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T-Cell-Independent Immunoglobulin G Responses In Vivo Are Elicited by Live-Virus Infection but Not by Immunization with Viral Proteins or Virus-Like Particles

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Immunoglobulin G (IgG) responses to viruses are generally assumed to be T-cell dependent (TD). Recently, however, polyomavirus (PyV) infection of T-cell-deficient (T-cell receptor β chain [TCR- β] $-/-$ or TCR- $\beta \times \delta -/-$) mice was shown to elicit a protective, T-cell-independent (TI) antiviral IgM and IgG response. A repetitive, highly organized antigenic structure common to many TI antigens is postulated to be important in the induction of antibody responses in the absence of helper T cells. To test whether the repetitive structure of viral antigens is essential and/or sufficient for the induction of TI antibodies, we compared the abilities of three forms of PyV antigens to induce IgM and IgG responses in T-cell-deficient mice: soluble capsid antigens (VP1), repetitive virus-like particles (VLPs), and live PyV. Immunization with each of the viral antigens resulted in IgM production. VLPs and PyV elicited 10-fold-higher IgM titers than VP1, indicating that the highly organized, repetitive antigens are more efficient in IgM induction. Antigen-specific TI IgG responses, however, were detected only in mice infected with live PyV, not in VP1- or VLP-immunized mice. These results suggest that the highly organized, repetitive nature of the viral antigens is insufficient to account for their ability to elicit TI IgG response and that signals generated by live-virus infection may be essential for the switch to IgG production in the absence of T cells. Germinal centers were not observed in T-cell-deficient PyV-infected mice, indicating that the germinal center pathway of B-cell differentiation is TD even in the context of a virus infection.

Although cognate T-cell–B-cell cooperation is essential for T-cell-dependent (TD) humoral immunity, antibody (immunoglobulin M [IgM] and IgG) responses to a variety of antigens in T-cell-deficient mice (14, 15, 17, 26) indicate that alternative, T-cell-independent (TI) mechanisms of B-cell activation, differentiation, and isotype switching also operate in vivo. The nature of these mechanisms and the essential characteristics of the antigens which activate the TI pathways in vivo, however, are not known. Many TI antigens (bacterial polysaccharides, polymerized flagellin, etc.) have a highly organized, repetitive antigen structure, which is thought to be essential for their ability to induce antibody responses in the absence of T-cell help. The repeating, identical epitopes can extensively cross-link the B-cell receptor, enabling these antigens to deliver strong activating signals to the B cells. The fact that both in vitro and in vivo B cells respond differently to the same antigenic epitope when it is presented in a nonrepetitive versus a highly organized, repetitive form (2, 21) supports this idea. It has also been suggested that there is a correlation between the repetitive structure of certain viruses and their ability to act as TI antigens (3, 4).

Polyomavirus (PyV) infection of $\alpha\beta$ T-cell- or $\alpha\beta$ and $\gamma\delta$ T-cell-deficient mice induces a TI IgM and IgG response, which provides resistance to the infection. T-cell-deficient mice survive PyV infection, whereas SCID mice have 100% acute

mortality (23, 24). Thus, PyV can effectively induce TI isotype switch in vivo. In this study, PyV-infected T-cell-deficient mice were used as a model to investigate the TI antiviral IgG responses and to analyze what role the nature of the antigen has in TI IgM and IgG induction. Comparing the abilities of soluble capsid proteins (VP1), repetitive virus-like particles (VLPs), and live PyV to induce TI antibodies, we showed that IgG responses, which require TI isotype switch, were elicited only by infection with live virus, not by immunization with VP1 or VLPs.

MATERIALS AND METHODS

Mice and immunizations. C57BL/6, CBA, and T-cell receptor β chain (TCR- β) $-/-$ and TCR- $\beta \times \delta -/-$ mice on a C57BL background were obtained from the Jackson Laboratory (Bar Harbor, Maine). Mice were immunized intraperitoneally (i.p.) with 10 μ g of purified VP1 of PyV strain RA produced by recombinant baculovirus (20) or with the same amount of recombinant VP1 protein assembled into VLPs in insect cultures (16). Infection with highly purified PyV strain RA (8) was done i.p., using 7×10^5 , 7×10^6 , or 7×10^7 PFU/mouse; in some experiments, unpurified PyV strain A2 was used.

VP1-specific ELISAs. To measure PyV capsid protein-specific antibody production in enzyme-linked immunosorbent assays (ELISAs), 96-well plates were coated with recombinant VP1 protein produced in *Escherichia coli* (11) (0.03 μ g/well). The serum samples were tested in duplicate. Biotinylated horse anti-mouse IgG and goat anti-mouse IgG and goat anti-mouse IgM plus streptavidin-horseradish peroxidase (HRP) (Vector Laboratories Inc., Burlingame, Calif.) were used to measure IgM and IgG, respectively. To detect IgG isotypes a Southern Biotech (Birmingham, Ala.) isotyping kit and HRP-labeled rat anti-mouse IgG2a from Pharmingen (San Diego, Calif.) were used. 3,3',5,5'-Tetramethyl-benzidine tablets (Sigma, St. Louis, Mo.) were used as the substrate to develop the enzyme reaction. Plates were read at an optical density of 450 nm (OD₄₅₀) by a THERMO_{MAX} plate reader and SoftMax 2.3 software (Molecular Devices Corp., Menlo Park, Calif.). The VP1 specificity of the ELISAs was tested with wells coated with proteins (0.03 μ g/well) derived from an *E. coli* lysate

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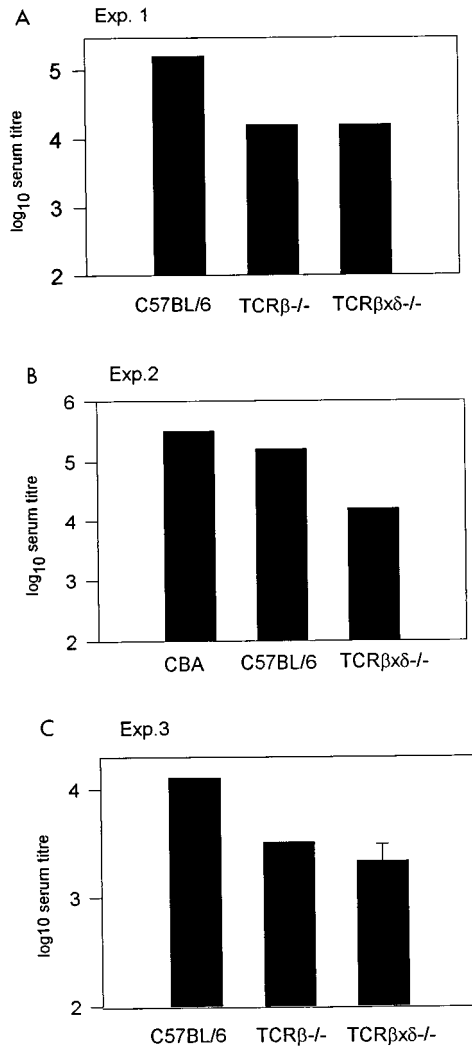


FIG. 1. Magnitude of IgG response to PyV in immunocompetent and T-cell-deficient mice. Shown are PyV VP1-specific serum IgG titers on day 14 postinfection. In experiments 1 and 2, the IgG titers of pooled serum samples ($n = 3$) from C57BL/6, CBA, TCR- $\beta^{-/-}$, and TCR- $\beta\times\delta^{-/-}$ mice infected i.p. with 7×10^6 PFU of purified PyV strain RA were determined by VP1-specific ELISAs and expressed as the reciprocal log₁₀ endpoint dilutions giving absorbance values at 450 nm above the background. Experiment 3 shows data obtained with mice infected with another PyV strain, A2. Pooled blood samples from C57BL/6 and TCR- $\beta^{-/-}$ mice ($n = 6$) and three individual samples from TCR- $\beta\times\delta^{-/-}$ mice were tested.

purified the same way as the recombinant VP1 protein. None of the serum samples tested gave OD readings above the background with this control antigen.

GC staining. Frozen, OCT-embedded spleen sections were fixed and stained with HRP-peanut agglutinin (PNA) (Vector Laboratories) to visualize germinal centers (GC) and counterstained with hematoxylin.

RESULTS

Magnitude and isotype profile of TI IgG response to PyV in T-cell-deficient mice. The magnitude of virus-specific TI IgG production in PyV-infected TCR- $\beta^{-/-}$ and TCR- $\beta\times\delta^{-/-}$ mice was evaluated by measuring PyV major capsid protein (VP1)-specific IgG endpoint titers in ELISAs. On day 14 following i.p. infection with 7×10^6 PFU of purified PyV strain RA, TCR- $\beta^{-/-}$ and TCR- $\beta\times\delta^{-/-}$ mice had VP1-specific serum IgG titers of 1.6×10^4 , which was 1 log lower than the

IgG values detected in wild-type C57BL/6 mice (1.6×10^5) (Fig. 1A and B). With another PyV strain, A2, the virus-specific IgG levels in T-cell-deficient mice were also ~10% of those measured in serum samples of immunocompetent mice (Fig. 1C). Analysis of the isotype distribution of the virus-specific IgG indicated that all four IgG subclasses were induced by PyV infection in wild-type C57BL/6 mice, with IgG2a and IgG2b being predominant. In TCR- $\beta\times\delta^{-/-}$ mice, no measurable VP1-specific IgG1 was observed, suggesting a T-cell dependence for the generation of this isotype (Fig. 2). In these T-cell-deficient mice with a C57BL/6 background, IgG2b was the predominant virus-specific IgG subclass, and various amounts of IgG2a and IgG3 were also consistently detected. Interestingly, TCR- $\beta^{-/-}$ and TCR- $\beta\times\delta^{-/-}$ mice with 129 \times C57BL background produced predominantly IgG2a in response to PyV infection (data not shown). TCR- $\beta^{-/-}$ and TCR- $\beta\times\delta^{-/-}$ mice did not show reproducible differences in the magnitude of VP1-specific IgG production, suggesting that $\gamma\delta$ T cells do not significantly influence TI antibody responses to PyV (Fig. 1C and 3).

TI IgM responses to viral capsomeres, VLPs, and PyV. PyV is a nonenveloped icosahedral DNA virus. The major capsid protein, VP1, forms pentameric capsomeres, and 72 capsomeres form the viral capsid (22). Because several known TI antigens have a highly organized, repetitive antigenic structure, it has been hypothesized that the structural repetitiveness is an essential characteristic which enables viruses to act as TI antigens (3, 4). The repetitive structure may function by cross-linking the B-cell receptors, thus providing signals activating antibody production.

To test whether the repetitive structure of PyV capsid is essential and/or sufficient for the induction of TI IgM and IgG antibody responses in vivo, three forms of the PyV VP1 antigen were used to immunize T-cell-deficient mice. Purified pentameric VP1 capsomeres were used as soluble protein antigens (11, 20). These capsomeres may randomly associate (19) but do not form highly organized structures. The source of highly organized, repetitive viral antigens were VLPs, which are empty viral capsids assembled in insect cell cultures after expression of the VP1 protein by using a recombinant baculovirus vector (16). The VLPs used in the experiments were morphologically intact judged by electron microscopy. Purified PyV virions were used as live viral antigens (8). The amount of protein antigens (VP1 and VLP) used in these studies was 10 μ g/mouse i.p., an antigen dose equivalent to 70 hemagglutination units (HAU) or 7×10^7 PFU of PyV. Live-virus infections were performed with highly purified PyV strain RA given i.p. in three different doses: 70, 7, and 0.7 HAU, corresponding to $\sim 7 \times 10^7$, 7×10^6 , and 7×10^5 PFU, respectively. VP1-specific IgM and IgG responses to the three forms of antigens were determined.

The VP1-specific IgM response peaked on day 4 in both wild-type C57BL/6 and TCR- $\beta\times\delta^{-/-}$ mice (Fig. 4A). On day 4, VP1-immunized immunocompetent and T-cell-deficient mice both had a low but measurable level of VP1-specific IgM. Both strains of mice, however, secreted significantly (10-fold) higher levels of IgM in response to the repetitive antigen, VLP (Fig. 4B and C). Infection with live, replicating PyV (7×10^6 and 7×10^5 PFU) led to two- to eightfold further increases in peak virus-specific IgM levels compared to that for VLP immunization. These results show that the IgM responses induced by viral proteins or live virus in the absence of T cells are similar in magnitude to the IgM production observed in immunocompetent mice, suggesting that the early IgM response is TI even in wild-type mice. The data also support the hypothesis that structural repetitiveness enhances the efficiency of TI

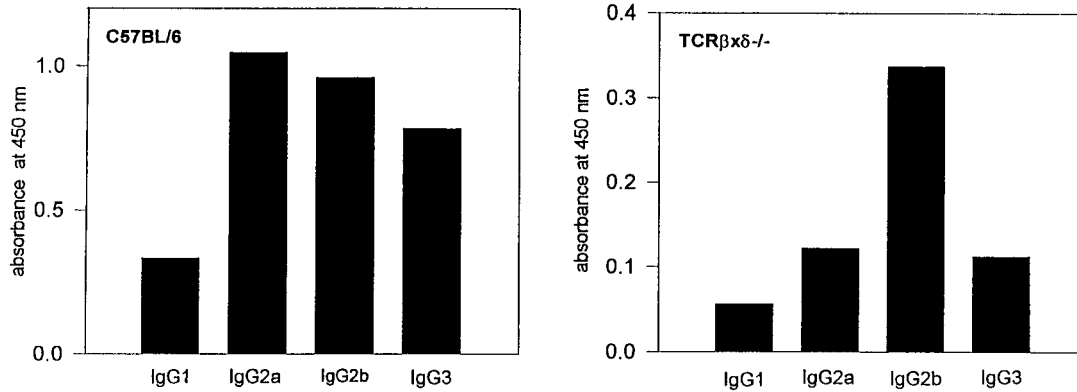


FIG. 2. Isotype distribution of VP1-specific IgG in immunocompetent and T-cell-deficient PyV-infected mice. VP1 antigen- and isotype-specific ELISAs were performed with pooled sera from C57BL/6 ($n = 5$) and TCR- $\beta \times \delta^{-/-}$ ($n = 5$) mice in 1:50 dilution. The serum samples were taken on day 21 following i.p. infection with PyV strain A2. The absorbance values of serum samples from uninfected mice were 0.048 to 0.06.

IgM induction (3, 4), consistent with similar findings for mice immunized with different forms of the vesicular stomatitis virus glycoprotein (1, 2, 7).

TI IgG responses are elicited by virus infection but not by immunization with viral capsomeres or VLPs. IgG responses to PyV were detected from day 7 in PyV-infected C57BL/6 and TCR- $\beta \times \delta^{-/-}$ mice, and the VP1-specific IgG levels progressively increased until day 21, when the experiment was terminated (Fig. 5). In the absence of T cells ($\alpha\beta$ or $\alpha\beta$ and $\gamma\delta$), neither the VP1 capsomeres nor the VLPs induced IgG production in T-cell-deficient mice (Fig. 5 and 6). In experiment 1, on day 14 postinfection the VP1-specific IgG endpoint titers were 10^4 in both TCR- $\beta^{-/-}$ and TCR- $\beta \times \delta^{-/-}$ PyV-infected mice. T-cell-deficient mice immunized with viral proteins did not have detectable IgG above 1:100 serum dilution (Fig. 6). In experiment 2, on day 21 after administration of antigens in wild-type C57BL/6 mice, both capsomeres and VLPs induced IgG responses with 3.2×10^3 and 1.2×10^4 endpoint titers, respectively. PyV-infected C57BL/6 mice had an IgG titer of 8×10^5 on day 21, whereas TCR- $\beta \times \delta^{-/-}$ mice had a titer of 4×10^4 , consistent with findings shown previously (Fig. 1 and 6). No VP1-specific IgG was detected, however, in T-cell-deficient mice immunized with VP1 proteins or VLPs (Fig. 6). These results strongly suggest that the highly organized repetitive antigen structure, which VLPs possess similarly to live virus, is not sufficient for the induction of a TI IgG response. We conclude that other signals generated in the context of live virus infection may be required for a TI isotype switch.

Simultaneous infection of VLP-immunized TCR- $\beta \times \delta^{-/-}$ mice with lymphocytic choriomeningitis virus (LCMV) did not result in inducing VP1-specific IgG production. Serum samples from all four mice on day 14 following infection with 5×10^4 PFU of LCMV and immunization with 70 HAU of VLPs i.p. had VP1-specific IgG levels below the background of detection in 1:100 dilution. Because LCMV infection can itself elicit antiviral TI IgG responses (5), this result suggests that systemic elevation of cytokines or activation of certain cell types induced by the virus infections is not sufficient to help the efficient TI isotype switch and argues for the importance of local signals.

Lack of GC formation in PyV-infected T-cell-deficient mice.

In the course of a primary antibody response to TD antigens, B cells follow one of two distinct pathways of differentiation. They differentiated into antibody-secreting plasma cells, or they migrate to the GC, where B cells proliferate, undergo

affinity maturation, and enter the B-cell memory pool (9, 12, 25). T-cell-B-cell interactions were shown to be essential for the GC induction in mice immunized with protein antigens (6, 10), and classical TI antigens typically do not induce GC development (17).

To test whether the GC pathway of B-cell differentiation requires T-cell-derived signals in the course of a virus infection, we tested GC formation in PyV-infected T-cell-deficient mice. GC B cells express a surface receptor for the lectin PNA, and this allows for the identification of GC (25). Frozen sections of spleens from day 14 PyV-infected TCR- $\beta^{-/-}$ and TCR- $\beta \times \delta^{-/-}$ as well as from C57BL/6 mice were analyzed for the presence of GC by immunohistochemistry. The formation of typical GC was not detectable in TCR- $\beta^{-/-}$ (Fig. 7B) and TCR- $\beta \delta^{-/-}$ (data not shown) mice, although small groups of weakly PNA-staining cells were seen in a fraction of the animals. The spleen sections from C57BL/6 mice exhibited numerous strongly PNA-staining areas characteristic of GC formation (Fig. 7A). These results suggest that the development of GC is TD even in the context of a virus infection.

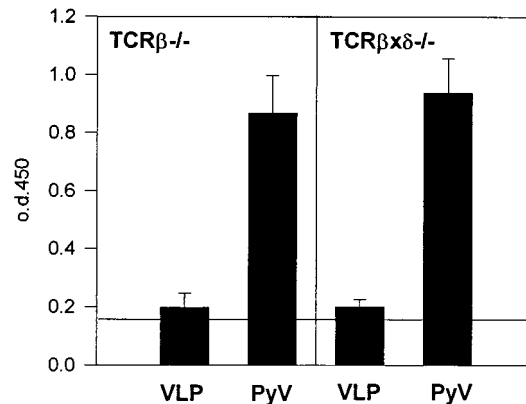


FIG. 3. VP1-specific IgG response to VLPs and PyV in TCR- $\beta^{-/-}$ and TCR- $\beta \times \delta^{-/-}$ mice. Serum samples obtained on day 14 following immunization with 70 HAU of VLPs or 70 HAU of PyV were assayed in 1:100 serum dilution. The absorbance (OD_{450}) values are means and standard deviations ($n = 3$ for VLP-immunized TCR- $\beta^{-/-}$ and TCR- $\beta \times \delta^{-/-}$ mice and PyV-infected TCR- $\beta^{-/-}$ mice; $n = 2$ for PyV-infected TCR- $\beta \times \delta^{-/-}$ mice). The horizontal line indicates the background absorbance obtained with uninfected mouse serum.

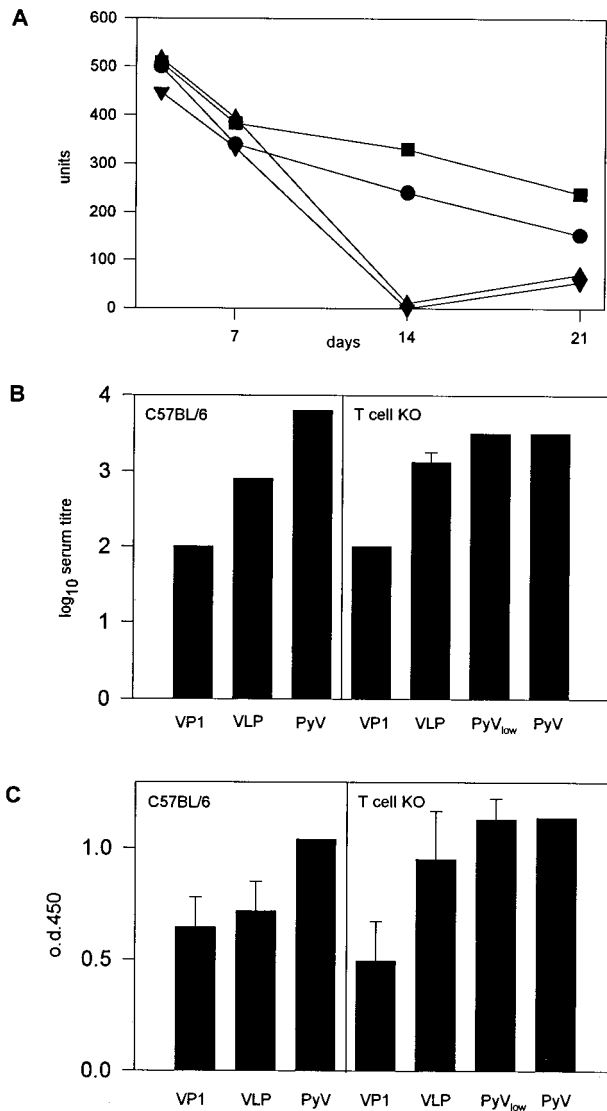


FIG. 4. Virus-specific IgM production in immunocompetent and T-cell-deficient mice in response to viral capsid proteins, VLPs, and live virus. (A) Time course of VP1-specific IgM response. To compare the IgM levels in serum samples obtained at different time points and assayed separately, the absorbance values were converted to relative units. Absorbance of a positive control serum (pooled serum from day 14 PyV-infected C57BL/6 mice, which was included in every assay) was used as reference and considered 100 U. Data obtained with serum samples (1:100 dilutions) from a C57BL/6 mouse (●) and a TCR-β×δ -/- mouse (■) injected with 7 × 10⁶ PFU of PyV (equivalent to 7 HAU) strain RA, or 7 HAU of PyV (n = 1); TCR-β -/- mice injected with 70 HAU of VP1 (n = 2) or 70 HAU of VLPs (n = 3); and TCR-β×δ -/- mice injected with 0.7 HAU of PyV (PyV_{low}; n = 3) or 7 HAU of PyV (n = 1). KO, knockout. (B) VP1-specific IgM titers on day 4 following immunization expressed as reciprocal log₁₀ of the endpoint dilutions giving positive values in ELISAs. The data shown were obtained from pooled sera from C57BL/6 mice immunized with 70 HAU of VP1 (n = 2), 70 HAU of VLPs (n = 3), or 7 HAU of PyV (n = 1); TCR-β -/- mice injected with 70 HAU of VP1 (n = 2) or 70 HAU of VLPs (n = 3); and TCR-β×δ -/- mice injected with 0.7 HAU of PyV (PyV_{low}; n = 3) or 7 HAU of PyV (n = 1). (C) VP1-specific IgM in 1:100 dilutions of sera obtained on day 7 following immunization of T-cell-deficient mice. The OD₄₅₀ values are means and standard deviations for C57BL/6 mice immunized with 70 HAU of VP1 (n = 2), 70 HAU of VLPs (n = 3), or 7 HAU of PyV (n = 1); TCR-β -/- mice injected with 70 HAU of VP1 (n = 2) or 70 HAU of VLPs (n = 3); and TCR-β×δ -/- mice injected with 0.7 HAU of PyV (PyV_{low}; n = 3) or 7 HAU of PyV (n = 1).

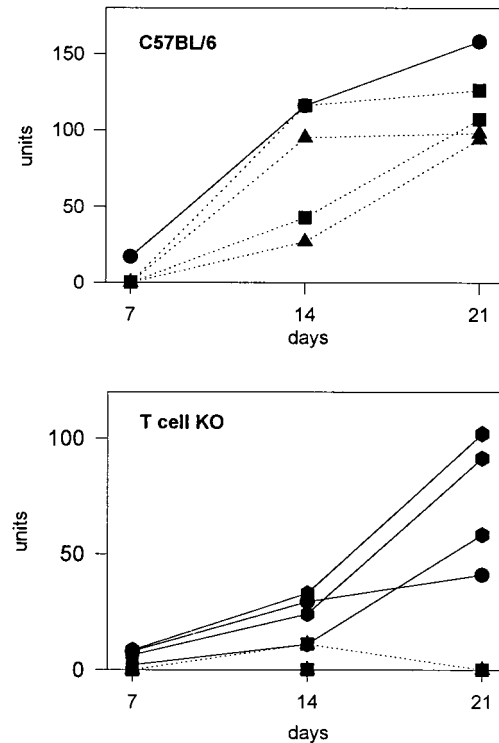


FIG. 5. Time course of VP1-specific IgG production in immunocompetent and T-cell-deficient mice in response to VP1, VLPs, and PyV. The IgG response, expressed in relative units, was calculated as described in the legend to Fig. 4. Each curve represents data obtained by sequential bleeding of a mouse immunized with 7 HAU of PyV (●), 0.7 HAU of PyV (●), 70 HAU of VLPs (■), or 70 HAU of VP1 (▲). KO, knockout.

DISCUSSION

This study demonstrates that virus infection can elicit not only IgM but also IgG responses without T-cell help, whereas IgG production depends on T-cell help when the mice are immunized with viral proteins. Repetitive antigens that strongly cross-link B-cell receptors (bacterial polysaccharides, haptenated polymers, and viruses) induce B-cell responses in the absence of T-cell help. It has been postulated that the highly structured organization of the epitope has a major role in the induction of TI antibodies. Our results indicate that the highly organized repetitive antigen structure of viruses indeed increases the efficiency of TI IgM responses but is not sufficient to induce antigen-specific IgG production. Therefore, we hypothesize that for TI switch to IgG, B cells require signals generated by live-virus infection.

Two observations suggest the importance of local signals in the immediate vicinity of the responding B cell: (i) the magnitude of TI IgG responses did not change with changing the infecting virus dose (from 7 × 10⁵ to 7 × 10⁷ PFU); and (ii) administration of VLP and a heterogeneous live virus did not result in a bystander induction of IgG response to the VLPs, indicating that systemic elevation of cytokines or activation of certain cell types is not the mechanism resulting in efficient isotype switch. Based on these findings, we suggest that the very high local antigen concentrations reached during infection with live, replicating virus, which may be present at high levels for a prolonged time, may contribute to the efficient induction of TI responses. Another factor may be the local inflammatory response accompanying virus infection, which results in the activation of NK cells, macrophages, and dendritic cells and

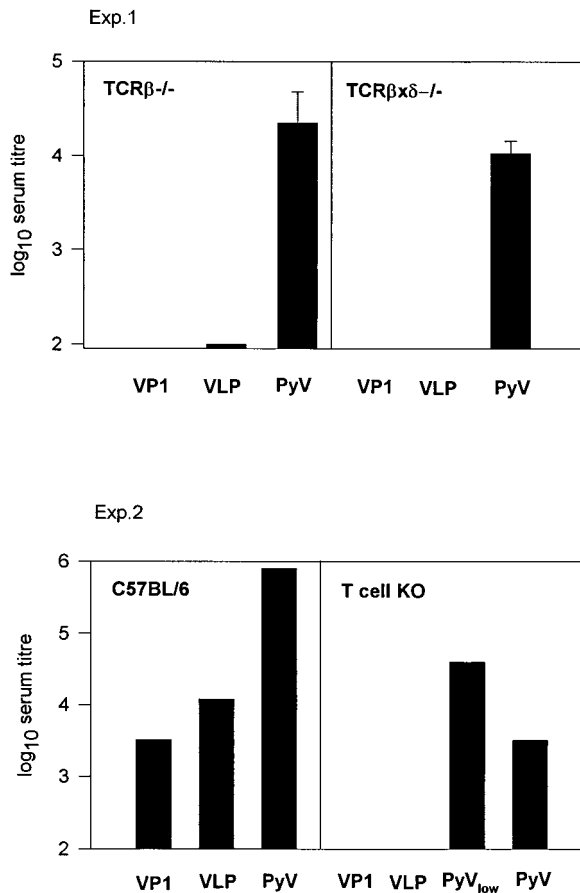


FIG. 6. VP1-specific IgG titers on days 14 and 21 following immunization. The IgG titers are expressed as log₁₀ of reciprocal endpoint dilutions giving positive values in ELISAs. In experiment 1, mean values and standard deviations for groups of mice (three per group) immunized with 70 HAU of VP1, 70 HAU of VLPs, or 7 HAU of PyV are shown. In experiment 2, pooled sera from C57BL/6 mice immunized with 70 HAU of VP1 ($n = 2$), 70 HAU of VLPs ($n = 3$), or 7 HAU of PyV ($n = 1$), TCR-β^{-/-} mice injected with 70 HAU of VP1 ($n = 2$) or 70 HAU of VLPs ($n = 3$), and TCR-β×δ^{-/-} mice injected with 0.7 HAU of PyV (PyV_{low}; $n = 3$) or 7 HAU of PyV ($n = 1$) were tested. KO, knockout.

the induction of a variety of cytokines. In this model, the infectious nature of the antigen would be indicated by the structure and the high local concentration, together with signals from the innate immune system, and the sum of these signals would trigger B-cell differentiation and isotype switch as well as IgM and IgG production even in the absence of T-cell help. These mechanisms would allow the generation of protective immunity against microbial pathogens even in an organism with impaired T-cell functions but would still safeguard against autoimmunity.

The antiviral IgG isotypes produced in PyV-infected TCR-β^{-/-} and TCR-β×δ^{-/-} mice were predominantly IgG2b, with various amounts of IgG2a and IgG3 also synthesized. No virus-specific IgG1 was detected in these mice, however, suggesting that the synthesis of IgG1 depends on αβ T-cell help. This finding is consistent with a report describing antiviral IgG2a, IgG2b, and IgG3 responses, but no IgG1 production, in LCMV-infected CD40L^{-/-} mice, which are deficient in a crucial component of T-cell help (27).

Isotype switching occurs in the periarteriolar lymphoid sheaths and in the GC. GC are also the sites associated with affinity

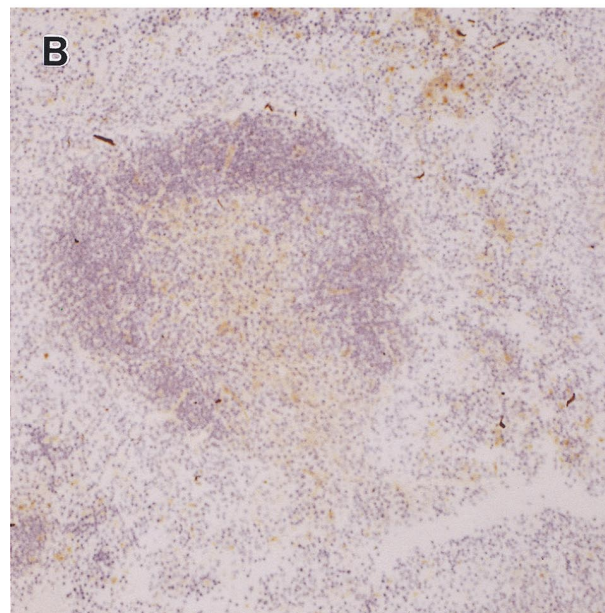
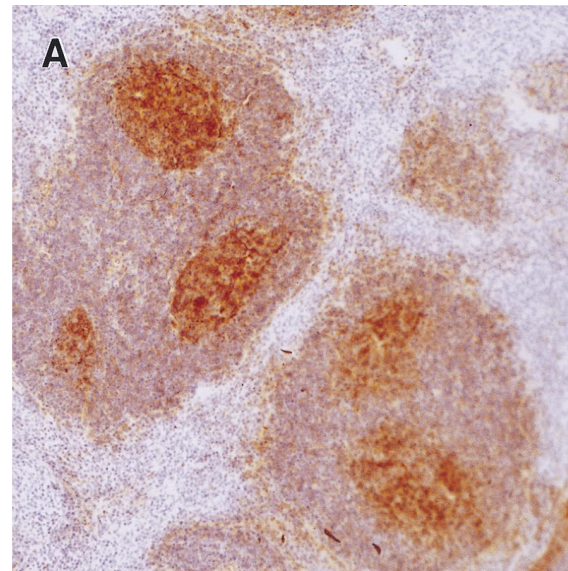


FIG. 7. Lack of GC formation in PyV-infected TCR-β^{-/-} and TCR-β×δ^{-/-} mice. Frozen sections of spleens were stained with HRP-PNA (brown) and counterstained with hematoxylin (blue). (A) Spleen of a C57BL/6 mouse on day 12 postinfection. The PNA-staining areas indicate the presence of GC. (B) Spleen of a TCR-β^{-/-} mouse on day 12 postinfection.

maturation and B-cell memory development (9). The lack of typical GC in the spleen of PyV-infected T-cell-deficient mice suggest that the GC pathway of B-cell differentiation is TD even in the context of a virus infection. Affinity maturation may not be absolutely dependent on the presence of GC, however, since in lymphotoxin-α-deficient mice, which fail to develop GC, affinity maturation of the antibody response following immunization with a very high antigen dose was found (13). It will be interesting therefore to test whether affinity maturation occurs in virus-infected T-cell-deficient mice.

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