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# The CD9 Tetraspanin Is Not Required for the Development of Peripheral B Cells or for Humoral Immunity[1]

**Annaiah Cariappa,* Tsipi Shoham,† Haoyuan Liu,* Shoshana Levy,† Claude Boucheix,‡ and Shiv Pillai[2]***

**The CD9 tetraspanin is known to be expressed at high levels on marginal zone (MZ) B cells, B-1 B cells, and plasma cells, and its expression is believed to be dependent on signals derived via Btk. In CD9 null mice, however, the development and survival of MZ B cells, B-1 B cells, and plasma cells all appear to be unaffected, and humoral immune responses to T-dependent and T-independent Ags are similar to those seen in wild-type littermate controls. In wild-type mice, CD9 levels may serve to distinguish between the presumed MZ precursor B cell population in the spleen and other IgD-expressing transitional B cells that express lower levels of CD21 and CD1d. These results suggest that CD9 is dispensable for B cell development and humoral immunity, but that this protein may serve as an additional marker for the presumed MZ precursor population of splenic B cells.** ***The Journal of Immunology,*** **2005, 175: 2925–2930.**

Tetraspanins are integral membrane proteins that contain four transmembrane domains and a conserved extracellular CCG motif. They have been implicated in many functions including cell adhesion, migration, signal transduction, and differentiation. These proteins serve as adaptors for the assembly of large conglomerates of plasma membrane proteins known as tetraspanin webs (reviewed in Refs. 1–3).

In lymphocytes, these tetraspanin webs include integrins as well as coreceptor proteins such as the CD4 and CD8 proteins in T cells, and the CD21/CD19 coreceptor complex in B cells (1–4). The tetraspanin that has been most extensively investigated in a lymphocyte context is CD81, which is part of the coreceptor complex in B cells (4–6). In the absence of CD81, T-dependent immune responses are initially diminished in magnitude but eventually resemble those of wild-type mice (6). Lymphocyte cell surface tetraspanin webs often contain a second prominent tetraspanin called CD9. The CD9 protein is known to be critical for sperm-egg fusion (7) and has also been implicated in myotube formation (8), cell motility (9), and neurite outgrowth (10). CD9 expression in tumors has also been inversely correlated with metastatic potential (11–15).

CD9 is known to be expressed in a very specific manner in certain populations of B lymphocytes (16). CD9 is expressed at high levels in marginal zone (MZ)[3] B cells, B-1 B cells, and in plasma cells, but this protein is barely detectable on resting recirculating follicular (FO) B cells, although it can be induced in these cells upon activation (16). Interestingly, many proteins that are part of tetraspanin webs or involved in integrin signaling are absolutely required for the development of MZ B cells. These include integrins (17), CD19 (18), Pyk2 (19), Lsc (20), and Dock-2 (21). It is particularly intriguing that although *Xid* mice, which harbor a defect in Bruton's tyrosine kinase (Btk), have normal numbers of MZ B cells (22–24), MZ B cells and plasma cells in these mice express little or no CD9 (16).

The high level expression of CD9 on selected B cell subsets and in plasma cells suggested that this protein might be critical either for the development or maintenance of these subsets or that it may be required, as has been suggested previously (16), for the generation of functional humoral immune responses. To address the role of CD9 in peripheral B cell development and in humoral immunity in general, we examined these issues in some detail in CD9 null mice.

## Materials and Methods

### *Mice*

CD9 null mice have been described earlier (7). Animal procedures were cleared by the Subcommittee on Research Animal Care at Massachusetts General Hospital and the Stanford University Committee on Animal Welfare.

### *Abs, staining, and flow cytometry (FCM)*

FCM was performed as described earlier (25, 26). Single-cell suspensions were made from spleen, bone marrow (BM), and peritoneal cavity. For surface staining, $1 \times 10^6$ cells were reacted with 2.4G2 (anti-CD16/CD32 (Fc$\gamma$ III/II receptor)), rat IgG2b, $\kappa$, and culture supernatant (BD Pharmingen) before staining with the following Abs: anti-IgD-biotin clone 11–26 and anti-IgM-PE clone R6-60 (Southern Biotechnology Associates); and anti-IgM$^a$-r-PE clone DS-1, anti-IgD$^a$-biotin clone AMS 9.1, anti-CD21/CD35-FITC clone 7G6, CD9-biotin clone KMC8, CD5-biotin clone 53–7.3, CD11b-allophycocyanin clone M1/70, CD45R-FITC/-PerCP clone RA3-6B2, CD19-PE/-biotin clone 1D3, CD43-FITC clone S7, and CD25-biotin clone 7D4 (all obtained from BD Pharmingen). Biotinylated Abs were revealed using streptavidin-allophycocyanin or -CyChrome (BD Pharmingen). Following staining, cells were analyzed without fixation except for BM cells, which were fixed with 2% paraformaldehyde in PBS (calcium- and magnesium-free).

Flow cytometric analysis was performed either on a dual laser FC500 (Beckman Coulter) or in the case of BM on a FACSCalibur (BD Immunocytometry Systems). Negative controls were used to set voltage, and single-color positive controls were used for electronic compensation. Background staining of the BM cells was determined by staining with isotype

*Massachusetts General Hospital Cancer Center, Harvard Medical School, Boston, MA 02129; †Department of Medicine, Division of Oncology, Stanford University Medical Center, Stanford, CA 94305; ‡Institut André Lwoff, Institut de la Santé et de la Recherche Médicale Unité 602, Hôpital Paul Brousse, Villejuif, France

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[2] Address correspondence and reprint requests to Dr. Shiv Pillai, Massachusetts General Hospital Cancer Center, Building 149, 13th Street, Charlestown, MA 02129. E-mail address: pillai@helix.mgh.harvard.edu

[3] Abbreviations used in this paper: MZ, marginal zone; FO, follicular; FCM, flow cytometry; BM, bone marrow; RT, room temperature; AFC, Ab-forming cell; MZP, MZ precursor.



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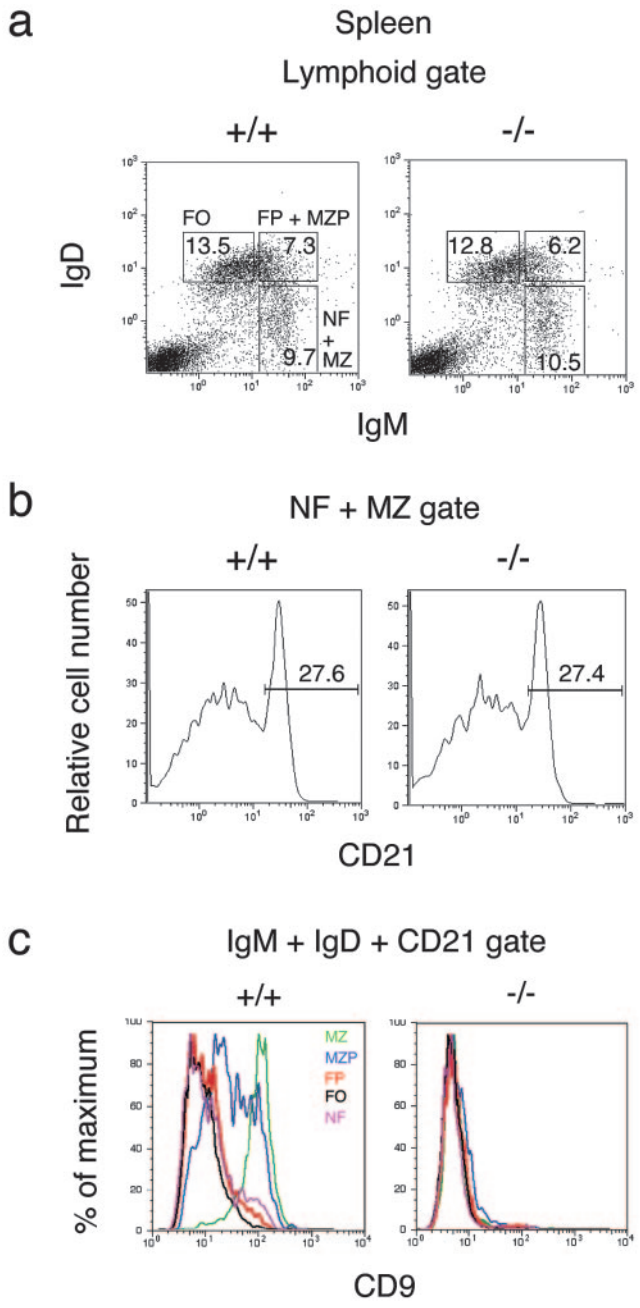


**FIGURE 1.** Newly formed, FO, and MZ B cells develop normally in CD9$^{-/-}$ mice. *a*, IgM/IgD profiles of littermate wild-type (+/+) and CD9 null (−/−) splenocytes are very similar, and no major difference was noted in the IgD$^{high}$IgM$^{low}$ mature FO B cell populations in wild-type and CD9 null mice. Numbers within figures represent percentage of the gate. See also Table I. NF refers to newly formed IgM$^{high}$IgD$^{low}$CD21$^{low}$ B cells. FP refers to IgD$^{high}$IgM$^{high}$CD21$^{int}$ FO precursor B cells, MZP refers to IgD$^{high}$IgM$^{high}$CD21$^{high}$ presumed MZP B cells. *b*, The development of IgM$^{high}$IgD$^{low}$CD21$^{high}$ MZ B cells is unaffected in CD9 null mice. *c*, CD9 is expressed in CD9$^{+/+}$ splenocytes but not in spleen cells from CD9 null mice. MZ B cells, and their putative precursors, MZP B cells express high levels of CD9. Representative results are shown. Three mice were analyzed in each group.

control Abs. Viable cells were determined by forward and side scatter characteristics, and $3 \times 10^4$ to $5 \times 10^4$ gated events were collected. Gates in the spleen for fractions I, II, and III were set according to Hardy et al. (27), and in the BM, gates were set according to Rolink and Melchers (28) and Hardy et al. (29). Processed samples were analyzed using RXP (Beck-

Table I. *Absolute numbers of splenic B cells in wild-type and CD9 null mice*

| Fraction | Absolute Number (mean ± SD) | |
|---|---|---|
| | +/+[a] | −/− |
| FO | $1.3 \times 10^7$ (0.100) | $1.0 \times 10^7$ (0.170) |
| T2-FP + T2-MZP | $6.2 \times 10^6$ (0.702) | $5.4 \times 10^6$ (0.208) |
| MZ | $2.1 \times 10^6$ (0.070) | $2.7 \times 10^6$ (0.212) |

[a] n = 3 mice in each group.

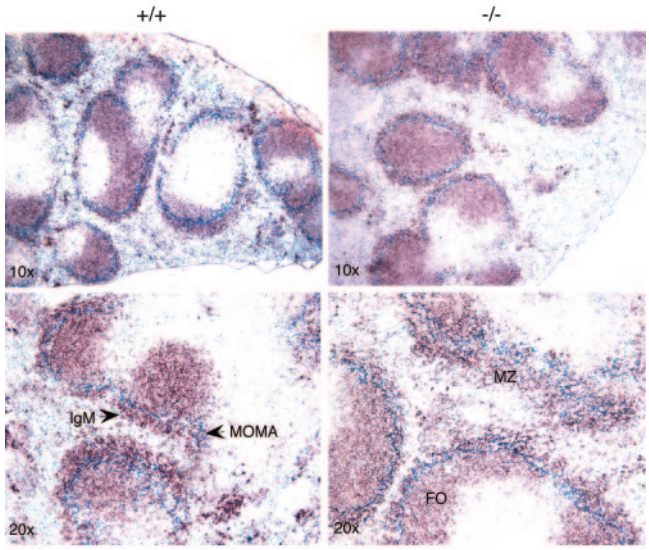


**FIGURE 2.** Immunohistochemical analysis of mouse spleen reveals normal FO and MZ B cell compartments in CD9$^{-/-}$ mice. Cells stained with MOMA represent MZ metallophilic macrophages.

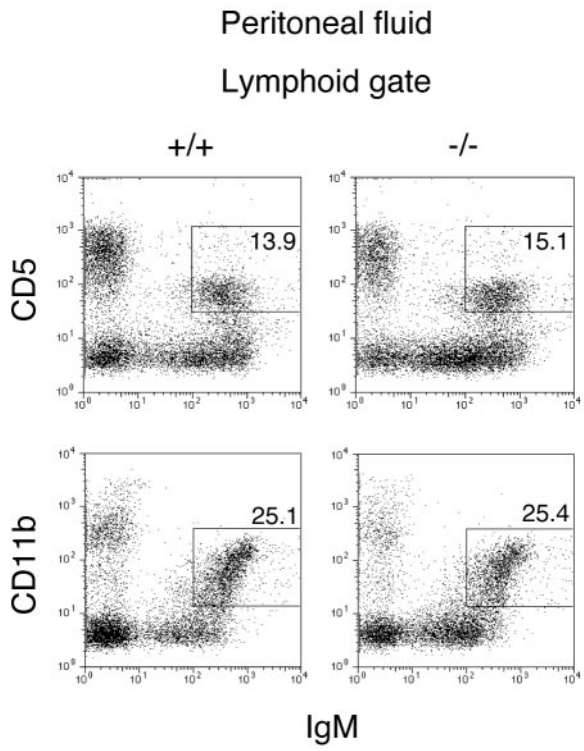


**FIGURE 3.** CD9 is not required for the generation of peritoneal B-1 B cells. No differences were noted in the IgM$^{high}$CD5$^{+}$ or IgM$^{high}$CD11b$^{+}$ peritoneal B cell populations in wild-type and CD9 null mice. Representative results are shown. Three mice were analyzed in each group.

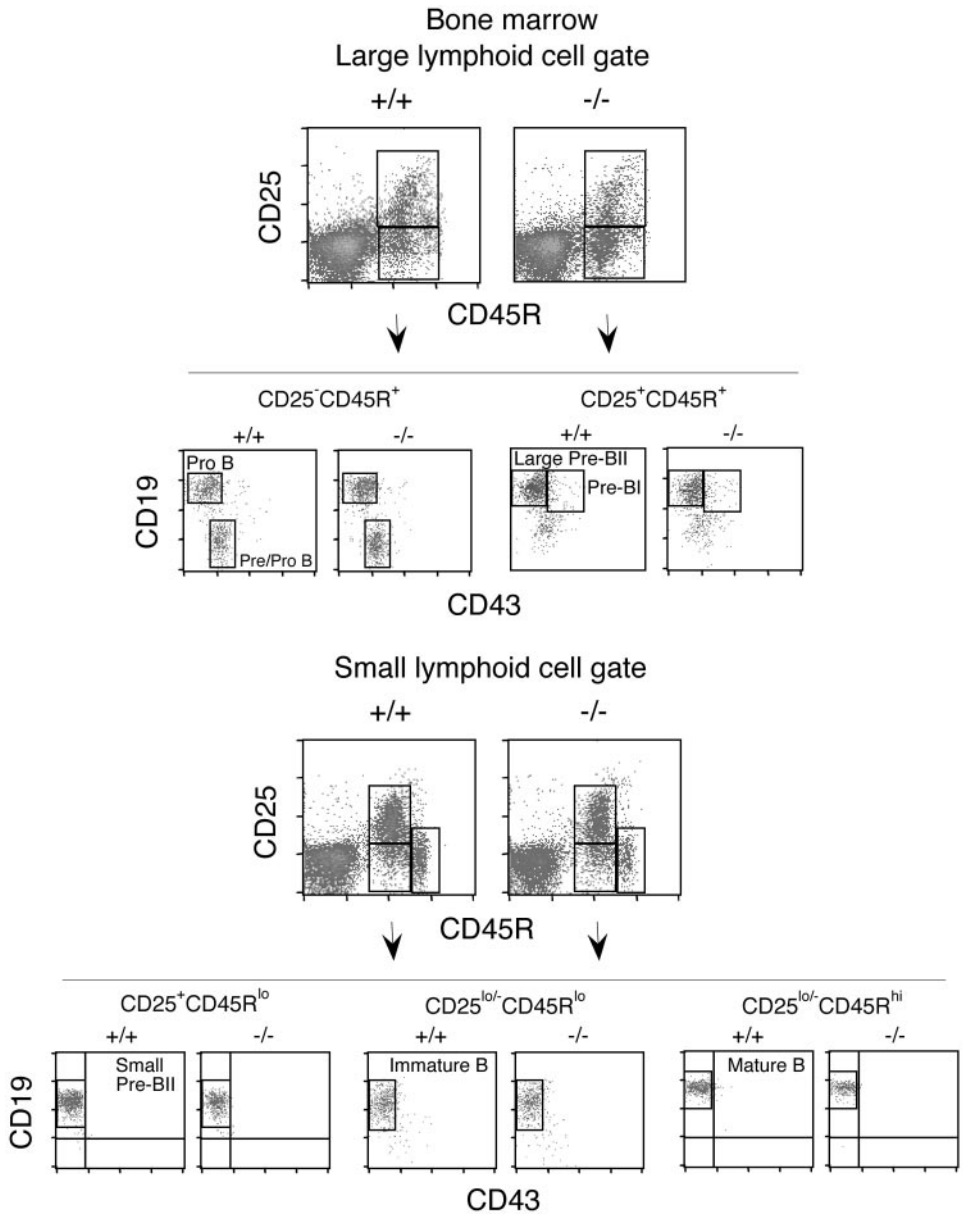


**FIGURE 4.** Normal BM B cell development in mice that lack CD9. Four-color flow cytometric analysis of BM B cell subsets in $CD9^{-/-}$ and $CD9^{+/+}$ mice was performed. Cells were first gated by forward scatter and side scatter as large or small cells. Gates were further set on the small and large cells delineating pre/pro-B (fraction A) cells as $CD43^{low}/B220^{low}/CD19^{-}/CD25^{-}$, pro-B cells (fractions B and C) as $CD43^{low}/B220^{low}/CD19^{+}/CD25^{-}$, small pre-BII cells (fraction D) as $CD43^{-}/B220^{+}/CD19^{+}/CD25^{+}$, immature B (fraction E) as $CD43^{-}/B220^{int}/CD19^{+}/CD25^{-}$, and mature B (fraction F) as $CD43^{-}/B220^{high}/CD19^{+}/CD25^{-}$. See also Table II. Representative results are shown. Three mice were analyzed in each group.

man Coulter), CellQuest (BD Biosciences), and FloJo v6.1.1 (Tree Star) analysis software.

### *Immunohistochemical studies on the spleen*

Immunohistochemistry was performed on frozen sections of the spleen using well-established techniques. Briefly, MZ metallophilic macrophages were stained with MOMA-1 (purified rat IgG2a; Serotec), followed in turn by rabbit $F(ab')_2$ anti-rat IgG (H+L) conjugated to alkaline phosphatase (Southern Biotechnology Associates), and Vector Blue alkaline phosphatase substrate kit III (Vector Laboratories). IgM-bearing B cells were stained with biotinylated rat anti-mouse IgM (clone R6-60.2; BD Pharmingen) followed in turn by Vectastain Elite ABC kit (Standard), and Vector NovaRED (both from Vector Laboratories).

### *IgM and IgG capture ELISPOT assay*

The 96-well filtration plates with Immobilon-P membranes (Millipore) were prewetted with 15 $\mu$l of 70% ethanol, washed thrice with sterile PBS, coated with purified goat anti-mouse IgM or IgG (both from Caltag Laboratories) at a concentration of 10 $\mu$g/ml in a volume of 100 $\mu$l of sterile PBS, and incubated overnight at 4°C. Before seeding, the plates were washed once with PBS, and then blocked with RPMI 1640 containing 10% FBS at 37°C for 2–3 h. A total of $0.5 \times 10^6$ splenocytes was plated in triplicate in a volume of 100 $\mu$l of the above medium used in the blocking step. As controls, triplicate wells without the capture Ab were included for

Table II. *Proportions of BM B lineage subsets in wild-type and CD9 null mice*

| Subset | Percentage (mean ± SD) +/+[a] | −/− |
|---|---|---|
| Pre/Pro-B $B220^{lo}/CD43^{lo}/CD19^{-}/CD25^{-}/IgM^{-}$ | 0.9 (0.036) | 1.1 (0.136) |
| Pro-B $B220^{lo}/CD43^{lo}/CD19^{+}/CD25^{-}/IgM^{-}$ | 1.0 (0.287) | 1.5 (0.499) |
| Pre-BI $B220^{lo}/CD43^{lo}/CD19^{+}/CD25^{+}/IgM^{-}$ | 0.3 (0.030) | 0.2 (0.020) |
| Large Pre-BII $B220^{+}/CD43^{-}/CD19^{+}/CD25^{+}/IgM^{-}$ | 1.7 (0.804) | 1.3 (0.176) |
| Small Pre-BII $B220^{int}/CD43^{-}/CD19^{+}/CD25^{+}/IgM^{-}$ | 4.1 (2.283) | 3.2 (0.715) |
| Immature B $B220^{int}/CD43^{-}/CD19^{+}/CD25^{-}/IgM^{+}$ | 6.9 (2.747) | 5.3 (0.852) |
| Mature B $B220^{hi}/CD43^{-}/CD19^{+}/CD25^{-}/IgM^{+}$ | 1.3 (0.148) | 0.7 (0.181) |

[a] n = 3 mice in each group.

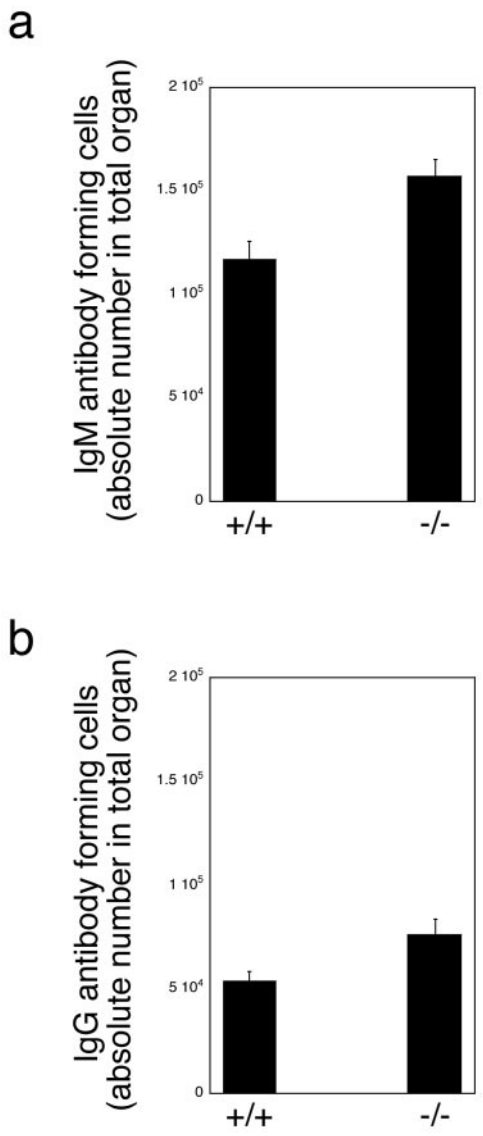


**FIGURE 5.** Plasma cell generation does not require CD9. *a*, IgM AFCs are present in unimmunized CD9 null mice as assessed by ELISPOT assay. *b*, IgG AFCs are also abundantly present in CD9 mutant mice. Error bars represent SEM. There were three mice per group per experiment.

each sample. Following an overnight incubation at 37°C, and 5% $CO_2$, the cells were discarded by flicking the plate. The wells were washed three times with PBS containing 0.05% Tween 20, and three times with PBS, and then incubated at 37°C for 2 h with 2 $\mu$g/ml (50 $\mu$l/well) of prefiltered biotin-conjugated goat anti-mouse IgM or IgG (H+L) mAb (Caltag Laboratories) in PBS containing 1% FBS. They were then washed three times with PBS containing 0.05% Tween 20, and then incubated for 1 h in the dark at room temperature (RT) with 100 $\mu$l/well of diluted Vectastain Elite ABC (Vector Laboratories). The wells were then washed in the same manner as described above, and spots developed with 100 $\mu$l/well of freshly prepared substrate (3-amino-9-ethylcarbazole; Vector Laboratories). The reaction was stopped 10 min later by washing with deionized water, and the plates were dried overnight in the dark at RT. The membranes were transferred onto TopSeal-A clear adhesive film (PerkinElmer Life and Analytical Sciences) using an Eli-Puncher kit (ZellNet Consulting). Spots were counted in a KS ELISPOT Automated Reader System (Carl Zeiss) driven by KS ELISPOT 4.5.21 software (performed by ZellNet Consulting). The number of IgM-specific Ab-forming cells (AFCs)/input cell number and the total organ cell count were used to calculate absolute numbers.

### *Histological staining of Peyer's patches*

Formalin-fixed sections of mouse intestine from wild-type and $CD9^{-/-}$ mice were stained using H&E following standard histological procedures.

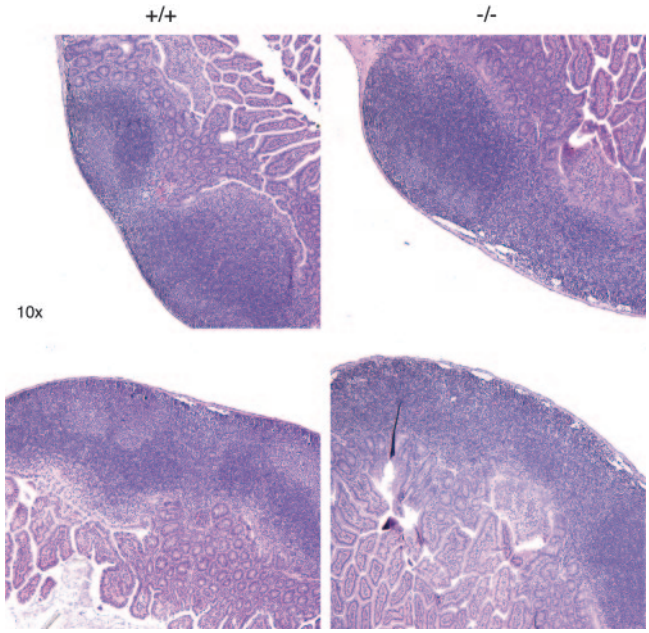


**FIGURE 6.** Peyer's patches from wild-type and $CD9^{-/-}$ mice contain similar distributions of germinal centers.

### *Immunization and assay for anti-TNP Abs*

Immunization and ELISAs were performed as described by Franzoso et al. (30). Briefly, 100 $\mu$g of TNP-KLH, 50 $\mu$g of TNP-LPS, and 25 $\mu$g of TNP-Ficoll (all from Biosearch Technologies) in 500 $\mu$l of sterile 1× PBS were injected i.p. into separate groups of C57BL/6 mice. Sera were collected on day 0 and day 14 postimmunization. Serial dilutions of serum in PBS-Tween were placed in TNP-BSA (Biosearch Technologies)-coated 96-well plates (Immunolon-4; Dynatech Laboratories) and refrigerated overnight. Following three washes with PBS-Tween, the plates were incubated for 1 h at RT with HRP-conjugated goat anti-mouse isotype-specific Abs (Southern Biotechnology Associates), washed three times, and then incubated at RT with the HRP substrate ABTS (Zymed Laboratories). The reaction was stopped with 1% SDS, and absorbance was measured at 405 nm.

### *Statistical analysis*

*p* values for differences between groups was determined by the Mann-Whitney *U* test using StatView version 5.0.1 (SAS Institute).

## Results

We sought to explore whether CD9 contributed to B cell development or humoral immunity. Examination of peripheral B cells in $CD9^{-/-}$ mice revealed essentially normal numbers of mature FO ($IgD^{high}IgM^{low}$, FO) B cells; transitional ($IgD^{high}IgM^{high}CD21^{int}$ T2-FP and $IgD^{high}IgM^{high}CD21^{high}$ T2-MZ precursor; MZP) B cells (Fig. 1*a*); and $IgM^{high}IgD^{low}CD21^{high}$ MZ B cells (Figs. 1*b*, and Table I). These nomenclatures are reviewed in Refs. 31 and 32. MZ B cells of wild-type mice showed the highest levels of CD9 by FCM (Fig. 1*c*, *left panel*), as reported in the literature (16). Interestingly, the levels of CD9 in MZPs, presumed precursors of MZ B cells (24, 32–34), which are FO CD23 and IgD-expressing B cells that express high levels of CD21 and CD1d, displayed the next highest levels of CD9. These data suggest that this tetraspanin may serve as another marker to define the MZP population, and provide indirect support to the notion that MZP B cells represent an intermediate stage during the generation of MZ B cells. The absence of CD9 expression in $CD9^{-/-}$ splenocytes was confirmed (Fig. 1*c*, *right panel*). FO and MZ architecture in the spleen were examined in CD9 null animals and wild-type controls, and no defect was observed in the absence of CD9 (Fig. 2). Peritoneal B-1a

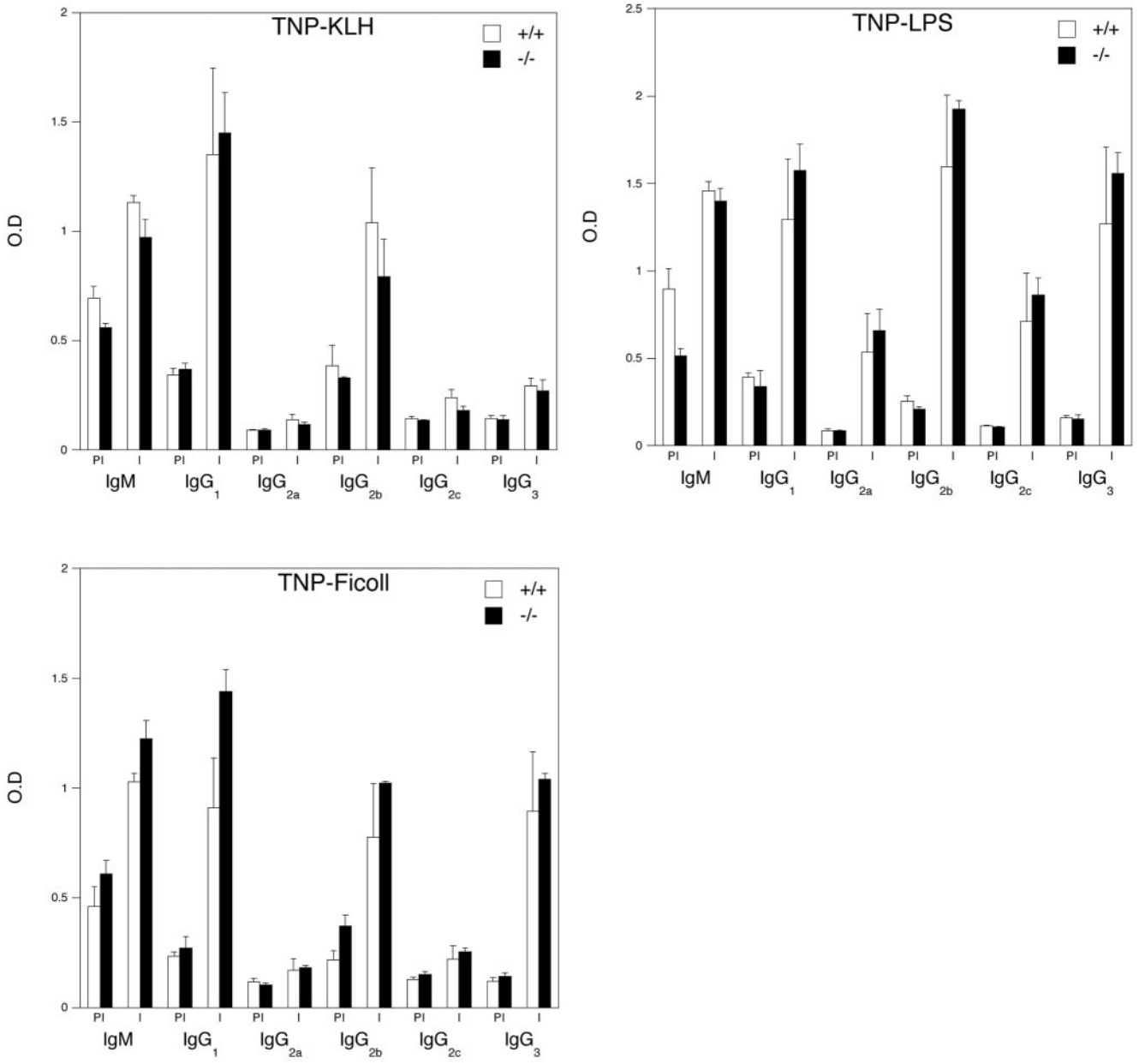


**FIGURE 7.** T-dependent and T-independent immune responses are unaffected in mice that lack CD9. Isotype-specific ELISAs for anti-TNP Abs were performed following immunization with the indicated Ags. PI refers to preimmunization, and I to postimmunization absorbance levels. There were three mice in each pre- and postimmunation group.

and B-1b B cell populations were quantitatively similar in wild-type and CD9 null mice (Fig. 3). Based on these analyses, it appears that CD9 is not required for the development or survival of FO, MZ, or B-1 B cells in mice. In addition, B lineage BM populations were essentially normal in mice lacking CD9 (Fig. 4, and Table II).

A defect in terminal B cell differentiation has been described in *Xid* mice (35), and as mentioned earlier, MZ B cells and plasma cells in these mice express very low levels of CD9 (16). Furthermore, it has been reported that *Xid* B cells lack the ability to terminally differentiate into plasma cells after antigenic triggering. In addition, CD9 is also known to be expressed at high levels in plasma cells. Given the possibility that CD9 might participate in the terminal stages of B cell differentiation, we examined plasma cell populations in the spleen and BM in wild-type and CD9 null mice. As seen in Fig. 5, the unimmunized spleens of both wild-type and CD9 null mice showed broadly similar numbers of IgM (Fig. 5*a*) and IgG (Fig. 5*b*) AFCs. Indeed, a slight increase in IgM ($p$ value $<0.05$) and IgG (statistically not significant) AFCs was observed in CD9 null mice. The relevance of this increase is not understood at this time because immunization studies revealed no differences in Ab responses between wild-type and mutant animals (see below, Fig. 7).

To determine whether germinal center formation in response to environmental Ags was defective in the absence of CD9, we examined sections of the ileum from unimmunized wild-type and CD9 null mice using standard histological approaches. As seen in Fig. 6, germinal centers within Peyer's patches did not vary with respect to frequency and size when comparing wild-type and mutant mice.

To further explore the possibility of a defect in the activation of B cells and Ab secretion in CD9 null mice, wild-type and mutant

mice were immunized with T-dependent and T-independent Ags. As seen in Fig. 7, Ab responses to TNP-KLH, TNP-Ficoll, and TNP-LPS were virtually identical in terms of magnitude and isotype specificity in both wild-type and CD9 null mice.

## Discussion

Our studies indicate that nonredundant functions cannot be ascribed to CD9 in any subset of mature peripheral B cells, because all aspects of B cell development and activation appear to be normal in CD9-deficient mice. CD9 is expressed at high levels in MZ B cells, B1 B cells, and plasma cells, but at extremely low to undetectable levels in mature FO B cells (16). It can be induced on activated FO B cells that are poised to differentiate into plasma cells, but is not, however, expressed in plasma cells from *xid* mice, suggesting that this tetraspanin may be induced downstream of the Ag receptor during T-independent humoral immune responses. These reasons led us to originally consider it likely that this tetraspanin might be required either for the development of specific B lymphocyte subsets or for the optimal generation of humoral immunity in vivo. Given that multiple tetraspanin proteins coexist in tetraspanin webs, it is possible that some function for CD9 in B lineage cells may be revealed in the future in mice lacking more than one tetraspanin.

These studies do suggest, however, that CD9 levels may be modulated during the generation of MZ B cells from FO precursors and suggest that this tetraspanin may represent a useful additional marker for the presumed MZP B cell population.

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## Disclosures

The authors have no financial conflict of interest.

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