CD1 Expression Defines Subsets of Follicular and Marginal Zone B Cells in the Spleen: β_2-Microglobulin-Dependent and Independent Forms[1]

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We have used multicolor FACS analysis, immunohistology, and functional assays to study the expression of CD1 on B cell subsets from normal and β_2m$^{-/-}$ mice. Two B cell subpopulations were identified that express high levels of CD1 in normal mice: splenic marginal zone B cells (IgM^{high} IgD^{low} CD21^{high} CD24^{intermediate} CD23$^-$ CD43$^-$) and a newly identified subpopulation of follicular B cells. The latter cells are unusual, because they are IgD^{high} CD23$^+$, like follicular B cells, but express high levels of CD21 and IgM, an expression pattern that is associated with marginal zone B cells. Therefore, the high-level expression of CD1 and CD21 was found to be closely associated on splenic B cells. Immunohistology confirmed the expression of CD1 on marginal zone B cells and on clusters of B cells in splenic follicles. Both the high-level CD1 expression by these cells and the low-level CD1 expression by subpopulations of B cells in the spleen, lymph node, peritoneal cavity, and bone marrow were markedly reduced in β_2m$^{-/-}$ mice. Despite this, a CD1-restricted T cell clone proliferated vigorously in response to LPS-activated spleen cells that had been obtained from both β_2m$^{-/-}$ and wild-type mice. This response was inhibited by the 3C11 anti-CD1 mAb. These results show the heterogeneity of B cell subsets in their expression of the β_2m-dependent form of CD1. They further suggest that a β_2m-independent form of CD1 is expressed on B cells that can stimulate T cells; however, this form is not easily visualized with the anti-CD1 mAb used here. *The Journal of Immunology,* 1998, 161: 1710–1717.**

C D1 is a nonpolymorphic, class I MHC-like molecule that associates noncovalently with β_2-microglobulin (β_2m)[3] (1–3). Two subgroups of CD1 proteins have been identified in humans; group 1 includes the closely related CD1a, CD1b, and CD1c proteins, and group 2 contains the more distantly related CD1d protein (3, 4). Only the homologue of the CD1d protein has been identified in mice (3). Although CD1 was first described as a surface marker on human thymocytes, APCs such as dendritic cells constitutively express high levels of CD1 (5). High levels of CD1a, CD1b, and CD1c can be induced on human monocytes by GM-CSF and IL-4 during the in vitro transition to dendritic-type cells with increased Ag-presenting functions (6). Subsets of normal human B cells and human B cell tumors reportedly express CD1c (7, 8), and mouse and human gastrointestinal epithelial cells express CD1d (9, 10). The predominant form of CD1d in the latter cells is a nonglycosylated β_2m-independent molecule, as judged by analyses of immunoprecipitates with the anti-CD1 mAb 3C11 (11).

Expression of a β_2m-independent form of CD1 has also been reported in transfected β_2m-deficient human melanoma cells (11).

CD1 molecules present Ags to T cells like class I MHC molecules. The CD1-associated Ags are unusual and include hydrophobic peptides as well as glycolipids (12–14). The CD1 molecule itself or in combination with endogenous Ags appears to be recognized by an autoreactive subset of T cells expressing the NK1.1 surface marker (15, 16). This T cell subset has a restricted TCR repertoire that is made up predominantly of an invariant rearrangement of the Vα14 and Jα281 gene segments associated with Vβ2, Vβ7, or Vβ8 receptors (16, 17). The NK1.1$^+$ T cell subset in the thymus is positively selected by CD4$^+$CD8$^+$ thymocytes that express CD1 (18). However, T cells that express neither the NK1.1 marker nor the Vα14 TCR are able to recognize CD1 on syngeneic APCs (19).

T cells are capable of recognizing both β_2m-dependent and independent forms of CD1 (19, 20). The TCR with the invariant Vα14-Jα281 rearrangement expressed by NK1.1$^+$ T cells recognizes only the β_2m-dependent form of CD1 on syngeneic B cells (20). However, non-Vα14 TCRs are able to recognize both the β_2m-dependent and -independent forms of CD1 on syngeneic B cells and other APCs as judged by the in vitro responses of T cell hybridomas (19, 20). The anti-CD1 mAb, 1B1, is able to inhibit T cell responses to the β_2m-dependent form of CD1 but not to the β_2m-independent form (20). This observation suggests that the 1B1 mAb recognizes only the β_2m-dependent form.

Previous studies indicated that almost all murine B cells express CD1 on their cell surface (20, 21). In the current study, we determined the levels of CD1 expression on subsets of B cells by multicolor immunofluorescent staining with two anti-CD1 mAbs, 1B1 (IgG2b) and 3C11 (IgM). Although dull staining of CD1 was observed on most T and B cells, two subsets of CD1^{high} B cells with characteristics of marginal zone and follicular B cells, respectively, were found in the

spleen. Staining with the two anti-CD1 mAbs showed a markedly reduced expression of CD1 on T and B cells from $\beta_2m^{-/-}$ mice, but a β_2m-independent form was detected on these cells by a T cell clone that recognized CD1. This T cell recognition of the β_2m-independent form was inhibited by the 3C11 mAb.

Materials and Methods

Animals and cell preparation

Male and female C57BL/6 and BALB/c mice of 8 to 12 wk of age were obtained from the Stanford University Department of Comparative Medicine (Stanford, CA). $\beta_2m^{-/-}$ C57BL/6 mice of similar age and sex were purchased from Taconix (Germantown, NY). The single-cell suspensions of spleens and peripheral lymph nodes (LNs) obtained from these mice were prepared by gentle passage through nylon mesh. E from the spleen were lysed by incubation in chilled 0.14 M NH_4Cl and 20 mM Tris (pH 7.4) for 2 min. Single-cell suspensions of bone marrow (BM) from the femur and tibia were prepared by flushing the marrow cavities with tissue culture medium.

Abs and cell lines

The following mAbs used in immunofluorescent staining were harvested from hybridomas, purified, and conjugated to the appropriate fluorochromes as described previously (22, 23): Cy7-allophycocyanin anti-IgD (1126), allophycocyanin anti-CD5 (53-7.3), Cascade Blue (CB) anti-IgM (331), CB anti-CD4 (G.K. 1.5), CB anti-CD8 (53-6.7), CB anti-CD3 (2C11), CB anti-F4/80 (F4/80), phycoerythrin (PE) anti-CD43 (S7), FITC anti-CD24 (heat stable Ag; 30F1), FITC anti-B220, and allophycocyanin anti-B220 (RB3-6B2). Biotinylated rat anti-CD1 (1B1), FITC anti-CD21/CD35 (complement receptor (CR)1/CR2;7G-6), PE anti-CD23 (low affinity IgER; B3B4), PE-streptavidin (SA), biotinylated control rat IgM and IgG2b, and unconjugated anti-CD16/32 (2.4G2) were purchased from PharMingen (San Diego, CA). Texas Red-SA (TR-SA) and tetramethylrhodamine isothiocyanate (TRITC)-SA were obtained from Southern Biotechnology (Birmingham, AL). Immunohistologic staining was performed with FITC anti-IgD (1126) and allophycocyanin anti-IgM (331) purified and conjugated as described previously (22). Another anti-CD1 mAb (3C11; rat IgM) was purified from the supernatants of hybridoma cells that were obtained from Dr. C. Terhorst (Harvard University, Cambridge, MA). The IgM fraction was isolated using an E-Z Sep bioreactor IgM size exclusion separator kit (Pharmacia, Uppsala, Sweden) followed by separation on Biogel A-5 m beads (Bio-Rad, Hercules, CA). Biotinylation was performed as described previously (23).

The cloned BALB/c $CD4^-CD8^-$ $V\beta9$ and $V\alpha4.4$ T cell lines (TLI-2.C4 and TLI-2.C7) that were used for the proliferation assays have been described previously (24). A nontransfected BALB/c B cell lymphoma line, A20 (25), and A20 cells transfected with CD1 cDNA were used as stimulator cells.

Immunofluorescent staining

Fresh single-cell suspensions of lymphoid cells were incubated with various combinations of fluorochrome- or biotin-conjugated Abs at saturation for 20 min at 4°C. In the case of the biotin-conjugated reagents, counterstaining for 15 min with fluorochrome-conjugated SA was performed. Stainings were performed in the presence of saturating concentrations of anti-CD16/CD32 mAb to block FcγRII/IIIRs. Stained samples were washed twice and resuspended in staining medium with calcium- and magnesium-free PBS containing 3% FBS (HyClone, Logan, UT) and 0.05% NaN_3 (Fisher Scientific, Fairlawn, NJ) with propidium iodide (PI) at 0.5 μg/ml (Sigma, St. Louis, MO).

Up to eight-color flow cytometric analysis was performed using a highly modified triple laser (488 nm argon, 408 nm krypton, 599 nm dye), FACStar (Becton Dickinson, Mountain View, CA), with four-decade logarithmic amplifiers (26). Data were analyzed with Flow Jo software (Green Star, San Carlos, CA) using 5% probability plots; cells staining for PI (dead cells) were excluded from analysis in all cases.

Immunohistology

Spleens were frozen in OCT compound (Miles, Elkhart, IN) and cut into 9-μm thick sections. Tissue sections were fixed at -20°C in acetone for 15 min and dried at room temperature. The nonspecific binding of staining Abs was blocked by incubating each slide with 10% rat serum in PBS for 15 min at room temperature. Abs were added to each slide and incubated for 1 h at room temperature. After washing out excess Abs, TRITC-SA was added to visualize biotinylated Abs. The slides were analyzed with a laser
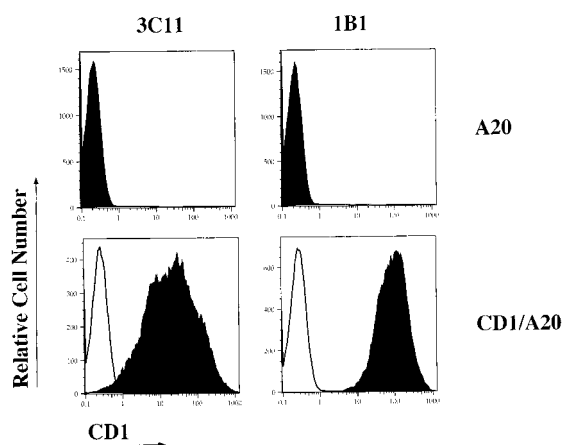

FIGURE 1. Staining of the BALB/c B cell tumor line, A20, which was transfected with CD1 cDNA (CD1/A20) and control nontransfected A20 cells for CD1 expression. One-color flow cytometric analysis is shown, with two anti-CD1 mAbs (3C11 and 1B1) in the shaded areas. Isotype control staining is shown in the open areas.

confocal microscope (CLSM2010, Molecular Dynamics, Sunnyvale, CA) at the Cell Science Imaging Facility at Stanford University.

T cell proliferation assays

Cloned $V\beta9/V\alpha4.4$ T cells (1×10^4) were incubated in flat-bottom, 96-well, plastic plates in triplicate cultures with graded numbers of nontransfected or CD1-transfected A20 B cells that had been irradiated in vitro (4000 cGy) immediately before culture. In other experiments, the cloned T cells were incubated with similarly irradiated spleen cells (5×10^4) from wild-type (wt) BALB/c, wt C57BL/6, or $\beta_2m^{-/-}$ C57BL/6 mice and stimulated for 48 h with 20 μg/ml LPS (Boivan type, Difco, Detroit, MI). Control cultures contained responder and stimulator cells alone. Cells were incubated for 48 h at 37°C in 5% CO_2 in RPMI 1640 tissue culture medium supplemented with 10% FBS (HyClone), 1×10^{-5} M 2-ME, 2 mM glutamine, and 100 μg/ml penicillin and streptomycin. [^{3}H]thymidine (1 μCi/well) (New England Nuclear, Boston, MA) was added to the cultures at 18 h before harvest. [^{3}H]thymidine incorporation was measured using a beta-plate counter (Beckmann Instruments, Fullerton, CA).

Results

Expression of CD1 on spleen cells

The specificity of two anti-CD1 mAbs, 3C11 (rat IgM) and 1B1 (rat IgG2b), was confirmed by immunofluorescent staining of a BALB/c B cell line (A20) that was nontransfected or had been transfected with CD1 cDNA (A20/CD1). Figure 1 shows the flow cytometric analysis of the two cell lines that had been stained with the two anti-CD1 mAbs and the isotype-matched control mAbs. The staining of the nontransfected A20 line with both anti-CD1 mAbs was similar to background controls, whereas the staining of the A20/CD1 line with those mAbs resulted in bright staining.

CD1 expression on normal C57BL/6 spleen cells was determined by simultaneously staining for B220 and CD1 using the two anti-CD1 mAbs and the isotype controls, as shown in Figure 2. All staining was performed in the presence of saturating concentrations of anti-FcγRII/III mAb. The analysis revealed higher levels of autofluorescence for $B220^+$ cells than for $B220^-$ cells. A subpopulation of B cells (enclosed in boxes), which accounted for 10.9 to 15.3% of all $B220^+$ cells, expressed high levels of CD1. Non-B cells ($B220^-$) stained dully with the 1B1 mAb but did not stain with the 3C11 mAb.

A similar analysis was performed on spleen cells from $\beta_2m^{-/-}$ mice on the C57BL/6 background (Fig. 2). A total of <1% of spleen B cells stained with 3C11 or 1B1 mAbs above the background thresholds that had been set using the isotype controls. In

3C11

1B1

FIGURE 2. Staining of spleen cells from C57BL/6 wt and β_2m$^{-/-}$ mice for B220 vs CD1. wt cells were stained with anti-B220 mAb vs rat IgM-biotin control, anti-CD1-biotin (3C11) mAb, rat IgG2b-biotin control, or anti-CD1-biotin (1B1) mAb. The boxes enclose B220$^+$ cells staining brightly for CD1, and the percentage for each box is shown. β_2m$^{-/-}$ cells were stained with the same reagents. TR-SA was used for counterstaining.
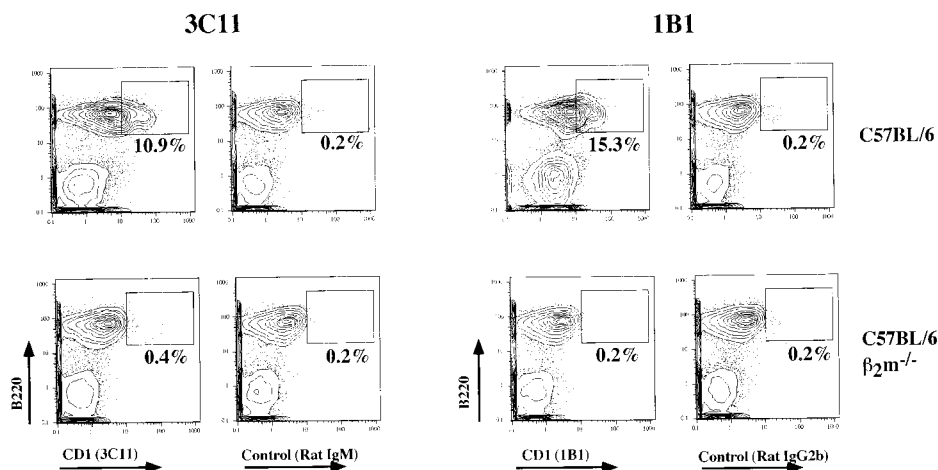
addition, the dull staining of the non-B cells that was observed with the 1B1 mAb in wt mice was not observed in the spleens of β_2m$^{-/-}$ mice. Thus, both the bright staining for CD1 on B cells and the dull staining on non-B cells in the spleen were markedly reduced in β_2m$^{-/-}$ mice.

Spleen cell staining for CD1 and B220 was compared with that of cells in the peritoneal cavity (PerC), LNs, and BM in Figure 3. To achieve an optimal resolution of CD1$^+$ and CD1$^-$ cells, counterstaining for the biotinylated 3C11 mAb was performed with a PE-SA conjugate instead of with the TR-SA conjugate used in Figure 2. This staining revealed the presence of B cells expressing high and low levels of CD1 staining (enclosed in boxes) in the spleens of wt C57BL/6 mice. The spleen cells from β_2m$^{-/-}$ mice showed some residual staining of dull cells (3.0% of B cells) and <1% of bright cells (Fig. 3). Background staining with control rat IgM showed staining of <1% of B cells in both the bright and dull boxes. Although, a bright CD1 (CD1^{high}) subset of B cells was found in the spleen, it accounted for 1% or fewer B cells in the PerC, LNs, or BM (Fig. 3). On the other hand, 14 to 51% of B cells in the three latter sources showed dull staining for CD1, which was clearly above background controls. In β_2m$^{-/-}$ mice, some residual CD1^{dull} cells were observed

in the three sources that accounted for ≤2.8% of all B cells (Fig. 3). Using the bright counterstain PE-SA, some dull staining of non-B cells was observed with the 3C11 mAb. This was not seen in β_2m$^{-/-}$ mice or with control IgM mAb (Fig. 3).

CD1^{high} splenic B cells express the marginal zone or follicular phenotype

To determine whether the CD1^{high} B cells represent a previously described B cell subset, seven-color fluorescent analysis was performed with mAbs that identify follicular, B-1 (Ly-1), immature, and marginal zone B cells (27–31). Figure 4 shows the phenotype of CD1^{high} splenic B cells that had been identified with either 3C11 (Fig. 4, *E* and *F*) or 1B1 mAb (Fig. 4, *H* and *I*) compared with the phenotype of total splenic B cells (Fig 4, *B* and *C*) from normal C57BL/6 mice. Fig. 4*B* shows the staining pattern of gated B220$^+$ cells (Fig. 4*A*, enclosed in the box) for CD23 vs CD24. The *upper box* in Figure 4*B* encloses CD23$^+$ B cells (73%), which are mostly CD24^{low} and consequently represent the follicular subset (27, 30, 31). The *lower box* encloses CD23$^-$CD24$^{low/intermediate}$ and CD23$^-$CD24^{high} B cells. The former contain marginal zone and B-1 cells, and the latter contain immature B cells (27, 31). As

FIGURE 3. Staining of B cells from different tissue sources of wt and β_2m$^{-/-}$ C57BL/6 mice for CD1. Spleens, axillary and cervical LNs, BM, and PerC cells were stained for B220 vs CD1 using the 3C11 mAb. Isotype control staining is shown as well. The boxes indicate the percentage of CD1^{dull} (*left*) and CD1^{high} (*right*) cells within the B (B220$^+$) cell population. PE-SA was used for counterstaining.
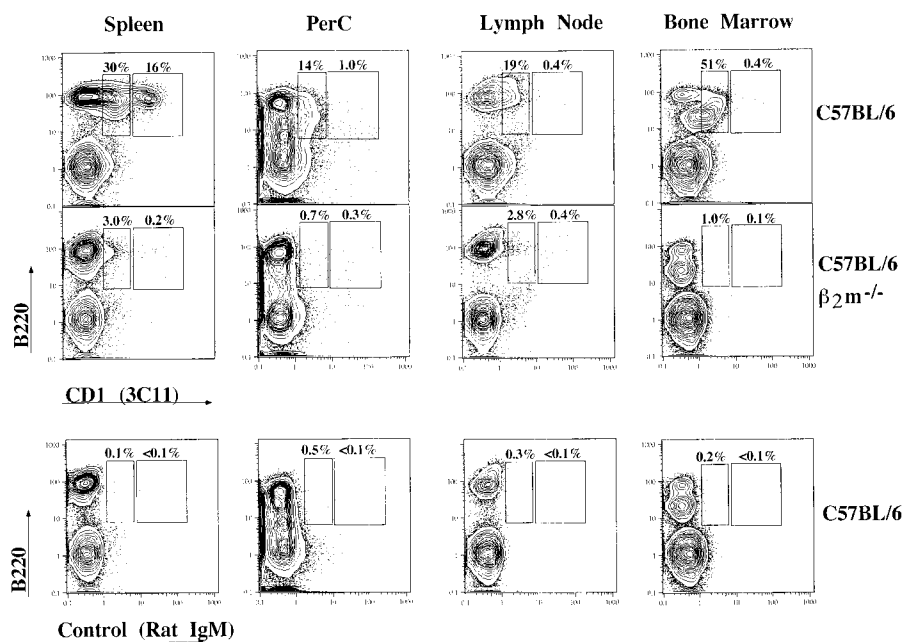
FIGURE 4. Surface phenotype of the CD1^{high} splenic B cell subset. *A*, *D*, and *G*, spleen cells from C57BL/6 wt mice were stained for B220 vs CD1; the boxes show the percentages of B220$^+$ or B220$^+$CD1^{high} cells among all nucleated cells. *B* and *C*, spleen cells were gated for B220$^+$ cells; staining patterns for either CD23 vs CD24 or IgM vs IgD, respectively, are shown. The boxes enclose subsets of B cells as described in the text, and the percentage of subsets among B cells is shown. *E* and *F*, spleen cells were gated for B220$^+$CD1^{high} cells (as shown in the box in *D*), and the expression of other surface markers was analyzed. *H* and *I*, the same analysis of gated B220$^+$CD1^{high} cells was performed, and the 1B1 mAb was used instead of 3C11. TR-SA was used for counterstaining.
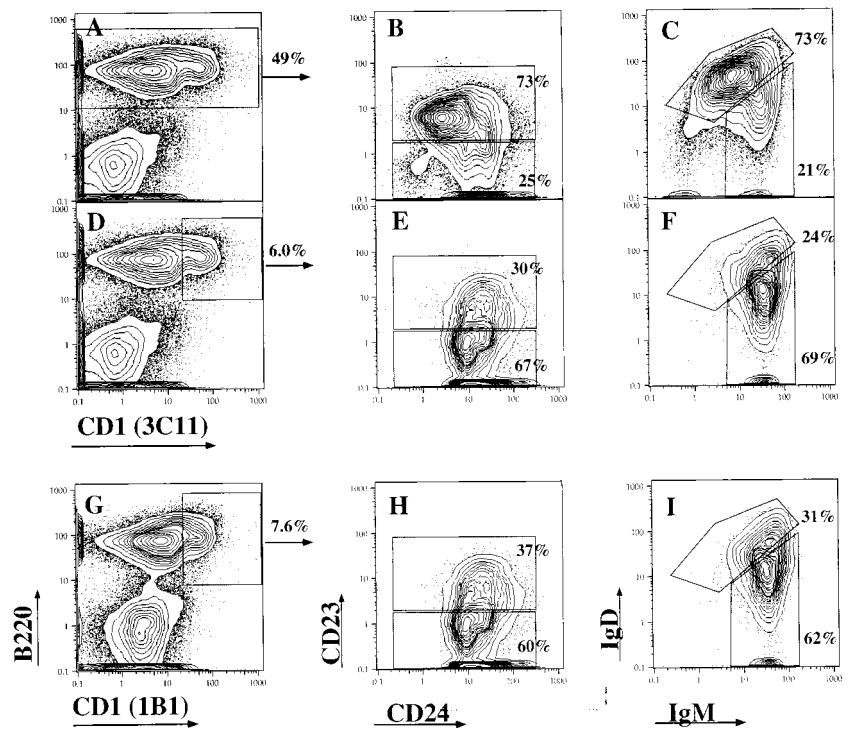
shown in Figure 4, *E* and *H*, B220$^+$CD1^{high} cells were enriched for CD23$^-$ cells, which represented 60% and 67% of all CD1^{high} cells. Most of the CD1^{high}CD23$^-$ cells were CD24^{intermediate}, which is consistent with them being either marginal zone or B-1 cells. Staining for IgD and IgM supported this notion by demonstrating a higher frequency (69% and 62%) of IgM^{high} IgD^{dull} cells among CD1^{high} B cells (Fig. 4, *F* and *I*, *lower box*) compared with splenic B cells. Follicular B cells are mainly IgM^{dull} IgD^{high} (Fig. 4*C*, *upper box*). Thus, roughly 70% of CD1^{high} B cells contained either immature, marginal zone, or B-1 cell subsets, and 30% contained

follicular B cells. Interestingly, most B220$^+$ CD1^{high} IgD^{high} (follicular) B cells also expressed high levels of IgM.

To further classify the CD1^{high} B cells, gated B220$^+$ or B220$^+$CD1^{high} cells were stained for CD43, a receptor that is expressed on both B-1a (CD5$^+$) and B-1b (CD5$^-$) cells (28, 29), and were also stained for CD5. Figure 5*B* shows that 0.4% of gated B220$^+$ cells (Fig. 5*A*, enclosed in the box) were CD43$^+$ and CD5$^+$. A similar percentage (0.3%) of gated CD1^{high} B cells (Fig. 5, *C* and *E*, enclosed in the boxes) were CD43$^+$ CD5$^+$ (Fig. 5, *D* and *F*, enclosed in the boxes).
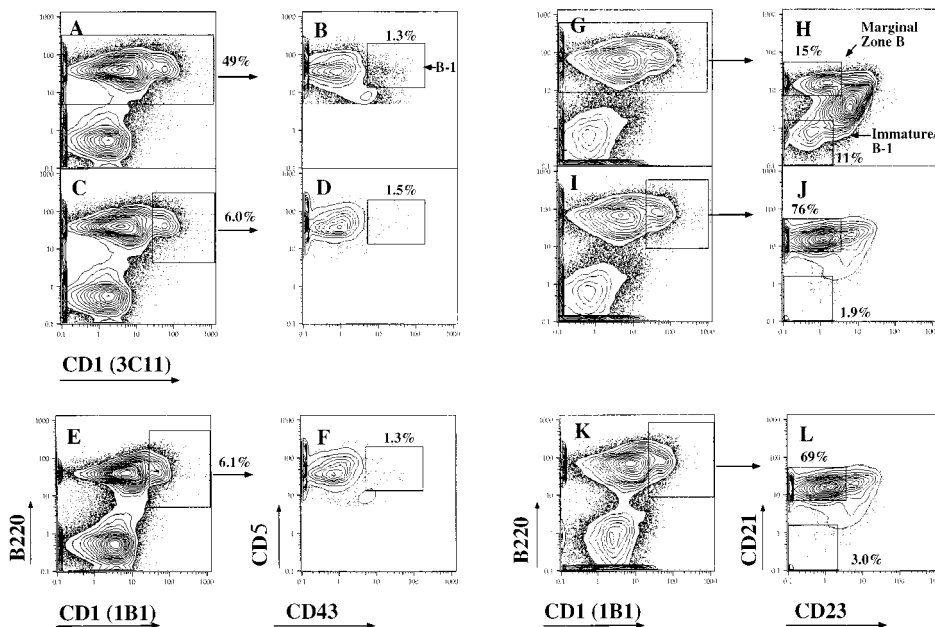
FIGURE 5. Surface phenotype of the CD1^{high} splenic B cell subset. In *A*, *C*, *E*, *G*, *I*, and *K*, C57BL/6 spleen cells were stained for CD1 vs B220. B220$^+$ cells were analyzed for the expression of CD5 vs CD43 in *B*. B220$^+$CD1^{high}-gated cells were analyzed for CD5 vs CD43 expression in *D* and *F*. The boxes in *B*, *D*, and *F* enclose B220$^+$ CD5$^+$ CD43$^+$ cells. B220$^+$ cells were analyzed for CD21 vs CD23 in *H*, and B220$^+$CD1^{high} cells were analyzed for CD21 vs CD23 in *J* and *L*.

FIGURE 6. Identification of splenic IgD^{high} IgM^{high} follicular B cells in C57BL/6 wt and β_2m$^{-/-}$ mice. Splenic CD3$^-$ CD4$^-$ CD8$^-$ F4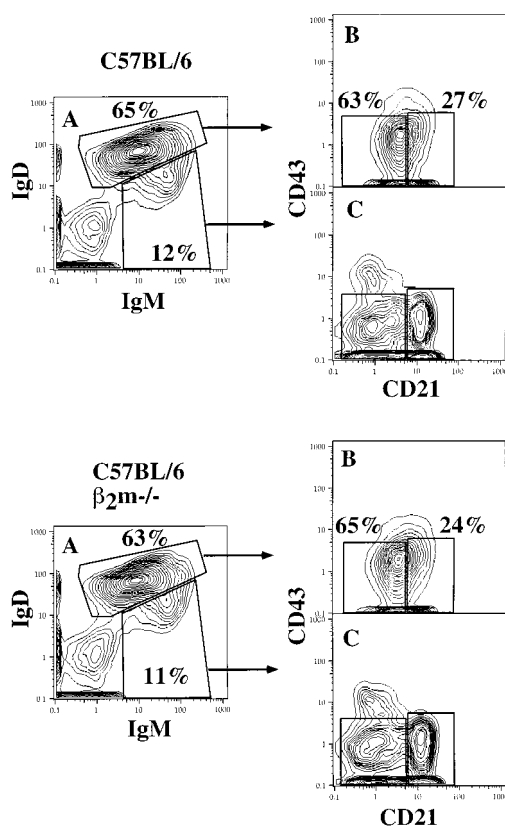/80$^-$ cells from wt and β_2m$^{-/-}$ mice were gated for the expression of IgM$^+$ IgD^{high} (follicular B cells) and IgM$^+$ IgD^{low} cells (A). The percentages of the gated populations are shown; the gates are indicated by boxes. Further gates separating CD43$^-$ CD21^{high} and CD43$^-$ CD21^{low} cells were set on the IgM$^+$ IgD^{low} cells (C) and applied to the follicular B cells (B). The frequencies of CD21^{high} and CD21^{low} follicular B cells are indicated.

B220$^+$CD1^{high}CD23$^-$ B cells were identified as marginal zone B cells by staining for CD21 (31). Figure 5H (*upper box*) shows that 15% of gated B220$^+$ cells (Fig. 5G, enclosed in the box) expressed high levels of CD21. CD21$^-$CD23$^-$ B cells that included immature and B-1 cells accounted for 11% (Fig. 5H, *lower box*) of B220$^+$ cells, and the remainder of B cells expressed intermediate levels of CD21. The latter cells were CD23$^+$ and were members of the follicular subset (27, 30, 31). Gated CD1^{high} B cells (Fig. 5, *I* and *K*, enclosed in the boxes) were predominantly (76%) CD21^{high}CD23$^-$ marginal zone B cells (Fig. 5J, enclosed in the *upper box*). Consistent with data shown in Figure 4, 25 to 30%, of CD1^{high} B cells were CD23$^+$ and therefore follicular B cells, but these cells also expressed high levels of CD21 (Fig. 5, *J* and *L*). A small percentage (1.9% and 3.0%) of the CD1^{high} B cells were CD21$^-$CD23$^-$ (Fig. 5, *J* and *L*). Gating on marginal zone B cells (B220$^+$CD23$^-$CD24$^{low/intermediate}$CD43$^-$) showed that ~50% were CD1^{high} (data not shown).

Next, we next determined the frequency of follicular CD23$^+$ IgM$^+$ IgD^{high} CD21^{high}CD43$^-$ B cells in wt and β_2m$^{-/-}$ C57BL/6 mice. The results shown in Figure 6 demonstrate that the percentage of follicular-type (IgD^{high}) B cells expressing high levels of CD21 and low levels of CD43 was similar in wt and β_2m$^{-/-}$ mice (27% and 24%, respectively). These CD21^{high} follicular cells had the same intensity of staining for CD21 and CD43 as the marginal zone B cells shown in Figure 6C for wt and β_2m$^{-/-}$ mice. Approximately 60% of the CD21^{high} follicular cells (15% of

B cells) from both mice also expressed high levels of IgM (data not shown) and therefore showed all of the characteristics of the marginal zone CD1^{high} subset. In normal mice, 20% of these follicular CD21^{high} cells (3% of B cells) coexpressed high levels of CD1 (data not shown).

Location of CD1^{high} B cells in tissue sections

To determine the location of CD1^{high} B cells in the spleen, laser confocal microscopy was employed. Figure 7 shows three-color immunofluorescent staining of frozen spleen sections from wt C57BL/6 mice (Fig. 7A) and β_2m$^{-/-}$ C57BL/6 mice (Fig. 7B). The primary follicles in Figure 7, *A* and *B*, stained blue-green, expressing high levels of IgD (green) and low levels of IgM (blue). CD1 (red) was expressed strongly on the outer rims of these primary follicles in wt C57BL/6 mice (Fig. 7A, arrows). These CD1$^+$ cells were IgD^{low} and IgM^{high}, and their location indicated that they are marginal zone B cells. A scattering of red-staining cell aggregates was seen in the follicles. The latter cells may correspond to the minority of CD1^{high}CD23$^+$CD21^{high} B cells identified by flow cytometry.

Similar three-color staining of spleens from β_2m$^{-/-}$ mice (Fig. 7B) showed that marginal zone B cells are still present as judged by IgD^{low} (faint green) and IgM^{high} (bright blue) cells at the follicular rims (arrows). However, CD1 expression was not detected. In the β_2m$^{-/-}$ mouse, the periarteriolar lymphatic sheaths (PALS) that were detected by staining with anti-Thy-1.2 mAb were enlarged as compared with the wt mouse (Fig. 7A) (data not shown). In Figure 7B, a central arteriole is indicated by the yellow arrowhead, and multiple B cell follicles can be seen around the PALS. Very strong staining with the 3C11 mAb was observed in the walls of blood vessels and on other scattered cells in the red pulp in both β_2m$^{-/-}$ and wt mice. The latter staining was specific as judged by background control staining with rat IgM myeloma protein (data not shown), but the nature of these positively stained cells was not determined.

T cell recognition of CD1 is not associated with β_2m

We previously established a panel of CD4$^-$CD8$^-$ T cell clones with invariant rearrangements of the Vβ7, Vβ9, or Vβ15 genes from the spleens of BALB/c mice (32). Clones with the invariant Vβ9 rearrangement were associated with an invariant Vα4.4-Ja24 rearrangement (24). Figure 8A shows that a Vβ9/Vα4.4 T cell clone recognized CD1, because it vigorously proliferated in response to the CD1-transfected B cell line A20 but did not proliferate in response to nontransfected A20 cells.

The T cell clone also proliferated in response to LPS-activated, wt BALB/c spleen cells. This response was inhibited by anti-CD1 mAb 3C11 (Fig. 8A) but not by an isotype-matched control mAb. Similarly, LPS-activated, wt C57BL/6 spleen cells induced T cell proliferation that was inhibited by the 3C11 mAb. In the absence of LPS activation, neither BALB/c nor C57BL/spleen cells stimulated a response above background (data not shown). Surprisingly, LPS-activated spleen cells obtained from β_2m$^{-/-}$ mice on the C57BL/6 background also stimulated a response that was inhibited by 3C11 mAb. The same concentration of 3C11 mAb failed to inhibit the [^{3}H]thymidine incorporation of the cloned T cells in response to various concentrations of rIL-2 (data not shown). The 1B1 mAb failed to inhibit the response of the cloned T cells to LPS-activated, wt, and β_2m$^{-/-}$ C57BL/6 spleen cells (data not shown).

Most LPS-activated, wt C57BL/6 spleen cells expressed CD1 (Fig. 8B), but <1% of LPS-activated cells from β_2m$^{-/-}$ mice stained above background (Fig. 8C). A similar staining pattern was observed with the 3C11 mAb, but the intensity of staining of the
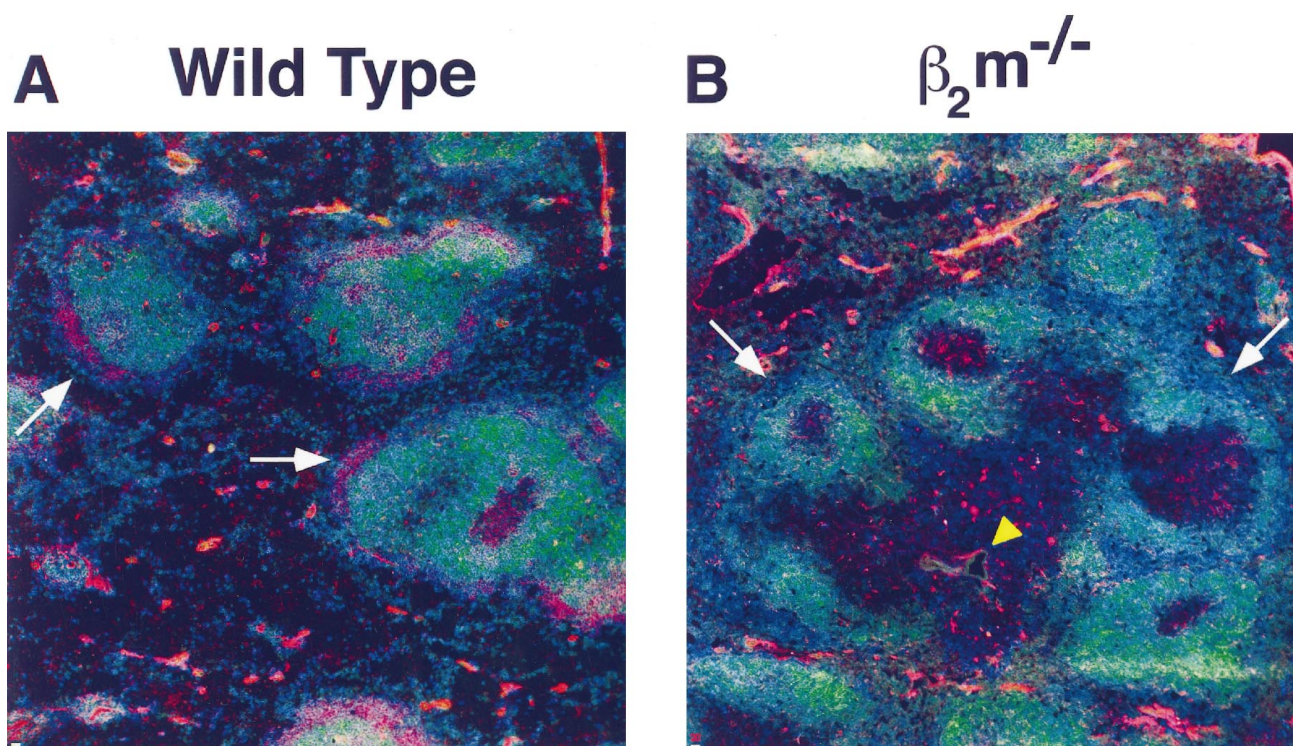
FIGURE 7. Immunohistologic staining of C57BL/6 wt (*A*) and $\beta_2 m^{-/-}$ (*B*) spleen tissue sections. Sections were stained with FITC anti-IgD mAb (green), allophycocyanin anti-IgM mAb (blue), biotinylated anti-CD1 (3C11) mAb, and SA-TRITC (red). The white arrows indicate the location of marginal zone cells, while the yellow arrowheads show the central artery of the PALS.

LPS-activated cells was not as high as that seen with the 1B1 mAb (data not shown).

Discussion

This study has identified two subpopulations of splenic B cells that express high levels of a $\beta_2 m$-dependent form of CD1: marginal zone B cells and a newly defined population of follicular (IgD^{high} CD23$^+$) B cells. This novel population, like the marginal zone B cell population, expresses high levels of CD21. The presence of a $\beta_2 m$-independent form of CD1 on activated murine B cells was indicated by the activation of CD1-restricted T cell clones by LPS-stimulated spleen cells.

The expression of CD1 on some B cell subsets in humans has been described previously (7, 8), and recent studies of both of CD1$^{-/-}$ and CD1$^{+/+}$ mice indicate that CD1 is expressed on most T and B lymphocytes in the spleen (20, 21). In the current study, we used two anti-CD1 (3C11 and 1B1) mAbs for the fluorescent analysis of various B cell populations. The specificity of these mAbs was confirmed by demonstrating their staining of CD1-transfected but not nontransfected B cell lymphoma A20 cells. A striking heterogeneity of CD1 expression was observed on B cell tumor lines, since another BALB/c B cell lymphoma line, BCL1, stained brightly for CD1 (33) in the absence of transfection with CD1 cDNA.

The staining of normal splenic B cells with both anti-CD1 mAbs identified a distinct population, accounting for ~15% of B (B220$^+$) cells, that stained brightly for CD1. The remaining B cells in the spleen, LNs, BM, and PerC showed either dull staining or no staining above background. The majority of non-B cells (mainly T cells) in the spleen stained above background using the 1B1 mAb, but this dull staining was less apparent when the 3C11 mAb was used. In all instances, saturating concentrations of unconjugated anti-FcR (anti-CD16/32) mAb were used to block nonspecific

staining via the FcR, and dead cells were excluded from analysis using PI staining. Furthermore, both bright and dull staining of B cells and dull staining of T cells, as seen with the 1B1 mAb, were markedly reduced in spleen cells from $\beta_2 m^{-/-}$ mice.

Multicolor, flow cytometric analysis demonstrated that ~70 to 75% of CD1^{high} B cells are marginal zone B cells. These cells expressed high levels of IgM, low levels of IgD, and lacked expression of CD23, a phenotype that is found on B-1, immature, and marginal zone B cells (27, 29, 30). Furthermore, the cells lacked expression of CD43 and CD5, which are markers for B-1 cells (28, 29), and expressed high levels of CD21, which is a CR marker found on marginal zone B cells but not on B-1 and immature B cells (31). Further evidence was obtained from immunohistology data, which showed that the majority of CD1^{high} IgM^{high} IgD^{low} B cells were localized to the marginal zone of spleen tissue sections. The staining of the tissue sections of $\beta_2 m^{-/-}$ mice showed that a rim of IgM^{high} IgD^{low} cells still surrounded the follicles; however, these cells did not stain for CD1, which is consistent with the failure to detect CD1^{high} B cells among spleen cells from these mice by FACS. Gating for marginal zone B cells (B220$^+$ CD24$^{low/intermediate}$ CD23$^-$ CD43$^-$) revealed that ~50% of these cells expressed high levels of CD1 (data not shown), and virtually all CD1^{high} cells were also CD21^{high} (Fig. 5).

Approximately 25 to 30% of B220$^+$CD1^{high} cells expressed the IgD^{high} CD23$^+$ phenotype of follicular B cells (27, 29). Interestingly, these cells also expressed high levels of CD21, and most cells were IgM^{high}, which is a phenotype not previously associated with follicular B cells (29, 31). Based on both their high expression of IgD and CD23 and on immunohistology data, which showed occasional aggregates of CD1^{high} cells in splenic primary follicles, we suggest that these CD1^{high} IgD^{high} IgM^{high} CD23$^+$CD21^{high} cells are a subset of follicular B cells. Further immunohistologic
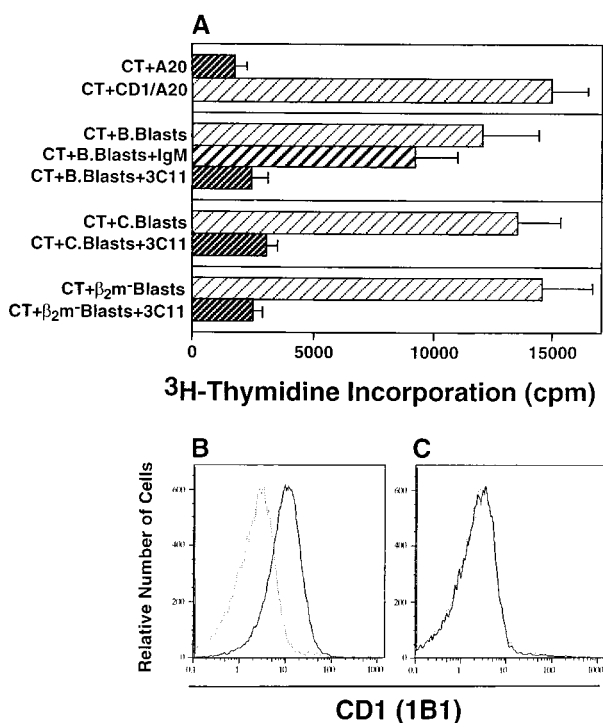
FIGURE 8. [^{3}H]Thymidine incorporation of cloned BALB/c Vβ9/Vα4.4 T cells after stimulation in vitro with B cell lines or LPS-activated spleen cells. *A*, cloned T cells (1×10^4) were incubated for 48 h with 5×10^4 irradiated (4000 cGy) BALB/c A20 B cells that had been transfected with CD1 cDNA or with nontransfected A20 cells. The bars represent the mean of triplicate values and the brackets indicate the SEs. In addition, cloned T cells (1×10^4) were incubated with 5×10^4 irradiated LPS-activated spleen cells from wt BALB/c, wt C57BL/6, or β_2m$^{-/-}$ C57BL/6 mice, respectively. 3C11 mAb (100 μg/ml) or the same concentration of the isotype control (rat IgM) was added to the culture media. The control values of T cells and B cells alone were $<2 \times 10^3$ cpm. *B* and *C*, LPS-activated C57BL/6 wt spleen cells and LPS-activated C57BL/6 β_2m$^{-/-}$ spleen cells were stained with anti-B220 mAb and anti-CD1 (1B1) mAb, respectively. An analysis of gated B220$^+$ cells is shown for the anti-CD1 mAb (solid line) or the isotype control (dotted line). CT = cloned T cells; B. Blasts = C57 LPS blasts; C. Blasts = BALB/c LPS blasts.

data are required to confirm the presence of B cells with this phenotype in splenic follicles. Thus, approximately two-thirds of CD1^{high} B cells expressed the phenotypic characteristics of marginal zone B cells, one-third expressed the characteristics of a novel follicular B cell subset, and the remainder included cells with characteristics of immature B cells, B-1 cells, and B cells that could not be identified with a previously described subset. Both, marginal zone B cells and CD21^{high} IgD^{high} IgM^{high} follicular B cells were found in comparable numbers in β_2m$^{-/-}$ mice, suggesting that the expression of CD1 might not be required for the development of these B cell populations. Very bright CD1-staining cells were also detected by immunohistology in the red pulp, which is consistent with previous reports of expression on blood vessels (34).

Although CD1 expression by splenic cells from β_2m$^{-/-}$ mice was markedly reduced (Figs. 2 and 3), LPS-activated spleen cells from these mice stimulated the proliferation of a CD1-restricted Vβ9/Vα4.4 T cell clone. This T cell proliferation was CD1-dependent, because it was inhibited by the anti-CD1 mAb 3C11 and was also induced by CD1-transfected but not nontransfected A20 cells. Thus, either a β_2m-independent form of CD1 itself or a molecule that cross-reacts with the 3C11 mAb is expressed on the

surface of LPS-activated B cells. This possibility is consistent with recent studies on anti-CD1 T cell hybridomas that have shown that some Vβ14$^-$-expressing T cells recognize a β_2m-independent form of CD1, whereas Vβ14$^+$ hybridomas only recognize a β_2m-dependent form (20). As T cell proliferation was not blocked by 1B1, which was the second anti-CD1 mAb used here, the current study confirms previous data showing that 1B1 recognizes only a β_2m-dependent form of CD1 (20) and indicates that the 3C11 mAb recognized both the β_2m-dependent and the β_2m-independent forms of CD1. The failure of 3C11 to demonstrate a significant expression of CD1 on B cells from wt or β_2m$^{-/-}$ mice as assessed by FACS could suggest that the β_2m-independent form is expressed below the threshold level of detection on most B cells, or that only a small subset ($<1\%$) of B cells in the spleen expresses this form. It is of note that 3% of splenic B cells in β_2m$^{-/-}$ mice showed dull staining for CD1 when a bright counterstain was used in conjunction with the 3C11 mAb (Fig. 3). A β_2m-independent form of CD1d has been reported previously in human gastrointestinal epithelial cells (11).

The T cell recognition of CD1 on the surface of B cells may play an important role in the pathogenesis of systemic lupus. CD4$^+$ and CD8$^+$ T cells expressing anti-CD1 TCR transgenes, which were obtained from the Vβ9/Vα4.4 T cell clone noted above, will induce lupus when transferred into syngeneic BALB/c nude hosts (33). These mice develop anti-dsDNA Abs, proteinuria, and ascites (33). Furthermore, the transgenic T cells can activate wt BALB/c B cells via the cross-linking of surface CD1 to secrete both IgM and IgG in vitro (33). Thus, the interaction between anti-CD1 T cells and B cells expressing surface CD1 leads to a mutual activation of both cell types that results in hypergammaglobulinemia and systemic autoimmunity in vivo (33). More recent studies have shown that the spontaneous secretion in vitro of both IgM and IgG by spleen cells from lupus-prone New Zealand Black/New Zealand White mice is mediated by the CD1^{high} subset of B cells (D. Zeng et al., manuscript in preparation).

It is of interest that the CD21 molecule, which is coexpressed with high levels of CD1 on marginal zone and follicular B cells according to this study, can trap polysaccharides from microorganisms after binding to complement (35). This mechanism, as well as that provided by the mannose receptor (36), may provide a pathway by which B cells expressing CD1 can trap nonprotein Ags and process them via a TAP-independent pathway for presentation on the cell surface via CD1 (37). T cells that recognize such nonprotein Ags in association with CD1 (13, 14) may interact in particular with the CD1/CD21-expressing marginal zone B cells so as to augment their Ab responses to these nonprotein Ags. Thus, the marginal zone may be a critical site for interactions between T and B cells for certain subsets of microbial Ags.

Acknowledgments

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