Brief Definitive Report

High Levels of CD44 Expression Distinguish Virgin from Antigen-primed B Cells

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Summary

The in vitro polyclonal stimulation of B cells through their surface immunoglobulin (Ig) induces substantial increases in CD44 protein levels within 24 hours, whereas other stimuli (e.g., lipopolysaccharide, phorbol 12,13 dibutyrate, and interleukin 4) fail to significantly upregulate CD44. The marked increase in CD44 protein expression on anti-Ig-treated B lymphocytes correlates with an increase in CD44-specific mRNA. Cell sorting experiments with B cells isolated from trinitrophenyl-keyhole limpet hemocyanin–immunized mice demonstrate that both short-term antigen-specific, IgG-secreting cells and long-term antigen-primed B cells are exclusively $CD44^{high}$. We speculate that the rapid and sustained increase in CD44 expression mediated by surface Ig stimulation may alter the homing properties of antigen-primed B cells.

Murine CD44 is a major cell surface molecule found on most primary leukocytes and leukocyte-derived cell lines (1). The functions of CD44 have yet to be clearly defined; however, CD44 has been implicated in the binding of lymphocytes to the postcapillary venules of lymph nodes and Peyer's patches (2). In addition, CD44 may play an important role in myelo- and lymphopoiesis in bone marrow (3). Such adhesions may involve interactions between CD44 and one or more of its known ligands, including hyaluronic acid (4), fibronectin (5), collagen types I and IV (6), and a mucosal vascular endothelium surface molecule (7).

Although most leukocytes express CD44, the level of expression can vary according to cell type and activation state. T cells upregulate their surface levels of CD44 upon antigenic stimulation (8). In this study, we have analyzed the expression of CD44 on B lymphocytes and lymphoblasts. Our results demonstrate that CD44 is upregulated on B cells after both membrane Ig–mediated polyclonal stimulation in vitro, and antigen-specific priming in vivo.

Materials and Methods

Immunization Protocols. 8–20-wk-old female CD_2F_1 mice (BALB/c × DBA/2)F_1 (Trudeau Institute, Saranac Lake, NY) were injected intraperitoneally with 100 μg of KLH (Calbiochem-Behring Corp., San Diego, CA) or TNP-KLH, emulsified in 100 μl CFA (Difco Laboratories, Detroit, MI).

Preparation of B and T Lymphocytes. B cells were prepared from the spleens of naive and TNP-KLH-immunized mice, using an anti-T cell cocktail followed by complement lysis (9). B cells were stained for cell sorting, as described below, or further depleted of adherent cells by passage over Sephadex G-10, and stimulated (2×10^6 cells/ml) with Sepharose-coupled αIg, El-4sn, 10 ng/ml phorbol 12,13 dibutyrate (PDBU) (Sigma Chemical Co., St. Louis, MO), or 40 μg/ml LPS (Difco Laboratories), for 12–64 h before flow cytometric analysis (9). T cells were enriched from 1-wk KLH-immunized spleens by depletion of B cells using a mAb specific for $CD45R_A$, RA3-3A1/6.1, followed by complement fixation. T cells were then irradiated (500 rad) and cultured as described below.

Fluorescence Flow Cytometry. Cells were stained as described (9) and analyzed on a FACScan®, or sorted on a FACStar Plus® (Becton Dickinson & Co., Mountain View, CA). In the case of naive and 2-mo immunized mice, the number of $CD44^{high}$ B cells was too small to sort exclusively (<5%). Therefore, the $CD44^{high}$ population was gated to include those cells expressing the 10–15% highest levels of CD44, whereas the $CD44^{low}$ population, representing the majority of B lymphocytes, was gated to include the 50% lowest CD44 expressors. In 1-wk immunized mice, 30–40% of the B cells were $CD44^{high}$ and gates were set accordingly.

In Vitro Antigen Stimulation and TNP-specific Antibody Assays. B cells were cultured at 4×10^6 cells/ml for 5 d in the presence or absence of 4×10^6 cells/ml carrier-primed T cells and antigen, in 96-well microtiter plates (100 μl/well). Test antigens included 30 ng/ml TNP-BSA, 30 ng/ml TNP-KLH, 4 μg/ml TNP-LPS (Sigma Chemical Co.), or 40 μg/ml LPS. Culture supernatants were assayed on 96-well Immulon plates (Dynatech, Chantilly, VA), previously coated with 20 μg/ml TNP-BSA. Alkaline phosphatase–conjugated isotype- and subclass-specific goat anti-mouse Igs (1:500 dilution) (Fisher Scientific, Pittsburgh, PA) were then added for 1 h. Plates were developed with *p*-nitrophenyl phosphate (Zymed, So. San Francisco, CA) and read on a plate reader at 410 nm (MR700; Dynatech).

RNA Preparations and Northern Blotting. RNA was extracted from freshly isolated B cells and 48-h αIg blasts, fractionated, and transferred to nitrocellulose (10). Two probes for CD44 were pre-

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pared from a full-length, 1.3-kb clone of CD44 cDNA (rlcp1): rlcp1.1 (258–1146) and rlcp1.2 (1–827) (numbered according to reference 11). We isolated rlcp1 by immunoselection of a eukaryotic expression library constructed from murine B blast mRNA, using the method described by Aruffo and Seed (12).

Results and Discussion

CD44 Protein and RNA Expression Are Upregulated in B Cells after Membrane Ig-mediated Activation In Vitro. Freshly isolated B lymphocytes express low levels of CD44. Upon in vitro stimulation with anti-Ig, this expression increases ~10-fold within 24 h (Fig. 1 *A*). This result is consistent with recent data showing the upregulation of B cell CD44 after 2 d of $F(ab')_2$ αIg stimulation (13). Maximal CD44 expression does not require the presence of exogenous lymphokines (Fig. 1 *A*). Other polyclonal stimuli (i.e., LPS, PDBU, El-4-sn, IL-4), which do not act through surface Ig, do not significantly increase CD44 expression at 24 h; although by 40 h, LPS induces a fivefold increase in CD44 (data not shown). In addition, B cells stimulated by co-culture with allogeneic T lymphoblasts increase CD44 expression 5–10-fold (14).

To determine if there is a concomitant increase in CD44 mRNA expression, we hybridized Northern blots with CD44-specific probes. Whereas resting B cells contain little CD44-specific RNA, αIg blasts contain substantial amounts of three distinct CD44 species of ~1.3, ~2.7, and ~3.9 kb in length (Fig. 1, *B–C*). We have also determined that all three CD44 species are exclusively encoded by poly(A)$^+$ RNA (Fig. 1 *C*), making it unlikely that the higher molecular weight forms are unspliced nuclear copies of CD44 RNA. The 1.3-kb species encodes a full-length message for CD44 (11, 15) (R.L. Camp and E. Puré, unpublished observations). The sequence of our 1.3-kb cDNA clone, derived from primary murine B lymphoblasts, closely matches the sequence of other CD44 clones isolated from B lymphoma, fibroblast, and macrophage cell lines (data not shown) (11, 15). Neither the sequence of the 2.7- and 3.9-kb molecules, nor their ability to be productively translated, has yet been documented.

Short- and Long-term Antigen-primed B Cells Are CD44^{high}. Whereas the vast majority (>95%) of splenic B cells in naive mice are CD44^{low}, a small number express 5–10-fold higher levels of CD44 (Fig. 2). Although the phenotype of these CD44^{high} B cells is heterogenous, the majority fit the profile of activated B lymphocytes (IgD^{low} and low buoyant density), and are primed to proliferate upon in vitro polyclonal stimulation (data not shown). The presence of small numbers of CD44^{high} B cells in naive mice is most likely a consequence of exposure to environmental antigens.

To follow the expression of CD44 on B lymphocytes stimulated with antigen in vivo, we primed mice with TNP-KLH. We then isolated splenic B cells from naive, 1-wk primed, or 1–4-mo primed mice, sorted them into CD44^{high} and

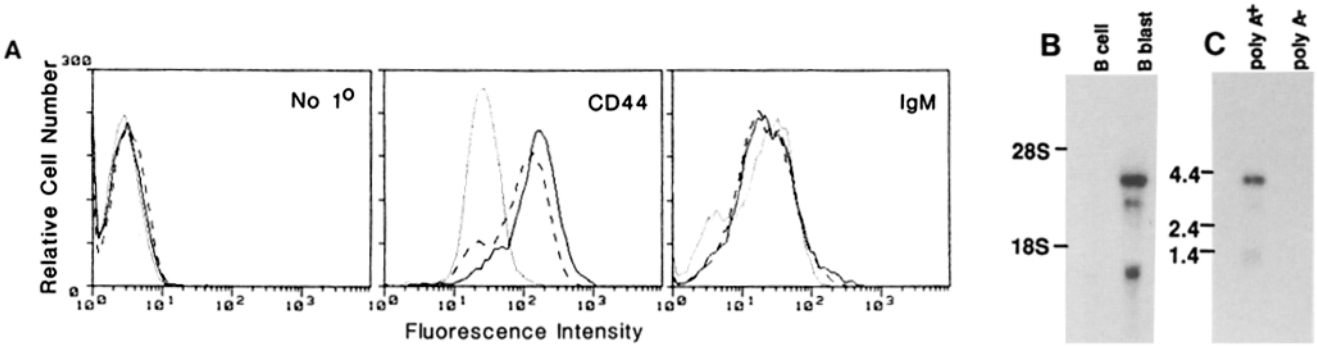

Figure 1. CD44 is upregulated at both the protein and RNA level on B cells polyclonally activated in vitro. (*A*) Freshly isolated B cells (.....), treated with Sepharose-coupled anti-Ig for 24 h in the presence (——) or absence (. . .) of supernatant from the El-4 thymoma cell line (El-4sn). Cells were stained with no primary, mAbs to CD44 (18C8), or surface IgM (Bet2). Stained cells were visualized using a FITC-conjugated secondary antibody, and analyzed on a FACScan®. (*B*) Comparison of 15 μg of total B cell RNA with an equivalent amount of total αIg blast RNA. This Northern blot was hybridized with the CD44-specific probe, rlcp1.2. (*C*) Poly(A)$^+$ and Poly(A)$^-$ fractions of RNA isolated from αIg blasts, hybridized with the CD44-specific probe, rlcp1.1. Standards are expressed in kilobases or as 28S and 18S ribosomal units.

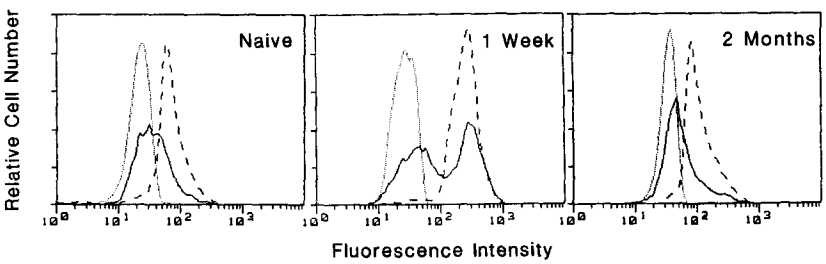

Figure 2. CD44^{high} and CD44^{low} subsets from naive and antigen-primed mice. Naive mice were primed with TNP-KLH in CFA, and splenic B cells were removed at 1 wk and 2 mo after injection. Cells were stained with the CD44-reactive mAb IM7.8.1, and visualized with FITC-conjugated mouse anti-rat Ig. Stained cells (——) were sorted into CD44^{low} (.....) and CD44^{high} (. . .) populations.

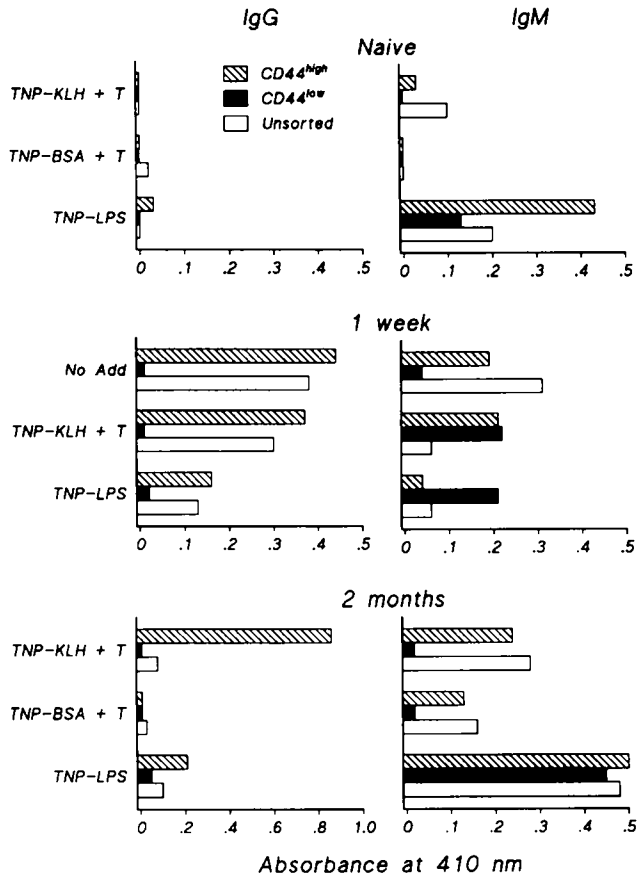

Figure 3. The CD44^{high} B cell subset contains antigen-primed B cells. Sorted cells from Fig. 2 were cultured in vitro, in the presence or absence of antigen (TNP-KLH or TNP-LPS), and KLH-primed T cells (containing APC). After 5 d, culture supernatants were tested for hapten-specific antibody in a TNP ELISA. Antibody titers are expressed in optical density units.

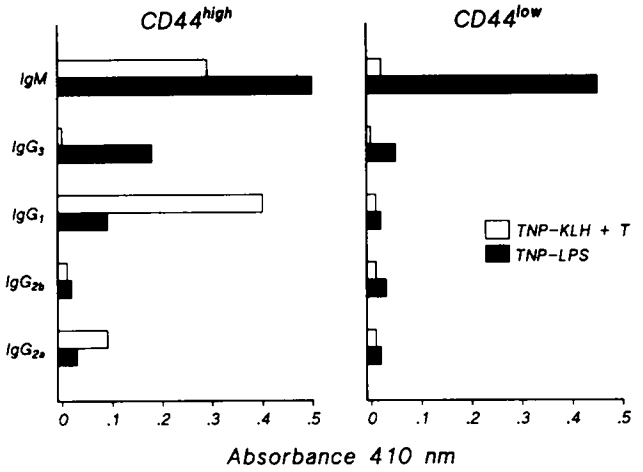

Figure 4. Isotype of TNP-KLH secondary immune response of B cells isolated from 2-mo primed mice. Sorted B cells were cultured, and secreted antibody was isotyped as in Fig. 3.

CD44^{low} subsets (Fig. 2), and compared their abilities to generate a secondary immune response in vitro. Sorted B cells were cultured in vitro, in the presence or absence of antigen (TNP-KLH) and a partially purified population of carrier primed T cells and APC, isolated from 1-wk KLH-primed mice. After in vitro stimulation, we analyzed the relative amount and isotype of TNP-specific antibody from the culture supernatants.

As expected, CD44^{high} and CD44^{low} B cells from naive mice fail to generate a T-dependent response upon in vitro stimulation with TNP-KLH plus KLH-primed T cells (Fig. 3). In contrast, both populations do respond to the T-independent antigen, TNP-LPS, by producing large quantities of TNP-specific IgM (Fig. 3).

Within 1 wk after immunization with TNP-KLH, the relative number of CD44^{high} B cells increases dramatically (Fig. 2). This effect is due in part to the immunostimulatory effects of CFA, since mice primed with CFA alone exhibit similar increases in their CD44^{high} population (data not shown). However, the CD44^{high} subset isolated from TNP-KLH-immunized mice contains virtually all of the IgG-secreting, TNP-primed B cells (Fig. 3). These cells produce high titers of antibody even in the absence of antigen or carrier-primed T cells in vitro, presumably because in vivo primed, antibody-secreting B cells are CD44^{high}. CD44^{low} B cells from 1-wk primed mice can secrete variable amounts of IgM in response to antigenic stimulation in vitro. At 2 mo post-immunization, the number of CD44^{high} B cells has returned to pre-immune levels (Fig. 2). Yet, despite the substantial loss of CD44^{high} cells, all of the surviving TNP-primed B cells remain CD44^{high} (Fig. 3). In contrast to the spontaneous production of TNP-specific antibody by CD44^{high} B cells at 1 wk, by 2 mo, the production of antibody is strictly dependent upon in vitro restimulation with antigen (TNP-KLH) in the presence of carrier-primed T cells and APC (Fig. 3). The majority of the TNP-specific IgG produced in response to TNP-KLH is IgG1, whereas stimulation with TNP-LPS results in the production of more IgG3 than IgG1, consistent with T-dependent and T-independent immune responses, respectively (Fig. 4) (16, 17). Although CD44^{low} B cells fail to respond to the T-dependent antigen (TNP-KLH) plus T cell help, they do produce large quantities of IgM in response to the T-independent conjugate, TNP-LPS (Fig. 3). By 4 mo post-immunization, the CD44^{high} subset still retains the remaining TNP-primed B cells; however, the ability of this subset to secrete IgG in response to in vitro priming with TNP-KLH and carrier-primed T cells is greatly diminished (data not shown).

Although decreases in surface IgD levels correlate with B cell activation (18), the ability of IgD to serve as a marker for antigen-primed B cells, particularly in the short term, is not as clear (19). Our results demonstrate that both short- and long-term antigen-primed B cells can be distinguished from naive cells by their high levels of CD44 expression. Thus, the separation of B cells according to CD44 phenotype, IgD expression, and buoyant density may provide an effective means of enriching for naive, short-term and long-term antigen-primed B cells.

The fact that both activated B and T cells express and maintain high levels of CD44 on their surface suggests that CD44

may play a common role in increasing the migratory potential of all lymphoblasts. This increased migratory capacity may be mediated by lymphocyte-endothelial interactions (7) or lymphocyte-extracellular matrix attachments (4). The importance of CD44 expression as it relates to B lymphocyte migration is not known. However, increased levels of CD44 expression may facilitate both the migration of antigen-activated B cells to lymph nodes as well as the subsequent dissemination of B lymphoblasts and plasma cells. Similarly, the presence of high levels of CD44 on memory B cells may expedite the generation of secondary immune responses by promoting the recirculation of antigen-primed B cells.

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