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Targeted Inactivation of the Tetraspanin CD37 Impairs T-Cell-Dependent B-Cell Response under Suboptimal Costimulatory Conditions

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CD37 is a membrane protein of the tetraspanin superfamily, which includes CD9, CD53, CD63, CD81, and CD82. Many of these molecules are expressed on leukocytes and have been implicated in signal transduction, cell-cell interactions, and cellular activation and development. We generated and analyzed mice deficient for CD37. Despite the high expression of CD37 on cells of the immune system, no changes in development and cellular composition of lymphoid organs were observed in mice lacking CD37. Analyses of humoral immune responses revealed a reduced level of immunoglobulin G1 (IgG1) in the sera of nonimmunized mice and an alteration of responses to T-cell-dependent antigens. Antibody responses to model antigen administered in the absence of adjuvant and to viral infections were generally poor in CD37-deficient mice. These poor antibody responses could be overcome by the immunization of antigen together with adjuvant. These results suggest a role for CD37 in T-cell–B-cell interactions which manifests itself under suboptimal costimulatory conditions.

CD37 belongs to the tetraspanin superfamily, which includes CD9, CD53, CD63, CD81, CD82, and a growing number of other proteins (18, 35). Molecules of the tetraspanin superfamily are characterized by the presence of four conserved transmembrane regions. Although more than 16 members with broad tissue distribution and high conservation among different species have been described so far, little is known about the molecular functions of these molecules in vivo.

CD37 was originally described as a cell surface glycoprotein expressed on mature human B cells but not on pro-B cells or plasma cells. T cells and monocytes express CD37 at low levels (28). Consistent with this observation, murine CD37 expression was shown to be restricted to lymphoid tissues and cell lines of lymphoid and myeloid origin, with the highest expression in a B-lymphoma cell line (32).

Many of the tetraspanins are detected in complexes with integrins, other tetraspanins, major histocompatibility complex (MHC) class II molecules, and costimulatory molecules. Tetraspanins are implicated in the regulation of cell-cell adhesion, signal transduction, and cellular activation. Human CD37 in particular can be coprecipitated with MHC class II molecules, components of the B-cell signal-transducing complex (CD19 and CD21), and other tetraspanins (CD81, CD82, and CD53) (1). CD37 is downregulated upon B-cell activation (27), and monoclonal antibodies against human CD37 were shown to modulate B-cell proliferation (14), suggesting an important role for CD37 in B-cell function. CD37 has also been found together with other tetraspanins, the costimulatory molecule B7.2 and MHC class II molecules, to be enriched in exosomes (7). These membrane-bound vesicles are secreted by B cells and are capable of effective antigen presentation to T cells (25, 36).

In order to gain more insight into CD37 function in vivo, we generated mice lacking CD37 gene expression by homologous recombination in embryonic stem cells. CD37-deficient mice show altered immune responses when challenged with viral and soluble model antigens. Although CD37 is differentially expressed during the maturation of human B cells, the analysis of CD37-deficient mice indicates that CD37 is not essential for B-cell development. Instead, our results suggest that CD37 plays an important role in B-cell function. The data indicate that CD37, like CD81, may act as a nonclassical costimulatory molecule or directly influence antigen presentation via complex formation with MHC class II molecules.

MATERIALS AND METHODS

Generation of CD37$^{-/-}$ mice. From a genomic clone of the CD37 gene derived from a 129sv λ-FixII Bank (Stratagene) (32), the target vector pPNTCD37 was generated. As a 3′ homolog, a 2-kb *Nco*I/*Nco*I fragment was cloned blunt ended in the *Xho*I site of pPNT (34). The plasmid was opened by *Eco*RI digestion, and a 2.5-kb *Xmn*I/*Nco*I fragment as a 5′ homolog was inserted by blunt end cloning. After linearization, 25 μg of the target vector was electroporated in E14 embryonic stem cells (11). Cells were grown under double selection (200 μg of G418 per ml, 2 μM ganciclovir), and genomic DNA from resistant colonies was tested for homologous recombination by Southern hybridization (30). From targeted embryonic stem cells, chimeras were generated by standard techniques (10). Upon germ line transmission, animals carrying the mutant CD37 allele were intercrossed. All assays were performed on mice of 129/Ola/C57BL/6 mixed background.

RNA analysis. Northern blot analysis was performed using standard techniques with total spleen RNA isolated by Trizol reagent (GIBCO BRL).

Antibodies and flow cytometry. Single-cell suspensions were prepared from the thymuses, spleens, bone marrow, and lymph nodes of 6- to 8-week-old mice. Peripheral blood was obtained by tail bleeding, and peritoneal cavity cells were obtained by peritoneal lavage with 0.34 M saccharose solution. All cells were subjected to hypotonic lysis of red blood cells by 12 min of incubation in a solution containing 150 mM NH_4Cl, 15 mM Na_2CO_3, and 0.1 mM EDTA (pH

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7.3), followed by washing in phosphate-buffered saline (PBS) containing 2% newborn calf serum, 0.1% NaN_3, and 2 mM EDTA and staining with antibodies against cell surface molecules. To generate B-cell blasts, spleen cells were stimulated for 72 h in vitro with lipopolysaccharide (LPS) (100 μg/ml) and dextran sulfate (40 μg/ml) (29). For flow cytometric analysis the unlabeled, biotinylated phycoerythrin-, allophycocyanin-, or fluorescein isothiocyanate-conjugated antibodies against the following cell surface molecules were used: CD3ε (145-2C11), CD4 (RM4-5), CD5 (53-7.3), CD8α (53-6.7), CD11b (M1/70), CD19 (1D3), CD23 (B3B4), CD24 (M1/69), CD25 (7D4), CD28 (37.51), CD16/32 (2.4G2), CD31 (MEC13.3), CD45R/B220 (RA3-6B2), immunoglobulin D (IgD) (11-26c.2a), IgM (R6-60.2), κ light chain (R5-240), I-A^b (25-9-17), CD43 (S7), TER119/Ly-76 (TER-119), syndecan (281-2), CD81 (2F7), pan-NK (DX5), Ly-6G (RB6-8C5), CD69 (H1.2F3), CD62L (MEL-14) (all from PharMingen), CD86 (GL1), CD80 (16-10A1) (Biosource International), F4/80 (CI:A3-1) (Serotec), λ light chain (goat polyclonal), and Ly-6C (HK1.4) (Southern Biotechnology). Biotinylated antibodies were visualized with fluorescein isothiocyanate- or allophycocyanin-conjugated (PharMingen) or phycoerythrin-conjugated (Biosource International) streptavidin. In order to prevent unspecific binding, all samples were preincubated with Fc-Block or unlabeled, isotype-matched unspecific antibodies (PharMingen). Samples were analyzed on a FACS-Calibur flow cytometer (Becton Dickinson) according to standard protocols. Gates on viable cells were set according to the exclusion of propidium iodide staining.

Preparation of antigens and immunization. Ficoll (Sigma) was carboxymethylated and amino groups were introduced as described previously (12). The preparation of trinitrophenol (TNP)-Ficoll, TNP-keyhole limpet hemocyanin (KLH), and TNP-bovine serum albumin (BSA) was carried out according to the method of Mäkelä and Seppälä (20). Eight-week-old sex-matched mice were immunized intraperitoneally (i.p.) with either 20 μg of TNP-Ficoll in PBS or 20 μg of TNP-KLH precipitated in alum on day 0 and were given boosters on day 21. Mice were also immunized subcutaneously (s.c.) with 10 μg of TNP-KLH in colonization factor antigen (CFA) or i.p. with 10 μg of TNP-KLH in PBS. Mice were bled on days 0 and 4 and then once weekly in all immunization experiments.

Serum immunoglobulin and antigen-specific immunoglobulin determination. We used a sandwich enzyme-linked immunosorbent assay (ELISA) to determine immunoglobulin isotypes in sera and TNP-specific antibodies after immunization. Ninety-six-well ELISA plates were coated with mouse immunoglobulin isotype-specific antibody anti-IgG1 (A85-1), anti-IgG2a (R19-15), anti-IgG2b (R12-3), anti-IgG3 (R40-82), anti-IgA (R5-140), anti-IgM (R6-60.2), or anti-IgE (R35-72) (all from PharMingen) or TNP-BSA diluted in 50 mM carbonate buffer (pH 9.6). After overnight incubation at 4°C, they were blocked with 1% BSA in PBS with 0.05% Tween 20. Mouse sera were diluted 1:100 and titrated 1:3 over eight dilution steps. Plates were incubated with biotinylated anti-κ (R8-140) and anti-λ (R26-46) antibodies (PharMingen) and then with horseradish peroxidase (HRP)-labeled streptavidin (Sigma). To detect hapten-specific antibodies, mouse IgM- or IgG-specific HRP-labeled goat antibodies or biotinylated goat anti-mouse IgG1, IgG2a, IgG2b, or IgG3 antibodies (all from Southern Biotechnology) were used. HRP-labeled rat anti-mouse IgE antibodies (R35-118) (PharMingen) were used for the quantification of IgE. For colorimetric detection, *o*-phenylenediamine (Sigma) was used as the substrate. Optical density was measured at 482 nm with the BioLumin 960 microassay reader (Molecular Dynamics). Titers were read at the dilution step of half-maximal optical density.

Infection with *Nippostrongylus brasiliensis*. To establish infection, mice were injected s.c. with 1,000 stage 3 larvae (kindly provided by K. Erb, Würzburg, Germany). Smears of peripheral blood were prepared from uninfected mice, on days 5 and 11 after infection, and stained with May-Grünwald and Giemsa (Pappenheim staining). Eosinophils were counted microscopically. Mesenteric lymph node cells (2×10^6/ml) were cultured in RPMI 1640 medium (Seromed) supplemented with 10% fetal calf serum (HyClone), L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Cell preparations were left untreated or stimulated with 5 μg of concanavalin A (Sigma) per ml. After 48 h, culture supernatants were tested for interleukin-4 (IL-4) and IL-5 in sandwich ELISAs with the following monoclonal antibodies recognizing two different epitopes of the respective cytokine: anti-mouse IL-4 (11B11), biotinylated anti-mouse IL-4 (BVD6-24G2), anti-mouse IL-5 (TRFK5), and biotinylated anti-mouse IL-5 (TRFK4) (all from PharMingen). Visualization was performed using streptavidin-HRP-labeled conjugate (Amersham-Buchler) and tetramethylbenzidine as a substrate. Absorbance was read at 405 nm in an ELISA microplate reader (Tecan). To quantify cytokines in culture supernatants, titrations were performed with murine IL-4 and IL-5 (PharMingen). At least three mice per group were used.

Infection with VSV and immunization with recombinant VSV antigens. Vesicular stomatitis virus (VSV) Indiana (Mudd-Summers isolate; gift of D. Kolakovsky, Geneva, Switzerland) was grown on BHK-21 cells. Recombinant vaccinia virus expressing VSV glycoprotein G (Vacc-G) was kindly provided by B. Moss, Bethesda, Md. Recombinant baculovirus expressing VSV-IND glycoprotein G (VSV-G) (gift of D. H. L. Bishop, Oxford, England) was derived from nuclear polyhedrosis virus and was grown at 28°C in *Spodoptera frugiperda* cells in spinner cultures in TC-100 medium. The titers of VSV-specific antibodies were measured by neutralization assay. To determine IgG titers, undiluted serum was pretreated with an equal volume of 0.1 mM 2-mercaptoethanol in saline. The sera of immunized mice were prediluted 40-fold in minimal essential medium containing 2% fetal calf serum. Serial twofold dilutions were mixed with equal volumes of VSV (500 PFU/ml) and incubated for 90 min at 37°C and in 5% CO_2. Subsequently, 100 μl of the serum-virus mixture was transferred onto Vero cell monolayers in 96-well plates and incubated for 1 h at 37°C. The monolayers were then overlaid with 100 μl of Dulbecco's modified Eagle medium containing 1% methylcellulose and incubated for 24 h at 37°C. The overlay was flicked off, and the monolayer was fixed and stained with crystal violet. The highest dilution of serum that reduced the number of plaques by 50% was taken as the titer.

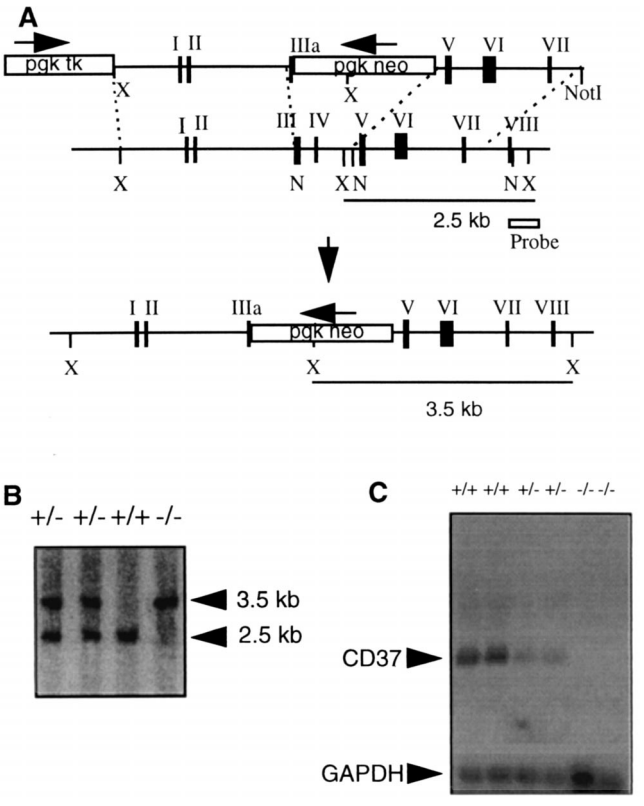


FIG. 1. CD37 gene inactivation. (A) Knockout strategy. Restriction maps are shown for the targeting vector (top), wild-type CD37 gene locus (middle), and mutated CD37 gene locus after homologous recombination (bottom). Exons are indicated as black boxes. The orientations of the neomycin (neo) resistance marker and the herpesvirus thymidinkinase (tk) under control of the phosphoglyceratekinase promoter (pgk) are shown by arrows. The target vector is linearized by *Not*I digestion. Restriction sites: X, *Xmn*I; N, *Nco*I. (B) Southern blot analysis of littermates from $CD37^{-/+}$ matings. A 300-bp *Xmn*I/*Nco*I fragment was used as a probe and detects a 2.5-kb wild-type and a 3.5-kb mutant hybridizable *Xmn*I fragment. (C) Northern blot analysis. Fifteen micrograms of total spleen RNA from wild-type (+/+), heterozygous (+/−), and mutant (−/−) mice was separated on an agarose gel, blotted, and hybridized with CD37 cDNA. After stripping, the filter was reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA.

Statistical analysis. The statistical differences between groups of mice in immunoglobulin levels in serum or immunoglobulin titers after immunization experiments, the numbers of eosinophils, and the levels of cytokines in cell supernatants were determined using a two-tailed Student *t* or alternate Welch *t* test.

RESULTS

In order to gain insight into the role of CD37 in vivo, CD37-deficient mice were generated. The structure of the CD37 gene locus and the targeting construct are shown in Fig. 1A. After homologous recombination, parts of exon III and the complete exon IV were replaced by the neomycin resistance gene. The deleted region encompasses parts of the second transmembrane domain, the short intracellular part of the protein between the second and third transmembrane domains, and the complete third transmembrane domain. Embryonic stem cell

TABLE 1. Frequency of lymphocytes in CD37-deficient mice

Tissue type	Phenotype	% of mice with lymphocyte phenotype[a]	
		$CD37^{+/+}$	$CD37^{-/-}$
Bone marrow	$B220^{+}$ IgM^{-}	41 ± 8	41 ± 11
	$B220^{dull}$ $CD43^{+}$	7 ± 2	8 ± 1
	IgM^{+} $B220^{low}$	19 ± 5	17 ± 5
	IgM^{+} $B220^{high}$	11 ± 3	12 ± 4
Spleen	$CD3^{+}$ $CD4^{+}$	25 ± 1	21 ± 2
	$CD3^{+}$ $CD8^{+}$	12 ± 1	10 ± 1
	$B220^{+}$ IgM^{high} IgD^{low}	8 ± 1	8 ± 2
	$B220^{+}$ IgM^{low} IgD^{high}	40 ± 7	41 ± 9
	$B220^{+}$ $CD5^{+}$	3 ± 1	2 ± 1
Lymph nodes	$CD3^{+}$ $CD4^{+}$	51 ± 1	49 ± 1
	$CD3^{+}$ $CD8^{+}$	27 ± 2	26 ± 1
	IgM^{+} $B220^{+}$	10 ± 5	15 ± 6
Peritoneum	$B220^{high}$ IgM^{int} $CD11^{-}$	20 ± 8	31 ± 9
	$B220^{int}$ IgM^{high} $CD11^{+}$	52 ± 10	35 ± 8
	$B220^{int}$ $CD11b^{+}$ $CD5^{+}$	19 ± 8	15 ± 9
	$B220^{int}$ $CD11b^{+}$ $CD5^{-}$	28 ± 7	25 ± 4

[a] Mean percentage ± standard error of the mean is given for groups of 6 to 11 animals analyzed in three or more independent experiments.

clones that had undergone homologous recombination were identified, and CD37-homozygous ($CD37^{-/-}$) mice were generated by standard techniques (Fig. 1B). Successful inactivation of the gene was proven by Northern blot hybridization of spleen RNA using CD37 cDNA as a probe, which showed the complete absence of CD37 mRNA (Fig. 1C). CD37-deficient mice showed no differences in growth, reproductive potential, health, or life span. $CD37^{-/-}$ mice had thymuses, spleens, lymph nodes, mucosa-associated lymphoid tissue, and bone marrow of normal size and architecture and did not show any detectable histological abnormalities, although a slight reduction in the number and size of germinal centers was observed in the splenic white pulp and lymph node cortex (data not shown). Lymphocyte development and the cellular composition of bone marrow, spleens, thymuses, and lymph nodes were determined for groups of at least eight 6- to 12-week-old mice by fluorescence-activated cell sorting. Analysis of bone marrow cells based on Ly6-C and CD31 expression (6), together with additional stainings for CD3, B220, Gr-1, and TER-119, showed unaltered numbers of all major cell populations: blast cells, early progenitors and stem cells ($CD31^{high}$ Ly-$6C^{-}$), lymphoid cells ($CD31^{int}$ Ly-$6C^{-}$), erythroid progenitors ($CD31^{-}$ Ly-$6C^{-}$), myeloid progenitors ($CD31^{+}$ Ly-$6C^{+}$), granulocytes ($CD31^{-}$ Ly-$6C^{int}$), and monocytes ($CD31^{-}$ Ly-$6C^{high}$) (data not shown).

The differential expression of human CD37 during the maturation of B cells (28) prompted us to analyze the B-cell compartments of $CD37^{-/-}$ mice in detail. As shown in Table 1, the frequency of $CD37^{-/-}$ pro-B cells expressing CD43 and that of immature and mature B cells in bone marrow expressing B220 and IgM are comparable with those in wild-type controls. The maturation of splenic B cells was unchanged, as revealed by IgM and IgD expression (Table 1). Likewise, CD19 and MHC class II antigen ($I\text{-}A^{b}$) expression on B cells from $CD37^{-/-}$ mice was unaffected (Fig. 2A). We also found normal expression of other surface markers, including CD23, CD40, CD86, CD80, CD43, and syndecan, as well as markers of activation (CD69 and CD62L) (data not shown). Even after in vitro stimulation with LPS and dextran sulfate, the upregulation of $I\text{-}A^{b}$, CD81, and CD86 (Fig. 2B), as well as CD23 and CD80, on $CD37^{-/-}$ B-cell blasts was indistinguishable from that in $CD37^{-/-}$ mice. Also, the in vitro proliferation of $CD37^{-/-}$ B cells stimulated with LPS, anti-IgM, or anti-CD40 plus IL-4 was unaltered (data not shown). There was also no significant difference in either the cellularity of peritoneal B cells or the frequency of B2, B1a, and B1b cells in $CD37^{-/-}$ mice. Furthermore, detailed fluorimetric analysis revealed normal numbers of all major T-cell subsets as well as unaltered expression

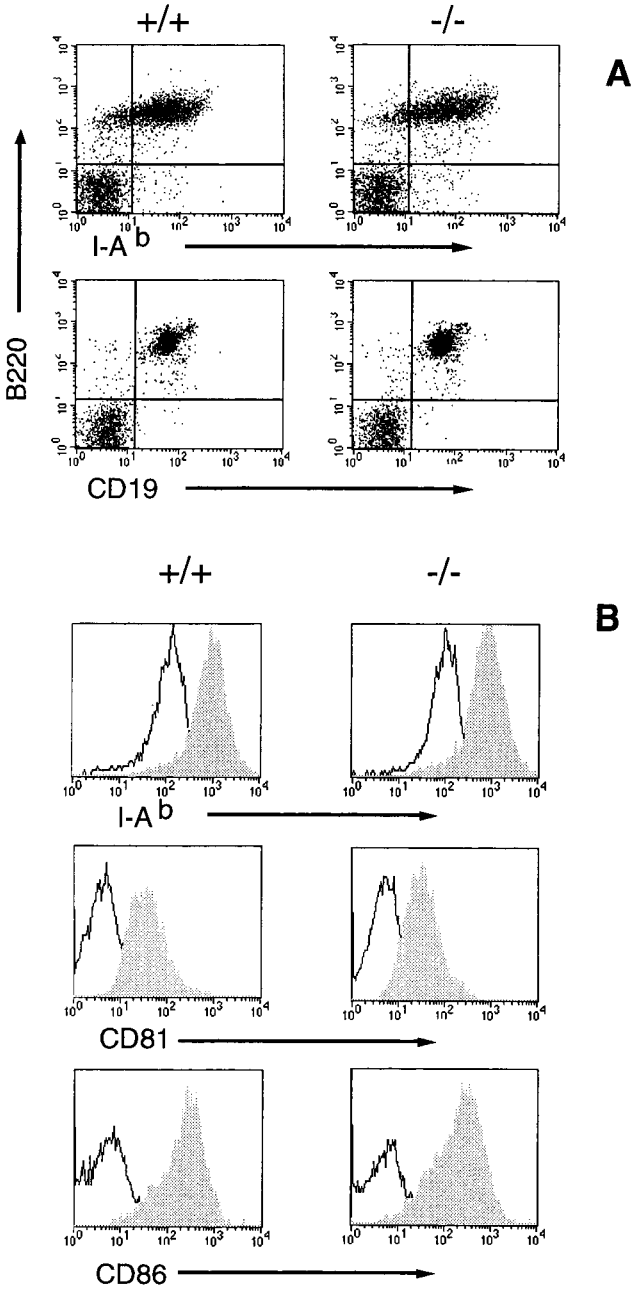
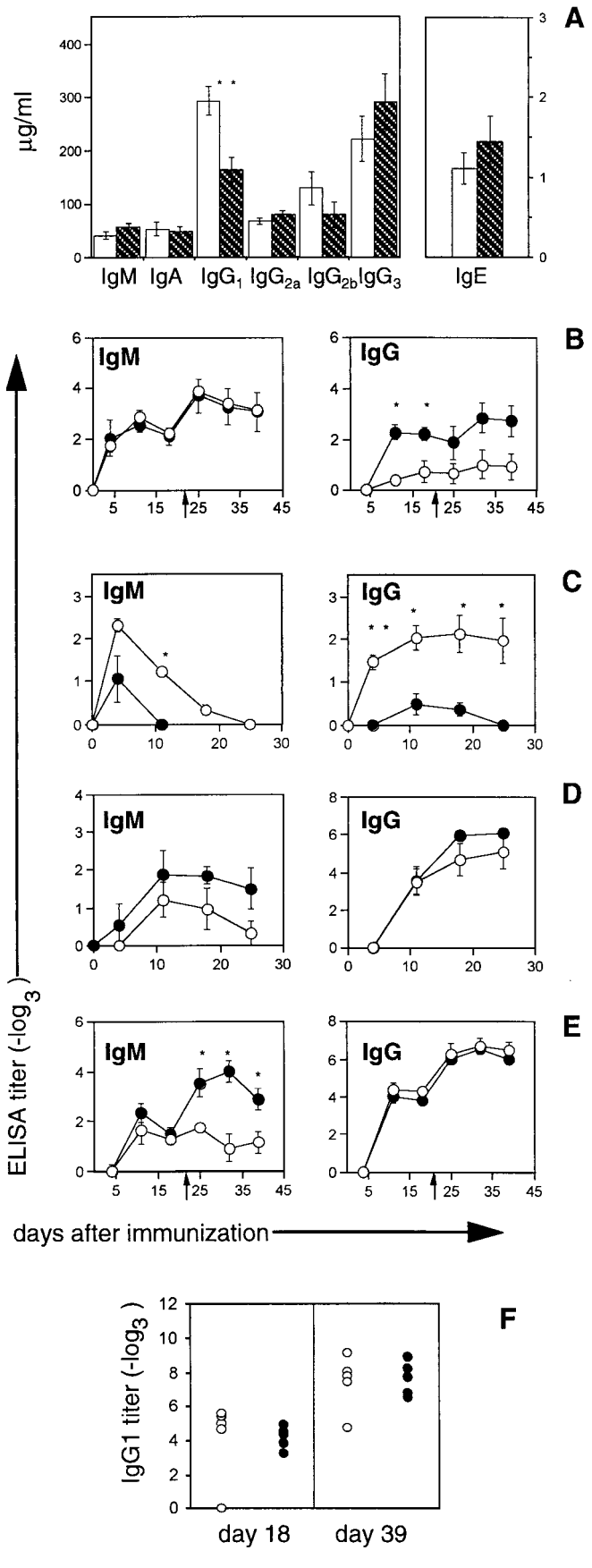


FIG. 2. Flow cytometric analysis of B lymphocytes in CD37-deficient mice (−/−) and wild-type littermates (+/+). (A) Spleen cells were stained for $I\text{-}A^{b}$ and CD19 versus B220. Living cells were gated by propidium iodide exclusion. (B) CD37-deficient B cells were able to upregulate $I\text{-}A^{b}$, CD81, and CD86 after stimulation with LPS and dextran sulfate. Histograms represent the expression levels of indicated molecules on $B220^{+}$ spleen cells after stimulation (shaded histograms) and on freshly isolated $B220^{+}$ spleen cells (white histograms).



of T-cell markers in CD37$^{-/-}$ mice, indicating that CD37 expression is not necessary for T-cell development (Table 1).

Together, these findings show that CD37 is dispensable in vivo for the development and activation of immune system cells, notably B cells, in spite of its prominent expression in these cells.

To examine B-cell functions, we analyzed immunoglobulin concentration and isotype distribution in the sera of nonimmunized mutant mice. In sera from 8-week-old CD37$^{-/-}$ mice, a statistically highly significant (1.8-fold) reduction of IgG1 was observed (Fig. 3A). This difference was not compensated for by the established age-related increase of serum immunoglobulins, as it persisted in the sera of 8-month-old mice (data not shown). In contrast, levels of IgM, IgG2a, IgG2b, IgG3, IgA, and notably IgE were unchanged, indicating a specific rather than general effect in immunoglobulin production due to the loss of CD37.

To investigate whether reduced IgG1 levels in CD37$^{-/-}$ mice were linked to deregulated TH1 and TH2 responses, CD37$^{-/-}$ mice were challenged with the intestinal parasitic nematode *N. brasiliensis*, which elicits a strong TH2 response. The induction of TH2 responses was monitored by examining IL-4 and IL-5 levels in supernatants of mesenteric lymph node cells, levels of IgE in sera, and numbers of eosinophils in peripheral blood. CD37$^{-/-}$ mice did not show any impairment in the upregulation of TH2-specific cytokines. Levels of IL-4 (1.32 ± 0.87 versus 1.90 ± 0.52 ng/ml) and IL-5 (20.00 ± 6.22 versus 26.01 ± 7.08 ng/ml) ($n = 3$) in wild-type and CD37$^{-/-}$ mice, respectively, were measured in the supernatants of mesenteric lymph node cells 5 days after infection. Moreover, the levels of IgE in serum and the numbers of eosinophils in peripheral blood did not differ in CD37$^{-/-}$ and control mice at any time point after infection (data not shown). Thus, the reduced IgG1 levels in nonimmunized CD37$^{-/-}$ mice seem not to be caused by an impairment of TH2 function, a conclusion further suggested by normal IgE levels in sera and normal TH2-dependent IgG1 response after immunization (see below).

We next investigated the role of CD37 in T-cell-independent humoral immune responses. To this end, we tested the primary and secondary antibody responses to TNP-Ficoll, a typical type II T-cell-independent antigen. These antigens induce the cross-linking of membrane immunoglobulins, thus leading to B-cell activation with IgM responses and subsequent differ-

FIG. 3. Antibody production in CD37$^{-/-}$ mice. (A) Reduced levels of IgG1 in nonimmunized CD37$^{-/-}$ mice. Serum IgG1 levels in 8-week-old wild-type (open bars) and CD37-deficient (hatched bars) mice were measured by isotype-specific ELISA. Error bars indicate standard errors of the means ($n = 8$ to 15 animals per group). **, $P < 0.001$. (B) Increased levels of IgG in CD37$^{-/-}$ mice in response to type II T-cell-independent antigen. CD37$^{+/+}$ (open symbols) and CD37$^{-/-}$ (solid symbols) mice were injected with 20 µg of the type II T-cell-independent antigen TNP-Ficoll in PBS i.p. on day 0 and given a booster on day 21, as indicated by an arrow. Four mice per group were immunized. After immunization, serum immunoglobulin titers were determined by TNP-specific ELISA. Error bars indicate the standard errors of the means. *, $P < 0.05$. One of two comparable experiments is shown. (C) Reduced IgM and IgG antibody responses in CD37$^{-/-}$ mice after immunization with the T-cell-dependent antigen TNP-KLH without adjuvants. Three mice per group were immunized with 10 µg of TNP-KLH in PBS i.p. *, $P < 0.05$; **, $P < 0.001$. (D) IgG and IgM responses of mice when immunized with TNP-KLH in alum as adjuvant. Five mice per group were injected i.p. with 20 µg of TNP-KLH precipitated in alum. *, $P < 0.05$. (E) IgM and IgG responses after immunization with TNP-KLH in CFA as adjuvant. Three mice per group were injected s.c. with 10 µg of TNP-KLH in CFA. Error bars indicate the standard errors of the means. (F) TNP-specific IgG1 levels in wild-type (open symbols) and CD37-deficient (solid symbols) mice after primary immunization and after a booster with 20 µg of TNP-KLH precipitated in alum.

entiation and immunoglobulin isotype switching to IgG (22). Wild-type and CD37-deficient mice were immunized twice with TNP-Ficoll, and TNP-specific IgM and IgG titers were determined. The priming of B cells, as a consequence of antigen engagement and subsequent IgM production in response to type II T-cell-independent antigen TNP-Ficoll, proceeded normally in $CD37^{-/-}$ mice, as revealed by unchanged IgM titers (Fig. 3B) and germinal center formation (data not shown). Their IgG titers were higher than those of wild-type animals (Fig. 3B). This increase was not subclass specific; both the IgG1 and IgG2a titers were elevated (data not shown). These findings imply a negative regulatory role for CD37 in the type II T-cell-independent IgG response, suggesting that CD37 exerts a regulatory role in B-cell humoral response triggered by B-cell receptor cross-linking.

In order to examine the antigen-specific T-cell-dependent immune responses in CD37-deficient mice, we immunized wild-type and $CD37^{-/-}$ mice with the soluble T-cell-dependent protein antigen TNP-KLH and compared the induction of TNP-specific serum immunoglobulins in vivo. After immunization with low doses of TNP-KLH without adjuvants, both IgM and IgG responses were significantly reduced in $CD37^{-/-}$ mice (Fig. 3C). This reduction was observed in both the IgG1 and IgG2a subclasses (data not shown). By contrast, when animals were immunized with an antigen in adjuvant (CFA or alum), CD37-deficient mice initially developed normal IgM levels. After a secondary challenge, mutant mice developed higher IgM titers than control animals (Fig. 3E), in which a secondary IgM response has been suggested to be T-cell dependent, unlike primary IgM production (13). As with IgM, the deficiency in the IgG responses of $CD37^{-/-}$ mice observed after immunization in the absence of adjuvant was reversed in the presence of alum or CFA (Fig. 3D and E). However, histological analysis on day 12 after immunization indicated a slight reduction in the number and size of germinal centers. IgG isotype distribution was normal in $CD37^{-/-}$ mice after immunization with antigen in adjuvants (data not shown), including IgG1 levels after a challenge in alum, which induces a strong TH2 response (Fig. 3F).

Collectively, these findings suggest a role for CD37 in mediating B- and T-cell interactions, which can best be observed under conditions of suboptimal stimulation when CD37-deficient mice are immunized in the absence of adjuvant.

To investigate whether the immune responses of $CD37^{-/-}$ mice are also altered after viral infections, antibody responses were tested after infection with VSV and recombinant Vacc-G and after immunization with recombinant VSV-G. Neutralizing antibody titers were analyzed at different time points. After infection with VSV, $CD37^{-/-}$ mice mounted normal VSV-neutralizing IgM levels and switched to IgG levels comparable to those in wild-type mice (Fig. 4). However, their T-cell-dependent IgG titers reached a plateau about 16-fold lower than titers in control animals. The antibody titers were subsequently sustained with comparable kinetics in wild-type and $CD37^{-/-}$ mice. Infection with Vacc-G, a T-cell-independent type II antigen, revealed a comparable antibody response with normal IgM titers but a slight reduction in the neutralizing IgG titers. After immunization with recombinant VSV-G alone, $CD37^{-/-}$ mice did not efficiently switch to IgG, in contrast to control animals. However, after a booster immunization with VSV-G, a potent, neutralizing IgG response indicated a prompt secondary antibody response. Thus, after infection with VSV and different recombinant VSV antigens, a normal T-cell-independent IgM response was observed, but the T-cell-dependent IgG antibody response was reduced.

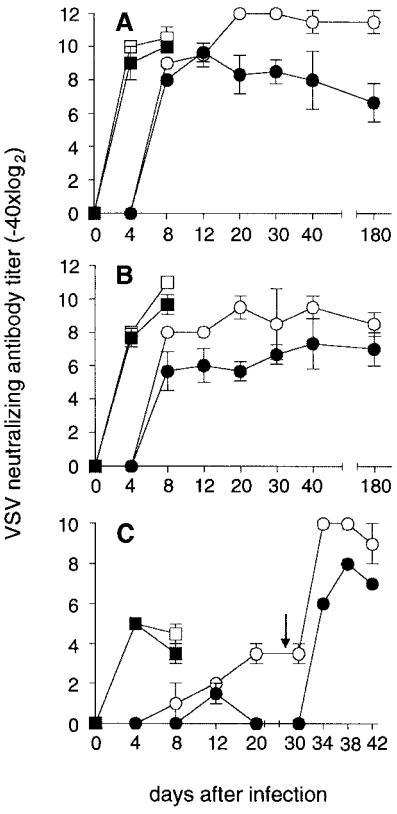


FIG. 4. Antibody responses after infection with VSV and recombinant VSV antigens in $CD37^{-/-}$ and $CD37^{+/+}$ mice. $CD37^{+/+}$ (open symbols) and $CD37^{-/-}$ (solid symbols) mice were infected intravenously with 2×10^6 PFU of VSV (A), 2×10^6 PFU of Vacc-G (B), and 10 μg of VSV-G (C). Neutralizing antibody titers were analyzed at the time point indicated. Thirty days after the initial immunization, $CD37^{-/-}$ and $CD37^{+/+}$ mice were given a booster with 10 μg of VSV-G, as indicated. The mean antibody titer of three animals per group is given. Rectangles, IgM responses; circles, IgG responses. Error bars indicate the standard deviations. One of two comparable experiments is shown.

DISCUSSION

We generated mice deficient in CD37 to gain insight into the functional role of CD37 in vivo. Immune cell development and humoral immune response after immunization with soluble antigens or infections with viruses were analyzed.

Surprisingly, the CD37 gene defect did not result in any detectable changes in the development and cellularity of the major cell populations in bone marrow, thymuses, spleens, or lymph nodes, although human CD37 is transiently expressed early in myeloid development and lost upon the culture of $CD34^+$ cells in the presence of IL-3 (26), which might have revealed a role for CD37 in hematopoiesis. Despite modulated expression during the differentiation of B cells, CD37-deficient mice did not display any apparent abnormalities of B-cell compartments. Thus, our findings suggest that CD37 is dispensable for B-cell development. The high expression of CD37 on mature human B cells led us to investigate whether the loss of CD37 could have functional consequences. This possibility was supported by the observation that IgG1 levels in nonimmunized $CD37^{-/-}$ mice were reduced. As isotype switching is largely controlled by the types of T-cell cytokines produced, we tested whether CD37-deficient mice, like the mice deficient for another tetraspanin, CD81, reveal a TH2 deficiency (17). As infection with *N. brasiliensis* did not result in differences in IL-4 or IL-5 production or IgE levels between mutant and wild-type

mice, the diminished IgG1 production in uninfected mice is not caused by a TH2 deficiency. This suggests that reduced IgG1 levels are caused by an intrinsic B-cell defect rather than T-cell-modulated B-cell function. The specific alteration of IgG1 production was also described for mice deficient for H2-O (15) and together with IgG2a for mice lacking all conventional MHC class II molecules (16).

CD37 has been described as a component of multimolecular complexes consisting of MHC class II molecules and other tetraspanins and is associated with the CD19–CD21–Leu-13 signal-transducing complex of B cells (1, 31). $CD37^{-/-}$ B cells could be activated in vitro, as measured by the upregulation of MHC class II molecules and costimulatory molecules (Fig. 2), and CD37 deficiency did not lead to changed MHC class II expression, as measured by the stimulation of alloreactive T cells in mixed lymphocyte reactions (data not shown). Thus, CD37 is dispensable for MHC class II expression on the cell surface. This does not exclude the possibility that CD37 might subtly influence antigen presentation and/or modulate the spectrum of presented peptides. Interaction between tetraspanins and the DM and DO molecules, which facilitate peptide loading for MHC class II molecules, has recently been described (9). Although CD37 and CD81 are associated as components of a tetraspanin complex that interacts with CD21 and CD19 (1), CD37 deficiency did not have any effect on CD81 surface expression on B cells. Moreover, while CD81-deficient B cells have reduced levels of CD19 (19, 21, 33), the loss of CD37 does not alter the expression of CD19 on the cell surface.

To determine whether CD37 contributes to the regulation of the humoral response, we immunized $CD37^{-/-}$ and $CD37^{+/+}$ mice with soluble type II T-cell-independent and T-cell-dependent antigen in the absence or presence of adjuvants. An unimpaired IgM response in $CD37^{-/-}$ mice suggests that the priming of B cells and initiation of response to TNP-Ficoll is CD37 independent. The IgG response to T-cell-independent antigens depends upon noncognate help (22) that is provided by cytokines produced by non-T cells, while T cells and T-cell-derived cytokines modify the outcome of class switching in response to T-cell-independent type II antigens (23). The hapten-specific IgG response indicates that in the absence of CD37, B cells can undergo T-cell-independent class switching. Nevertheless, CD37 deficiency leads to a higher IgG response to T-cell-independent type II antigens. This effect is also observed in CD81-deficient mice (33) and might be explained by altered costimulatory signals after the antigen uptake or the lack of appropriate downregulation of costimulatory signals after successful B-cell priming. However, when CD37-deficient mice were challenged with Vacc-G as a self-replicating T-cell-independent type II antigen, reduced IgG responses were observed, indicating that the dysregulation of IgG response is antigen dependent.

Different routes of immunization or different adjuvants can lead to the utilization of distinct antigen-presenting cell (APC) subpopulations like dendritic cells and B cells, in which the expression kinetics and regulation of costimulatory molecules may differ (4, 5). Antigen applied s.c. in CFA induces additional local inflammation, which leads to nonantigen-mediated upregulation of costimulatory molecules on the APCs, which in turn activates naive TH cells and mostly favors the development of TH1 responses (2). After the local application of antigen with CFA, CD37-deficient mice show no impairment of immunoglobulin response or immunoglobulin isotype composition. TNP-KLH systemically applied in CD37-deficient mice leads to a distinct reduction of both IgM and IgG responses. In contrast, the same antigen under more effective costimulatory conditions induced strong IgM and IgG responses in $CD37^{-/-}$ mice. Furthermore, the induction of normal titers of hapten-specific IgG1 occurs after immunization under conditions inducing a strong TH2 response. This indicates that an impaired humoral response caused by the absence of CD37 can be overcome or compensated for by additional costimulatory effects provided by adjuvants or by the recruitment of APCs other than those elicited by immunization with antigen alone.

While many aspects of the immune response can be studied by immunization with both soluble model antigens and infectious agents, factors like replication kinetics, cell tropism, cytopathogenicity, time-dependent amplification, anatomical localization, and the triggering of the responses of the innate immune system are unique to infectious-disease models and may affect the generation of specific immune responses (24). The recovery of mice from primary VSV infection and resistance to reinfection depend on the induction of VSV-specific T-cell help and neutralizing IgG antibody responses (3). The early IgM response to VSV is T-cell independent type I (8), and then VSV induces a rapid and strong T-cell-dependent, neutralizing IgG response starting around days 6 to 7 after infection and reaching a plateau after 3 weeks. $CD37^{-/-}$ mice showed normal T-cell-independent type I IgM responses after infection with VSV and normal T-cell-independent type II IgM responses after infection with Vacc-G, suggesting that the availability and precursor frequency of VSV-specific B cells are not affected by the lack of CD37. In contrast, CD37 deficiency leads to a slower rise of T-cell-dependent IgG titers in response to VSV and slightly lower IgG levels after infection with Vacc-G. This effect was even more pronounced after immunization with soluble recombinant VSV-G, following which CD37-deficient mice were unable to switch efficiently to IgG after primary immunization despite a normal IgM response. These results indicate that in the absence of CD37, the T-cell–B-cell interaction is impaired.

Taken together, the results suggest that CD37 is important for T-cell–B-cell interaction, as CD37-deficient mice immunized with antigen alone or infected with virus tend to mount poor T-cell-dependent antibody responses. However, the defect caused by CD37 deficiency is observed only under suboptimal costimulatory conditions and can be overcome by the administration of antigen with adjuvant.

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