Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 11558–11562, December 1995
Immunology

The CD19 signal transduction molecule is a response regulator of B-lymphocyte differentiation

(transgenic mice/immunoglobulin/humoral immunity)

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Communicated by Gordon G. Hammes, Duke University Medical Center, Durham, NC, September 5, 1995

ABSTRACT **The phenotypes of CD19-deficient ($CD19^{-/-}$) mice, and human CD19-transgenic (hCD19TG) mice that overexpress CD19 indicate that CD19 is a response regulator of B-lymphocyte surface receptor signaling. To further characterize the function of CD19 during B-cell differentiation, humoral immune responses to a T-cell-independent type 1 [trinitrophenyl-lipopolysaccharide (TNP-LPS)], a T-cell-independent type 2 [dinitrophenyl (DNP)-Ficoll], and a T-cell-dependent [DNP-keyhole limpet hemocyanin (KLH)] antigen were assessed in $CD19^{-/-}$ and hCD19TG mice. B cells from $CD19^{-/-}$ mice differentiated and underwent immunoglobulin isotype switching *in vitro* in response to mitogens and cytokines. *In vivo*, $CD19^{-/-}$ mice generated humoral responses to TNP-LPS and DNP-KLH that were dramatically lower than those of wild-type littermates. Surprisingly, the humoral response to DNP-Ficoll was significantly greater in $CD19^{-/-}$ mice. In contrast, hCD19TG mice were hyperresponsive to TNP-LPS and DNP-KLH immunization but were hyporesponsive to DNP-Ficoll. These results demonstrate that CD19 is not required for B-cell differentiation and isotype switching but serves as a response regulator which modulates B-cell differentiation. Since humoral responses to both T-cell-dependent and T-cell-independent antigens were similarly affected by alterations in CD19 expression, these differences are most likely to result from intrinsic changes in B-cell function rather than from the selective disruption of B-cell interactions with T cells.**

A hallmark of B-lymphocyte maturation is the expression of surface immunoglobulin (Ig) receptors which provide B cells with the ability to respond to antigen (1). B cells also express other cell surface molecules which regulate antigen responses, development, tolerance induction, proliferation, and differentiation. Among these molecules, CD19 serves as a coreceptor which modifies signals generated through other cell surface receptors (2, 3). CD19 is a M_r 95,000 glycoprotein expressed by early pre-B cells from the time of Ig heavy-chain gene rearrangement until plasma cell differentiation, and by follicular dendritic cells (4–6). Ligation of CD19 *in vitro* initiates a complex series of biological responses linked to B-cell signaling and/or growth regulation (7–11). The extensive cytoplasmic domain of CD19 (6) is likely to be involved in signal transduction, as it associates with the Lyn, Lck, Fyn, and Vav protein-tyrosine kinases (12–14) and also contains kinase insert regions that, when phosphorylated, mediate the binding and activation of phosphatidylinositol 3-kinase (15). Since coligation of CD19 with cell surface Ig can alter the threshold for B-cell signaling induced by anti-Ig antibodies (9, 16–19), the CD19 complex may, in part, deliver protein-tyrosine kinases to the B-cell antigen receptor complex upon crosslinking.

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The phenotypes observed in CD19-deficient ($CD19^{-/-}$) mice and in human CD19-transgenic (hCD19TG) mice that overexpress CD19 suggest that CD19 is a response regulator of cell surface receptor signaling that modulates signaling thresholds for both early negative selection and later clonal expansion of the peripheral B-cell pool (20–22). $CD19^{-/-}$ mice have no defects in B-cell generation in the bone marrow but have a significant reduction in the number of peripheral B cells (20). B cells from $CD19^{-/-}$ mice exhibit markedly decreased proliferative responses to mitogens and have an overall 77% reduction in serum Ig levels, with the most dramatic losses in IgG1 and IgG2a isotypes. In contrast, the development of immature and mature B cells in the bone marrow of hCD19TG mice is significantly impaired, resulting in a substantial decrease (>80%) in peripheral B-cell numbers (21). However, B cells from hCD19TG mice display augmented mitogenic responses to transmembrane signals, and these mice have significantly elevated serum Ig levels (≈40% greater) when compared with wild-type mice, with the greatest effects in IgG2a and IgG2b levels (20, 21). While these findings suggest that CD19 regulates B-lymphocyte selection and activation, the observed alterations in the endogenous serum Ig levels of $CD19^{-/-}$ and hCD19TG mice suggest that CD19 also regulates B-lymphocyte differentiation.

There is a significant decrease in the number of peripheral B cells in $CD19^{-/-}$ mice (20), with the most striking loss in B-1 cells which are the predominant B-lymphocyte subpopulation found in the peritoneal cavity of adult mice (22–25). This suggests that positive signals generated through CD19 are necessary for the generation or maintenance of peripheral B cells, particularly the B-1 population. Although the precise function of B-1 cells in normal mice is unknown (26), they have been reported to be the source for most serum IgM (27). To determine the effect that loss of the B-1 population of cells has on the humoral immune response and to further characterize the function of CD19 during B-cell differentiation, humoral responses to a T-cell-independent type 1 (TI-1), a TI type 2 (TI-2), and a T cell-dependent (TD) antigen were assessed in $CD19^{-/-}$ and hCD19TG mice.

MATERIALS AND METHODS

Mice. $CD19^{-/-}$ mice were generated as described (20). The hCD19TG mouse line h19-1, which expresses 2- to 3-fold higher levels of CD19, was as described (21). All mice were housed under identical conditions in a viral pathogen-free facility.

Abbreviations: DNP, 2,4-dinitrophenyl; TNP, 2,4,6-trinitrophenyl; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide; IL-4, interleukin 4; TI, T-cell-independent; TD, T-cell-dependent; hCD19TG, human CD19-transgenic.

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B-Cell Differentiation Assays. Single-cell suspensions of B cells were isolated from spleens and purified by complement-mediated lysis of T cells with an anti-T-cell receptor monoclonal antibody, H57-597 (American Type Culture Collection). B cells (1×10^5 per well in 96-well flat-bottomed culture plates) were cultured in 0.2 ml of RPMI 1640 medium (GIBCO/BRL) containing 10% fetal bovine serum either alone, with lipopolysaccharide (LPS, *Escherichia coli* serotype 0111:B4; Sigma), or in the presence of LPS plus recombinant mouse interleukin 4 (IL-4; PharMingen) for 5 days. Ig secreted into the culture medium was determined by ELISA.

Immunization of Mice. Two-month-old mice were immunized i.p. with 100 μg of 2,4-dinitrophenyl-conjugated keyhole limpet hemocyanin (DNP-KLH; Calbiochem–Novabiochem, La Jolla, CA) in complete Freund's adjuvant and were boosted 21–23 days later. Also, mice were immunized i.p. with 50 μg of 2,4,6-trinitrophenyl (TNP)-LPS (Sigma) or 25 μg of DNP-Ficoll (Biosearch) in saline. Mice were bled before and after immunization, and hapten-specific serum antibody levels were measured by ELISA.

Mouse Ig Isotype-Specific ELISA. Antibody levels in individual samples of serum or culture medium were determined in duplicate by ELISA as described (20). DNP- or TNP-specific antibody titers were measured similarly by using ELISA plates coated with DNP-bovine serum albumin (DNP-BSA; Calbiochem–Novabiochem) or TNP-BSA (Biosearch). Relative levels of antigen-specific IgM and IgG were determined for each group of mice by using pooled serum samples. Sera were diluted at logarithmic intervals (1:10 to $1:10^6$) and assessed for relative Ig levels as above except that the results were plotted as OD versus dilution (logarithmic scale). The dilutions of sera giving half-maximal OD values were determined by linear regression analysis, thus generating values in arbitrary units/ml that could be compared between sets of sera.

Immunohistochemistry. Serial frozen tissue sections of spleen were fixed in acetone and incubated with biotinylated peanut agglutinin (Sigma), goat anti-mouse IgM-biotin (Southern Biotechnology Associates), or rat anti-mouse IgDb-biotin [Ig(5b)6.3; American Type Culture Collection] at predetermined optimal dilutions in saline with 2% fetal bovine serum for 30 min at room temperature. The sections were washed and the primary reagent was detected by use of an ABC alkaline phosphatase kit (Vector Laboratories).

Statistical Analysis. All data are shown as mean values ± SEM. Analysis of variance (ANOVA) was used to analyze the data, and Student's *t* test was used to determine the level of significance of differences in population means.

RESULTS

***In Vitro* Differentiation of B Lymphocytes from CD19$^{-/-}$ Mice.** The capacity of purified B cells from CD19$^{-/-}$ and wild-type littermates to differentiate in response to LPS stimulation was compared. Background levels of Ig secretion were similar for CD19$^{-/-}$ and wild-type B cells cultured in medium alone (Fig. 1). Stimulation of B cells from seven CD19$^{-/-}$ mice with LPS resulted in IgM secretion that was 43 ± 14% lower than with wild-type B cells. When B cells were cultured with LPS and IL-4, there was a predominant switch to IgG1 production (Fig. 1) which was only 11 ± 34% lower (n = 5 mice) in CD19$^{-/-}$ B cells. Therefore, loss of CD19 expression does not block the ability of B cells to differentiate or switch isotypes when they receive the appropriate stimulus.

Humoral Response of CD19$^{-/-}$ and hCD19TG Mice to a TI-1 Antigen. The influence of CD19 on immune responses was assessed by immunizing mice with TNP-LPS. Hapten-specific IgM levels were 69–75% lower ($P < 0.04$) for CD19$^{-/-}$ mice when compared with wild-type littermates, both before and following immunization (Table 1; Fig. 2*A*). Although the IgG3 response was initially more vigorous in CD19$^{-/-}$ mice, the overall IgG response at day 14 was 94% lower in CD19$^{-/-}$ mice (Table 1), with lower IgG1 and IgG2b responses (Fig. 2*A*). The IgA response of CD19$^{-/-}$ mice was similar to that of control littermates.

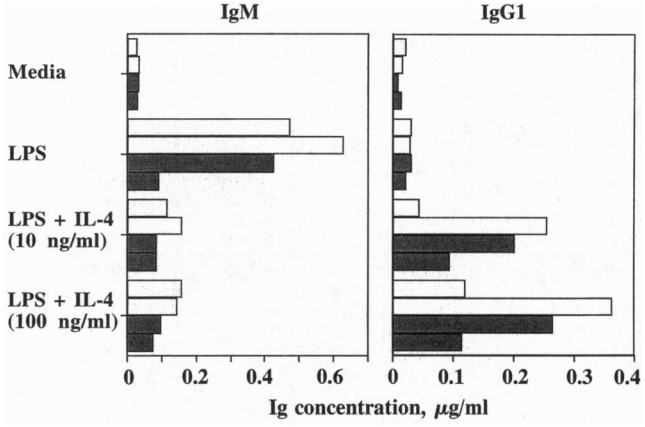

FIG. 1. *In vitro* differentiation of B cells from CD19$^{-/-}$ mice. Purified spleen B cells from two CD19$^{-/-}$ (filled bars) and two wild-type (open bars) littermates were cultured with LPS (20 μg/ml) and IL-4 as indicated. Values represent the mean Ig levels obtained from duplicate cultures. Results represent those obtained with five to seven pairs of mice of the indicated genotypes.

hCD19TG mice had an overall increase in serum Ig levels in response to TNP-LPS immunization (Fig. 2*B*) despite their dramatic reduction in peripheral B-cell numbers (21). On average, baseline hapten-specific IgM levels were higher in hCD19TG mice than in wild-type littermates and were 2-fold higher ($P < 0.03$) following immunization (Table 1). The IgG response was similar to that of wild-type littermates, with significantly higher IgG1 (1 week, $P < 0.05$), IgG2b ($P \leq 0.01$), and IgA ($P < 0.05$) responses. Thus, B cells that overexpress CD19 were hyperresponsive to this TI-1 antigen, whereas CD19$^{-/-}$ mice were hyporesponsive.

Humoral Response of CD19$^{-/-}$ and hCD19TG Mice to a TI-2 Antigen. The hapten-specific IgM response of CD19$^{-/-}$ mice to immunization with DNP-Ficoll was 4-fold higher (P = 0.03) than in wild-type littermates 2 weeks after immunization (Table 1; Fig. 3*A*). Similarly, the IgG response of CD19$^{-/-}$ mice was 20-fold higher ($P < 0.01$) on day 14 (Table 1). Significantly increased ($P \leq 0.03$) serum responses for IgG1, IgG2a, IgG2b, and IgG3 were observed in CD19$^{-/-}$ mice in comparison with wild-type littermates, whereas IgA responses were modest in all mice (Fig. 3*A*). Therefore, the humoral response to DNP-Ficoll was significantly enhanced in the absence of CD19.

Table 1. Ig levels in CD19$^{-/-}$ and hCD19TG mice relative to wild-type (WT) littermates

		CD19$^{-/-}$/WT, %		hCD19TG/WT, %	
Immunogen	Day	IgM	IgG	IgM	IgG
None*	0	25	21	120	148
TNP-LPS	14	30	6	198	80
DNP-Ficoll	14	414	2087	30	33
DNP-KLH	16	5	8	274	51
DNP-KLH	28–30	17	4	216	80

Values represent the percentage of each Ig isotype relative to wild-type littermates as determined by ELISA. ELISA data were analyzed as described in *Materials and Methods* to generate arbitrary units/ml which could be directly compared between test and wild-type mice. Ig levels were determined from pooled samples of the sera shown in Figs. 2–4.

*Values for unimmunized mice were as published (20).

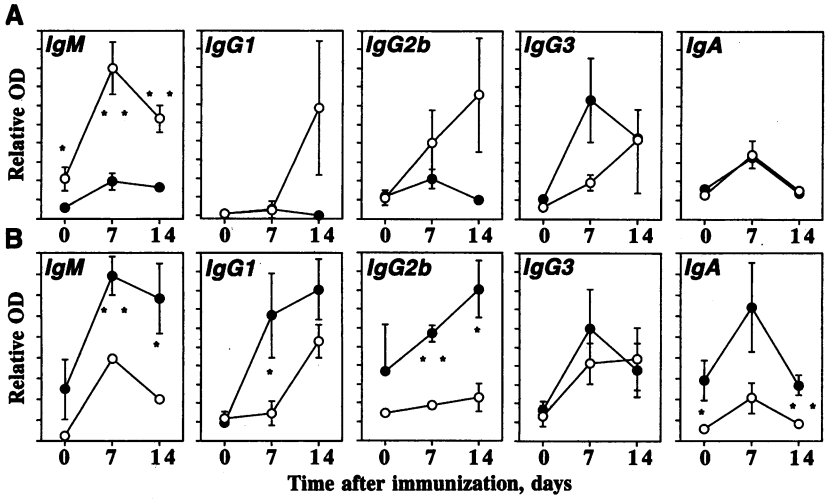

FIG. 2. Humoral immune responses of CD19$^{-/-}$ and hCD19TG mice to immunization with a TI-1 antigen. Two-month-old CD19$^{-/-}$ (five mice, ●) and wild-type (five mice, ○) littermates (*A*) or 2-month-old hCD19TG (four mice, ●) and wild-type (five mice, ○) littermates (*B*) were injected i.p. with 50 μg of TNP-LPS on day 0 and bled on the indicated days. Values are mean (± SEM) OD units from ELISAs. Similar results were obtained with wild-type mice in *A* and *B*, but the results are shown on different relative scales to highlight the differences between test and control mice. IgG2a responses were low and are therefore not shown. Differences between wild-type littermates and CD19$^{-/-}$ or hCD19TG mice were significant: *, $P < 0.05$; **, $P < 0.01$.

hCD19TG mice had only a modest humoral response to DNP-Ficoll, with ≈70% lower IgM and IgG responses than wild-type littermates (Table 1; Fig. 3*B*). The IgG3 response was lower by a factor of 100 in hCD19TG mice (Fig. 3*B*). Thus, overexpression of CD19 inhibited humoral responses induced by DNP-Ficoll and significantly skewed the IgG3 response.

Humoral Response of CD19$^{-/-}$ and hCD19TG Mice to a TD Antigen. CD19$^{-/-}$ mice immunized with DNP-KLH mounted only modest primary IgM, IgG, and IgA responses which were reduced by a factor ≥10 ($P \leq 0.01$) when compared with wild-type littermate responses (Table 1; Fig. 4*A*). Secondary IgM, IgG, and IgA responses following antigen challenge were also significantly lower ($P \leq 0.01$) than those in wild-type littermates. Therefore, mice do respond to TD antigens in the absence of CD19, although at significantly reduced levels.

hCD19TG mice had significantly increased primary and secondary IgM responses (2- to 3-fold, $P < 0.02$) to DNP-KLH when compared with wild-type littermates (Table 1; Fig. 4*B*). Although hapten-specific IgG1 and IgA responses were similar to those observed in wild-type littermates, primary IgG2a, IgG2b, and IgG3 responses were less than half of those induced in wild-type mice (Fig. 4*B*). The IgG3 response of hCD19TG mice was also low following secondary challenge. Thus, CD19 overexpression enhanced the humoral response to a TD antigen yet significantly skewed the production of different Ig isotypes.

Germinal-Center Formation in CD19$^{-/-}$ and hCD19TG Mice. The frequency of germinal centers that developed in spleen lymphoid follicles following immunization was assessed by immunohistochemical staining with peanut agglutinin. Consistent with the general hyporesponsive nature of CD19$^{-/-}$ mice, their spleens had a significantly ($P < 0.04$) lower frequency of germinal centers than wild-type littermates, both before and after immunization (Table 2). Although germinal centers were identified in CD19$^{-/-}$ mice, they were consistently smaller than those of wild-type littermates (Fig. 5). The frequency of germinal centers in unimmunized hCD19TG mice was similar to that observed in wild-type littermates (Table 2). However, the number of germinal centers in hCD19TG mice did not increase in response to immunization and they were smaller than those observed in wild-type littermates (Fig. 5).

DISCUSSION

The observed effects of CD19 deficiency and CD19 overexpression on B-cell responses provide considerable insight into CD19 function *in vivo*. Although B cells from CD19$^{-/-}$ mice are generally hyporesponsive to mitogenic signals, they were able to differentiate *in vitro* in response to LPS stimulation (Fig. 1). Further, LPS plus IL-4 induced Ig isotype switching with IgG1 secretion similar to that observed with wild-type B cells (Fig. 1). Consistent with this, CD19$^{-/-}$ mice generated humoral responses to TI and TD antigens (Figs. 2–4) despite their 55% decrease in peripheral B-cell numbers and their overall decrease in serum Ig levels (20). Although immune responses to TNP-LPS and DNP-KLH were modest (Table 1), CD19$^{-/-}$ mice generated a humoral response to DNP-Ficoll that was dramatically greater than that of wild-type littermates (Fig. 3*A*; Table 1). In contrast, hCD19TG mice were hyperresponsive to immunization with TNP-LPS (Fig. 2*B*) and DNP-KLH (Fig. 4*B*) but were hyporesponsive to DNP-Ficoll

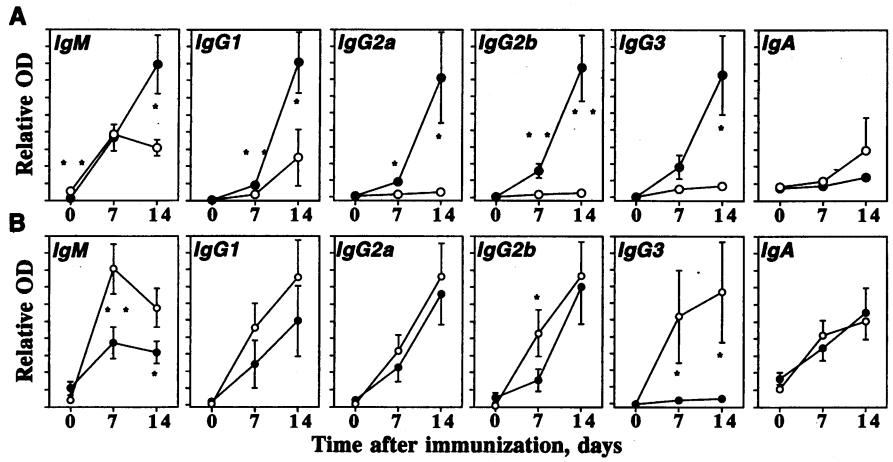

FIG. 3. Humoral immune responses of CD19$^{-/-}$ and hCD19TG mice to immunization with a TI-2 antigen. Two-month-old CD19$^{-/-}$ (5 mice, ●) and wild-type (5 mice, ○) littermates (*A*) or 2-month-old hCD19TG (10 mice, ●) and wild-type (9 mice, ○) littermates (*B*) were injected i.p. with 25 μg of DNP-Ficoll on day 0 and bled at the indicated times. Results are mean (± SEM) OD units from ELISAs. Similar results were obtained with wild-type mice in *A* and *B*, but the results are shown on different relative scales to highlight the differences between test and control mice. Differences between wild-type littermates and CD19$^{-/-}$ or hCD19TG mice were significant; *, $P < 0.05$; **, $P < 0.01$.

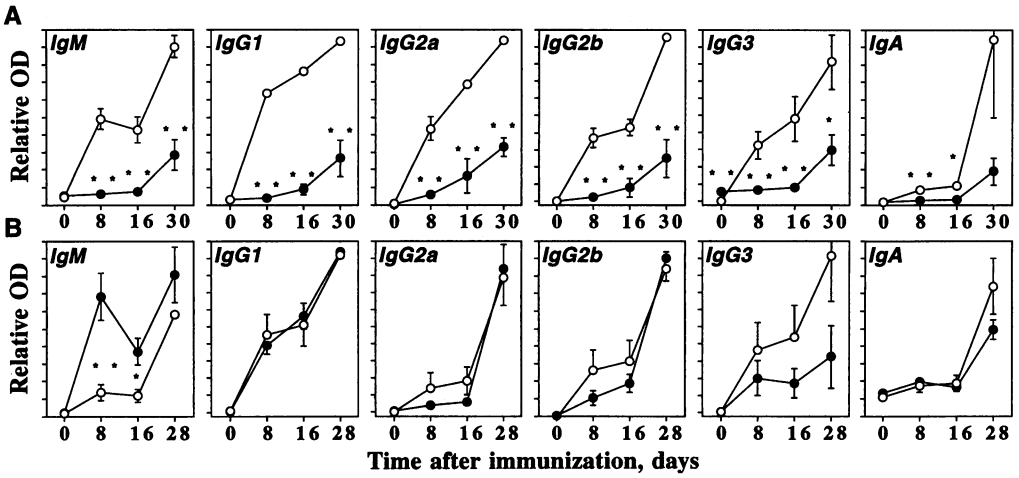

FIG. 4. Humoral immune responses of CD19$^{-/-}$ and hCD19TG mice to immunization with a TD antigen. Two-month-old CD19$^{-/-}$ (five mice, ●) and wild-type (seven mice, ○) littermates (*A*) or hCD19TG (five mice, ●) and wild-type (five mice, ○) littermates (*B*) were injected i.p. with 100 μg of DNP-KLH in complete Freund's adjuvant on day 0 and bled on the indicated days. All mice were challenged with antigen on day 23 (*A*) or 21 (*B*). Results are mean (± SEM) OD units from ELISAs. Similar results were obtained with wild-type mice in *A* and *B*, but the results are shown on different relative scales to highlight the differences between test and control mice. Differences between wild-type littermates and CD19$^{-/-}$ or hCD19TG mice were significant; *, $P < 0.05$; **, $P < 0.01$.

(Fig. 3*B*). Therefore, it appears that CD19 is not required for B-cell differentiation or isotype switching but, rather, serves as a response regulator that ultimately controls the magnitude of B-cell differentiation.

The molecular basis for the observation that CD19$^{-/-}$ mice were hyperresponsive to DNP-Ficoll immunization while hCD19TG mice were hyporesponsive to this antigen is unknown (Table 1) but may relate to the fact that TI-2 antigens induce B-cell proliferation and differentiation through fundamentally different signal transduction mechanisms than TI-1 or TD antigens (28). The loss or overexpression of CD19 may have radically different effects on these signaling pathways. Alternatively, the unexpected humoral response to DNP-Ficoll could correlate with the hypothesized role for CD19 in early B-cell tolerance induction (2). If CD19 is involved in establishing the signaling thresholds necessary for clonal selection in the bone marrow, a lack of CD19 expression could allow a larger number of B cells with low-affinity surface receptors for Ficoll to mature and enter the circulation, resulting in increased peripheral responses. In the case of hCD19TG mice, overexpression of CD19 could enhance clonal deletion during early B-cell development and diminish specific humoral responses. However, since spleen marginal-zone B cells are thought to be the primary source of TI-2 responses (28), a critical analysis of the effects of altered CD19 expression on this unique population of B cells is warranted.

Table 2. Germinal-center formation in CD19$^{-/-}$ and hCD19TG mice

	Frequency, %			
	CD19$^{-/-}$ littermates		hCD19TG littermates	
Immunogen	Wild type	CD19$^{-/-}$	Wild type	hCD19TG
Control	22 ± 7	0.5 ± 0.3*	12 ± 3	15 ± 4
TNP-LPS	30 ± 10	0.3 ± 0.3*	9 ± 3	13 ± 7
DNP-Ficoll	35 ± 8	4 ± 2**	19 ± 2	17 ± 1
DNP-KLH	71 ± 3	4 ± 2**	57 ± 9	18 ± 1*

Spleens from three or four mice of each genotype were assessed for frequency (%) of germinal centers among follicles. Serial tissue sections from three separate regions of each spleen were evaluated. Values represent the mean percentage ± SEM. Differences between wild-type littermates and CD19$^{-/-}$ or hCD19TG mice were significant: *, $P < 0.05$; **, $P < 0.01$.

Formation of germinal centers is important for the development of secondary immune responses, isotype switching, and affinity maturation of B cells. Although the number of follicles in the spleen of CD19$^{-/-}$ mice is not significantly changed (20), germinal centers in the spleens of unimmunized or immunized CD19$^{-/-}$ mice were significantly reduced in frequency (>95%, Table 2) and size (Fig. 5). The frequency of germinal centers in unimmunized hCD19TG mice was similar to that observed in control littermates (Table 2). However, the germinal centers were smaller than those of wild-type littermates and the number of germinal centers did not increase with DNP-KLH immunization, despite augmented Ig production. Therefore, overexpression of CD19 appears to significantly enhance Ig production without significant increases in germinal-center formation. This may result from CD19 extending the lifetime of antibody-secreting cells *in vivo*, as reported earlier for cultured B cells (20), resulting in increased overall Ig production without a need for significant clonal expansion. Alternatively, overexpression of CD19 may augment transmembrane signals so that cell–cell interactions provided in germinal centers, which are necessary for isotype switching and affinity maturation, are not required to the same extent. Although a requirement for CD19 in follicular dendritic cell function is unknown, it is also possible that some of the effects manifest in CD19$^{-/-}$ mice result from alterations in follicular dendritic cell function.

Although the striking loss of the B-1 population of cells within the peritoneum of CD19$^{-/-}$ mice (20, 22) would be expected to have a significant effect on serum IgM levels, the levels of other Ig isotypes which are thought to be predominantly produced by B-2 cells are reduced to a similar extent (20). Skewed Ig isotype responses were also observed in CD19$^{-/-}$ and hCD19TG mice following immunization (Figs. 2–4). These differences did not correlate directly with the loss of a particular known B-cell function or single cytokine response but clearly suggest that T cell–B cell interactions or cytokines which foster the generation of these isotypes may be particularly affected by loss or overexpression of CD19. In view of the critical role that the B-cell-activating agent plays in determining the effects that individual lymphokines have on Ig production and on isotype selection (29), it is not surprising that the alterations in susceptibility to transmembrane signals manifest in CD19$^{-/-}$ and hCD19TG mice skew the humoral response in such unpredicted ways.

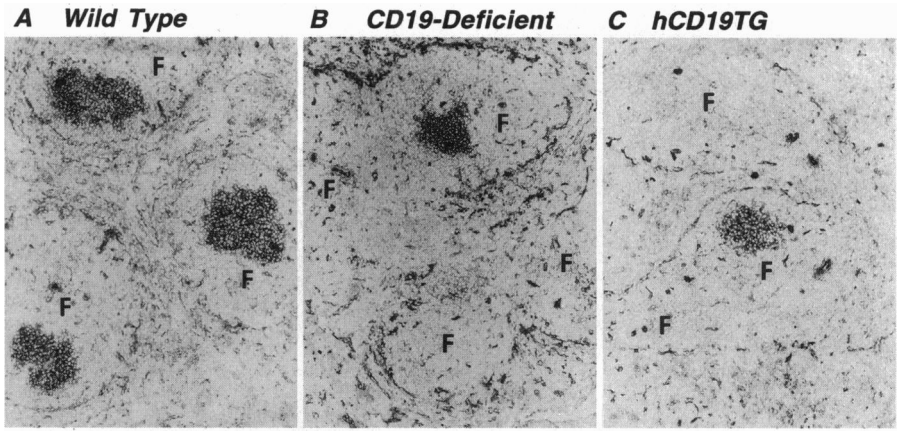

FIG. 5. Germinal-center formation in CD19$^{-/-}$ and hCD19TG mice. Wild-type (*A*), CD19$^{-/-}$, (*B*) or hCD19TG (*C*) mice were immunized with DNP-KLH as in Fig. 4 and their spleens were harvested on days 28–30 for immunohistochemistry. Sections were stained with peanut agglutinin to detect germinal-center B cells. Follicles (F) were identified by staining serial sections with anti-IgM or anti-IgD antibodies (data not shown). Results are representative of those obtained with spleens from unimmunized mice and mice immunized with DNP-Ficoll and TNP-LPS. (×100.)

Some differences in results have been reported by the two groups that have generated CD19$^{-/-}$ mice (20, 22). In contrast to the mice described in this report, CD19$^{-/-}$ mice generated by Rickert *et al.* (22) have normal numbers of spleen and lymph node B cells, normal humoral responses to a TI-2 antigen (4-hydroxy-3-nitrophenylacetyl-Ficoll), and no germinal-center formation, and B cells from their mice respond normally to B-cell mitogens (22). On the basis of these data, Rickert and colleagues have concluded that the loss of CD19 selectively impairs TD responses. Although the basis for these differences is not known, our findings that hCD19TG mice which overexpress CD19 generate reciprocal results from those obtained with our CD19$^{-/-}$ mice provides additional support to the conclusions of our studies. Nonetheless, the results of this study suggest that decreased serum Ig levels in CD19$^{-/-}$ mice result from decreased B-cell responses to external stimuli rather than the inability of stimulated cells to terminally differentiate. Since humoral responses to both TI and TD antigens were affected similarly by alterations in CD19 expression, the variations in CD19$^{-/-}$ mice are most likely to result from intrinsic changes in B-cell function rather than the selective disruption of T cell–B cell interactions. Therefore, taken together, our results confirm the crucial role of CD19 in B-cell activation but suggest that variations in isotype switching, antibody production, and germinal-center formation may result from alterations in signaling thresholds rather than a strict requirement for CD19 in these processes.

We thank Drs. Pablo Engel, Liang-Ji Zhou, and Michael McHeyzer-Williams for helpful discussions. This work was supported by National Institutes of Health Grants AI26872, HL50985, CA34183, and CA54464 and funding from the North Carolina Biotechnology Center. T.F.T. is a Stohlman Scholar of the Leukemia Society of America.

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