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Antiviral B Cell Memory in the Absence of Mature Follicular Dendritic Cell Networks and Classical Germinal Centers in TNFR1$^{-/-}$ Mice[1]

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TNFR1$^{-/-}$ mice have been shown to lack networks of mature follicular dendritic cells (FDCs) and they do not form germinal centers. With nonreplicating Ags, IgG titers were inefficiently induced and not maintained. In this study, the neutralizing Ab response and the establishment of B cell memory in TNFR1$^{-/-}$ mice after infection with vesicular stomatitis virus (VSV) were analyzed histologically and functionally. Immunization with VSV-derived protein Ags without adjuvant induced only IgM but no IgG Abs in TNFR1$^{-/-}$ mice, whereas VSV glycoprotein emulsified in CFA or IFA induced IgM and IgG responses that were short-lived and of moderate titer. However, infection with live VSV induced excellent neutralizing IgM and IgG responses in TNFR1$^{-/-}$ mice, and adoptively transferable B cell memory was generated and persisted for more than 300 days. In contrast, IgG levels and Ab-forming cells in the bone marrow declined within 300 days by 90–95% compared with controls. These findings suggest that 1) increased Ag dose and time of Ag availability can substitute for FDC-stored Ab-complexed Ag in the induction of efficient IgG responses in TNFR1$^{-/-}$ mice devoid of classical germinal centers; 2) the induction and maintenance of adoptively transferable B cell memory can occur in the absence of Ag bound to mature FDCs; and 3) the long-term maintenance of elevated IgG titers is largely dependent on FDC-associated persisting Ag. However, about 5–10% of the Ab production remained in the absence of detectable persisting Ag in TNFR1$^{-/-}$ mice, probably either due to immature FDCs being partially functional and/or due to long-lived plasma cells. *The Journal of Immunology,* **2000, 164: 768–778.**

B cell memory is usually defined by three major characteristics: 1) The persistence of elevated levels of specific Ab. 2) The presence of memory B cells that are often isotype switched and recirculating through the lymphatic system. 3) Secondary Ab responses after reexposure to Ag are faster, lead to higher Ab titers, and produce Ab of higher affinities than primary Ab responses (1–3). However, some memory B cells do not recirculate and some have been shown to be of the IgM isotype (4–6). Maintenance of elevated Ab titers vs survival of memory B cells seems to be regulated by distinct mechanisms. A large part of the maintenance of memory Ig titers is thought to be dependent on the presence of Ag persisting on follicular dendritic cells (FDCs)[3] in secondary lymphoid organs, indicating that at least some memory B cells are activated by Ag to produce Abs (7–10). It has recently been shown that plasma cells in the BM can be long-lived (with a $t_{1/2}$ of 90–130 days), contributing to the maintenance of memory IgG titers in an Ag-independent fashion (11, 12). However, the relative contribution of the Ag-independent vs Ag-dependent Ab production for the maintenance of memory Ig titers is still a matter of debate.

The regulation of memory B cell survival occurs differently. Early experiments suggested that survival of memory B cells was Ag dependent, since they rapidly died after transfer into an Ag-free host (13, 14). However, other groups have shown that memory B cells divide rarely and have a prolonged life span without the obvious need of continuous Ag contact (3, 15). Although Ab-forming cells (AFCs) colocalize with persisting Ag within secondary lymphoid organs, most memory B cells recirculate throughout the lymphatic system, further indicating that they do not need continuous contact with persisting Ag (9). Thus, most of the current data suggests that memory B cells do not require persistent Ag for survival, but whether elevated specific Ig titers in the serum are largely Ag dependent or not is unclear.

Germinal centers (GCs) are specialized sites in secondary lymphoid organs where memory B cells are generated and selected for enhanced survival and for expression of high affinity surface IgG by Ag persisting on FDCs (2, 16–18). If the GC reaction is inhibited either by depletion of the complement component C3 or by blocking the interaction of CD40 with CD40L, memory B cells and secondary Ab responses are not generated (19–23). However,

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[3] Abbreviations used in this paper: FDC, follicular dendritic cell; AFC, Ab-forming cell; BM, bone marrow; GC, germinal center; LN, lymph node; MLN, mesenteric lymph node; MZ, marginal zone; PALS, periarteriolar lymphoid sheath; PNA, peanut agglutinin; TD, T cell dependent; TI, T help-independent; VaccIND$_G$, vaccinia virus recombinant for VSV-G; VSV, vesicular stomatitis virus; VSV IND, VSV Indiana strain; VSV NJ, VSV New Jersey strain; VSV-G, glycoprotein of VSV IND; CD40L, CD40 ligand.

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isotype switch can occur outside the follicle and before GCs are generated (24), and surprisingly also affinity maturation seems not to be strictly dependent on a GC reaction (25).

Mice deficient for TNF-α or for TNFR1 (p55, CD120a) have been shown to lack mature FDCs and primary B cell follicles and, upon immunization with T cell-dependent (TD) Ags, GC formation was absent (26–28). The humoral immune response of TNF-α-deficient and TNFR1-deficient mice to nonviral TD Ag was reduced, and the isotype switch from IgM to IgG occurred only with delay and was reduced in magnitude (27, 28). However, none of these studies addressed the fate of B cell memory in the absence of FDCs and GCs.

In this study, the primary B cell response and the development of B cell memory in TNFR1$^{-/-}$ mice after infection with vesicular stomatitis virus (VSV) serotype Indiana (VSV IND) were analyzed. VSV is a cytolytic virus that does not persist in an infectious form after peripheral infection of adult mice (29, 30). VSV induces an early, T help-independent (TI) neutralizing IgM response by day 4, followed by a neutralizing IgG response peaking by day 12 that is dependent on cognate interaction of Th cells and B cells (31–33). Although TI Ags are usually poor inducers of GC and B cell memory, it has been shown that VSV induced long-lived GCs in the spleen (>day 100) and life-long neutralizing IgG titers in normal mice (9, 10). Neutralizing Abs to VSV IND are not cross-reactive to the VSV-New Jersey (NJ) serotype (34), and they are specific for one antigenic site on the glycoprotein, as defined by surface-binding competition experiments (35, 36). This exclusive specificity for multiple identical determinants expressed on intact VSV particles also allows specific amplification of the Ab-binding signal, and therefore the staining of Ag-specific GC on frozen sections, as determined earlier (9).

We report in this work that live VSV induced excellent in vivo neutralizing IgM and IgG responses of intermediate to high affinity in TNFR1$^{-/-}$ mice. However, UV-inactivated VSV or rVSV-glycoprotein preparations without adjuvant induced only an IgM but no IgG response in TNFR1$^{-/-}$ mice, suggesting that T-B collaboration was reduced in TNFR1$^{-/-}$ mice. Adoptively transferable B cell memory persisted in TNFR1$^{-/-}$ mice and in controls over 300 days. In contrast, memory IgG titers were reduced by 95%, indicating that a large proportion of specific memory Ig is dependent on Ag persisting on FDCs, which allows continued induction of plasma cells.

Materials and Methods

Mice and animal experiments

TNFR1$^{-/-}$ mice as well as (C57BL/6 $\times$ 129/Sv)F_1 and C57BL/6 mice used as normal controls were bred and kept under specific pathogen-free conditions. The generation of TNFR1$^{-/-}$ and IFN-$\alpha\beta$R$^{-/-}$ mice has been described in detail elsewhere (37, 38). In all experiments presented in this study, no significant differences between the C57BL/6, 129/Sv, and (C57BL/6 $\times$ 129/SV)F_1 mouse strains used as normal controls have been detected. Therefore, data from one control strain (usually C57BL/6) are presented. TNFR1$^{-/-}$ mice and (C57BL/6 $\times$ 129/Sv)F_1 mice were obtained from BRL (Füllinsdorf, Switzerland). C57BL/6 mice were purchased from the breeding colony of the Institut für Labortierkunde (University of Zürich, Zürich, Switzerland). All animal experiments were performed with age- and sex-matched mice of 7–14 wk of age with permission of the veterinary office, according to cantonal and federal law requiring the use of minimal numbers of experimental animals.

Viruses

VSV, VSV IND (Mudd-Summers isolate), and VSV NJ (Pringle isolate), originally obtained from Dr. D. Kolakofsky (University of Geneva), were grown on BHK-21 cells infected at low multiplicity and plaqued on Vero cells (39). Recombinant vaccinia virus expressing the glycoprotein of VSV IND ($VaccIND_G$) was a generous gift of Dr. B. Moss, Laboratory of Viral Diseases, National Institutes of Health (Bethesda, MD) (40). Vaccinia virus was grown at a low multiplicity of infection on BSC 40 cells and plaqued on BSC 40 cells. The recombinant baculovirus expressing the glycoprotein of VSV IND (VSV-G) was a generous gift of Dr. D. H. L. Bishop, NERC Institute of Virology (Oxford, U.K.). The recombinant baculovirus was derived from nuclear polyhedrosis virus and was grown at 28°C in *Spodoptera frugiperda* cells in spinner cultures using TC-100 medium. Recombinant protein was produced as described (41).

Immunohistology

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 TBS (pH 7.4) containing 5% normal mouse serum. All other dilutions were made in TBS alone. Incubations were done at room temperature for 30 min; TBS was used for all washing steps. Alkaline phosphatase was visualized using naphthol AS-BI phosphate and New Fuchsin as substrate, which yields a red precipitate. Only for the detection of VSV-Ag-coupled alkaline phosphatase the more sensitive NBT/BCIP reaction was used. Endogenous alkaline phosphatase was blocked by levamisole. All color reactions were performed at room temperature for 15 min with reagents from Sigma (St. Louis, MO). Sections were counterstained with hemalum. Coverslips were mounted with glycerol and gelatin.

Staining for viral and cell differentiation markers. Rehydrated tissue sections were incubated with the following rat primary mAb: anti-CD4 (YTS 191) (42), anti-CD45RO/B220 (RA3-6B2; PharMingen, San Diego, CA), and antifollicular dendritic cells FDC-M1 (43) and anti-CD35 (8C12; PharMingen) (44). Primary rat mAb were revealed by a 2-fold sequential incubation with rabbit anti-rat Ig and rat APAAP (alkaline phosphatase antialkaline phosphatase) complex (Dako, Glostrup, Denmark). GCs were stained with peanut agglutinin (PNA), as previously described (9).

Staining for Ig. Sections were incubated with biotinylated monoclonal rat anti-mouse IgM (R6-60.2) or rat anti-mouse IgD^b (217-170) (PharMingen), followed by alkaline phosphatase-labeled avidin-biotin complexes (prepared according to the instructions of the manufacturer; Dako). The detection of VSV-Ag and VSV-specific B cells has been described previously (10).

Serum neutralization test

Neutralizing titers of sera and hybridoma supernatants were determined as described (45). In brief, serial 2-fold dilutions of 40-fold prediluted (supplemented MEM) and heat-inactivated (30 min at 56°C) sera or undiluted supernatants were mixed with equal volumes of VSV IND diluted to contain 500 PFU/ml. The mixture was incubated for 90 min at 37°C in an atmosphere containing 5% CO_2. A total of 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 DMEM containing 1% methylcellulose. After incubation for 24 h at 37°C, the overlay was removed and the monolayer was fixed and stained with 0.5% crystal violet. The highest dilution of the serum or supernatant that reduced the number of plaques by 50% was taken as the neutralizing titer. To determine IgG titers in the sera, undiluted serum was first pretreated with an equal volume of 0.1 M 2-ME in saline. Such treatment has been shown to completely abrogate IgM-mediated VSV neutralization of IgM mAbs and of polyclonal IgM antisera (day 4 after VSV infection) (34, 36). Titers represent $\log_2$ steps of 40-fold prediluted sera.

Neutralizing activity is expressed as standardized VSV-neutralizing titer of purified mAbs with a starting concentration of 1 μg/ml.

Avidity measurement

Avidity was determined directly from the purified hybridoma supernatants by an ELISA solid-phase method, as described (36, 46, 47). In brief, ELISA plates were coated with purified VSV at three different densities, and Ab concentrations leading to half-maximal absorbance were determined. The three values for Ab input concentration needed for half-maximal binding allowed the determination of free Ab concentration required for half-maximal binding.

Enumeration of AFC

VSV IND-specific AFC were enumerated as described (48). In brief, 25-well plates (Petra Plastik, Chur, Switzerland) were coated overnight with purified UV-inactivated VSV IND. Because largely intact virus particles are exposed, predominantly virus-neutralizing Abs can bind (9). After a blocking step, 5-fold dilutions of single spleen cell suspensions were added

and incubated for 5 h at 37°C. Plates were washed and incubated with goat anti-mouse IgG Abs, then washed and incubated with alkaline phosphatase-labeled donkey anti-goat IgG Abs. Ab spots were developed with alkaline buffer solution (Sigma) containing 1 mg/ml BCIP mixed with 3% agarose in distilled water (4:1).

Adoptive transfer for B cell memory detection

A total of 10^7 spleen cells of VSV IND- or VSV-G-primed TNFR1$^{-/-}$ and control mice (C57BL/6 and (C57BL/6 × SV129)F_1) was adoptively transferred into sublethally irradiated (4.5 Gy, day −1) C57BL/6 recipients. To allow for excess T help, 10^7 spleen cells from C57BL/6 mice immunized with 2×10^6 VSV NJ 6 days previously were cotransferred. VSV NJ has been shown to cross-prime T help for anti-VSV IND-neutralizing IgG, whereas the neutralizing anti-VSV Abs are not cross-reactive between the two VSV serotypes (45). This has also been demonstrated to be the case in TNFR1$^{-/-}$ mice (not shown). Twelve to twenty-four hours after adoptive transfer, the recipients were immunized with 2×10^6 PFU VSV IND, and the neutralizing IgG titers were determined at the indicated time points.

Detection of infectious VSV by adoptive transfer of organ homogenates into VSV-susceptible IFN-αβR$^{-/-}$ mice

TNFR1$^{-/-}$ mice and control wt 129 mice were infected i.v. with 2×10^6 PFU VSV, and 1 or 20 days later, brain, spinal cord, spleen, liver, and kidney were removed. Organs were homogenized and brain + spinal cord, spleen, and liver + kidney were pooled from two mice before i.p. inoculation into one IFN-αβR$^{-/-}$ mouse. Survival of the animals was monitored twice daily.

Generation of VSV IND-specific hybridomas

For the generation of VSV IND-specific monoclonal B cell hybridomas, TNFR1$^{-/-}$ mice and (C57BL/6 × 129Sv)F_1 control mice were immunized with 2×10^6 PFU VSV IND and boosted 9 or 16 days later with the same Ag and dose. Three days after the boosting, 10^7 spleen cells were fused with 10^7 cells of the Ig-nonproducing myeloma line X63Ag8.653. The supernatants of the growing hybridomas were screened for specific VSV binding with ELISA on VSV-coated plastic plates. VSV-binding supernatants were tested for VSV neutralization, and the positive clones were selected and subcloned by limiting dilution. Supernatants from the subclones were purified over a protein G column and then used for isotype determination, avidity measurements, and the determination of the neutralizing activity against VSV IND.

Results

No development of classical GCs in the spleen and the mesenteric lymph node (MLN) in TNFR1$^{-/-}$ mice after immunization with live VSV IND or with VSV-infected cells

TNFR1$^{-/-}$ mice and control mice were infected with 2×10^6 PFU live VSV IND i.v. or s.c. to immunohistochemically monitor the development of VSV-specific GCs in the spleen or in the draining LN. Alternatively, to allow the direct visualization of VSV-Ag, which is methodologically not possible after infection with 2×10^6 PFU live VSV IND (9, 10), mice were immunized i.p. with UV-inactivated cell lysates of VSV-infected BHK cells. It has been shown previously that both immunization protocols lead to an intensive GC reaction in the spleen of normal mice beginning at about day 6 after immunization, peaking at day 12, and persisting for more than 100 days (9, 10).

On day 1 after BHK-VSV administration, VSV-Ag was detectable in the red pulp and in the MZ of the spleen and in the marginal sinuses of MLN of TNFR1$^{-/-}$ mice and controls to a similar degree; the Ag localization correlated with the presence of resident macrophages (Fig. 1, *A* and *B*). By day 4, the red pulp and the MZ were already cleared of VSV-Ag. By then, VSV-Ag was colocalized with FDCs within B cell follicles of the spleen (Fig. 1*D*) and the MLN (not shown) in control mice. VSV-specific B cells were found in B cell foci in the red pulp and the MZ, but also within B cell follicles, where the GC formation started (not shown). In contrast, in the spleen and the MLN (not shown) of TNFR1$^{-/-}$ mice, no VSV-Ag (Fig. 1, *C* vs *D*, d4, and *E* and *F*, d12) and no mature FDC networks (Fig. 1, *G* vs *H*, d12, and *a*, *b* vs *e*, *f*) were detectable. VSV-specific B cell foci in the red pulp and scattered VSV-specific B cells were detectable in similar amounts as in controls (not shown).

By day 12 after immunization, when the GC reaction after VSV infection is maximal, all VSV-Ag detectable in control mice was localized within the GC (Fig. 1*F*) associated with FDCs (Fig. 1*H*) and VSV-specific B cells (Fig. 1*K*), thus suggesting that VSV was bound to the FDCs driving this GC reaction. In addition, some VSV-specific B cell foci in the red pulp and the MZ and some scattered VSV-specific plasma cells were detectable in control mice (Fig. 1*K*). VSV-specific plasma cells were characterized by very strong cytoplasmatic staining with VSV, whereas VSV-specific B cells show surface staining with no significant cytoplasmatic staining (9, 10). In contrast, VSV-Ag was not detectable by immunohistochemistry in spleens and MLN of TNFR1$^{-/-}$ mice (Fig. 1*E*). This suggested that mature FDCs were needed for efficient trapping of Ag and that they were lacking in TNFR1$^{-/-}$ mice also after infection with VSV, as defined by no observable cell staining with the FDC-specific Ab FDC-M1 (Fig. 1, *G* and *e*). Absence of networks of mature FDCs in classical GCs in TNFR1$^{-/-}$ mice upon immunization with VSV was confirmed using the 8C12 Ab (Fig. 1, *b* vs *f*), which recognizes complement receptor 1 or CD35 (44).

Moreover, VSV-specific B cell foci and plasma cells were detectable to a similar degree as in control mice (Fig. 1*I*). In addition, particular accumulations of VSV-specific B cells that were PNA positive (Fig. 1, *I* and *L*) were detected very rarely (1–2 per section) within the T cell area around the central arteriole of the spleen, but not in MLN (not shown). A similar PNA-positive structure has recently been described in MLN of TNFR1$^{-/-}$ mice (49). Due to their aberrant localization in T areas and lack of organized structure exhibiting a dark and a light zone, the PNA$^+$ B cell clusters are clearly different from classical GCs. Moreover, because PNA is generally up-regulated on recently activated B cells and also on B cells forming AFC foci in T regions (50), these pseudo-GCs most likely reflect aggregates of VSV-specific AFCs. At day 30 after infection, few VSV-specific B cells were still scattered in the red pulp and the MZ of the spleen of TNFR1$^{-/-}$ mice (Fig. 1*T*), but they gradually disappeared at earlier time points than in controls (Fig. 1*U*).

After infection with 2×10^6 PFU live VSV IND i.v., no GC were induced in TNFR1$^{-/-}$ mice at any time point. The quantity and distribution of VSV-specific B cells were comparable in TNFR1$^{-/-}$ and control mice with that after i.p. immunization with UV-inactivated BHK cell lysates, including the mentioned pseudo-GCs (not shown).

As additionally shown in Fig. 1, *X* and *Y*, IgD$^+$ B cells accumulate within the inner marginal zone in TNFR1$^{-/-}$ mice and they did not form primary or secondary B cell follicles. This has been shown previously for TNF-α-deficient mice (28).

Normal neutralizing IgM and IgG responses in TNFR1$^{-/-}$ mice after infection with live VSV IND and VaccIND$_G$

To evaluate the consequences of the lack of FDC and the inability to form GC in TNFR1$^{-/-}$ mice for TI and TD B cell responses, TNFR1$^{-/-}$ and control mice were infected with 2×10^6 live VSV IND or VaccIND$_G$ i.v. (Fig. 2). It has been shown that the neutralizing IgM response after infection with VSV IND is of the TI-1 type, and the one after infection with VaccIND$_G$ of the TI-2 type (51). The neutralizing IgG response is largely TD (32, 33, 52).

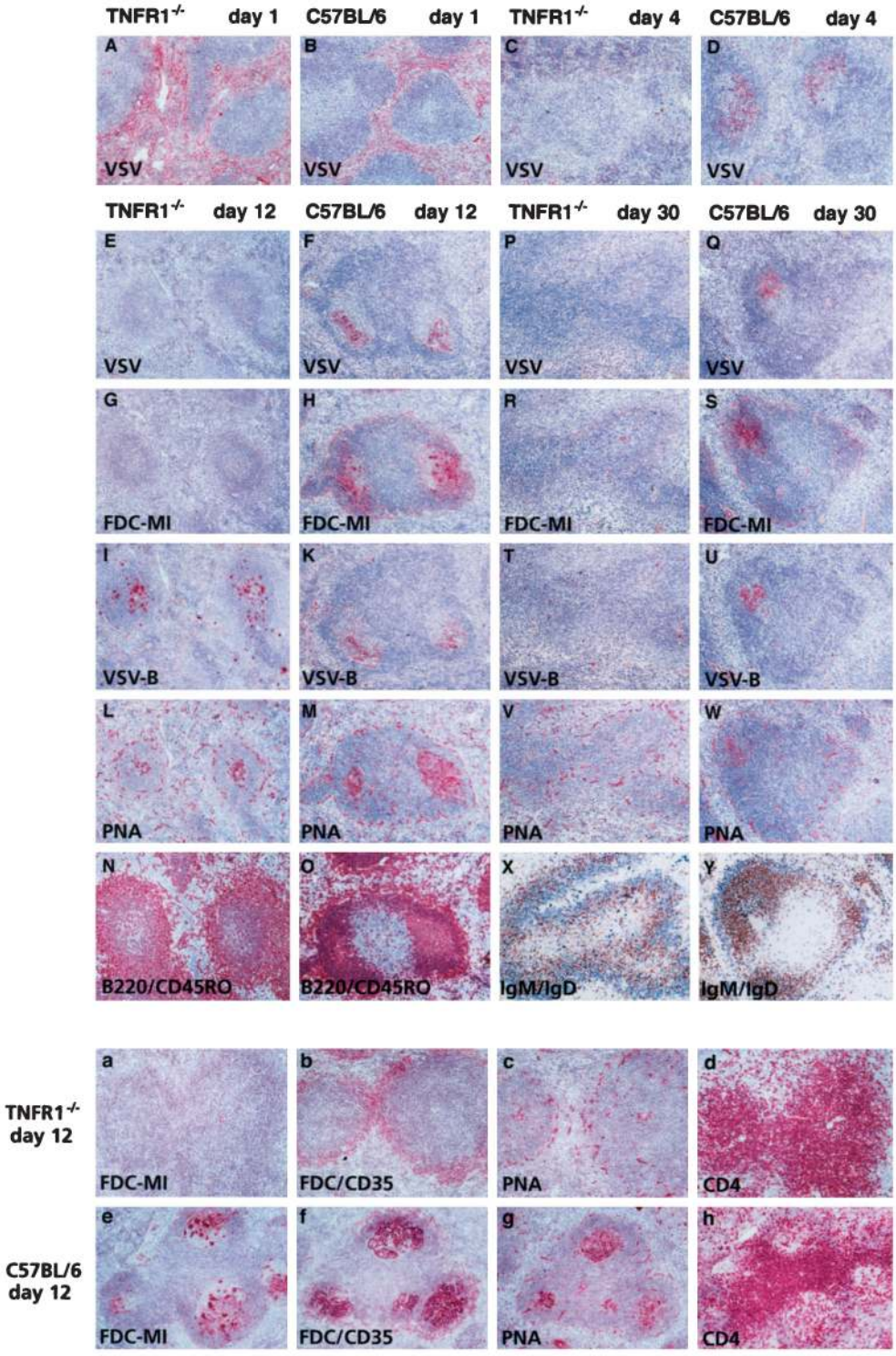

FIGURE 1. Immunohistological analysis of the VSV-specific B cell response of TNFR1$^{-/-}$ and control mice. Mice were immunized with VSV-infected and UV-inactivated cell lysates i.p., and the spleen sections were stained with the indicated quality. The distribution and persistence of VSV-Ag were monitored at days 1, 4, 12, and 30 after immunization, and the VSV-specific GC reaction was visualized on consecutive sections at days 12 and 30 after immunization. VSV, VSV-Ag; FDC-M1, follicular dendritic cells (FDC-M1 Ab); FDC/CD35, follicular dendritic cells (anti-CD35 Ab, 8C12); VSV-B, VSV-specific B cells; PNA, peanut hemagglutinin binding; B220/CD45RO, B220-positive B cells; IgM/IgD, double staining for IgM-positive cells (blue) and IgD-positive cells (brown); CD4, CD4-positive T cells.

The neutralizing IgM response was similar in TNFR1$^{-/-}$ mice and in controls after infection with live VSV IND. After infection with the TI-2 Ag VaccIND$_G$, the neutralizing IgM response was even more pronounced in TNFR1$^{-/-}$ mice compared with controls probably because vaccinia virus replicated slightly better in TNFR1$^{-/-}$ mice (53). Surprisingly, also the neutralizing IgG response after immunization with both viruses was similar in TNFR1$^{-/-}$ mice and controls, indicating that FDCs and GCs are not a prerequisite for the isotype switch from IgM to IgG after these viral infections.

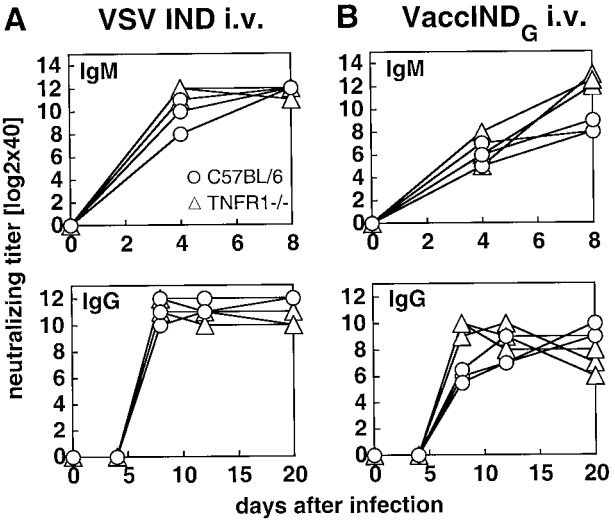

FIGURE 2. VSV-neutralizing IgM and IgG responses after infection with 2×10^6 PFU live VSV IND i.v. (*A*) or with 2×10^6 PFU VaccIND$_G$ i.v. (*B*). Blood was taken at the indicated time points, and the neutralizing IgM (*upper panels*) or IgG titers (*lower panels*) were determined in the sera. Titers represent $\log_2$ dilutions of 40-fold prediluted sera. △, TNFR1$^{-/-}$ mice; ○, C57BL/6 control mice. Each line represents one individual mouse. One of three equivalent experiments is shown.

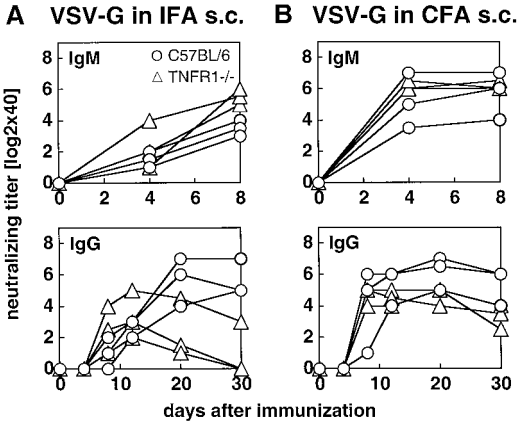

FIGURE 4. VSV-neutralizing primary IgM and IgG responses after immunization with 10 μg VSV-G emulsified in IFA (*A*) or CFA s.c. (*B*) at the base of the tail. Mice were immunized at day 0 and blood was taken at the indicated time points, and the neutralizing IgM (*upper panels*) or IgG titers (*lower panels*) were determined in the sera. Titers represent $\log_2$ dilutions of 40-fold prediluted sera. △, TNFR1$^{-/-}$ mice; ○, C57BL/6 mice. Each line represents one individual mouse. One of three equivalent experiments is shown.

Nonreplicating Ags without adjuvant fail to induce isotype switch in TNFR1$^{-/-}$ mice

UV-inactivated VSV or rVSV-G represent VSV-Ag preparations that do not replicate and, without an adjuvant, they fail to induce a significant inflammatory reaction. Both Ags induce a strong TI-neutralizing IgM response in normal mice, reflecting direct B cell activation, whereas the neutralizing IgG response is T dependent (51). The neutralizing IgM response after immunization of TNFR1$^{-/-}$ mice with these nonreplicating VSV-Ags was comparable with controls (Fig. 3). In contrast, TNFR1$^{-/-}$ mice were not able to switch to IgG after either immunization protocol. Even priming with VSV-G and boosting 40 days later did not lead to measurable neutralizing IgG titers, and the response after boosting showed no characteristics of a secondary response (Fig. 3, *B* and *C*).

If TNFR1$^{-/-}$ mice were immunized s.c. with 10 μg VSV-G emulsified in IFA, leading to a slow Ag release and a modest inflammation, the neutralizing IgM response was again comparable with controls, but the switch to IgG occurred only in about 50% of the TNFR1$^{-/-}$ mice. In addition, the titers were lower than in controls (Fig. 4*A*). If VSV-G, emulsified in CFA to promote slow Ag release and considerable inflammation, was used for immunization, all TNFR1$^{-/-}$ mice produced neutralizing IgM and IgG titers comparable with controls (Fig. 4*B*).

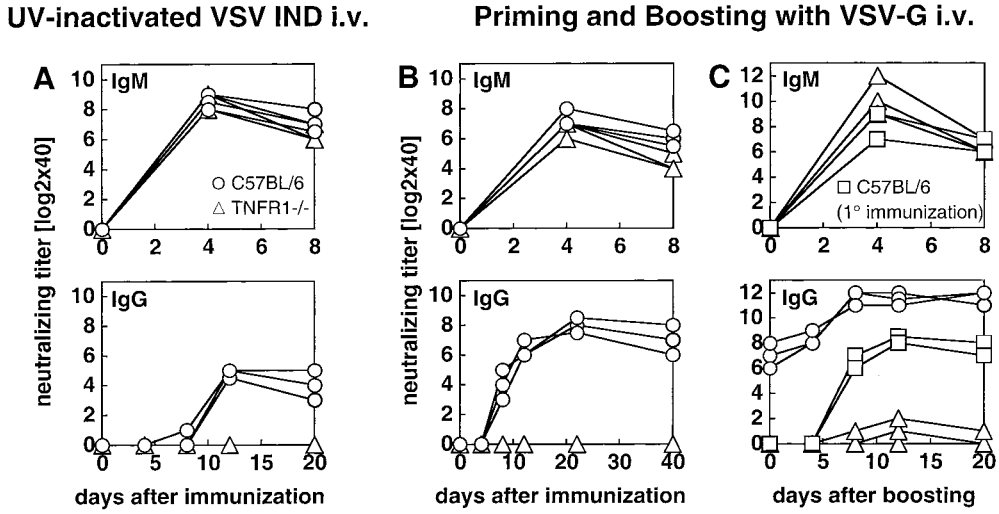

FIGURE 3. VSV-neutralizing primary IgM and IgG responses after immunization with 10^8 PFU UV-inactivated VSV IND i.v. (*A*) or with 10 μg VSV-G in BSS i.v. (*B*) and secondary neutralizing Ab responses after priming and boosting at day 40 with 10 μg VSV-G in BSS i.v. (*C*). Blood was taken at the indicated time points, and the neutralizing IgM (*upper panels*) or IgG titers (*lower panels*) were determined in the sera. IgM titers were absent at the time point of boosting. Titers represent $\log_2$ dilutions of 40-fold prediluted sera. △, TNFR1$^{-/-}$ mice; ○, C57BL/6 mice; □, control naive C57BL/6 mice receiving 10 μg VSV-G in BSS i.v. Each line represents one individual mouse. One of two equivalent experiments is shown.

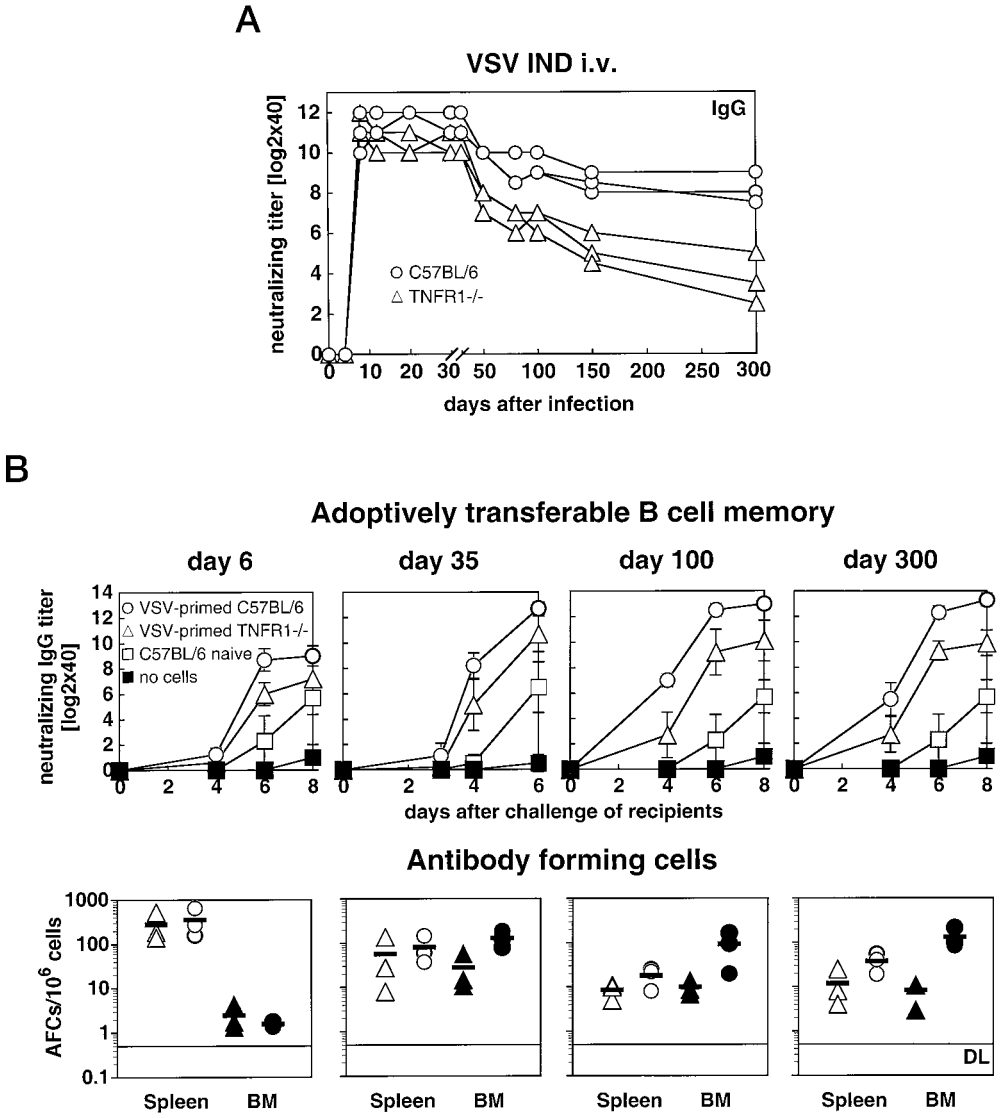

FIGURE 5. *A*, VSV-neutralizing memory IgG response after infection with 2×10^6 PFU live VSV IND i.v. Blood was taken at the indicated time points, and the neutralizing IgG titers were determined in the sera. Titers represent $\log_2$ dilutions of 40-fold prediluted sera. △, TNFR1$^{-/-}$ mice; ○, C57BL/6 control mice. Each line represents one individual mouse. One of two equivalent experiments is shown. *B*, Detection of adoptively transferable B cell memory in the spleen (*upper panel*) and of AFCs in the spleen and the BM (*lower panel*) at different time points after infection with 2×10^6 PFU live VSV IND i.v. For detection of transferable B cell memory, 10^7 spleen cells of VSV-infected donor mice were adoptively transferred at the indicated time points after infection into irradiated (4.5 Gy) C57BL/6 recipient mice. The recipients were challenged with 2×10^6 PFU live VSV IND i.v., and the neutralizing IgG response was monitored. Each line represents the mean ± SD of at least six recipients per experimental group (two to three recipient mice/donor mouse). Donor mice: △, VSV-primed TNFR1$^{-/-}$ mice; ○, VSV-primed C57BL/6 mice; □, naive C57BL/6 mice; ■, no cells transferred. VSV-specific AFCs were determined in the spleen (open symbols) and the BM (filled symbols) of the same donor mice used for detection of transferable B cell memory at the indicated time points after VSV infection. Each point represents one individual mouse. Horizontal bars indicate the mean of three mice. The horizontal line indicates the detection limit of the assay. △ and ▲, TNFR1$^{-/-}$ mice; ○ and ●, C57BL/6 mice. DL, detection limit. One of two equivalent experiments is shown.

Kinetics of the decline of neutralizing serum IgG, of adoptively transferable B cell memory, and of AFCs in the spleen and the BM in TNFR1$^{-/-}$ mice after infection with live VSV IND

The role of Ag persisting on mature FDC networks and of GCs for the maintenance of VSV-specific IgG-titers, of adoptively transferable B cell memory, and of AFCs in the spleen and the BM was evaluated by i.v. infection of TNFR1$^{-/-}$ mice or control mice with 2×10^6 VSV IND (Fig. 5*A*). The neutralizing IgG titer of TNFR1$^{-/-}$ mice was as high as in controls until day 30. At day 50, the IgG titer of TNFR1$^{-/-}$ mice was about 5-fold reduced compared with controls. This represents a significant difference in the VSV neutralization assay. A gradual decrease in neutralizing IgG occurred in control and TNFR1$^{-/-}$ mice until day 150, which was slightly more pronounced in TNFR1$^{-/-}$ mice. Thereafter, a constant and high neutralizing IgG titer was maintained in controls until day 300 after immunization, whereas in TNFR1$^{-/-}$ mice a further decline of circulating neutralizing IgG occurred. By day 300, the difference in neutralizing IgG between TNFR1$^{-/-}$ mice and controls was about 16- to 32-fold (4–5 $\log_2$-scale steps in Fig. 5*A*); thus, there was a decline of about 90–97% of the neutralizing IgG titer in TNFR1$^{-/-}$ mice. The same observations as for the VSV-neutralizing IgG response were made over a 300-day period for VSV-G- or VSV-N-binding IgG titers, which were determined by ELISA (data not shown).

Table I. *Replicating VSV does not persist in TNFR1$^{-/-}$ mice nor in control wt 129 mice*[a]

	Organ Homogenates Transferred into IFN$\alpha\beta$R$^{-/-}$ Recipients					
	Brain + spinal cord		Spleen		Liver + kidney	
Donors	TNFR1$^{-/-}$	wt 129	TNFR1$^{-/-}$	wt 129	TNFR1$^{-/-}$	wt 129
d1	6/6	6/6	6/6	6/6	6/6	6/6
d20	0/4	0/4	0/4	0/4	0/4	0/4

[a] Values indicate numbers of very sick mice per total number of recipients. TNFR1$^{-/-}$ mice and control wt 129 mice were infected i.v. with 2×10^6 PFU VSV and 1 or 20 days laer the indicated organs were removed and homogenized. Homogenized organs were pooled from two mice before i.p. injection into one VSV-susceptible IFN$\alpha\beta$R$^{-/-}$ mouse. Survival of the recipients was followed twice daily. All recipients of d1 organ homogenates became sick and were killed before death 4 days after inoculation, whereas recipients of d20 organ homogenates did not shown any signs of disease. VSV titers in the brains of IFN$\alpha\beta$R$^{-/-}$ mice were high in recipients of d1 organ homogenates and were not detectable in recipients of d20 organ homogenates (not shown).

A possible explanation for the relatively long-lived IgG titers in TNFR1$^{-/-}$ mice could be that VSV persists as infectious virus for prolonged time periods. To exclude this possibility, TNFR1$^{-/-}$ mice and control mice were infected with VSV. One or twenty days later, brain, spinal cord, spleen, liver, and kidney were removed. The organs were homogenized, and organ homogenates were inoculated i.p. into VSV-susceptible IFN-$\alpha\beta$R$^{-/-}$ mice. It has been shown that infection with as few as 50 PFU of live VSV is lethal in IFN-$\alpha\beta$R$^{-/-}$ mice (54). Using this sensitive assay to assess viral persistence, it was possible to show that infectious VSV could be recovered from organ homogenates 1 day after VSV infection, but not 20 days after VSV infection of TNFR1$^{-/-}$ and control mice (Table I). Because organ homogenates from two mice were pooled for infection of one recipient animal, the detection limit of this assay was at about 25 infectious virus particles per organ.

In parallel with the decline of circulating VSV-neutralizing IgG titer, the numbers of VSV-specific AFCs in the spleen and especially in the BM declined faster in TNFR1$^{-/-}$ mice than in controls (Fig. 5*B*). Because the BM has been shown to be the major source of circulating IgG late after immunization (9, 55–58), the more prominent reduction in the number of AFCs in the BM is probably responsible for the decline of circulating VSV-neutralizing IgG titers in TNFR1$^{-/-}$ mice. On day 6 (Fig. 5*B*) and day 8 (not shown), most of the AFCs were present in the spleen and only few in the BM in both TNFR1$^{-/-}$ and control mice. By day 35, AFCs in the BM, but not in the spleen, were about 5-fold reduced in TNFR1$^{-/-}$ mice compared with controls ($28 \pm 20/10^6$ BM cells vs $130 \pm 53/10^6$ BM cells). Whether this reflects less efficient generation of AFCs in the spleen or LNs of TNFR1$^{-/-}$ mice or reduced homing capacity of AFCs to the BM is not known. Whereas AFCs in the BM of control mice remained at this level until day 300, a further 2–3-fold decline occurred in TNFR1$^{-/-}$ mice. By day 300, there was a 16-fold difference of AFCs in the BM ($8 \pm 5/10^6$ in TNFR1$^{-/-}$ vs $133 \pm 71/10^6$ BM cells in control mice) and a 3-fold difference in the spleen ($12 \pm 13/10^6$ vs $38 \pm 18/10^6$ spleen cells) between TNFR1$^{-/-}$ and control mice (Fig. 5*B*).

In addition, the maintenance of adoptively transferable B cell memory was monitored in these mice after infection with 2×10^6 PFU live VSV IND using a previously described adoptive transfer system (Fig. 5*B*) (9). If memory B cells were present in the adoptively transferred spleen cells, a neutralizing IgG response after challenge infection with VSV IND was found to arise early in the recipients. This strong early IgG response by the transferred B cell memory was dependent on Ag challenge because adoptive transfer of B cell memory without subsequent VSV challenge did not induce a VSV-specific IgG response (not shown). The magnitude of the early memory VSV IND-specific IgG response correlated with the number of adoptively transferred memory B cells; i.e., the less memory B cells transferred, the lower the magnitude of the early memory IgG titer (Fig. 6).

Because these adoptive transfer experiments were performed in H-2 class II-compatible, backcrossed, but not truly syngeneic recipients, we had to exclude that unexpected allogeneic effects would influence the outcome of the experiments (59, 60). First, transfer of partially allogeneic VSV-primed spleen cells without challenge infection of the recipient did not result in a measurable neutralizing IgG response. Second, transfer of naive syngeneic (C57BL/6) or partially allogeneic ((C57BL/6 $\times$ SV129)F_1) spleen cells and subsequent VSV challenge of the recipients revealed a similar neutralizing IgG response 8–12 days after challenge. Third, after adoptive transfer of primed syngeneic or primed partially allogeneic spleen cells from both strains of control mice, there was no measurable difference in the neuralizing IgG response of the recipients. This control was performed at all time points after priming (not shown). Thus, the minor undefined histocompatibility differences are unlikely to influence the outcome of the experiment.

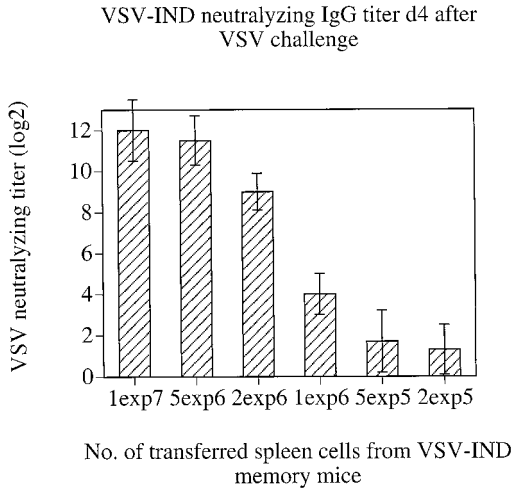

FIGURE 6. Correlation between the adoptive memory B cell response and the number of transferred memory B cells. Graded numbers of spleen cells originating from a VSV memory mouse (day 150 after VSV infection) were adoptively transferred into irradiated (4.5 Gy) C57BL/6 recipient mice. The recipients were challenged with 2×10^6 PFU live VSV IND i.v., and the neutralizing IgG response was monitored at day 4 after challenge infection. Each bar represents the mean $\pm$ SD of at least four recipients per experimental group.

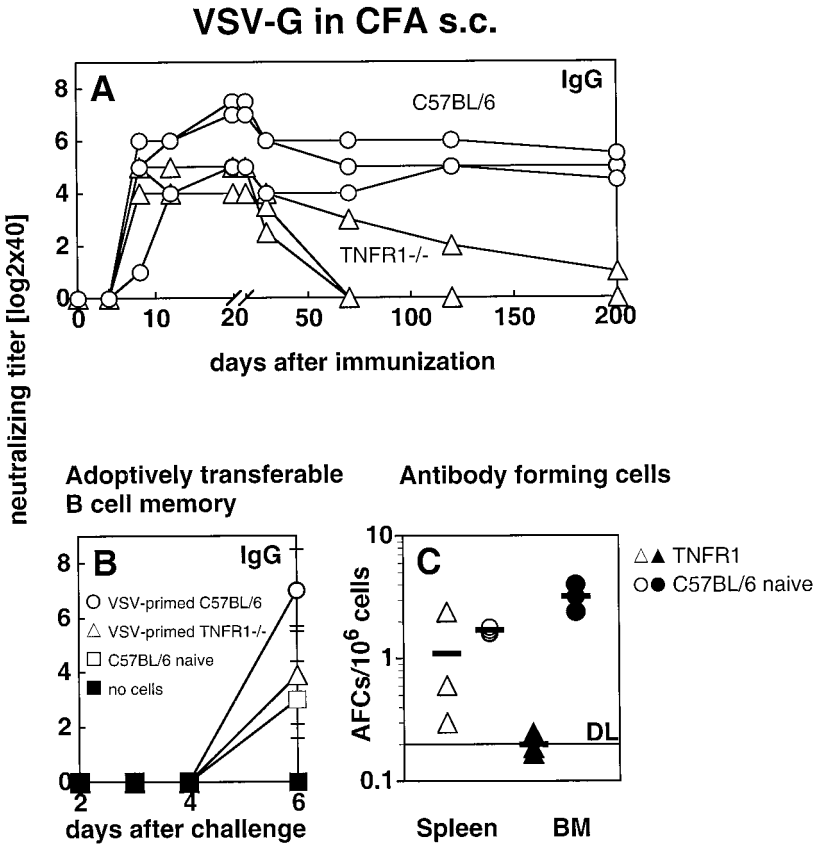

FIGURE 7. VSV-neutralizing IgG response (*A*), adoptively transferable VSV-specific B cell memory in the spleen (*B*), and AFCs in the spleen and the BM (*C*) after immunization with 10 μg VSV-G in CFA s.c. into the base of the tail. Mice were immunized at day 0 and blood was taken at the indicated time points, and the neutralizing IgG titers were determined in the sera (*A*). Titers represent $\log_2$ dilutions of 40-fold prediluted sera. △, TNFR1$^{-/-}$ mice; ○, C57BL/6 mice. Each line represents one individual mouse. For detection of transferable B cell memory (*B*), 10^7 spleen cells of VSV-G-immunized donor mice were adoptively transferred at day 220 after immunization into irradiated (4.5 Gy) C57BL/6 recipient mice. The recipients were challenged 1 day later with 2×10^6 PFU live VSV IND i.v., and the neutralizing IgG response was monitored. Each line represents the mean ± SD of at least six recipients per experimental group (two to three recipient mice/donor mouse). Donor cell origin: △, TNFR1$^{-/-}$ mice; ○, C57BL/6 mice; □, naive C57BL/6 mice; ■, no cells transferred. *C*, VSV-specific AFCs were determined ex vivo in the spleen (open symbols), and the BM (filled symbols) of the same donor mice used detection of transferable B cell memory at day 220 after VSV-G immunization. Each point represents one individual mouse. Horizontal bars indicate the mean of three mice. The horizontal line indicates the detection limit of the assay. △ and ▲, TNFR1$^{-/-}$ mice; ○ and ●, C57BL/6 mice. DL, detection limit. One of two equivalent experiments is shown.

In all adoptive transfer experiments, 2×10^7 spleen cells from C57BL/6 mice that had been infected with VSV NJ 6 days previously were cotransferred with the spleen cells containing VSV-IND-specific B cell memory. This cotransfer was performed to assure nonlimiting T help for the transferred VSV IND-specific memory B cells. It has clearly been shown previously that cross-reactivity between the two VSV serotypes IND and NJ exist on the helper cell epitope level, but absolutely no cross-reactivity could be observed between these two serotypes at the level of the neutralizing B cell response (9, 45, 61). This has been confirmed for TNFR1$^{-/-}$ mice (not shown).

As shown in Fig. 5*B*, adoptively transferable B cell memory was present in both donor mouse strains at day 6 after priming. At day 35, there was no significant difference in the magnitude of transferable B cell memory between TNFR1$^{-/-}$ mice and controls. Only by day 100 after infection, the controls exhibited more pronounced recirculating B cell memory in their spleens as compared with TNFR1$^{-/-}$ mice, reflected by a stronger IgG response of the recipients after challenge. This difference did not significantly change until day 300. This indicated that the induction and the maintenance of transferable B cell memory in TNFR1$^{-/-}$ mice were largely independent of Ag persisting on FDCs and of the GC reaction. The substantial maintenance of transferable B cell memory in TNFR1$^{-/-}$ mice over more than 300 days agrees with previous reports that have shown that the transferable memory B cell pool was long-lived in mice in the absence of Ag (3, 10).

Loss of circulating VSV-specific IgG, of adoptively transferable B cell memory, and of AFC in the BM of TNFR1$^{-/-}$ mice after s.c. immunization with rVSV-G in CFA

To evaluate whether these different parameters of B cell memory could also be observed in TNFR1$^{-/-}$ mice after immunization with a protein Ag, mice were immunized s.c. with 10 μg VSV-G in CFA (Fig. 7). CFA was used as adjuvant because TNFR1$^{-/-}$ mice failed to switch to IgG similar to controls, except after immunization with VSV-G in CFA (Fig. 4*B*). As shown in Fig. 7*A*, the neutralizing IgG response reached similar titers in TNFR1$^{-/-}$ mice and controls at day 12, and only slight differences were established until day 30. However, two of three TNFR1$^{-/-}$ mice lost their IgG titers by day 70, and the other mouse showed a gradual and almost complete decline until day 200. In contrast, the IgG titers of control mice remained at a constant level from day 30 until day 200. Adoptively transferable B cell memory in the spleen was below detection level in TNFR1$^{-/-}$ mice 200 days after immunization, whereas in controls it was still detectable at significant but low levels (Fig. 7*B*). AFCs in the BM were below detection level, whereas some AFCs could still be detected in the spleen of TNFR1$^{-/-}$ mice 200 days after immunization, but values from individual mice varied markedly compared with control mice (Fig. 7*C*).

Generation of high avidity neutralizing IgG in the spleen of TNFR1$^{-/-}$ mice

To test whether the quality of the neutralizing Ab response against VSV was different in TNFR1$^{-/-}$ mice and controls, B cell hybridomas were generated at two time points after infection with 2×10^6 PFU VSV IND (day 12 and day 19). The mice received a booster injection of 2×10^6 PFU VSV IND 3 days before the fusion. Previous studies had shown that these immunization protocols lead to the generation of high avidity neutralizing IgG in normal mice with a high neutralizing activity (36, 62). After VSV infection, the neutralizing activity of VSV-specific IgG mAbs can be used as an indicator for somatic mutation, because for some V_H families the in vitro neutralizing activity of germline Abs may be 30–100-fold lower than those of somatically mutated Abs (62). The increase in avidity for VSV binding between germline IgGs

Table II. *Binding avidities and neutralizing capacities of VSV neutralizing Igs secreted by hybridomas isolated 12 or 19 days after boosted VSV infection*[a]

Clone	Origin	Immunization 2×10^6 PFU VSV i.v.	Ig Subclass	Binding Avidity ($\times 10^8$ M^{-1})	Neutralizing Capacity (titer $\times$ $mg^{-1} \times L^{-1}$)
20H6	TNFR1$^{-/-}$	Day 16/19	IgM	1.8	0.7
23D5	B6 $\times$ 129	Day 16/19	IgM		0.2
15D8	TNFR1$^{-/-}$	Day 16/19	*IgG*^{b}	1.8	1
15C3	TNFR1$^{-/-}$	Day 16/19	*IgG*^{b}	3.8	11.8
14F12	TNFR1$^{-/-}$	Day 16/19	*IgG*^{b}	6.7	0.22
13D1	TNFR1$^{-/-}$	Day 16/19	IgG3	17	19
15D12	TNFR1$^{-/-}$	Day 16/19	IgG2a	18	20.2
13H3	TNFR1$^{-/-}$	Day 16/19	IgG1	48	1.7
11F7	B6 $\times$ 129	Day 9/12	IgG3	2.6	2.7
16D11	B6 $\times$ 129	Day 16/19	IgG2a	10	97
8B12	B6 $\times$ 129	Day 9/12	IgG3	12	48
23D7	B6 $\times$ 129	Day 16/19	*IgG*^{b}	18	12.5
7E9	B6 $\times$ 129	Day 9/12	IgG1	47	126
8A4	B6 $\times$ 129	Day 9/12	IgG1	84	144

[a] TNFR1$^{-/-}$ mice or C57BL/6 $\times$ wt129 (B6 $\times$ 129) mice were infected intravenously with 2×10^6 PFU VSV and 9 or 16 days later they were boosted with the same dose of VSV. Three days after boosting (d 12 or d 19, respectively), spleen cells were fused to the myeloma cell line X63Ag8.653. VSV-binding avidities and VSV-neutralizing capacities were determined by using purified Igs secreted by subcloned hybridomas.

[b] Isotype could not be determined.

(that exhibit average avidities of 10^{-8} to 10^{-9} M^{-1}) and somatically mutated IgGs was only about 2–5-fold (62). Therefore, the increase in avidity is probably not a good indicator for the maturation of the B cell response after VSV infection. From two independent fusions of spleen cells from TNFR1$^{-/-}$ mice and controls, we isolated 14 VSV-neutralizing Ab-producing clones, 7 of each mouse strain (Table II). Two isolated clones produced low avidity IgM Abs. All of the remaining 6 clones from the controls produced IgG of high avidity. Assessment of the neutralizing activity of equivalent concentrations of these monoclonal IgGs showed the following results: IgGs from 2 clones (11F7 and 23D7) from the control mice exhibited intermediate neutralizing activities, whereas IgGs from the 4 other clones showed high neutralizing activities. Interestingly, the IgG Abs from the clones derived from TNFR1$^{-/-}$ mice exhibited only low to intermediate, but no high neutralizing activities, suggesting that the maturation of the VSV-specific IgG response was less efficient in TNFR1$^{-/-}$ mice than in controls. It has to be noted that the low to intermediate neutralizing activities of TNFR1$^{-/-}$-derived clones were not outside the range of neutralizing activities observed in clones derived from C57BL/6 mice. However, this discrepancy of the neutralizing activity of monoclonal IgG in vitro was not detectable in the polyclonal VSV-neutralizing IgG response after VSV infection in vivo (Fig. 2). Although we performed sequence analysis of the V_H regions of some of these mAbs, we were not able to identify point mutations that would clearly indicate affinity maturation in TNFR1$^{-/-}$ mice.

Discussion

In this study, we show that after a viral infection B cells are efficiently activated and that the generation of adoptively transferable B cell memory does occur in the absence of a detectable classical GC reaction in TNFR1$^{-/-}$ mice. A strict correlation between GC formation and establishment or maintenance of B cell memory has been previously questioned by the finding that GC-like structures are generated in response to haptenated LPS (a type 1 TI Ag) that do not support memory responses (63). In addition, GCs present in aged mice or in young mice treated with anti-B7.2 Ab do not support hypermutation or the generation of B cell memory (64). Nevertheless, it should be emphasized that our data do not demonstrate that B cell memory is usually induced in normal mice in a GC-independent fashion; they simply indicate that B cell memory can occur in the absence of classical GCs under certain conditions such as the lack of mature FDC networks in TNFR1$^{-/-}$ mice.

Our data are compatible with the notion that hypermutation of V region Ig genes is inefficient in the absence of classical GCs in TNFR1$^{-/-}$ mice. Although the average affinity of neutralizing Abs does barely increase during the course of VSV infection, point mutations of the V regions of Ig do occur in the maturation of the Ab response against VSV. These point mutations often lead to an increase of the in vitro neutralizing activity up to 100-fold (62). The finding that mAbs isolated from TNFR1$^{-/-}$ mice exhibited low to intermediate neutralizing activities despite rather high avidities is compatible with hypermutation being impaired in the absence of classical GC formation. This is consistent with findings in other experimental systems that somatic hypermutation is impaired although not completely absent in the absence of GCs after immunization of LTα-deficient mice (lacking FDCs and GCs) with high protein Ag doses in adjuvant (25).

The maintenance of Ag-specific Ab titers, plasma cells, and especially of adoptively transferable B cell memory was surprisingly independent of detectable persisting Ag. However, because 90–95% of circulating neutralizing IgG and of VSV-specific AFCs in the BM disappeared within about 1 yr in TNFR1$^{-/-}$ mice, these first two parameters of B cell memory seem to be more Ag dependent than the maintenance of adoptively transferable B cell memory.

Because T-B interaction was shown to be necessary for memory B cell induction, the following two anatomical sites are likely to support T-B interaction and memory B cell induction in TNFR1$^{-/-}$ mice. 1) Outer periarteriolar lymphoid sheath (PALS): Within the outer PALS, first contact of Ag-specific B cells with primed Th cells occurs and isotype switch is induced in TD hapten-specific Ab responses (65). However, it has been shown in TNF-$\alpha^{-/-}$ mice that naive B cells were not able to enter the outer PALS, but accumulated in the MZ, and consequently, no primary B cell follicles were formed (28). A similar scenario is suggested

by the immunohistochemical analysis of the spleen of TNFR1$^{-/-}$ mice (Fig. 1, *X* and *Y*). Whether primed B cells can migrate into the outer PALS to seek for T help in these mutant mice has not been analyzed yet. However, the histological analysis did not show large numbers of VSV-specific B cells at this site.

2) Inner PALS: In Fig. 1 (*I* and *L*), we showed the colocalization of VSV-specific B cells with PNA$^+$ and B220$^+$ B cells in very close proximity to CD4$^+$ Th cells and to the central arteriole of the spleen, an anatomical site in which B cells are usually not present. We were able to identify such a structure in several sections taken 12 days after infection with VSV, although they were much rarer (15–20 times) than VSV-specific GCs in normal mice after the same immunization protocol. A similar structure was recently described in the MLN of TNFR1$^{-/-}$ mice (49). Additional experiments will help to clarify whether these structures have a functional role as pseudo-GC in these mutant mice. However, absence of defined dark and light zones in addition to its aberrant localization in the T cell area and the plasma cell-like appearance of the B cells do not suggest a functional role of these pseudo-GCs.

After the induction of adoptively transferable B cell memory had occurred in TNFR1$^{-/-}$ mice, it remained detectable in TNFR1$^{-/-}$ mice up to day 300 after infection. This indicates that the population of recirculating memory B cells is probably long-lived and rather independent of persisting Ag, as it was suggested by previous studies (3, 10, 16). Although 90–97% of circulating VSV-specific IgG and AFCs in the BM were lost within 1 yr after infection in TNFR1$^{-/-}$ mice, both parameters persisted for surprisingly long periods after infection with replicating VSV, but not after immunization with rVSV-G in CFA. The decline of circulating VSV-specific IgG and AFCs in the BM between day 100 and 300 was very slow in TNFR1$^{-/-}$ mice, indicating a $t_{1/2}$ of about 90 days for AFCs in the BM of TNFR1$^{-/-}$ mice. In recent studies, the $t_{1/2}$ of AFCs in the BM was estimated to be about 130 days (11, 12), and thus, once AFCs are induced after primary infection, this might be sufficient for the maintenance of a rather stable IgG titer for the first 2–3 mo after infection. The slow but steady decline of VSV-specific IgG titers between day 100 and 300 in TNFR1$^{-/-}$ mice might support the concept of long-lived plasma cells in the BM. In contrast, the constant IgG titer between day 100 and 300 observed in control mice argues for a continuous replenishment of the AFC pool by Ag-stimulated memory B cells, the Ag being trapped and presented on FDCs (10, 16, 57). Because this Ag-storage mechanism is lacking in TNFR1$^{-/-}$ mice, the most efficiently restimulating Ag pool seems to be hampered. An alternative explanation for the decline of VSV-specific AFCs in the BM of TNFR1$^{-/-}$ mice could be that signaling via TNFR1 is important for the survival of AFCs in the BM.

However, it remains to be explained why a much more efficient and long-lasting IgG response, a better induction of AFCs and of adoptively transferable B cell memory was observed in TNFR1$^{-/-}$ mice infected with live VSV as compared with immunization with VSV protein in CFA. One could speculate that VSV persists at low levels in the form of infectious virus, and would thus represent a low but constant amount of Ag present for restimulation of memory B cells. Although mice were infected with replicating VSV, the persistence of live VSV seems unlikely because no infectious virus could be recovered from multiple organs of VSV-infected TNFR1$^{-/-}$ mice 20 days after infection (Table I). In addition, VSV is a cytopathic virus and was shown to replicate only to a very limited extent extraneuronally after peripheral infection of normal mice (29, 30). Alternatively, it could be possible that VSV-Ag persists as immune complexes bound to cells different from classical mature FDCs within secondary lymphoid organs, e.g., bound to interdigitating cells or to few immature FDC-like cells that have been shown to exhibit very slow Ag processing. Such low level Ag persistence may not be detected by immunohistochemistry. VSV is a highly cytopathic virus that induces a very potent inflammatory response. It is therefore possible that VSV may directly induce the generation of mature FDCs from precursors in the absence of TNFR1 signaling. Although sufficient for stimulation of some plasma cell formation, these few mature and unorganized FDCs may escape detection by immunohistochemistry. Finally, one could argue that infectious VSV induces a qualitatively and quantitatively better immune response than the respective protein Ags in adjuvant. Therefore, it could be that live VSV initially induces a sufficiently high number of long-lived AFCs able to maintain high but slowly declining levels of circulating IgG up to 300 days after infection, whereas such potent AFC induction is not achieved after protein immunization, thus resulting in short-lived IgG titers.

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References

1. Eisen, H. N., and G. W. Siskind. 1964. Variations in affinities of antibodies during the immune response. *Biochemistry 3:996.*
2. Siskind, G. W., and B. Benacerraf. 1969. Cell selection by antigen in the immune response. *Adv. Immunol. 10:1.*
3. Schittek, B., and K. Rajewsky. 1990. Maintenance of B-cell memory by long-lived cells generated from proliferating precursors. *Nature 346:749.*
4. Liu, Y. J., S. Oldfield, and I. MacLennan. 1988. Memory B cells in T cell-dependent antibody responses colonize the splenic marginal zones. *Eur. J. Immunol. 18:355.*
5. Liu, Y.-J., C. Barthélémy, O. de Bouteiller, C. Arpin, I. Durand, and J. Banchereau. 1995. Memory B cells from human tonsils colonize mucosal epithelium and directly present antigen to T cells by rapid up-regulation of B7-1 and B7-2. *Immunity 2:239.*
6. Klein, U., T. Goossens, M. Fischer, H. Kanzler, A. Braeuninger, K. Rajewski, and R. Kuppers. 1998. Somatic hypermutation in normal and transformed human B cells. *Immunol. Rev. 162:261.*
7. Tew, J. G., R. P. Phipps, and T. E. Mandel. 1980. The maintenance and regulation of the humoral immune response: persisting antigen and the role of follicular antigen-binding dendritic cells as accessory cells. *Immunol. Rev. 53:175.*
8. Tew, J. G., M. H. Kosco, G. F. Burton, and A. K. Szakal. 1990. Follicular dendritic cells as accessory cells. *Immunol. Rev. 117:185.*
9. Bachmann, M. F., T. M. Kündig, B. Odermatt, H. Hengartner, and R. M. Zinkernagel. 1994. Free recirculation of memory B cells versus antigen-dependent differentiation to antibody forming cells. *J. Immunol. 153:3386.*
10. Bachmann, M. F., B. Odermatt, H. Hengartner, and R. M. Zinkernagel. 1996. Induction of long-lived germinal centers associated with persisting antigen after viral infection. *J. Exp. Med. 183:2259.*
11. Manz, R. A., A. Thiel, and A. Radbruch. 1997. Lifetime of plasma cells in the bone marrow. *Nature 388:133.*
12. Slifka, M. K., R. Antia, J. K. Whitmire, and R. Ahmed. 1998. Humoral immunity due to long-lived plasma cells. *Immunity 8:363.*
13. Celada, F. 1971. The cellular basis of the immunologic memory. *Prog. Allergy 15:223.*
14. Gray, D., and H. Skarvall. 1988. B-cell memory is short-lived in the absence of antigen. *Nature 336:70.*
15. Sprent, J., and D. F. Tough. 1994. Lymphocyte life-span and memory. *Science 265:1395.*
16. Thorbecke, G. J. 1990. Focusing: The dilemma of interpreting sharp images on a blurred background. *J. Immunol. 145:2779.*
17. MacLennan, I. C. M. 1994. Germinal centers. *Annu. Rev. Immunol. 12:117.*
18. Ahmed, R., and D. Gray. 1996. Immunological memory and protective immunity: understanding their relation. *Science 272:54.*
19. Klaus, G. G. B., and J. H. Humphrey. 1977. The generation of memory cells. I. The role of C3 in the generation of memory cells. *Immunology 33:31.*
20. Foy, T. M., J. D. Laman, J. A. Ledbetter, A. Aruffo, E. Claassen, and R. J. Noelle. 1994. gp39-CD40 interactions are essential for germinal center formation and the development of B cell memory. *J. Exp. Med. 180:157.*
21. Gray, D., P. Dullforce, and S. Jainandunsing. 1994. Memory B cell development but not germinal center formation is impaired by in vivo blockade of CD40-CD40 ligand interaction. *J. Exp. Med. 180:141.*
22. Renshaw, B. R., W. C. Fanslow, R. J. Armitage, K. A. Campbell, D. Liggitt, B. Wright, B. L. Davison, and C. R. Maliszewski. 1994. Humoral immune responses in CD40 ligand-deficient mice. *J. Exp. Med. 180:1889.*
23. Kawabe, T., T. Naka, K. Yoshida, T. Tanaka, H. Fujiwara, S. Suematsu, N. Yoshida, T. Kishimoto, and H. Kikutani. 1994. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity 1:167.*

24. Toellner, K.-M., A. Gulbranson-Judge, D. R. Taylor, D. M.-Y. Sze, and I. C. M. MacLennan. 1996. Immunoglobulin switch transcript production in vivo related to the site and time of antigen-specific B-cell activation. *J. Exp. Med. 183:2303.*
25. Matsumoto, M., S. F. Lo, C. J. L. Carruthers, J. Min, S. Mariathasan, G. Huang, D. R. Plas, S. M. Martin, R. S. Geha, M. H. Nahm, and D. D. Chaplin. 1996. Affinity maturation without germinal centers in lymphotoxin-α-deficient mice. *Nature 382:462.*
26. Matsumoto, M., S. Mariathasan, M. H. Nahm, F. Baranyay, J. J. Peschon, and D. D. Chaplin. 1996. Role of lymphotoxin and the type I TNF receptor in the formation of germinal centers. *Science 271:1289.*
27. Le Hir, M., H. Bluethmann, M. H. Kosco-Vilbois, M. Müller, F. Di Padova, M. Moore, B. Ryffel, and H. Eugster. 1996. Differentiation of follicular dendritic cells and full antibody responses require tumor necrosis factor receptor-1 signaling. *J. Exp. Med. 183:2367.*
28. Pasparakis, M., L. Alexopoulou, V. Episkopou, and G. Kollias. 1996. Immune and inflammatory responses in TNFα-deficient mice: a critical requirement for TNFα in the formation of primary B-cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. *J. Exp. Med. 184:1397.*
29. Hecht, T. T., and W. P. Paul. 1981. Replication of vesicular stomatitis virus in mouse spleen cells. *Infect. Immun. 32:1014.*
30. Wagner, R. R. 1987. *The Rhabdoviruses*. Plenum Press, New York, p. 1.
31. Burns, W. H., L. C. Billups, and A. L. Notkins. 1975. Thymus dependence of viral antigens. *Nature 256:654.*
32. Leist, T. P., S. P. Cobbold, H. Waldmann, M. Aguet, and R. M. Zinkernagel. 1987. Functional analysis of T lymphocyte subsets in antiviral host defense. *J. Immunol. 138:2278.*
33. Oxenius, A., K. A. Campbell, C. R. Maliszewski, T. Kishimoto, H. Kikutani, H. Hengartner, R. M. Zinkernagel, and M. F. Bachmann. 1996. CD40-CD40 ligand interactions are critical in T-B cooperation but not for other anti-viral CD4$^+$ T-cell functions. *J. Exp. Med. 183:2209.*
34. Charan, S., H. Hengartner, and R. M. Zinkernagel. 1986. Primary antibody responses to a well-defined and unique hapten are not enhanced by preimmunization with carrier: analysis in a viral model. *Proc. Natl. Acad. Sci. USA 83:2604.*
35. Roost, H. P., A. Haag, C. Burkhart, R. M. Zinkernagel, and H. Hengartner. 1996. Mapping of the dominant neutralizing antigenic site of a virus using infected cells. *J. Immunol. Methods 189:233.*
36. Roost, H. P., M. F. Bachmann, A. Haag, U. Kalinke, V. Pliska, H. Hengartner, and R. M. Zinkernagel. 1995. Early high affinity neutralizing anti-viral IgG responses without further overall improvements of affinity. *Proc. Natl. Acad. Sci. USA 92:1257.*
37. Rothe, J., W. Lesslauer, H. Lötscher, Y. Lang, P. Koebel, F. Köntgen, A. Althage, R. M. Zinkernagel, M. Steinmetz, and H. Bluethmann. 1993. Mice lacking the tumor necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by *Listeria monocytogenes*. *Nature 364:798.*
38. Müller, U., U. Steinhoff, L. Reis, S. Hemmi, J. Pavlovic, R. M. Zinkernagel, and M. Aguet. 1994. Functional role of type I and type II interferons in antiviral defense. *Science 264:1918.*
39. McCaren, L. C., J. J. Holland, and J. T. Syverton. 1959. The mammalian cell-virus relationship. I. Attachment of poliovirus to cultivated cells of primate and non-primate origin. *J. Exp. Med. 109:475.*
40. Mackett, M., T. Yilma, J. K. Rose, and B. Moss. 1985. Vaccinia virus recombinants: expression of VSV genes and protective immunization of mice and cattle. *Science 227:433.*
41. Bailey, M. J., D. A. McLeod, C. Y. Kang, and D. Bishop. 1989. Glycosylation is not required for the fusion activity of the G protein of vesicular stomatitis virus in insect cells. *Virology 169:323.*
42. Cobbold, S. P., A. Jayasuriya, A. Nash, T. D. Prospero, and H. Waldmann. 1984. Therapy with monoclonal antibodies by elimination of T cell subsets in vivo. *Nature 312:548.*
43. Gray, D., M. Kosco, and B. Stockinger. 1991. Novel pathways of antigen presentation for the maintenance of memory. *Int. Immunol. 3:141.*
44. Kinoshita, T., J. Takeda, K. Hong, H. Kozono, H. Sakai, and K. Inoue. 1988. Monoclonal antibodies to mouse complement receptor type 1 (CR1): their use in a distribution study showing that mouse erythrocytes and platelets are CR1-negative. *J. Immunol. 140:3066.*
45. Roost, H. P., S. Charan, and R. M. Zinkernagel. 1990. Analysis of the kinetics of antiviral memory T help in vivo: characterization of short lived cross-reactive T help. *Eur. J. Immunol. 20:2547.*
46. Beatty, J. D., B. G. Beatty, W. G. Vlahos, and L. R. Hill. 1987. Method of analysis of non-competitive enzyme immunoassays for antibody quantification. *J. Immunol. Methods 100:161.*
47. Beatty, J. D., B. G. Beatty, and W. G. Vlahos. 1987. Measurement of monoclonal antibody affinity by non-competitive enzyme immunoassay. *J. Immunol. Methods 100:173.*
48. Sedgwick, J. D., and P. G. Holt. 1983. A solid-phase immunoenzymatic technique for the enumeration of specific antibody-secreting cells. *J. Immunol. Methods 57:301.*
49. Fu, Y.-X., G. Huang, M. Matsumoto, H. Molina, and D. D. Chaplin. 1997. Independent signals regulate development of primary and secondary follicle structure in spleen and mesenteric lymph node. *Proc. Natl. Acad. Sci. USA 94:5739.*
50. McHeyzer-Williams, M., M. J. McLean, P. A. Lalor, and J. V. Nossal. 1993. Antigen-driven B cell differentiation in vivo. *J. Exp. Med. 178:295.*
51. Bachmann, M. F., H. Hengartner, and R. M. Zinkernagel. 1995. T helper cell-independent neutralizing B cell response against vesicular stomatitis virus: role of antigen patterns in B cell induction? *Eur. J. Immunol. 25:3445.*
52. Freer, G., C. Burkhart, I. Ciernik, M. F. Bachmann, H. Hengartner, and R. M. Zinkernagel. 1994. Vesicular stomatitis virus Indiana (VSV-IND) glycoprotein as a T cell-dependent and -independent antigen. *J. Virol. 68:3650.*
53. McKall- Faienza, K. J., K. Kawai, T. M. Kündig, B. Odermatt, M. F. Bachmann, A. Zakarian, T. W. Mak, and P. S. Ohashi. 1998. Absence of TCFRp55 influences virus-induced autoimmunity despite efficient lymphocyte infiltration. *Int. Immunol. 10:405.*
54. Steinhoff, U., U. Müller, A. Schertler, H. Hengartner, M. Aguet, and R. M. Zinkernagel. 1995. Antiviral protection by VSV-specific antibodies in α/β interferon receptor-deficient mice. *J. Virol. 69:2153.*
55. Benner, R., W. Hijmans, and J. J. Haaijman. 1981. The bone marrow: the major source of serum immunoglobulins, but still a neglected site of antibody formation. *Clin. Exp. Immunol. 46:1.*
56. DiLosa, R. M., K. Maeda, A. Masuda, A. Szakal, and J. G. Tew. 1991. Germinal center B cells and antibody production in the bone marrow. *J. Immunol. 146: 4071.*
57. Tew, J. G., R. M. DiLosa, G. F. Burton, M. H. Kosco, L. I. Kupp, A. Masuda, and A. K. Szakal. 1992. Germinal centers and antibody production in bone marrow. *Immunol. Rev. 126:99.*
58. Slifka, M. K., M. Matloubian, and R. Ahmed. 1995. Bone marrow is a major site of long-term antibody production after acute viral infection. *J. Virol. 69:1895.*
59. Katz, D. H. 1972. The allogeneic effect on immune responses: model for regulatory influences of T lymphocytes on the immune system. *Transplant. Rev. 12:141.*
60. Rajewsky, K., G. E. Roelants, and B. A. Askonas. 1972. Carrier specificity and the allogeneic effect in mice. *Eur. J. Immunol. 2:592.*
61. Lefrancois, L., and D. S. Lyles. 1982. The interaction of antibody with the major surface glycoprotein of vesicular stomatitis virus. *Virology 121:157.*
62. Kalinke, U., E. M. Bucher, B. Ernst, A. Oxenius, H.-P. Roost, S. Geley, R. Kofler, R. M. Zinkernagel, and H. Hengartner. 1996. The role of somatic mutation in the generation of the protective humoral immune response against vesicular stomatitis virus. *Immunity 5:639.*
63. Liu, Y. J., J. Zhang, P. J. L. Lane, E. Y. Chan, and I. C. MacLennan. 1991. Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens. *Eur. J. Immunol. 21:2951.*
64. Miller, C., and G. Kelsoe. 1995. Ig V_H hypermutation is absent in germinal centers of aged mice. *J. Immunol. 155:3377.*
65. MacLennan, I. C. M., A. Gulbranson-Judge, K.-M. Toellner, M. Casamayor-Palleja, D. M.-Y. Sze, S. Luther, and H. Acha Orbea. 1997. The changing preference of T- and B-cells for partners as T-dependent antibody responses develop. *Immunol. Rev. 156:53.*