NP, (4-hydroxy-3-nitrophenyl)acetyl; KLH, keyhole limpet hemocyanin; PNA, peanut agglutinin; AP, alkaline phosphatase.

0022-1767/00/$02.00

Blimp-1 acts as a direct transcriptional repressor of the c-*myc* gene (24) and the *CIITA* gene[4] in B cells.

Although studies in cell lines suggest that Blimp-1 is likely to play a key role in plasma cell development, the expression pattern of Blimp-1 in normal B cells has not been studied. The data reported here show that Blimp-1 is expressed in plasma cells resulting from TI Ags, in plasma cells from both primary and secondary responses to TD Ags, and in long-lived plasma cells resident in the bone marrow. This is consistent with its previously suggested role as a regulator of terminal B cell differentiation. In addition, we show that Blimp-1 is not expressed in memory cells but is expressed in a small subset of GC B cells that have a partial plasma cell phenotype. These data suggest that expression of Blimp-1 is important for the commitment of GC B cells to a plasma cell fate.

Materials and Methods

Immunization

C57BL/6 (The Jackson Laboratory, Bar Harbor, ME) mice 8–10 wk old were immunized i.p. with 25 μg of (4-hydroxy-3-nitrophenyl)acetyl (NP)-Ficoll in 0.1 ml of saline (0.85% NaCl) or 100 μg of NP-keyhole limpet hemocyanin (KLH) alum precipitated (Biosearch Technologies, Novato, CA) in 0.2 ml of saline. Secondary immunization was done with 100 μg of alum-precipitated NP-KLH. Four weeks elapsed between primary and secondary immunization.

Antibodies

Rabbit polyclonal anti-Blimp-1 was kindly provided by Mark Davis (20). Anti-CD138 (antimouse syndecan-1) and anti-B220 were purchased from PharMingen (San Diego, CA); anti-peanut agglutinin (PNA) and anti-PNA-biotin were obtained from Vector Laboratories (Burlingame, CA); anti-Ki-67 (clone TEC-3), anti-CD10 (clone 56C6), anti-CD20 (clone L26), anti-CD138 (antihuman syndecan-1), and anti-CD23 were purchased from Serotec; HRP-streptavidin and alkaline phosphatase (AP)-streptavidin were obtained from Dako (Carpinteria, CA); anti-λ and all secondary Abs, biotin or AP conjugated, were purchased from Southern Biotechnology Associates (Birmingham, AL); anti-CD3-biotin and anti-CD20-FITC were obtained from Becton Dickinson (Mountain View, CA); anti-CD38-biotin was obtained from Caltag (South San Francisco, CA); and anti-IgD-FITC was purchased from Dako.

Immunohistochemistry

Spleens were removed at indicated times after immunization and fixed in 1% paraformaldehyde overnight at 4°C, embedded in paraffin blocks, and processed by routine methods. Dewaxed sections were microwaved in 10 mM EDTA (pH 8) for 15 min (26), cooled, blocked with 3% human AB serum (Sigma, St. Louis, MO), and incubated overnight with appropriate dilutions of primary Abs and control sera. Sections were stained by a double-immunoenzyme technique using the biotin-avidin-peroxidase system and AP system. First, a rabbit anti-mouse Blimp-1 polyclonal (1:1000) or control serum was used. The sections were washed in washing buffer (TBS: 50 mM Tris (pH 7.5) and 0.1% Tween 20) and counterstained with 1:200 diluted, biotin-conjugated, mouse and human serum-adsorbed goat anti-rabbit Ab (Southern Biotechnology Associates). Finally, HRP-streptavidin was added and, after washing, developed with aminoethylcarbazole (Sigma). After color development the sections were incubated with the second primary Ab for 2–16 h at room temperature, at the dilutions suggested by manufacturers. Following washing, sections were incubated with the appropriate secondary Ab either biotin or AP conjugated (1:200 dilutions); in the former case, sections were finally incubated with streptavidin-AP (1:300 dilution). AP was developed by fast blue and napthol AsBi-phosphate (Sigma) substrate. Slides were lightly counterstained with hematoxylin.

Double immunofluorescence to detect Blimp-1 and Ki-67

Following treatment of paraffin-embedded spleen sections as described for immunohistochemistry, sections were blocked in TBS-3% serum for 10 min at room temperature. Blimp-1 antiserum (1:1000 dilution) was incubated overnight. Sections were washed five times with TBS-0.1%Tween 20 and incubated for 45 min with anti-rabbit IgG-biotin conjugated; following quenching of endogenous peroxidase, streptavidin-HRP (1:500 dilution) was added for 45 min. The Blimp-1 signal was enhanced with the tyramide amplification system (NEN Life Science Products, Boston, MA); sections were incubated with TBS-1% BSA prior addition of tyramide-biotin diluted 1:500 into Holmes buffer for 30 min. At this step, rat anti-Ki-67 (1 μg/ml) was added for overnight incubation. After washing, sections were incubated with FITC (tetramethylrhodamine isothiocyanate)-conjugated goat anti-rat (red color) Ig. A second round of primary and secondary Ab incubation (1 h each) was performed, followed by a 45-min incubation with streptavidin-7-amino-4-methylcoumarin-3-acetic acid to detect Blimp-1 (blue color). After final washings, coverslips were applied on sections using Vectashield (Vector Laboratories).

Double immunofluorescence to detect Blimp-1 and IRF4

Double immunofluorescence was performed on human tonsil sections by incubating fixed slides with the two primary Abs: rabbit anti-Blimp-1 and mouse anti-IRF4 (27). After overnight incubation, sections were washed and stained with both tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit Ab and FITC-conjugated goat anti-mouse Ab. A second round of primary and secondary Ab incubation (1 h each) was performed to enhance specific staining. After final washings, coverslips were applied on sections using Vectashield (Vector Laboratories).

In situ TUNEL assay

The TUNEL assay was performed using an in situ detection kit following the manufacturer's instructions (Boehringer Mannheim GmbH, Mannheim, Germany) with the following modifications. Tonsil sections were fixed and stained with Blimp-1 Ab as described in immunohistochemistry methods. Sections were then labeled with 50 μl (3-fold diluted in appropriate buffer) of the TUNEL reaction mixture for 60 min at 37°C, washed, and then incubated for 30 min with 50 μl of converter AP. The slides were rinsed and incubated for 10 min at room temperature with 100 μl of fast blue/napthol AsBi phosphate solution.

Isolation of IgD$^-$, CD38$^-$, CD20$^+$ memory cells

Human tonsils taken from patients during routine tonsillectomy were finely minced and the resulting cell suspension was subject to depletion of T cells by rosetting with sheep RBC. Partially purified B cells were incubated for 20 min with biotin-conjugated mouse Abs against CD3, CD38, and IgD and with FITC-conjugated mouse Ab against CD20. Following washes, cells were incubated for 15 min with streptavidin-APC. FITC-CD20$^+$, APC-(CD38/CD3/IgD)$^-$, FITC-CD20$^-$, and APC-(CD38/CD3/IgD)^{high} were sorted using FACStar (Becton Dickinson Immunocytometry Systems, San Jose, CA). Sorted cells were cytocentrifuged for 5 min at 800 rpm on microscope slides, dried at room temperature for 1 h, fixed for 10 min in 10% buffered Formalin and 10 min in methanol, and then stained with Blimp-1 polyclonal Ab as described for immunohistochemistry.

Results

Blimp-1 is expressed in plasma cells formed during a TI response

We wished to confirm Blimp-1 expression in plasma cells formed in vivo during an immune response and also wished to determine whether Blimp-1 expression varied in plasma cells formed during a TI or TD response. A recent report using in vitro culture has suggested that Blimp-1 expression is limited to TI responses (28). To determine whether Blimp-1 is expressed in short-lived, low-affinity plasma cells generated in response to a TI Ag, mice were immunized with NP (3) coupled to Ficoll (NP-Ficoll). This immunogen produces a TI response which is genetically restricted in C57BL/6 mice to production of Abs that predominately contain λ light chains (14).

Mice were sacrificed 10 days after immunization and paraffin-embedded spleen sections were stained with Abs recognizing Blimp-1, λ chains, and syndecan-1 (Fig. 1). The specificity of Blimp-1 antiserum used in all our experiments was confirmed by blocking with recombinant Blimp-1 protein before immunohistochemistry (data not shown). Blimp-1$^+$ (nuclear staining shown in red) and syndecan-1$^+$ (surface staining shown in blue) cells were detected in PALS of the spleen where foci of AFC are normally found (Fig. 1*A*). Ninety-four percent of the syndecan$^+$ cells present in the PALS were Blimp-1$^+$ and 97% of Blimp-1$^+$ cells

[4] J. Piskurich, Y. Lin, Y. Wang, K. Lin, J. Ting, and K. Calame. *Submitted for publication*.

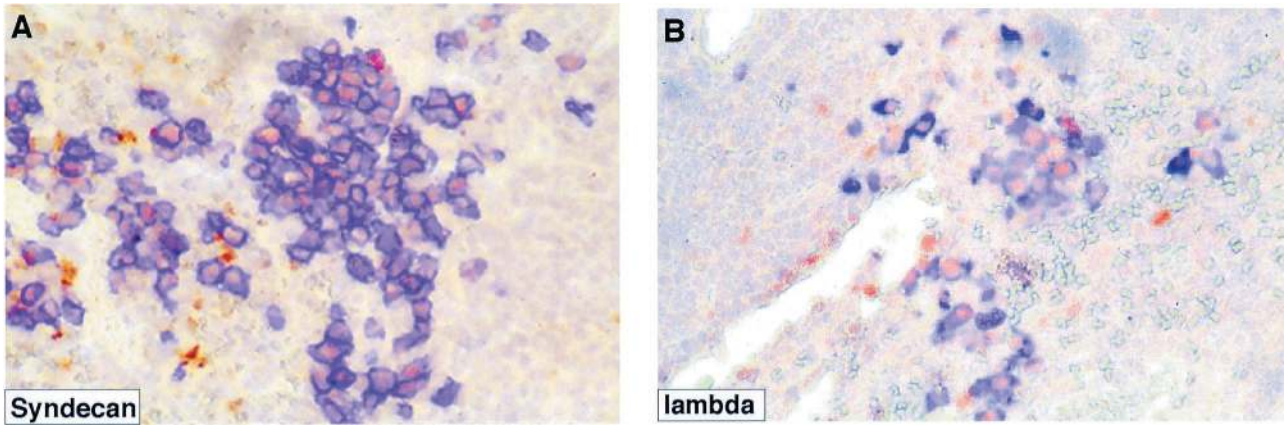

FIGURE 1. Immunohistochemical staining for Blimp-1 in spleen sections of mice immunized with TI Ag NP-Ficoll. *A*, Nuclear Blimp-1 staining (red) and surface syndecan-1staining (blue) detect double-positive plasma cells. *B*, Blimp-1 (red) and λ (blue) staining detects double-positive cells.

were found to be syndecan$^+$. These cells represent Ig-secreting plasma cells (29). When spleen sections were stained for expression of λ light chain, large clusters of λ$^+$ cells (blue staining) were detected in the PALS of immunized mice but not in control mice, where λ$^+$ cells represented <5% of Ig-expressing cells (data not shown). Thus, the λ$^+$ cells represent plasma cells that resulted from immunization with the TI Ag NP-Ficoll. Nuclear Blimp-1 expression was detected in 85% the λ$^+$ cells (red nucleus surrounded by cytoplasmic staining for λ) and 94% of the syndecan$^+$ cells (Fig. 1*B*). Analysis of serial sections showed that some cells appear to be Blimp-1$^-$ because the plane of the section does not pass through the center of the nucleus, thus not allowing detection of Blimp-1. These data show that Blimp-1 is expressed in plasma cells derived from a TI response to NP-Ficoll.

Blimp-1 is expressed in plasma cells formed during a TD immune response

To investigate the expression of Blimp-1 in plasma cells resulting from a TD Ag, C57BL/6 mice were immunized with NP hapten conjugated with KLH. Splenic sections were analyzed by immunohistochemistry at intervals during the response.

Ten days after immunization, clusters of Blimp-1$^+$ cells (nuclear staining shown in red) were observed in PALS and red pulp of the spleen. Ninety-seven percent of Blimp-1$^+$ cells were syndecan-1$^+$ (Fig. 2*A*). B cells showing strong expression of B220 (B220^{high}) (surface staining shown in blue) were negative for Blimp-1 expression (*left,* Fig. 2*B*), whereas B220$^{-/low}$ cells were Blimp-1$^+$(Fig. 2*B*), consistent with a plasma cell phenotype (17).

The immune response to NP-KLH in C57BL/6 mice is genetically restricted (30, 31): the majority of primary Abs bear the λ light chain (32) and use the V186.2 V_H gene segment (30). The pattern of the response is also well characterized (5). Six days after immunization, foci of λ$^+$ cells appear in the extrafollicular areas of lymphoid organs along one face of the T cell-rich areas of the PALS; these represent short-lived plasma cells that survive for a few days and secrete low-affinity IgM (5). These foci decrease in size after day 8; the cells become increasingly dispersed and lose their intimate association with the PALS (5).

When spleen sections obtained from mice 6 days after immunization were analyzed by immunohistochemistry, clusters of λ$^+$ Blimp-1$^+$ cells were observed along the PALS (Fig. 2*C*). These cells are also syndecan-1$^+$ (Fig. 2*D*) and most likely represent short-lived plasma cells that arise during the early phase of a TD response (17). λ$^+$ Blimp-1$^+$ cells were also detected 12 and 15 days after immunization in PALS (data not shown) and red pulp when the λ$^+$ cells start to lose intimate association with PALS (5). Thus, Blimp-1 is expressed in the short-lived terminally differentiated plasma cells that arise during the primary immune response to the TD Ag NP-KLH. Spleen sections from immunized animals were also stained to detect cytoplasmic expression of non-IgM isotypes in plasma cells. Blimp-1 protein was observed in the nuclei of IgG1$^+$ and IgA$^+$ plasma cells (data not shown). Thus, Blimp-1 expression is not limited to the IgM-secreting cells and Blimp-1 is expressed in plasma cells that have undergone isotype switching. Blimp-1 was also observed in syndecan$^+$ plasma cells in human tonsils (see Fig. 7), adenoids (data not shown), and lymph nodes (Fig. 2*E*).

Plasma cells that arise from the GC reaction migrate to the bone marrow where they survive without undergoing any further proliferation and are responsible for long-term Ab production (14, 16, 33). Mouse bone marrow cells and sections from human bone marrow were stained with Blimp-1 and syndecan-1 Abs. Blimp-1$^+$ (nuclear staining in blue) syndecan$^+$ (surface staining in red) cells were detected both in mouse (data not shown) and human bone marrow (Fig. 2*F*). Thus, Blimp-1 is expressed in long-lived plasma cells that home to the bone marrow.

Blimp-1 is expressed in plasma cells formed during a secondary immune response but not in memory B cells

The GC reaction produces memory B cells as well as plasma cells. Upon secondary stimulation with Ag, memory cells proliferate and differentiate into Ig-secreting plasma cells. To understand the involvement of Blimp-1 in response to secondary stimulation, C57BL/6 mice, previously immunized with NP-KLH, were boosted 1 mo after primary immunization. This immunization protocol gives a strong secondary IgG1 response (34). Spleen sections from boosted animals, along with spleen sections from control mice (not boosted), were analyzed for cytoplasmic IgG1 (blue) and nuclear Blimp-1 (red). Spleens from animals receiving a secondary immunization show large clusters of IgG1$^+$ plasma cells (Fig. 3) not observed in control mice (data not shown). These clusters of IgG1$^+$ cells, most likely derived from secondary immune response, were mainly Blimp-1$^+$ (Fig. 3, higher magnification). Thus, Blimp-1 is expressed in plasma cells that arise from a secondary immune response to NP-KLH.

We also wished to determine whether Blimp-1 was expressed in memory cells before differentiation in response to secondary antigenic challenge. To address this question, we analyzed B cells in human tonsils, where the localization and surface phenotype of

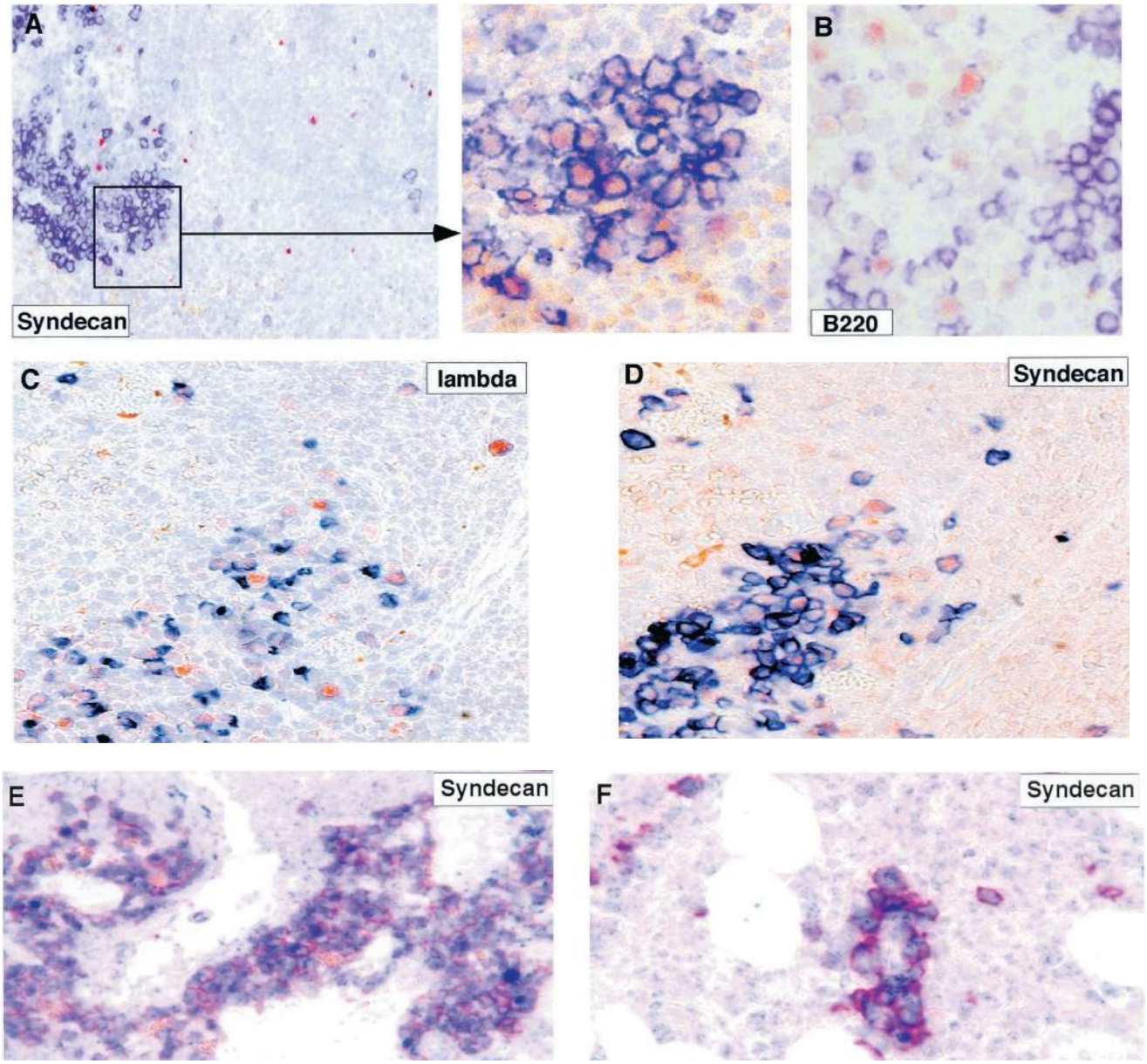

FIGURE 2. Immunohistochemical staining for Blimp-1 in spleen sections of mice immunized with TD Ag NP-KLH. *A*, Blimp-1 (red) and syndecan-1 (blue) staining in spleen PALS detects double-positive plasma cells; these cells are also B220 (blue) negative or low consistent with their plasma cell phenotype (*B*). *C*, Blimp-1(red) and λ (blue) staining on spleen sections obtained from mice at day 6 after immunization show clusters of double-positive cells; these cells are also syndecan-1$^+$ (blue) according to their plasma cell phenotype (*D*). Blimp-1 (blue nuclear staining) and syndecan-1 (surface red staining) (note reversal of colors) identify plasma cells in human lymph nodes (*E*). Blimp-1 (blue nuclear staining) and syndecan-1 (surface red staining) (note reversal of colors) identify clusters of plasma cells in human bone marrow (*F*).

memory B cells have been defined more clearly than in mice (35). Human memory B cells are CD20$^+$, CD138$^-$ (syndecan-1), CD23$^-$CD10$^-$, and IgD$^-$; this phenotype distinguishes them from naive B cells (CD20$^+$CD23$^+$IgD$^+$), GC B cells (CD20$^+$CD38$^+$CD10$^+$), and plasma cells (CD20$^-$CD38$^+$CD138$^+$) (35). Another distinguishing characteristic of human memory B cells is their localization to specific areas within the tonsillar epithelium (35).

Serial sections of tonsils were stained with Abs against the surface markers CD10, CD138, CD23, CD20, and Blimp-1 as indicated in Fig. 4. Since Blimp-1 is abundantly expressed in epithelial cells (D. Chang and C. Angelin-Duclos, unpublished data), the tonsillar epithelial regions were easily identified by strong staining for Blimp-1 (in red). In Fig. 4*A*, the section was stained with a mixture of antisera against CD10, CD138, CD23 (surface staining in blue), and Blimp-1 (nuclear staining in red). Cells that stain blue on the surface with this mixture of Abs are naive, GC, or secreting B cells but are not memory B cells. As expected, some of these cells express Blimp-1. In Fig. 4*B*, the section was stained with anti-CD20 (surface staining in blue) and anti-Blimp-1 (nuclear staining in red). Clusters of CD20$^+$ cells within the tonsillar epithelium (see *boxed region*, Fig. 4*B*), that were negative for CD10, CD138, and CD23 (see *boxed region*, Fig. 4*A*) have a memory cell phenotype (35). These cells do not express Blimp-1 although Blimp-1$^+$CD20$^-$ cells, presumably plasma cells, were observed in other areas of the section.

We also sorted memory (CD20$^+$IgD$^-$CD38$^-$CD3$^-$) cells and a fraction enriched for plasma cells (CD38^{high}CD20$^-$) from purified tonsillar B cells (36) using flow cytometry. The sorted cells (Fig. 5) were stained for expression of Blimp-1. The IgD$^+$ CD3$^+$CD38$^+$CD20$^-$ fraction contained Blimp-1$^+$ cells which are

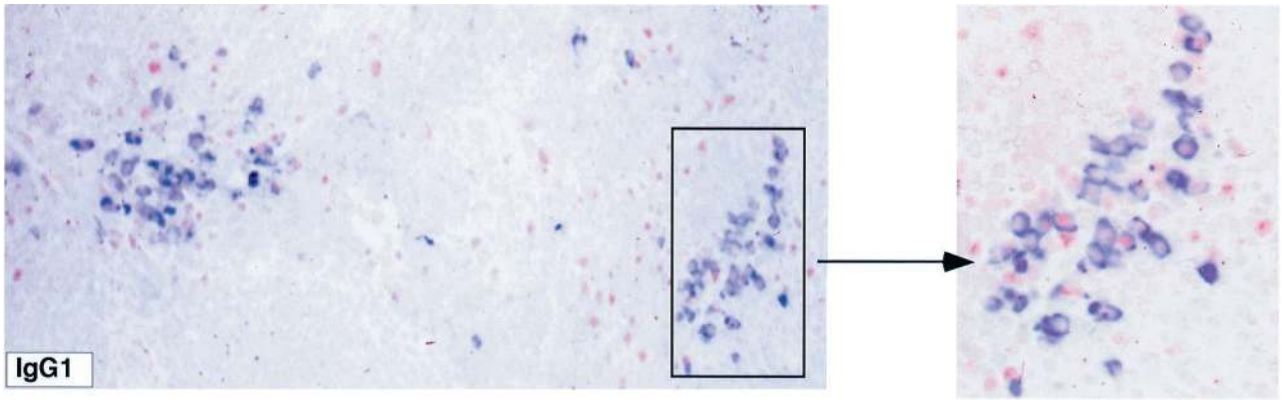

FIGURE 3. Immunohistochemical staining for Blimp-1 (red) and IgG1 (blue) in sections of spleen from mice following a secondary immunization with NP-KLH. *Left panel*, large clusters of IgG1$^+$ plasma cells that are also Blimp-1$^+$ in the enlarged region on the *right side* of the *panel*.

probably plasma cells (CD38$^+$CD20$^-$) and provide a positive control in this experiment; however, no Blimp-1$^+$ cells were observed in the CD20$^+$IgD$^-$CD38$^-$ fraction. Thus, we conclude that Blimp-1 is undetectable, and unlikely to be expressed, in memory B cells.

Blimp-1 is expressed in a subset of GC B cells that have a partial plasma cell phenotype

During a TD response, plasma cells (that express Blimp-1) and memory cells (that do not express Blimp-1) are produced by GCs. When murine GCs were analyzed, we found that a small but reproducible subset of the PNA$^+$ GC B cells was Blimp-1$^+$ (Fig. 6*A*). During the primary immune response to NP-KLH, GCs are observed in the spleen at day 5, reach maximum expansion at day 12, and persist until at least day 20 (5, 37). To determine when Blimp-1$^+$ cells appeared during GC formation, spleen sections from mice immunized with NP-KLH were analyzed 5, 8, 12, 15, and 22 days after immunization for expression of Blimp-1 (red nuclear staining) and PNA (blue surface staining). Both the total number of Blimp-1$^+$ cells per GC and the frequency of Blimp-1$^+$ cells in GCs were low at days 5 and 8, high at day 12, and then fell rapidly by day 15 (Table I). This pattern suggests that Blimp-1$^+$ cells, once formed, do not remain in the GC.

Further analysis of GCs in spleens 12 days after immunization revealed Blimp-1$^+$ (red nuclear staining) λ^+ (blue staining) cells (Fig. 6*B*), confirming that these cells resulted from immunization with NP-KLH. In the Blimp-1$^+$ λ^+ cells, λ staining was stronger than in the majority of GC cells and appeared to be localized to the cytoplasm rather than on the surface, showing that these cells were secreting Ig.

GC centroblasts undergo very active proliferation whereas centrocytes do not divide. Based on the Blimp-1-dependent repression of c-*myc* transcription and cessation of proliferation observed in the BCL-1 cell model, we expected that Blimp-1 expression in the GC would be limited to centrocytes. To test this hypothesis, we used Ab to Ki-67, a nucleolar protein present only in dividing cells (38) to distinguish GC centroblasts (large cells) from centrocytes (small cells, Ki-67$^-$). Surprisingly, two-color immunofluorescence, using Blimp-1 and Ki-67 Abs, revealed that at least half of the Blimp-1$^+$ GC cells were Ki-67$^+$ (Fig. 6, *C–E*). Thus, our data show that some of the Blimp-1$^+$ cells in the GC are proliferating centroblasts.

GCs in human tonsils and adenoids were also examined, and Blimp-1 expression was observed in ~15% of CD10$^+$ GC cells. To characterize these cells further, human tonsils were stained for expression of CD10 and Bcl-6 (characteristic of GC B cells);

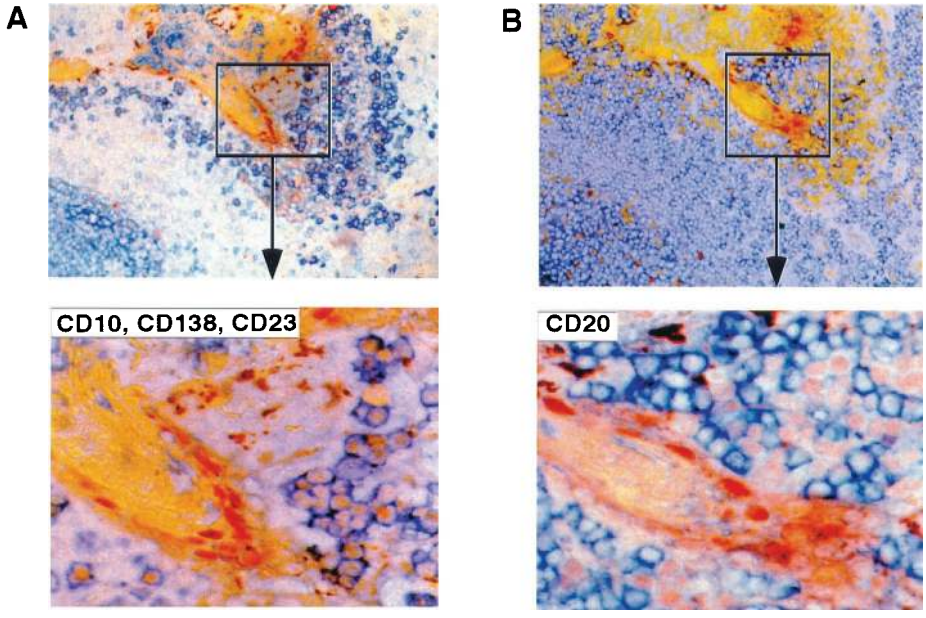

FIGURE 4. Immunohistochemical staining for Blimp-1 in human tonsils. Serial sections were stained for Blimp-1(red) and a mixture of CD10, CD138, CD23 (blue, *A*), or Blimp-1 and CD20 (*B*). Tonsillar epithelial regions are boxed (*upper panels*) and enlarged in the *lower panels;* epithelial cells are detected by strong Blimp-1$^+$ staining. *Boxed regions* show clusters of CD10$^-$, CD138$^-$, CD23$^-$ (*A*), CD20$^+$(*B*), and Blimp-1$^-$ memory cells.

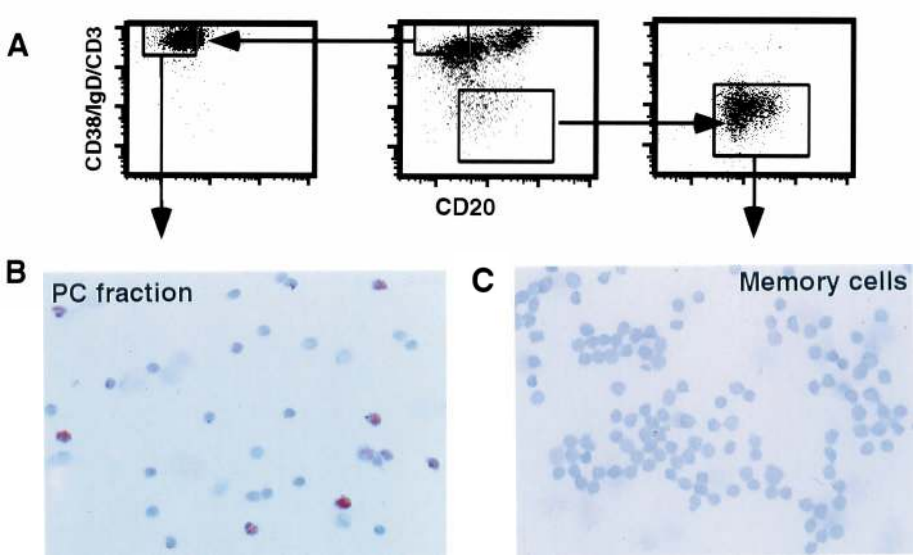

FIGURE 5. *A*, Flow cytometry analysis of tonsillar cells stained with anti-IgD, anti-CD38, and anti-CD3-APC and anti-CD20-FITC. *B*, A population enriched for plasma cells was sorted by gating on $CD20^-IgD^+CD38^+CD3^+$ cells. *C*, Memory cells were sorted by gating on $CD20^+IgD^-CD38^-CD3^-$ cells. Sorted populations were cytocentrifuged, immunostained for Blimp-1 (red), and counterstained with hematoxylin (blue).

CD138 and IRF4 (characteristic of Ig-secreting plasma cells), CD23 (characteristic of mature mantle B cells and follicular dendritic cells), and CD20 (characteristic of pre-B, resting, activated, or memory B cells, but not plasma cells) (39–41). Fig. 7, *A–D*, shows serial sections of a tonsillar GC and extrafollicular regions stained for Blimp-1 (red) and for CD10, CD138, CD20, or CD23 (in blue), and Fig. 7 (*E–G*) shows a tonsillar GC stained for Blimp-1 and IRF-4. Blimp-1^+ plasma cells were observed outside the follicles (Fig. 7*B*); in addition, ~15% of the $CD10^+$ GC cells were Blimp-1^+ (Fig. 7*A*). Frequently the Blimp-1^+ cells in human GCs were organized in clusters, possibly around a follicular dendritic cell (see *boxed regions* in *A–D*). These clustered Blimp-1^+ cells are $CD10^+$ (Fig. 7*A*), $CD138^+$ (Fig. 7*B*), $CD20^-$ (although a few are positive) (Fig. 7*C*), $CD23^-$ (Fig. 7*D*), $IRF4^+$ (Fig. 7, *E–G*), and Bcl-6^- (data not shown). Expression of CD138 and IRF4 and loss of expression of Bcl-6 and CD20 are characteristic of plasma cells rather than GC cells or memory cells.

Enforced expression of Blimp-1 in cultured lines representing pre-B or mature B cell developmental stages causes apoptosis (24, 42). Therefore, we wondered whether Blimp-1 expression in GC B cells was associated with apoptosis. In situ TUNEL assay was performed on the human tonsil sections in combination with Blimp-1 staining. Most Blimp-1^+ (red nuclear staining) cells were not $TUNEL^+$ (Fig. 7*H*). Because we were technically unable to detect both Blimp-1 and dUTP incorporation using double immunofluorescence, we cannot rule out the possibility that some $TUNEL^+$ cells may be Blimp-1^+. However, by analyzing serial sections that were single stained with Blimp-1 or TUNEL (data not

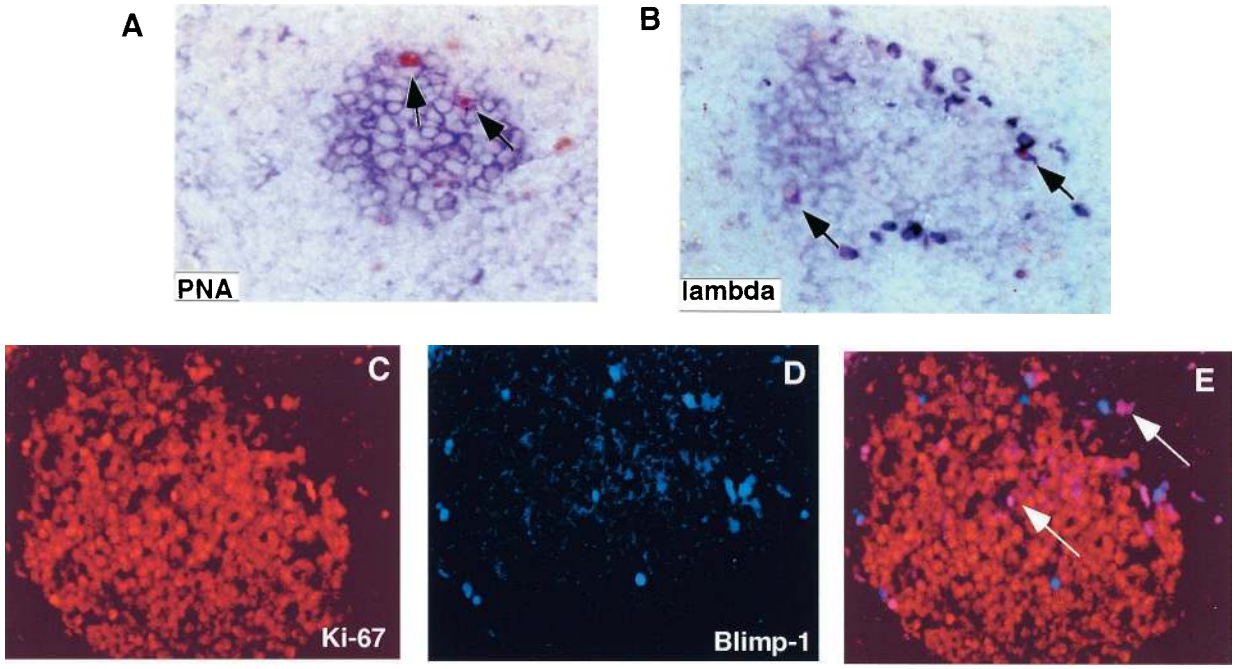

FIGURE 6. Immunohistochemical (*A* and *B*) and immunofluorescent (*C–E*) staining for Blimp-1 in GCs of mice immunized with TD Ag. *A*, Nuclear Blimp-1 staining (red) and PNA surface staining (blue) detects Blimp-1^+PNA^+ cells in GC (double-positive cells are indicated by the arrows); some of the GC Blimp-1^+ cells, indicated by the arrows, are λ^+ (blue, *B*). *C*, Nucleolar proliferating marker Ki-67 (nuclear red fluorescence); Blimp-1 is detected in the same section (nuclear blue fluorescence). *D*, Blimp-1 and Ki-67 double-positive cells are detected in purple (*E*).

Table I. *Count of Blimp-1$^+$ cells in GC of mice immunized with NP-KLH*[a]

	Day 5[b]		Day 8		Day 12		Day 15		Day 22	
Section	1	2	1	2	1	2	1	2	1	2
No. of GC/section	16	17	7	12	20	34	17	27	14	19
No. of PNA$^+$/GC	10–50	10–50	20–100	20–60	20–300	15–200	30–70	25–80	30–80	15–60
Total PNA$^+$/section	370	400	250	300	1300	1400	500	920	700	650
Total Blimp$^+$ in GC/section	3	2	1	4	41	62	3	10	2	2
% Blimp$^+$PNA$^+$	0.8	0.5	0.4	1.3	3.1	4.4	0.6	1	0.28	0.3

[a] The count of Blimp-1$^+$ cells in GC was performed on spleen sections of two mice for each time point after immunization. Number of GC and PNA$^+$ cells/GCs were counted in each section. Numbers of PNA$^+$ cells of each GC in a specific section were pooled together as well as for Blimp-1$^+$ cells. Percentage of Blimp-1$^+$PNA$^+$ cells was calculated on the total numbers of PNA$^+$ cells per section.

[b] Count of PNA$^+$ cells at day 5 was performed in two sections per mouse.

shown), we conclude that many/most Blimp-1$^+$ cells are not TUNEL$^+$. In addition, none of the Blimp-1$^+$ cells have an apoptotic morphology. Therefore, the data show that expression of Blimp-1 in GC B cells is not obligatorily associated with apoptosis.

In summary, we show that a subset of GC B cells expresses Blimp-1 in both mouse and human. These cells are not apoptotic and some of them are proliferating. In mice, their numbers peak at day 12 following immunization with NP-KLH and they do not accumulate in the GC thereafter. Although GC markers, PNA, or CD10 are expressed on these cells, they also express CD138 and IRF4, but not Bcl-6 and appear to be secreting Ig, suggesting acquisition of a partial plasma cell phenotype.

Discussion

Blimp-1 was first identified as a master regulator B cell terminal differentiation in the BCL-1 culture model (20) and subsequent studies have further defined its role and mechanism of action in cultured cells (24, 42, 43). To understand the role of Blimp-1 in the development of B cells in vivo, we have studied the expression pattern of Blimp-1 during immune responses of mice to the well-characterized hapten NP and in human lymphoid tissues. We show that Blimp-1 is expressed in plasma cells of all types, including those formed in a primary response to NP presented as a TI or a TD Ag, in those formed in a secondary response to NP presented as a TD Ag and in long-lived plasma cells that home to the bone marrow. There was no evidence of Blimp-1 expression in memory cells. Intriguingly, Blimp-1 was found in a subset of GC B cells in human tonsils and murine spleen. The phenotype of these cells is intermediate between GC B cells and plasma cells, suggesting involvement of Blimp-1 in commitment of GC B cells to a plasma cell fate.

Blimp-1 is present in plasma cells but not in memory cells

During a primary response, two types of plasma cells are formed. Outside of the follicles, short-lived plasma cells producing IgM with low affinity for Ag (29) provide the first response to TD Ags and the only response to TI Ags. In response to TD Ags, longer-lived plasma cells secreting IgG or IgA with higher affinity for Ag are formed after B cells undergo affinity maturation and isotype switching. Long-lived plasma cells in the bone marrow are thought to be derived from plasma blasts that exit the GC (14, 43, 44). High-affinity isotype-switched plasma cells are also formed from memory cells in a secondary immune response. Blimp-1 was present in plasma cells in the PALS, resulting from a primary response to either TI or TD Ags, and in plasma cells arising from a secondary response to a TD Ag. In addition, the presence of Blimp-1 in GC B cells (Fig. 6) further confirms that Blimp-1 is induced during a TD response.

The small percentage of Blimp-1$^+$ syndecan$^-$ cells may represent precursors of the AFC in which the syndecan marker is not yet expressed. A similar precursor can be found in the GC where a small percentage of Blimp-1$^+$ syndecan$^-$ cells is also observed. Blimp-1 expression in these cells may represent one of the early signals required for differentiation into short-lived AFC.

Blimp-1 was also present in the long-lived plasma cells resident in the bone marrow. It has been suggested that Blimp-1 in short-lived plasma cells is associated with apoptosis due to repression of the anti-apoptotic gene *A1* (43), and this possibility is consistent with our data. However, since Blimp-1 is also expressed in long-lived plasma cells, its expression is not necessarily associated with apoptosis in all plasma cells. Expression of Blimp-1 in all types of plasma cells is consistent with the presence of Blimp-1 in murine plasmacytoma and human myeloma cell lines and with its induction during terminal differentiation of BCL-1 cells to a plasma cell phenotype.

However, our results differ from a previous report in which an antisense strategy was used to inhibit Blimp-1 expression in cultured splenocytes (28). In this study, Blimp-1 appeared to be required for a TI but not for a TD response when splenocytes were treated in vitro with anti-CD40 mAb and IL-4. It seems likely that differences between stimulation of splenocytes in culture and an immune response in vivo account for the apparent discrepancy, particularly since the role of CD40 in a TD immune response and its role in plasma cell terminal differentiation is still debated (46–50). It is also formally possible that even though Blimp-1 is expressed in plasma cells formed during a TD response in vivo, it is not required for that response. The final resolution of this question awaits B cell-specific deletion of the *Blimp-1* gene in vivo.

Our studies on human tonsils revealed no evidence for Blimp-1 in CD20$^+$CD23$^-$CD138$^-$CD10$^-$ memory cells found in the epithelial area of tonsils (Fig. 4) or in purified populations of memory cells (Fig. 5). Thus, it is clear that Blimp-1 is not expressed in most memory cells. This finding is similar to that of Nagumo et al. (51), who also reported lack of Blimp-1 in CD20$^+$ cells purified from human PBLs. Given our understanding that Blimp-1 induces Ig secretion and represses c-*myc* and class II MHC expression, it is reasonable that memory cells, which retain surface Ig and class II MHC expression (35, 52) and retain the ability to proliferate, do not express Blimp-1.

Blimp-1$^+$ cells in the GC may be committed to a plasma cell fate

Finding Blimp-1 in a subset of GC B cells, in both murine spleen and human tonsils and adenoids, was unexpected based on the previously observed expression pattern of Blimp-1 in B cell lines. The majority of Blimp-1$^+$ GC cells are viable and some are positive for Ki-67, a marker of proliferating cells. Thus, Blimp-1 does

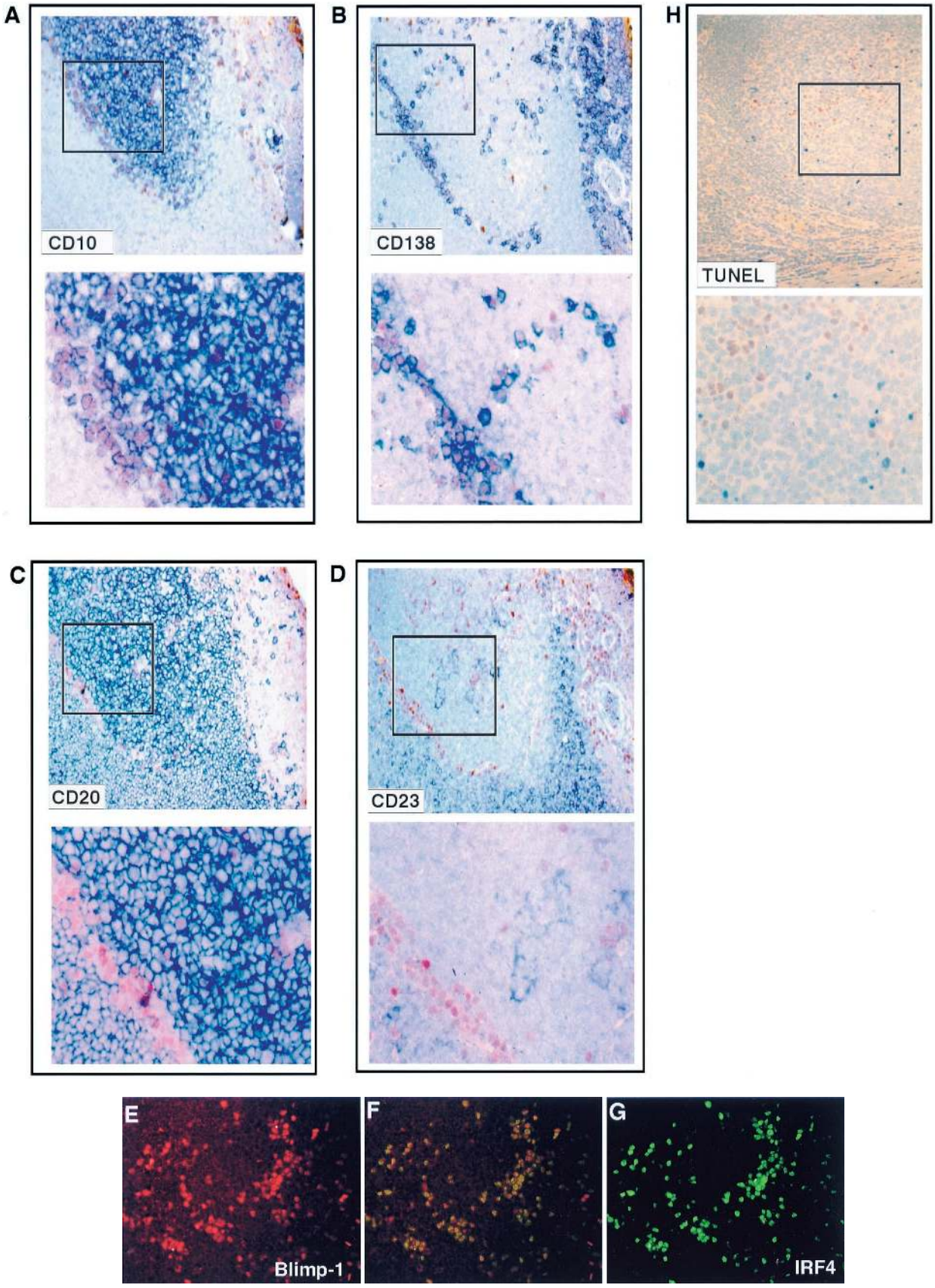

FIGURE 7. Immunohistochemical staining for Blimp-1 in GCs of human tonsils. Serial sections were stained for Blimp-1 (red) and for surface markers CD10, CD138, CD20, and CD23 (blue) as indicated (*A–D*). *Boxed regions* of each panel are enlarged to show the phenotypic features of the Blimp-1$^+$ cells in the GCs. They mostly are CD10$^+$, CD138$^+$, CD20$^-$, CD23$^-$. *E–G*, *E* shows in red, fluorescent nuclear staining for Blimp-1 in tonsillar GC; IRF-4 is detected in the same section with a green fluorescence (*G*); Blimp-1$^+$IRF-4$^+$ cells are detected in yellow (*F*). *H*, TUNEL assay on human tonsils; the section was stained for TUNEL (blue) and Blimp-1 (red), *bottom panel*; an enlarged region of this panel shows that most of the Blimp-1$^+$ cells are not TUNEL$^+$.

not cause apoptosis in these cells as it does when ectopically expressed in B cell lines representing earlier stages of development (24, 42).

Centroblasts are highly proliferative and probably receive multiple mitogenic signals that activate c-*myc* transcription. We hypothesize that Blimp-1-dependent repression of c-*myc* transcription may be overcome by these signals so that centroblasts express Blimp-1 as well as c-Myc. In centroblasts, Blimp-1 may regulate other target genes, such as *A1* (43) or *CIITA*.[4] Once B cells leave the dark zone or the GC, strong proliferative signals that activate c-*myc* transcription may be lost, allowing Blimp-1 to repress c-*myc* transcription, leading to cessation of cell cycle that accompanies terminal differentiation.

Blimp-1^+ cells in the GC appear to represent a dynamic transition from a GC phenotype ($CD10^+$ or PNA^+, $CD20^+$, $CD138^-$, surface Ig) to an AFC phenotype ($CD10^-$ or PNA^-, $CD20^-$, Bcl-6^-, $IRF4^+$, $CD138^+$, Ig secreting). In both human and murine GCs, the Blimp-1^+ cells were predominately syndecan-1^+ (70%); in the mice immunized with NP-KLH, λ expression in Blimp-1^+ GC cells was cytoplasmic, consistent with cells secreting Ig rather than expressing Ig on the surface. The Blimp-1^+CD10^+ GC cells in tonsils were predominately $CD138^+IRF4^+CD20^-$Bcl-6^-CD23^-, a phenotype associated with AFC. AFC have been observed in the GC previously (53–57). More recently IRF4, which is required for formation of Ig-secreting cells (58), was shown to be expressed in a subset of GC B cells with an AFC phenotype (27). Indeed, we found that IRF4 and Blimp-1 are expressed in the same subset of GC cells. The existence in the GC of this Blimp-1^+IRF4^+ subset with an AFC phenotype implies that the fate decision between memory cells and AFC occurs before B cells leave the GC.

Blimp-1^+ cells appear in the GC early in the primary response and peak at day 12; thereafter, they disappear rapidly (Table I). This timing is consistent with previous reports that AFC are produced in the GC earlier than the bulk of memory cells (54, 59–62). The data also suggest that, once formed, Blimp-1^+ cells do not accumulate in the GC but exit rapidly. This observation leads to the interesting possibility that expression of Blimp-1 is important for the decision of an AFC to exit the GC as well as with commitment to an AFC phenotype.

Further study on the role and regulated expression of Blimp-1 is likely to illuminate important aspects of AFC differentiation in the GC. In particular, it will be important to determine whether induction of Blimp-1 in the GC is stochastic or instructed. Cytokine-dependent induction of *Blimp*-1 mRNA in cell culture models suggests that it may be instructed (20, 24). Furthermore, studies with primary cultures of human tonsillar B cells suggest that CD40/CD40 ligand signals favor memory cell differentiation and block plasma cell differentiation (36). Consistent with an instructive model, treatment with CD40 ligand blocks Blimp-1 induction as well as plasma cell differentiation in the CH12 cell line (50). Plasma cell differentiation appears to be driven by signals from OX40 ligand, CD27, and cytokines, including IL-10, IL-3, and IL-6 (10, 51, 63, 64). In addition, a recent report suggests that a threshold level of Ag affinity is required for development of AFC but not memory cells (62) although another study did not observe this requirement (65). Thus, signaling via B cell receptor may be important for Blimp-1 induction. It will be important to learn if one or a combination of these signals induces expression of Blimp-1 in GC B cells.

Acknowledgments

We are grateful to Dr. Garnett Kelsoe for critically reading this manuscript and to Dr. Alan Stall and our colleagues in the Calame laboratory for helpful discussions. The antiserum to Blimp-1 was a kind gift from Dr. Mark Davis.

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