Antiviral Protection and Germinal Center Formation, But Impaired B Cell Memory in the Absence of CD19

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Summary

Coligation of CD19, a molecule expressed during all stages of B cell development except plasmacytes, lowers the threshold for B cell activation with anti-IgM by a factor of 100. The cytoplasmic tail of CD19 contains nine tyrosine residues as possible phosphorylation sites and is postulated to function as the signal transducing element for complement receptor (CR)2. Generation and analysis of CD19 gene–targeted mice revealed that T cell–dependent (TD) antibody responses to proteinaceous antigens were impaired, whereas those to T cell–independent (TI) type 2 antigens were normal or even augmented. These results are compatible with earlier complement depletion studies and the postulated function of CD19. To analyze the role of CD19 in antiviral antibody responses, we immunized $CD19^{-/-}$ mice with viral antigens of TI-1, TI-2, and TD type. The effect of CD19 on TI responses was more dependent on antigen dose and replicative capacity than on antigen type. CR blocking experiments confirmed the role of CD19 as B cell signal transducer for complement. In contrast to immunization with protein antigens, infection of $CD19^{-/-}$ mice with replicating virus led to generation of specific germinal centers, which persisted for >100 d, whereas maintenance of memory antibody titers as well as circulating memory B cells was fully dependent on CD19. Thus, our study confirms a costimulatory role of CD19 on B cells under limiting antigen conditions and indicates an important role for B cell memory.

Key words: CD19 • antiviral immunity • complement • germinal center • B cell memory

CD19 is a 95-kD transmembrane protein of the Ig superfamily. It is specifically expressed on B cells, from early progenitors in the bone marrow to memory cells, except for antibody-forming plasma cells (1). Therefore, it is used as a major target for immunotherapy approaches to B cell leukemias (2). CD19 exhibits a highly conserved structure of two extracellular Ig domains and a 243–amino acid long cytoplasmic tail with nine tyrosine residues as possible phosphorylation sites (3, 4). These structural features contribute to its known signaling ability. Upon cross-linking, CD19 associates with the protein tyrosine kinases lyn (5) and fyn (6), and signaling via vav/ras/mitogen-activated protein kinase and the phosphatidylinositol 3-kinase pathways are initiated (7). Phospholipase Cγ is also activated (8), leading to the generation of inositol triphosphate (9) and to Ca^{2+} mobilization (10).

On mature B cells, CD19 associates in vitro and in vivo with three different molecules to form a tetrameric complex: CR2 (CD21), target of the antiproliferative antibody 1 (CD81), and Leu-13 (11, 12). These associations have led to the concept that CD19 is the signal transducer for CR2, which may not possess intrinsic signaling properties since it has a short cytoplasmic domain. In addition, CD19 can associate directly with the B cell receptor (13, 14). CD19 is necessary for formation of the peritoneal B-1 cell population (15–18). Treatment of pregnant mice with an anti-CD19 antibody led to the loss of B-1 cells in the offspring (16). This finding correlates with a severe and selective B-1 cell deficiency in $CD19^{-/-}$ mice (17, 18). In mature B lymphocytes, CD19 mediates homotypic aggregation (19) and functions as a B cell costimulator, since coligation of CD19 lowers the threshold for B cell activation with anti-IgM antibody by a factor of 100 (20).

Complement, especially its activated component C3d, is

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/98/07/145/11 $2.00
Volume 188, Number 1, July 6, 1998 145–155
http://www.jem.org

known to exert B cell costimulatory activity in antibody responses to T cell–dependent (TD)[1] antigens (21) and in general, under limiting antigen doses (22, 23). This was shown first by complement depletion experiments with cobra venom factor (CVF [24]), later by treatment of mice with anti-complement receptor (CR) antibodies (25, 26) or soluble CD21 (27), and most recently by the generation of C3- and CR2-deficient mice (28–30). We demonstrated recently that neutralizing IgM antibodies to vesicular stomatitis virus (VSV) were mostly complement-independent except when limiting antigen doses were used. In contrast, the TD switch to IgG was dependent on complement (31). Binding of C3 to antigen, which has been demonstrated for VSV (32), may allow coligation of surface (s)Ig and CR ("dual antigen recognition" [33]). The recent observation that immunization with a recombinant hen egg lysozyme containing one to three C3d domains led to greatly enhanced antibody titers (34) suggests an adjuvant function for C3d (35) and supports this corecognition model.

The generation and analysis of CD19-deficient mice revealed that TD antibody responses and germinal center (GC) formation were impaired (17, 18). In contrast, antibody titers to polymeric T cell–independent (TI) type 2 antigens were normal (17) or even augmented (36). B cell proliferation upon LPS stimulation was normal in one $CD19^{-/-}$ strain (17) and reduced in the other (18), and IgM titers to the TI-1 antigen LPS-TNP were reduced (36). Transgenic mice expressing the human CD19 molecule could complement the murine CD19 defect and perhaps lead to hyperresponsiveness (37).

Immunizations with proteinaceous antigens in adjuvant and live virus infections differ in various respects (38, 39). Therefore, we wanted to analyze whether the results on the role of CD19 obtained in in vitro experiments as well as by immunizations with inert haptenated antigens could be generalized to models of infection. We used VSV, a member of the Rhabdovirus family, and lymphocytic choriomeningitis virus (LCMV), a representative of the Arenavirus family, as inducer of TI and TD antibody responses, respectively (40). Replicating and nonreplicating forms and various application routes were compared, and the role of complement was studied by CR blocking experiments. Finally, by memory antibody titers, plasma cell frequencies, memory B cells transfers, and immunohistology, we looked for a role of CD19 in B cell memory.

Materials and Methods

Mice. $CD19^{-/-}$, Monitor, KINDG, 129Sv, and C57/BL6 mice were obtained from the breeding colony of the Institut für Labortierkunde, Veterinary Hospital (Zurich, Switzerland). Breedings were performed under specific pathogen–free conditions, whereas experiments were done in conventional animal facilities. Mice were used at 8–12 wk of age.

Viruses. VSV serotype Indiana (VSV-IND; Mudd-Summers isolate) was obtained originally from Prof. D. Kolakowsky, University of Geneva (Geneva, Switzerland). It was grown on BHK cells in MEM supplemented with 5% FCS at 37°C. LCMV (isolate WE) had been obtained originally from Dr. F. Lehmann-Grube, Heinrich Pette Institut für Experimentelle Virologie und Immunologie, University of Hamburg (Hamburg, Germany). The recombinant baculoviruses expressing the glycoprotein of VSV (VSV-G) and the nucleoprotein of LCMV (LCMV-NP) were a gift from Dr. D.H.L. Bishop, NERC Institute of Virology (Oxford, UK). They were derived from nuclear polyhedrosis virus, and were grown at 28°C in *Spodoptera frugiperda* cells in spinner cultures in TC-100 medium (41).

CVF and Anti-CR Antibody. Purified CVF (*Naja naja*) was obtained from Sigma Chemie (233550; Buchs, Switzerland). We injected twice 1.2 μg of CVF diluted in 200 μl of balanced salt solution (BSS) with an interval of 8–10 h, 16 h before immunization. The anti-CR antibody 7G6 was a gift of Prof. T. Kinoshita, Department of Immunoregulation, Research Institute for Microbial Diseases, Osaka University (Osaka, Japan). It cross-reacts extensively on CR2 and CR1 and induces a downregulation of these receptors for ~7 d. 200 μg of antibody was diluted in 200 μl of BSS and injected once intravenously 1 d before immunization (25).

Immunizations. For immunizations with live virus or baculovirus-derived VSV-G (Bac VSV-G), 2×10^6 PFU of VSV or 10 μg of Bac VSV-G, respectively, were injected in 200 μl of BSS i.v. or i.p. Alternatively, the same amount of virus or protein was mixed 1:1 with IFA and then injected subcutaneously at the base of tail.

Formalin inactivation of VSV-IND was performed by addition of 16 μl of 4% formalin to 1 ml of VSV-IND at a concentration of 10^9 PFU/ml (final formalin concentration 0.0625% [42]). The mixture was incubated at 4°C for 16h. Before injection, inactivated virus was diluted with BSS so that 200 μl could be injected intravenously in every experiment.

VSV Neutralization Assay. Neutralizing titers of sera were determined as described (43). In brief, the sera were prediluted 40-fold in MEM supplemented with 5% FCS, then heat inactivated for 30 min at 56°C. Serial twofold dilutions were mixed with equal volumes of VSV diluted to contain 500 PFU/ml. The mixture was incubated for 90 min at 37°C in an atmosphere with 5% CO_2. 100 μl of serum–virus mixture was transferred onto Vero cell monolayers in 96-well plates and incubated for 1 h at 37°C. An overlay of 100 μl DMEM containing 1% methylcellulose was added. After incubation for 24 h at 37°C, the overlay was flicked off, and the monolayer was fixed and stained with 0.5% crystal violet. The highest dilution of serum that reduced the amounts of plaques by 50% was taken as the neutralizing titer. Titers are indicated as $-\log_2$ of 40-fold prediluted sera. To determine IgG titers, undiluted serum was first pretreated with an equal volume of 0.1 M 2-mercaptoethanol in saline (44).

LCMV-NP–specific ELISA. We used a sandwich ELISA with the following steps: (*a*) coating with baculovirus-derived LCMV-NP (1 μg/ml); (*b*) blocking with 2% BSA (Fluka AG, Buchs, Switzerland) in PBS; (*c*) 20-fold prediluted mouse serum, titrated 1:2 over 10 dilution steps; (*d*) IgM- or IgG-specific horseradish peroxidase–labeled goat anti–mouse antibodies (0.5 μg/ml; Southern Biotechnology Associates, Inc., Birmingham, AL); and (*e*) substrate ABTS (2.2′-azino-bis-[3-ethylbenzthiazoline-6-sulfonate]); Boeh-

[1]*Abbreviations used in this paper:* AFC, antibody-forming cell; Bac VSV-G, baculovirus-derived VSV-G; BSS, balanced salt solution; CVF, cobra venom factor; FDC, follicular dendritic cell; GC, germinal center; LCMV, lymphocytic choriomeningitis virus; LCMV-NP, LCMV nucleoprotein; PNA, peanut agglutinin; RT, room temperature; sIg, surface Ig; TD, T cell–dependent; TI, T cell–independent; vacc VSV-G, recombinant vaccinia virus expressing VSV-G; VSV, vesicular stomatitis virus; VSV-G, VSV glycoprotein; VSV-IND, VSV Indiana serotype.

ringer Mannheim, Mannheim, Germany) and H_2O_2 (Fluka AG). Plates were coated overnight at 4°C; all other incubations were for 60–90 min at room temperature (RT). Between incubations, plates were washed three times with PBS containing 0.5 ml Tween 20 per liter. OD was measured at 405 nm in an ELISA reader.

Enzyme-linked Immunospot Assay for VSV-specific Antibody-forming Cells. Antibody-forming cell (AFC) frequencies were determined as described (45, 46). In brief, 25–square well polystyrene plates were coated with purified VSV ($\sim 5 \times 10^8$ PFU/ml). On the next day, plates were blocked with 2% BSA in PBS (see ELISA, above) for 2 h. Titrated amounts of spleen or bone marrow cells (titration 1:5 over four dilution steps starting with 5×10^6 cells/well) were added in 2% MEM and incubated for 5 h at 37°C. After washing with PBS-Tween (see ELISA, above), goat anti–mouse IgG antibody (2 μg/ml; E·Y Labs, San Mateo, CA) was added, and plates were incubated for 2 h at 37°C. After washing with PBS-Tween, alkaline phosphatase–labeled donkey anti–goat antibody (1 μg/ml; Jackson ImmunoResearch Labs, West Grove, PA) was added, and plates were incubated overnight at RT. On the next day, plates were washed, and the substrate solution (5-bromo-4-chloro-3-indolyl phosphate at 1 mg/ml in 0.6% agarose) was added to develop blue color spots. AFC numbers were then calculated per 10^6 nucleated cells.

Adoptive Transfer Experiments. Single cell suspensions of splenocytes from VSV-IND–primed normal or $CD19^{-/-}$ mice were injected intravenously into irradiated (450 rad) recipients. Donor cells were pooled from two individuals, and 10^7 spleen cells were transferred to each host (groups of three animals). Recipient mice were then challenged 24 h after transfer with 2×10^6 PFU of formalin-inactivated VSV. Blood was taken on day 3 after challenge to determine neutralizing IgG titers.

Immunohistochemistry. Freshly removed organs were immersed in HBSS and snap frozen in liquid nitrogen. Tissue sections of 5-μm thickness were cut in a cryostat, placed on siliconized glass slides, air dried, fixed with acetone for 10 min, and stored at −70°C. Secondary affinity-purified polyclonal anti-Ig antisera were diluted in Tris-buffered saline (TBS, pH 7.4) containing 5% mouse serum. All other dilutions were made in TBS alone. Incubations were done at RT for 30 min; TBS was used for all washing steps. Alkaline phosphatase was visualized using naphthol AS-BI (6-bromo-2-hydroxy-3-naphtholic acid-2-methoxy anilide) phosphate and new fuchsin as substrate. Endogenous alkaline phosphatase was blocked by levamisole. All color reactions were performed at RT for 15 min with reagents from Sigma Chemical Co. (St. Louis, MO). Sections were counterstained with hemalum. Coverslips were mounted with glycerol and gelatin.

Staining for VSV-specific B cells was done as described (47). For staining of cell differentiation markers, rehydrated tissue sections were incubated with the rat primary mAbs anti-FDC (4C11 [48]) and anti-B220 (RA3-6B2; PharMingen, San Diego, CA). Primary rat mAbs were revealed by a twofold sequential incubation with rabbit anti–rat Ig and rat alkaline phosphatase anti–alkaline phosphatase complex (Dakopatts A/S, Glostrup, Denmark). GCs were stained with peanut agglutinin (PNA [47]).

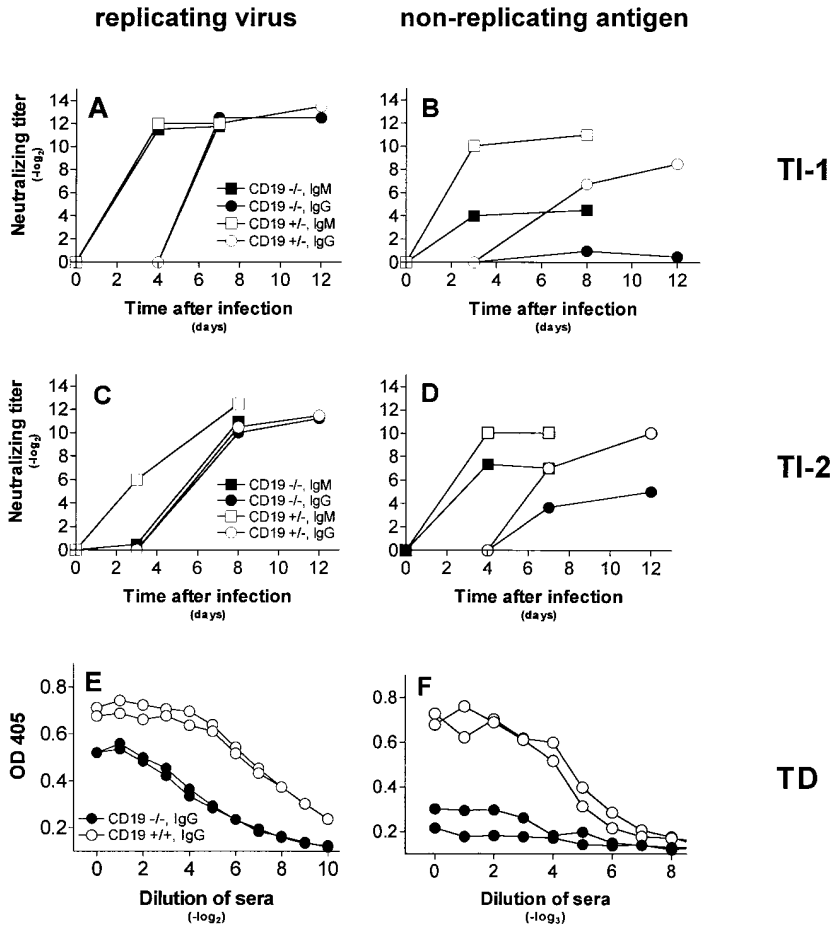

Figure 1. Antibody responses to replicating and nonreplicating viral antigens of TI-1, TI-2, and TD type. (*A–D*) Mice were immunized intravenously with either 2×10^6 PFU of live (*A*) or formalin-inactivated VSV (*B*), vacc VSV-G (*C*), or 10 μg of Bac VSV-G, and sera for determination of VSV-neutralizing titers were taken at the indicated time points. Each data point represents the mean of two to three mice. (*E* and *F*) Mice were immunized with 200 PFU of live LCMV intravenously (*E*) or 300 μl of purified LCMV in CFA subcutaneously (*F*), and sera were taken on day 16 after immunization to test for LCMV-NP–binding antibodies in ELISA. Each line indicates an individual mouse. All experiments were done with two to three mice per group and repeated at least once. *Filled symbols*, $CD19^{-/-}$; *open symbols*, $CD19^{+/+}$; *squares*, IgM; *circles*, IgG.

Table 1. *Dependence of VSV Neutralizing Antibodies on Application Route*

Mouse strain:	$CD19^{+/+}$		$CD19^{-/-}$	
Neutralizing antibody:	IgM, day 4	IgG, day 12	IgM, day 4	IgG, day 12
VSV live				
i.v.	9/8/8*	12/12/11	9/9/8	12/10/9
s.c.	8/7/7	12/10/9	9/7/4	12/11/8
i.p.	9/8	13/13	**7/6/5**	**10/9/7**
VSV form				
i.v.	10/9	13/12	7/6/5	10/9/7
i.p.	7/7/6	4/2/1	**0/0/0 (day 4)** **3/1/1 (day 12)**	**0/0/0**
Bac VSV-G				
i.v.	8/8/7	9/9/7	6/5/5	4/3/2
s.c.	6/5	5/4	4/1	1/1
i.p.	8/8/8	9/9/8	7/7/7	8/7/6

*Neutralizing titers are indicated as $-\log_2$ of 40-fold prediluted sera. Each number indicates the titer of an individual mouse. Each experiment was repeated once or twice.

Results

Normal Antibody Titers against Replicating, But Reduced Titers against Nonreplicating TI-1 or TI-2 VSV-G Antigens. Primary neutralizing antibody responses to four different forms of VSV-G were tested (Fig. 1, *A–D*). VSV-G exposed in a highly repetitive order on live or formalin-inactivated viral particles has been shown to induce neutralizing IgM responses completely independent of T cells, i.e., as a TI-1 antigen. In contrast, VSV-G on the membrane of cells infected with a recombinant vaccinia virus expressing VSV-G (vacc VSV-G) and Bac VSV-G are TI-2 antigens (49). For all types of VSV-G antigens, the IgG response is TD (50).

$CD19^{-/-}$ mice immunized with 2×10^6 PFU of live VSV (Fig. 1 *A*) or vacc VSV-G (Fig. 1 *C*) exhibited normal neutralizing VSV-G–specific antibody titers, although in the latter case, induction of IgM antibodies was delayed 3 d compared with control mice. $CD19^{-/-}$ mice survived these infections normally without displaying any morbidity. In contrast, antibodies to formalin-inactivated VSV (Fig. 1 *B*) and Bac VSV-G protein (Fig. 1 *D*) were reduced markedly. This result differs from recent experiments with haptenated antigens, where the TI-2 response to DNP-Ficoll (36) but not to NP-Ficoll (17) was enhanced.

TD Anti-LCMV Antibodies Are CD19-dependent for Replicating and Nonreplicating Antigen. Antibodies to LCMV have been shown to be dependent on T helper cells for the IgM and IgG response. Therefore, we tested $CD19^{-/-}$ mice for their capacity to mount LCMV-NP–specific antibodies after immunization with either live virus intravenously (100 PFU LCMV strain WE; Fig. 1 *E*) or with UV-inactivated virus subcutaneously (300 μl of purified LCMV in CFA corresponding to ~20 μg of protein; Fig. 1 *F*). IgG (Fig. 1, *E* and *F*) as well as IgM (data not shown) to both types of antigens were decreased when measured in an NP-specific ELISA. This result confirms the published severe defect of these mice in TD antibody responses (17, 18). But there is a striking difference between a low dose of replicating and a high dose of inactivated virus in $CD19^{-/-}$ mice; only the latter nonreplicating antigen failed to induce IgG antibodies in $CD19^{-/-}$ mice.

The Route of Antigen Administration Selectively Influences Antiviral Antibody Responses in $CD19^{-/-}$ Mice. B cell development in $CD19^{-/-}$ mice has been shown to be virtually normal except for a severe reduction of peritoneal B-1 cells (17, 18). To see whether this has any consequence for antiviral antibodies, we immunized $CD19^{-/-}$ and control mice with live VSV, formalin-inactivated VSV, or Bac VSV-G via three different application routes (Table 1): intravenously, subcutaneously in IFA, or intraperitoneally. In the case of Bac VSV-G, the reduction in neutralizing titers in $CD19^{-/-}$ mice was comparable for all three application routes, although it was slightly more pronounced after subcutaneous immunization; this indicated that IFA could not compensate for a CD19-mediated costimulation of B cells.

In contrast, after intraperitoneal immunization with live or, with a much more pronounced effect, formalin-inactivated virus, neutralizing titers were reduced by a factor of 8–100, suggesting a role for B-1 cells in early anti-VSV immune responses after intraperitoneal infection.

Interactions between CD19 and the Complement System. To test the hypothesis that CD19 functions as a signal transducer for CR2, we looked for the ability of $CD19^{-/-}$ mice to produce VSV-specific neutralizing antibodies after interference with the complement system in two different

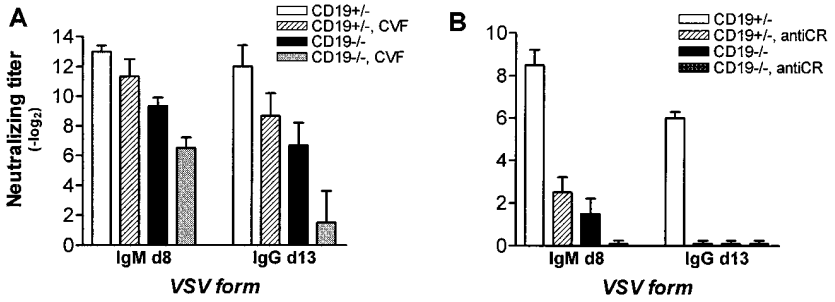

Figure 2. Antibody responses to formalin-inactivated VSV after treatment with CVF or anti-CR antibody. On day −1, CD19$^{-/-}$ or control mice were injected twice with CVF (*A*) or once with anti-CR antibody 7G6 (*B*). All mice were then immunized on day 0 with 2×10^6 PFU of formalin-inactivated VSV, and VSV-neutralizing titers were determined on day 8 (*IgM d8*) or day 13 (*IgG d13*). Each bar represents the mean of two to three mice. The experiments were repeated once.

ways: either the complement component C3 was depleted by CVF (Fig. 2 *A*), or the complement receptors CR1 and CR2 were blocked by the mAb 7G6 (Fig. 2 *B*). Mice were then immunized with 2×10^6 PFU of formalin-inactivated VSV.

Both treatments led to more pronounced reduction of IgG than IgM responses, confirming earlier results showing that VSV activates B cells to produce IgM mostly independent of additional signals, whereas switch to IgG depends on CD4$^+$ T cells and complement (31). In addition, Fig. 2 shows that (*a*) the titers in CD19$^{-/-}$ mice were lower than in C3-depleted or (less pronounced) in anti-CR–treated controls, and (*b*) C3 depletion led to a further reduction in CD19$^{-/-}$ mice. This second finding suggests that complement has effects on B cells that are not CD19 mediated, as for example an opsonizing function of complement for enhanced antigen presentation (51). The first finding could be explained by incomplete blocking of the complement system with CVF or anti-CR, or it may indicate a direct complement-independent function of CD19 on B cells. Whether this needs triggering via a separate potential CD19 ligand is not known. But direct association of CD19 with sIgM on B cells has already been demonstrated (13, 14), and may indicate a direct influence on sIg signaling by CD19 (52).

CD19 Is of Crucial Importance for Antiviral B Cell Memory. More than 20 years ago, an important role of complement for maintenance of B cell memory was postulated (53) and later confirmed (54, 55). By opsonization of antigen or immune complexes, complement would either enhance the persistence of antigen on follicular dendritic cells (FDCs) or deliver special signals to memory cells via CRs. Since CR2 is thought to transduce signals through CD19, we looked at B cell memory in CD19$^{-/-}$ and control animals. These mice were immunized with a standard dose of 2×10^6 PFU of VSV, which is known to induce life-long immunity in mice (47). To assess B cell memory, we chose three different readouts. (*a*) First, we looked at maintenance of neutralizing IgG titers over ~100 d after immunization with live VSV (Fig. 3 *A*) or Bac VSV-G (Fig. 3 *D*); after live VSV infection of CD19$^{-/-}$ mice, neutralizing antibody titers dropped from 10 to 2 within 105 d, and after immunization with Bac VSV-G, they dropped to 1 within 30 and to 0 within 120 d. In both experiments, control animals maintained the titers obtained by day 20 within a factor of two to four (or within one to two titer steps). (*b*) As a readout for memory B cells, we took spleen cells from animals of the same experiment as in Fig. 3 *A* at three different time points and adoptively transferred them into irradiated recipient mice. 1 d after transfer, the recipients were challenged with 2×10^6 PFU of formalin-inactivated virus, and neutralizing IgG titers 3 d after challenge were taken as a readout of B cell memory (Fig. 3 *B*). In control mice, the memory titers after challenge did not drop significantly over 105 d, whereas in CD19$^{-/-}$ mice, a significantly lower titer than in controls could be measured as early as day 12. This titer had disappeared completely by the later time points tested. In a similar experiment (Fig. 3 *E*), memory spleen cells were transferred into irradiated transgenic recipients expressing the VSV-G either as a soluble protein in the serum (Monitor [56]) or under a class I promoter on the membrane of most cells (KINDG [57]). The antigenicity of these transgenes has been shown previously to restimulate memory B cells (58). Although in Monitor mice, only CD19-competent cells were able to mount a small neutralizing titer, in KINDG mice, CD19$^{-/-}$ cells also produced some neutralizing antibody, but at least 100-fold lower than control cells. (*c*) As a readout for VSV-specific AFCs (Fig. 3 *E*), enzyme-linked immunospot assays of bone marrow and spleen cells were performed, again from animals of the experiment described in Fig. 3 *A*. Plasma cell frequencies were determined at three different time points. Although neutralizing antibody titers in the serum did not differ between CD19$^{-/-}$ and control mice 12 d after immunization, AFC frequencies in CD19$^{-/-}$ mice were already lower by a factor of eight in the spleen and almost undetectable in the bone marrow. After 46 and 105 d, AFC numbers in CD19$^{-/-}$ mice were below the detection level, whereas in CD19-competent mice, significant numbers of plasma cells could be measured in spleen and bone marrow for up to 105 d.

CD19 Is Not Necessary for the Generation of VSV-specific GCs. A severe defect in the generation of GCs after immunization with TD antigens has been described for CD19$^{-/-}$ (17, 36) as well as C3$^{-/-}$ (59) and CR$^{-/-}$ mice (60). Since maintenance of B cell memory is thought to be governed by persisting antigen in immune complexes on FDCs in GCs (61), we wanted to see whether the loss of VSV-specific B cell memory in CD19$^{-/-}$ mice was due to the failure to generate GCs. From the same mice shown in Fig. 3, *A–C*, spleen sections were prepared and stained immunohistochemically for VSV-specific B cells (see Materials and Methods, and reference 47; Fig. 4, *A* and *B*), GC B cells (PNA; Fig. 4, *C* and *D*), FDCs (4C11; Fig. 4, *E* and *F*),

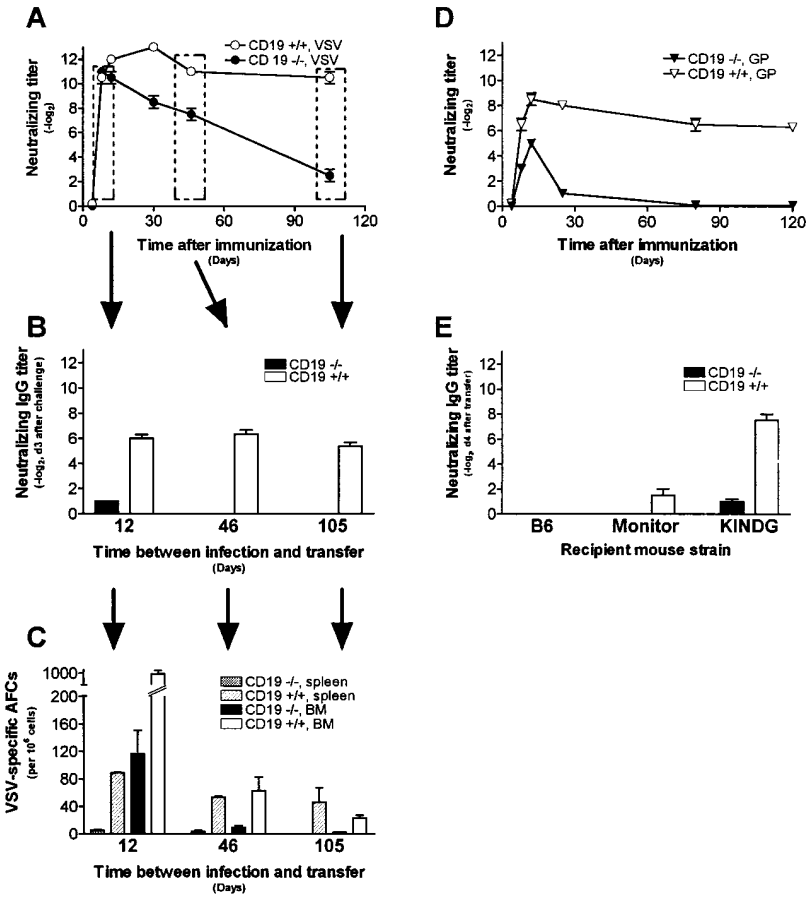

Figure 3. Analysis of B cell memory after VSV infection. $CD19^{-/-}$ and control mice were immunized with 2×10^6 PFU of live VSV (*A*) or 10 μg of Bac VSV-G (*D*), and neutralizing IgG titers followed over the indicated time period. On day 12, 46, and 105, spleen and bone marrow cells of two mice from each group (as in *A*) were taken and analyzed for frequency of VSV-specific AFCs (*C*), or spleen cells were transferred into irradiated recipients, challenged with inactivated VSV, and assessed for neutralizing IgG titers 3 d after challenge (*B*). In another experiment (*E*), day 30 VSV–immune memory spleen cells were transferred into VSV-G transgenic Monitor or KINDG mice, and neutralizing IgG titers were measured 4 d after transfer. For each time point, two donor mice and three recipient mice were used. The experiment was performed twice with similar results.

and total B cells (B220; Fig. 4, *G* and *H*). Fig. 4 shows spleen sections derived from the group of mice killed 46 d after immunization. Surprisingly, in $CD19^{-/-}$ as well as in CD19-competent mice, VSV-specific GCs could be observed until day 105; despite this, the former mice lost their neutralizing antibody titers. Quantitative analysis (Table 2) showed a difference in numbers of GCs, with the $CD19^{-/-}$ mice about twofold lower than the controls. In addition, GCs of $CD19^{-/-}$ mice appeared smaller in size (diameter reduction of ~30%). Quantitative analysis of overall GC numbers yields a reduction of maximally sevenfold [$(1.5)^3 \times 2 = 6.75$]. This reduction is substantial, but cannot explain the total loss of VSV-specific neutralizing antibodies solely on a quantitative basis. In general, CD19 was apparently not necessary for generation of antigen-specific GCs to VSV-G–neutralizing epitopes.

Discussion

The role of CD19 and associated CR2 for costimulation of B cells has been clearly demonstrated in vitro as well as with $CD19^{-/-}$ (17, 18) and $CR^{-/-}$ mice (29, 30) in vivo. Complement has been postulated to have a bridging role between innate and adaptive immunity (35). This study first looked at B cell responses to live virus infections in $CD19^{-/-}$ mice. Mice were immunized with VSV, which induces TI-1 IgM antibody response and is controlled by the subsequent TD IgG response; alternatively, vacc VSV-G was used, which induces TI-2 antibodies and is controlled by cytokines and T cells, or LCMV, an Arenavirus that induces strictly TD antibody responses and is controlled by cytotoxic T cells. $CD19^{-/-}$ mice were protected against all of these infections and did not show any morbidity. Consistent with published results from studies with haptenated protein antigens, the TD antibody responses were reduced markedly. But in contrast to another study (15), TI-2 responses were not enhanced, and TI-1 responses were only reduced when nonreplicating antigen was used. This finding may be explained by the following: (*a*) immunization with live virus, even if only an abortive replication cycle occurs, may produce a greater antigen load. This correlates with published studies that show decreasing dependence of B cells upon costimulation via complement (and probably CD19) with increasing antigen dose (22, 23, 31). (*b*) In contrast to protein antigens, immunization with live virus always leads to nonspecific inflammatory reactions and secretion of cytokines (62), among which IFN-α has been shown to bind to CR2 (63) and to provide survival signals for GC B cells (64). Whether this mechanism has any relevance during infections needs to be further investigated. Our results at least indicate that such a stimulation via CR2 would be independent of CD19.

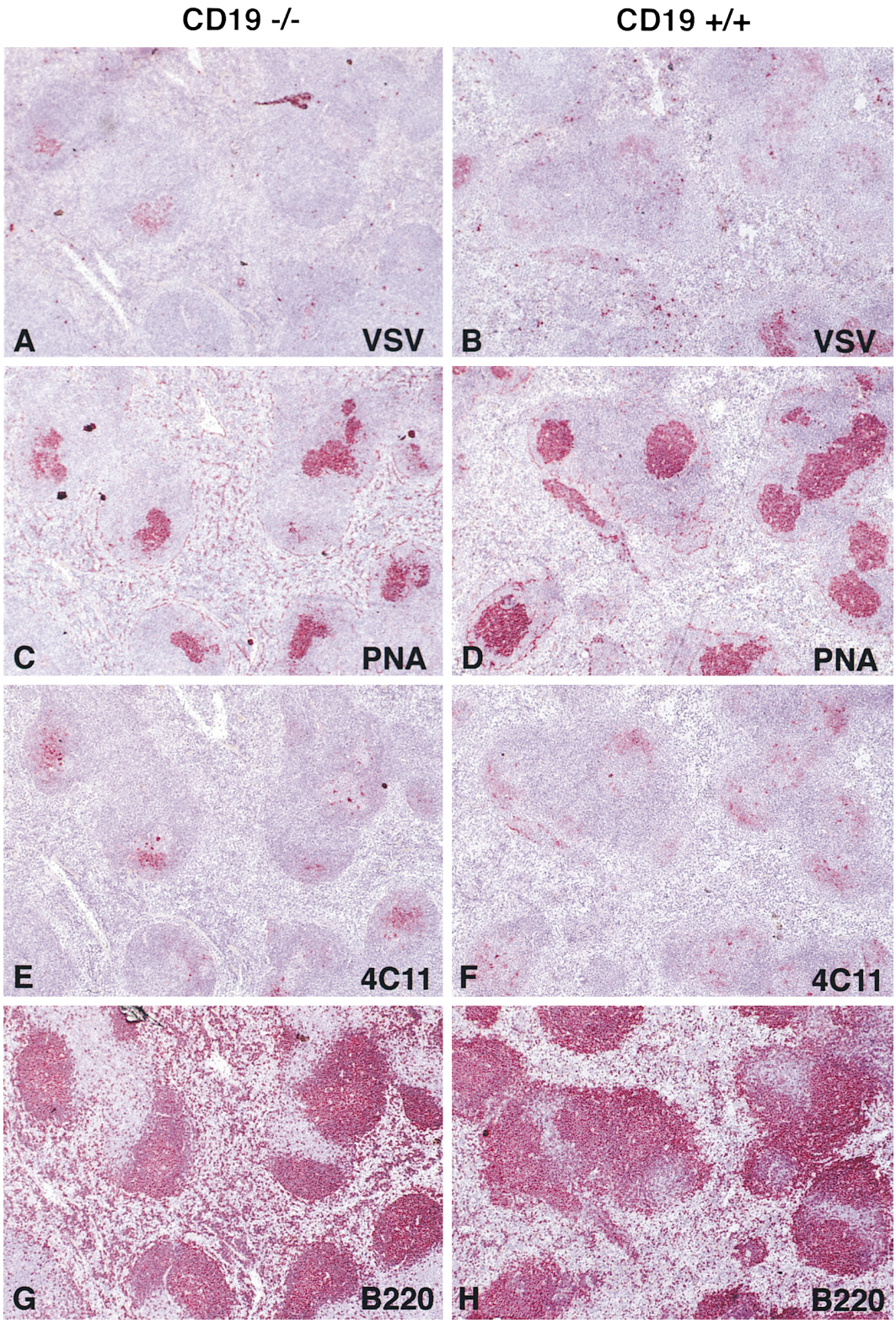

Figure 4. Immunohistochemical staining of memory spleen sections after VSV infection. Spleen sections of the same mice as in Fig. 3, *A–C* (time point day 46), were analyzed by immunohistochemistry and stained for VSV-specific B cells (*A* and *B*), GC B cells (PNA; *C* and *D*), FDCs (4C11; *E* and *F*), and B cells in general (B220; *G* and *H*). Original magnifications are ~80-fold.

Table 2. *Quantitative Analysis of GCs in CD19$^{-/-}$ and Control Mice after Immunization with VSV*

Days after immunization	Mouse strain	No. of PNA^{+} GCs*	No. of 4C11^{+} GCs*	No. of VSV-specific GCs*
12	CD19$^{+/+}$	32 ± 8	27 ± 2	15 ± 4
	CD19$^{-/-}$	27 ± 2	21 ± 5	10 ± 5
46	CD19$^{+/+}$	40 ± 6	21 ± 7	10 ± 3
	CD19$^{-/-}$	16 ± 1	16 ± 1	5 ± 3
105	CD19$^{+/+}$	59 ± 2	32 ± 3	7 ± 4
	CD19$^{-/-}$	25 ± 11	16 ± 7	3 ± 1

*Numbers ± SD indicate the amount of GCs counted per 10 visual fields at a magnification of 10.

CD19$^{-/-}$ animals have a severe defect in the formation of B-1 B cells, whereas B-2 cells are normal or only slightly reduced. B-1 cells are thought to produce natural antibodies that may also be involved in autoimmunity (65) as well as in early defense mechanisms against pathogens. Whether the absence of CD19 and the defect in peritoneal B-1 cells have consequences for antiviral immunity was investigated here by comparing immunizations with VSV via various infection routes. Indeed, when the virus was given intraperitoneally, neutralizing antibody titers were reduced. This difference was even more drastic when the virus was inactivated. But in the case of live virus infection, it did not have any effect on protection of CD19$^{-/-}$ animals. These results may indicate a crucial role of the route of antigen application, or they reveal a biological function of the B-1 cell compartment.

GC formation in CD19$^{-/-}$ mice has been shown to be severely impaired after immunization with haptenated protein antigens (17, 36). But consequences for generation of B cell memory have not been analyzed. These results indicate that memory antibody titers, AFC frequencies, and memory B cells detected by adoptive transfer are severely impaired in these mice. Most probably, the generation of memory B cells is already affected, because—although antibody titers 12 d after infection were comparable to control mice—AFC and memory B cells were already clearly reduced at that time. This finding was independent of the generation of GCs, which could be found in both CD19$^{-/-}$ and control animals, even at >100 d after infection, as described for the latter (47). These findings question the strict correlation between GC formation and establishment or possibly maintenance of B cell memory (66). GCs are thought to be the structural environment where somatic mutation occurs, high-affinity B cells are selected (67), and potentially autoreactive B cells are eliminated (68). Our results suggest that GC formation may be necessary but not sufficient for generation or maintenance of B cell memory. This supports evidence obtained in earlier studies using haptenated LPS (69), aged mice (70), or blocking of B7-2 in established GCs, which impaired B cell memory (71). CD19 may provide additional signals for generation and survival of memory B cells. Earlier studies that demonstrate the binding of CD19 to CD77, a marker of GC B cells, would be in line with this hypothesis (72, 73).

We would like to thank M.F. Bachmann and U. Karrer for stimulating discussions, A. Oxenius for providing purified LCMV, A. Althage for excellent technical assistance, L. Vek for immunohistology, and H. Nef and N. Wey for photographs.

This work was supported by the Swiss National Science Foundation (grant 31-32195.91 to R.M. Zinkernagel), the Kanton of Zürich, the Deutsche Forschungsgemeinschaft (SFB243), and the Human Frontier Science Program.

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Received for publication 10 December 1997 and in revised form 6 April 1998.

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