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RESEARCH ARTICLE

Long-Lasting T Cell-Independent IgG Responses Require MyD88-Mediated Pathways and Are Maintained by High Levels of Virus Persistence

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ABSTRACT **Many viruses induce acute T cell-independent (TI) B cell responses due to their repetitive epitopes and the induction of innate cytokines. Nevertheless, T cell help is thought necessary for the development of long-lasting antiviral antibody responses in the form of long-lived plasma cells and memory B cells. We found that T cell-deficient (T cell receptor β and δ chain [TCR$\beta\delta$] knockout [KO]) mice persistently infected with polyomavirus (PyV) had long-lasting antiviral serum IgG, and we questioned whether they could generate TI B cell memory. TCR$\beta\delta$ KO mice did not form germinal centers after PyV infection, lacked long-lived IgG-secreting plasma cells in bone marrow, and did not have detectable memory B cell responses. Mice deficient in CD4$^+$ T cells had a lower persisting virus load than TCR$\beta\delta$ KO mice, and these mice had short-lived antiviral IgG responses, suggesting that a high virus load is required to activate naive B cells continuously, and maintain the long-lasting serum IgG levels. Developing B cells in bone marrow encounter high levels of viral antigens, which can cross-link both their B cell receptor (BCR) and Toll-like receptors (TLRs), and this dual engagement may lead to a loss of their tolerance. Consistent with this hypothesis, antiviral serum IgG levels were greatly diminished in TCR$\beta\delta$ KO/MyD88$^{-/-}$ mice. We conclude that high persisting antigen levels and innate signaling can lead to the maintenance of long-lasting IgG responses even in the absence of T cell help.**

IMPORTANCE **Lifelong control of persistent virus infections is essential for host survival. Several members of the polyomavirus family are prevalent in humans, persisting at low levels in most people without clinical manifestations, but causing rare morbidity/mortality in the severely immune compromised. Studying the multiple mechanisms that control viral persistence in a mouse model, we previously found that murine polyomavirus (PyV) induces protective T cell-independent (TI) antiviral IgG. TI antibody (Ab) responses are usually short-lived, but T cell-deficient PyV-infected mice can live for many months. This study investigates how protective IgG is maintained under these circumstances and shows that these mice lack both forms of B cell memory, but they still have sustained antiviral IgG responses if they have high levels of persisting virus and intact MyD88-mediated pathways. These requirements may ensure life-saving protection against pathogens even in the absence of T cells, but they prevent the continuous generation of TI IgG against harmless antigens.**

Received 30 September 2013 **Accepted** 15 October 2013 **Published** 5 November 2013

Citation Raval FM, Mishra R, Garcea RL, Welsh RM, Szomolanyi-Tsuda E. 2013. Long-lasting T cell-independent IgG responses require MyD88-mediated pathways and are maintained by high levels of virus persistence. mBio 4(6):e00812-13. doi:10.1128/mBio.00812-13.

Editor Herbert Virgin, Washington University School of Medicine

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Serological memory, the long-term maintenance of virus-specific antibody (Ab) in serum, plays an important role in the control of persistent infections by inhibiting viral recrudescence. Two types of long-lived antigen (Ag)-specific B cell populations are responsible for the sustained serum Ab levels: the long-lived plasma cells (LL PCs) and memory B cells (B_{MEM}). LL PCs are terminally differentiated cells fully committed to the secretion of Abs; they reside in the bone marrow where they receive survival signals continuously. B_{MEM} cells, on the other hand, do not secrete immunoglobulins (Ig), but they are Ag-experienced cells that can rapidly secrete large amounts of Ab upon restimulation. Both of these long-lived B cell populations are derived from germinal centers (GC) and thought to be dependent on T cell help (1). Ab responses can also be generated without T cell help, and these T cell-independent (TI) Ab responses are usually short-lived (2). The typical TI Ags, such as 4-hydroxy-3-nitrophenylacetyl (NP)-Ficoll or bacterial polysaccharides, are not proteins, and thus cannot be presented by Ag-presenting cells (APCs) as peptides to activate helper CD4$^+$ T cells. Therefore, these TI Ags usually do not induce GC formation and subsequent LL PC and recall B_{MEM} generation.

Polyomavirus (PyV) is a small double-stranded DNA virus that causes a lifelong low-level persistent infection in mice (3). This virus is well controlled and does not cause disease in immunocompetent animals but leads to tumor development after many months in T cell-deficient mice (3, 4). Previously we found that PyV infection can induce a potent TI IgG response in T cell-deficient mice. These TI Ab responses are protective (5); they reduce the viral load and prevent virus-induced lethal acute myeloproliferative disease, observed in PyV-infected T and B cell-

deficient SCID mice (6). TI IgG responses to PyV are mostly specific for the major capsid protein, VP1, and are predominantly of the IgG2a/c and IgG2b isotypes (7). This response is in contrast to the TI Ab responses induced by typical TI polysaccharide Ags, which are mainly IgM and IgG3 (8, 9). Testing the capacity of various forms of viral Ags (live PyV, VP1 protein, or virus-like particles) to induce TI Ab responses, we found that TI IgG is induced only if T cell-deficient mice are infected with live PyV (10). This observation suggests an important role for innate and inflammatory signals induced by the live, replicating virus in the generation of TI IgG specific to this infection (11).

T cell receptor β chain (TCRβ) knockout (KO) mice, which lack $\alpha\beta$ T cells, and TCR$\beta\delta$ KO mice, which lack both $\alpha\beta$ T and $\gamma\delta$ T cells, survive PyV infection for many months but maintain ~10-fold-higher persisting virus loads than wild-type C57BL/6 (B6) mice (4). Although the level of PyV persistence is not different in TCRβ KO and TCR$\beta\delta$ KO mice, these mice differ greatly in their tumor susceptibility. Whereas most TCR$\beta\delta$ KO mice develop PyV-induced salivary gland tumors between 5 and 8 months postinfection, TCRβ KO mice live well beyond this time without apparent pathology (4, 5). The relatively long life span of PyV-infected T cell-deficient mice, which have only B cell-derived adaptive immune responses, suggests that the TI IgG responses may be long-lived, because mice which lack both T and B cells die rapidly after infection.

In the studies reported here, we have investigated the longevity of antiviral IgG responses in PyV-infected T cell-deficient mice. We also questioned how PyV-specific IgG responses are maintained in the absence of T cells and whether innate immune pathways are involved in the generation and maintenance of TI antiviral IgG. Here, we provide evidence that PyV-infected T cell-deficient mice have long-lasting antiviral serum IgG responses, but the longevity of these responses is not due to the presence of LL PC in the bone marrow or B_{MEM}. Instead, the TI IgG is likely to be continuously generated by short-lived B cells and requires high persisting virus load and intact MyD88-mediated innate immune pathways.

RESULTS

Sustained antiviral IgG responses in the sera of PyV-infected T cell-deficient mice. To test the longevity of T cell-independent (TI) Ab responses, antiviral serum IgG levels of TCR$\beta\delta$ KO mice and control immunocompetent C57BL/6 (B6) mice were measured at various time points after PyV infection. At 3 months postinfection, TCR$\beta\delta$ KO mice maintained VP1-specific IgG levels in serum, similar to wild-type B6 mice (Fig. 1). Pooled serum samples taken from TCR$\beta\delta$ KO mice sequentially at various time points in an independent experiment also showed the maintenance of virus-specific IgG responses up until 6 months postinfection, the latest time point tested. The VP1-specific IgG endpoint titers were approximately 50-fold higher in pooled sera from B6 mice (1.6×10^6) compared to TCR$\beta\delta$ KO mice (3.2×10^4) at 5 months postinfection, and these data on the magnitude of TI IgG responses were consistent with published experiments from our laboratory (10).

Lack of GC B cells, bone marrow PCs, and recall memory B cell responses in T cell-deficient mice. Long-lived Ab responses are usually derived from long-lived plasma cells (LL PCs) and from the activation of memory B cells (B_{MEM}), and both of these cell types are normally generated from T cell-dependent (TD)

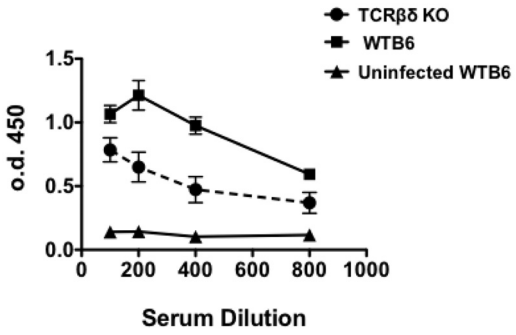


FIG 1 Serum IgG responses to PyV infection are long-lived in TCR$\beta\delta$ KO mice. VP1-specific IgG in sera of TCR$\beta\delta$ KO mice ($n = 5$) and B6 mice ($n = 2$) 3 months postinfection is compared to pooled sera of uninfected B6 mice. Mean values of absorbance (optical density at 450 nm [o.d. 450]) ± standard deviations (SD) (error bars) are shown at various serum dilutions. The results of one of three experiments are shown. WTB6, wild-type B6.

germinal center (GC) responses. However, there are reports of TI GC formation (12, 13). Moreover, PyV (and other TI viral Ags) differ from the prototypical polysaccharide TI Ags, as they are proteins and induce mostly TI IgG2a/c, not IgM and IgG3. Therefore, we tested whether T cell-deficient mice form GC upon PyV infection. Splenocytes harvested from TCR$\beta\delta$ KO mice and wild-type controls 8 days after intraperitoneal (i.p.) PyV infection were examined by flow cytometry for GC B cells. The splenocytes were gated on $CD19^+$ $B220^+$ cells and $GL\text{-}7^+$ and Fas^{high} were considered GC B cells. The level of GC B cells in the spleens of B6 mice increased from 0.11% in uninfected mice (background) to 2.05% by day 8. TCR$\beta\delta$ KO mice, however, did not have GC B cells above the frequencies in naive controls (Fig. 2). These data show that T cell-deficient mice are not able to generate GC B cells in response to PyV infection and are consistent with our previously published findings showing that the spleens of PyV-infected TCRβ KO mice lacked peanut agglutinin (PNA)-staining GC visualized by immunohistochemistry (10).

PyV has been characterized as a TI type II Ag, as it fails to induce antibody responses in T cell-deficient Xid mice, which have B cells with Btk kinase mutation (14). It has been reported that some TI type II Ags can generate memory B cell responses. Hence, we tested whether the presence of long-lasting antiviral serum IgG in PyV-infected T cell-deficient mice was due to LL PCs in bone marrow and/or B_{MEM} cells. TCR$\beta\delta$ KO mice and wild-type B6 controls were infected with PyV, and at various time points, their spleens and bone marrow were tested for VP1-specific antibody-secreting cells (ASCs) by enzyme-linked immunosorbent spot (ELISPOT) assays. Wild-type mice, as shown in Fig. 3, had IgG-producing ASC frequencies of 40 to 140 per 10^6 spleen cells at all three time points after infection. Similarly, T cell-deficient mice had VP1-specific ASCs in their spleens at all three time points, and over time the frequency of ASCs increased. ASCs in bone marrow represent LL PCs that usually appear a few weeks postinfection (15). At 6 weeks postinfection, virus-specific ASC frequencies in bone marrow were readily detected in wild-type mice (frequencies of ~40/10^6 bone marrow cells), and their frequency increased by 6 months. In contrast, we did not observe ASCs in the bone marrow of T cell-deficient mice 6 months postinfection (Fig. 3A), suggesting that T cell-deficient mice are not able to generate IgG-secreting LL PCs.

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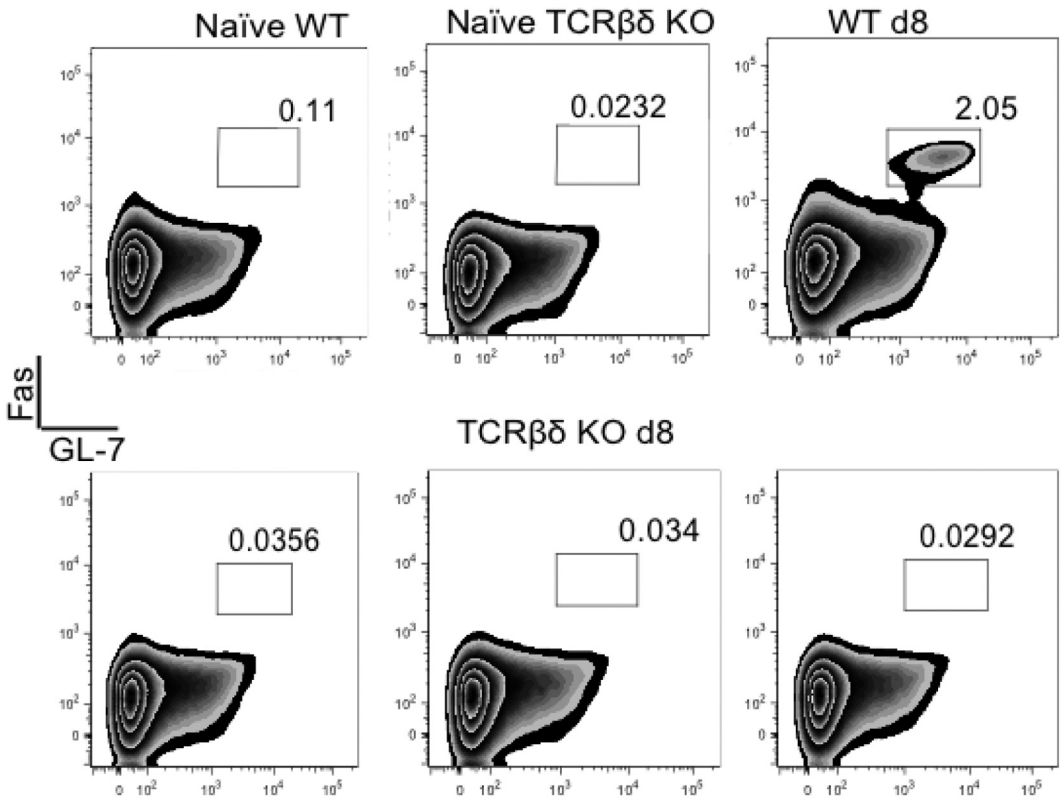


FIG 2 PyV-infected TCR$\beta\delta$ KO mice are defective in GC B cell formation. The spleens of naive and PyV-infected TCR$\beta\delta$ KO and B6 mice on day 8 (d8) after infection were examined by flow cytometry. The fluorescence-activated cell sorting (FACS) plots were gated on $B220^+$ $CD19^+$ B cells; the box gates indicate $Fas^{high}/GL7^+$ GC B cells. WT, wild type.

B_{MEM} cells that can be reactivated also contribute to long-term humoral immunity. Studying B_{MEM} in mice is a difficult task, because there is no surface marker available that can unambiguously identify murine B_{MEM} (16, 17). Therefore, we have developed and utilized a functional adoptive transfer assay in order to study murine B_{MEM} IgG responses. This assay was based on demonstrating early (day 5 to 7) recall IgG responses to PyV. Spleen cells from PyV-immune B6 mice (groups 1 and 2) or naive mice

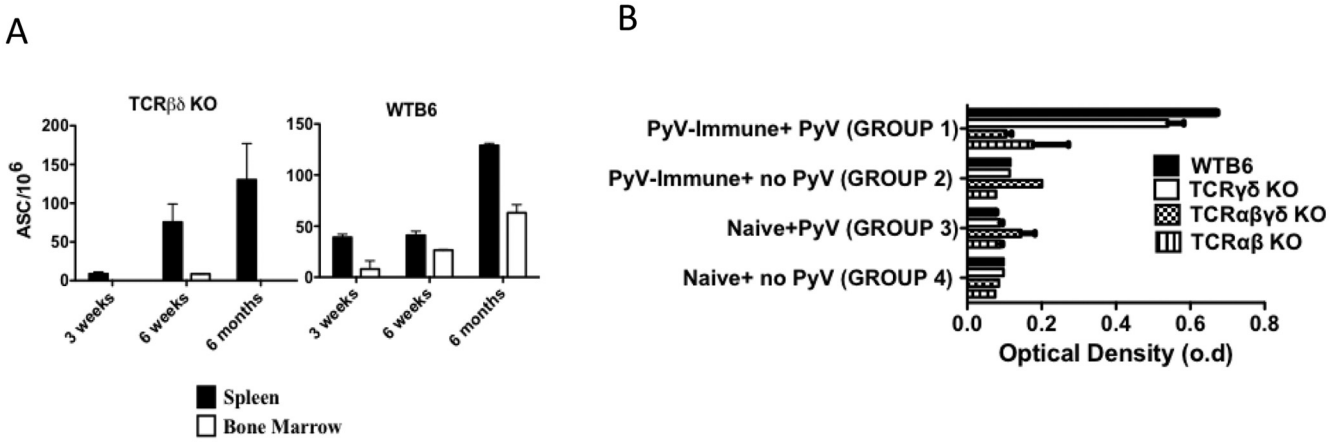


FIG 3 TCR$\beta\delta$ KO mice lack bone marrow PC and B_{MEM} after PyV infection. (A) TCR$\beta\delta$ KO and B6 mice were examined for VP1-specific IgG Ab-secreting cells (ASC) in their spleen and bone marrow at 3 weeks, 6 weeks, and 6 months after PyV infection. Mean values ± SD are shown. The figure shows the results of a representative experiment (three experiments were performed). (B) VP1-specific serum IgG ELISAs of SCID mice reconstituted with PyV-immune (groups 1 and 2) or naive (groups 3 and 4) spleen cells from wild-type B6 (WTB6), TCRδ KO (white bars), TCR$\beta\delta$ KO (checkered bars) or TCRβ KO (striped bars) mice. SCID mice in group 1 reconstituted with PyV-immune splenocytes were infected with PyV 1 day after cell transfer, and mice in group 2 were left uninfected. Similarly, mice in group 3 that received naive cells were infected with PyV, and mice in group 4 were left uninfected. The ELISA was done on serum samples collected 7 days postinfection at a serum dilution of 1:200. The results of a representative experiment are shown (three experiments were performed).

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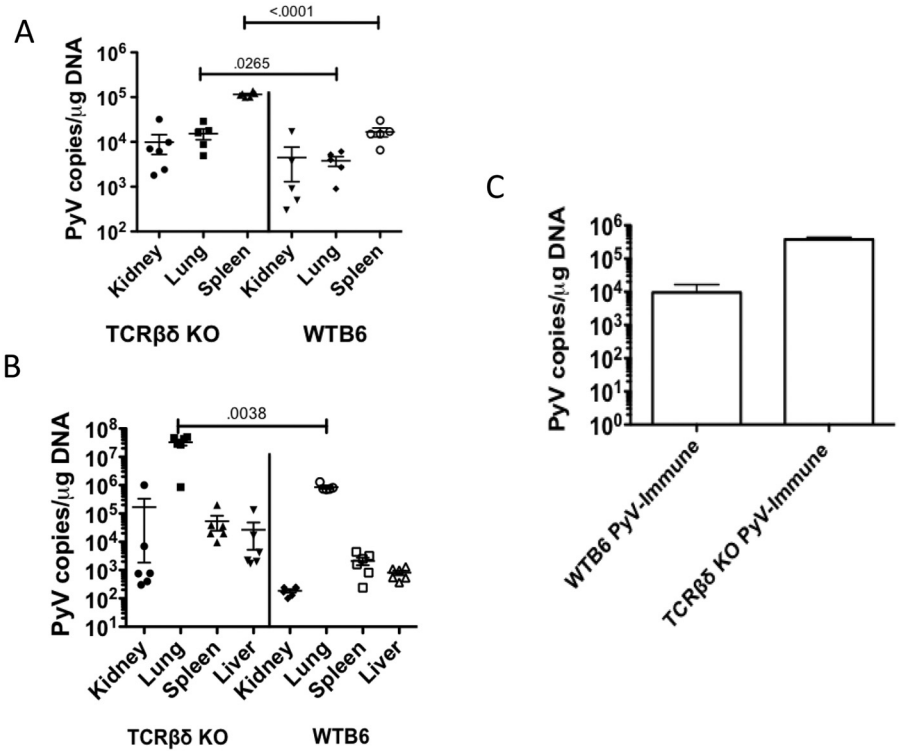


FIG 4 Higher levels of PyV persistence are seen in the organs of T cell-deficient mice compared to B6 mice. (A and B) PyV load in the organs of TCR$\beta\delta$ KO and wild-type B6 mice 14 days after intraperitoneal (A) or intranasal (B) infection. Each symbol represents the value for an individual mouse. The mean values (horizontal bar) ± SD (error bar) for the groups are indicated. Values that are statistically significantly different are indicated by horizontal bars and *P* values. (C) PyV load in the bone marrow of B6 and TCR$\beta\delta$ KO mice 3 months after i.p. infection ($n = 3$).

(groups 3 and 4) were transferred into naive SCID mice, which were infected with PyV (groups 1 and 3) or mock infected (groups 2 and 4) on the next day. Mice that received nonimmune spleen cells and were infected with PyV (group3), or mice that received PyV-immune splenocytes but were not infected with PyV (group 2), as well as mice which received nonimmune splenocytes and were left uninfected (group 4), had very low or undetectable VP1-specific IgG in their sera at this early time point. SCID mice which received PyV-immune spleen cells and PyV infection (group 1), however, had measurable antiviral IgG responses by days 5 to 7 postinfection. To test whether T cell-deficient mice are capable of having IgG recall responses, these functional memory B cell assays were performed with spleen cells of PyV-immune and naive TCRβ KO, TCRδ KO, and TCR$\beta\delta$ KO mice along with wild-type B6 mice. SCID mice given virus-immune wild-type B6 or TCRδ KO spleen cells and secondary PyV infection were able to generate good recall (B_{MEM}) responses to PyV. On the other hand, SCID mice reconstituted with virus-immune TCRβ KO or TCR$\beta\delta$ KO spleen cells along with a PyV infection were unable to generate B_{MEM} responses (Fig. 3B). These data demonstrated that B_{MEM} recall responses depend on $\alpha\beta$ T cells. Our findings therefore indicated that the long-lasting serum IgG responses in T cell-deficient mice are not due to the presence of LL PCs or B_{MEM}.

The persisting viral load is higher in T cell-deficient mice than in wild-type mice. $CD4^+$ T cell-deficient mice ($I\text{-}A^{b-/-}$) infected with PyV generated short-term Ab responses to PyV and, similar to TCR$\beta\delta$ KO mice, lacked LL PCs in bone marrow (18). On day 8 postinfection, VP1-specific IgG levels were similar in wild-type B6 and $I\text{-}A^{b-/-}$ mice, but by 23 days postinfection, they decreased in $I\text{-}A^{b-/-}$ mice compared to wild-type B6 mice, and by day 86, they were almost undetectable. These data demonstrated that TI Ab responses are not maintained in mice that lack only $CD4^+$ T cells (18). In contrast, mice that lack both $CD8^+$ and $CD4^+$ T cells maintain TI antiviral serum IgG responses. PyV causes a persistent infection, and $CD8^+$ T cells are known to effectively control the PyV load (19, 20). We reasoned that an elevated virus load in mice that lack all $\alpha\beta$ T cells, including $CD8^+$ cytotoxic T lymphocytes (CTL), compared to major histocompatibility complex (MHC) class II-deficient mice ($I\text{-}A^b$), which have normal CTL responses to PyV, may play a role in maintaining long-lived Ab responses in T cell-deficient mice. PyV levels in the kidney, lung, and spleen of TCR$\beta\delta$ KO mice and wild-type B6 controls were determined on day 14 following PyV infection. Indeed, TCR$\beta\delta$ KO mice had 10-fold-higher levels of PyV than wild-type mice did (Fig. 4A and B), whether the mice were infected i.p. or intranasally (i.n.). Thus, the elevated virus load in TCR$\beta\delta$ KO mice, compared to B6 mice, may contribute to the maintenance of long-lasting TI Ab responses in these mice.

Decrease in virus load after transfer of $CD8^+$ T cells into TCR$\beta\delta$ KO mice is associated with rapidly declining antiviral serum IgG levels. We questioned whether high levels of persistent PyV are responsible for the long-lasting Ab responses in T cell-deficient mice. Naive $CD8^+$ T cells, obtained by magnetic cell separation (MACS) enrichment, from the spleens of uninfected B6 mice were transferred into TCR$\beta\delta$ KO mice. The following day (day 1), the $CD8^+$ T cell-reconstituted TCR$\beta\delta$ KO mice were

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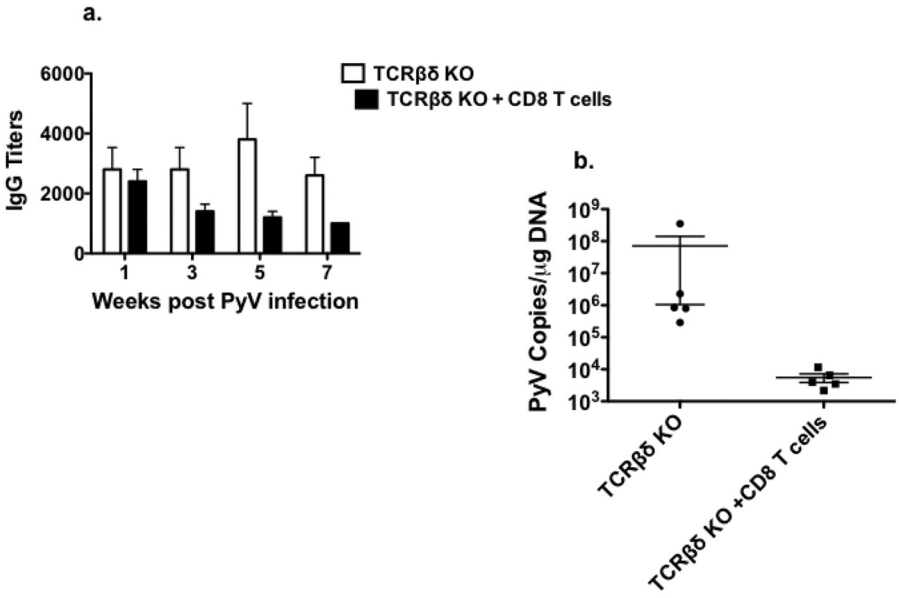


FIG 5 Reduced levels of PyV persistence are associated with rapidly decreasing serum IgG titers. (a) TCR$\beta\delta$ KO mice that received CD8$^+$ T cells (black bars) or no cell transfer (white bars) were tested for their VP1-specific serum IgG at various time points postinfection. The endpoint titers of groups of mice (5 mice in each group) are shown. (b) PyV load of TCR$\beta\delta$ KO mice which were reconstituted with CD8$^+$ T cells 1 day prior to infection or left alone (no cell transfer). The number of PyV genome copies per microgram of DNA from spleen tissue at 7 weeks postinfection is shown.

infected with PyV, as was a control group of T cell-deficient mice that received no cell transfer. Serum samples were collected, and PyV-specific IgG levels were determined at 1, 3, 5, and 7 weeks postinfection (Fig. 5a). At 1 week postinfection, the VP1-specific IgG responses were similar in the two groups. However, over time, TCR$\beta\delta$ KO mice that had received CD8$^+$ T cells generated lower levels of virus-specific IgG. PyV viral loads were also measured at the last time point of the study (7 weeks postinfection) and were ~3 orders of magnitude higher in the spleens of TCR$\beta\delta$ KO mice without cell transfer than in mice that received CD8$^+$ T cells (Fig. 5b). This experiment established a correlation between the presence of CD8$^+$ T cells, lower persisting virus load, and lower Ab responses.

Diminished antiviral IgG responses in T cell-deficient MyD88 KO mice. On the basis of the results described above, we hypothesized that a high level of persistent PyV antigen in TCR$\beta\delta$ KO mice may activate naive B cell populations that continuously emerge from the bone marrow, thereby maintaining the long-lasting IgG responses in T cell-deficient mice. This mechanism raises the question of whether B cells encounter PyV during their development in the bone marrow, and if so, how can they bypass tolerance? We first tested whether PyV was present in the bone marrow of TCR$\beta\delta$ KO mice. TCR$\beta\delta$ KO mice and wild-type controls were infected with PyV, and 3 to 4 months postinfection, we harvested bone marrow cells from these mice and performed quantitative PCR (qPCR) to determine the numbers of PyV genome copies in these samples. Bone marrow cells from TCR$\beta\delta$ KO mice harbored high levels of PyV, indicating that viral Ag was present at the site of B cell development (Fig. 4C).

The high virus load in bone marrow suggested that B cells that were developing there encountered PyV, but this encounter may not lead to tolerance, as newly emerging mature B cells seemed to continue to generate Ab to PyV in the periphery. A report by Berland et al. (21) suggested that "dual specificity" antigens, which ligate the B cell receptor (BCR) and endosomal Toll-like receptors (TLRs) such as TLR7/8 and TLR9 may break B cell tolerance. In that study, B cells with a transgenic BCR specific for the autoantigens RNA, double-stranded DNA (dsDNA), and nucleosomes, showed a TLR7-dependent loss of B cell tolerance. PyV can also be considered an antigen capable of "dual engagement" of BCR and innate receptors, as we have found that PyV infection activates MyD88-mediated pathways initiated by TLRs (22; F. M. Raval, unpublished data), and PyV antigens also engage the BCR of some B cells. Therefore, we hypothesized that TCR$\beta\delta$ KO mice, with high levels of PyV in bone marrow, may lose their tolerance to PyV and that this disruption of tolerance may be MyD88 dependent. In this case, it would follow that in T cell-deficient MyD88 KO mice, tolerance would be developed, and new B cells emerging from the bone marrow would not respond to persisting PyV, leading to diminished antiviral serum IgG. To see whether MyD88-mediated pathways are needed for continuous generation of TI IgG, we infected TCR$\beta\delta$ MyD88 triple-knockout mice (TCR$\beta\delta$ KO/MyD88$^{-/-}$) and mice that are T cell deficient but MyD88$^{+/+}$ (TCR$\beta\delta$ KO) with PyV. VP1-specific IgG responses were tested at 1, 2, and 4 weeks after PyV infection (Fig. 6). The virus-specific IgG levels were similar in TCR$\beta\delta$ KO/MyD88$^{-/-}$ mice and TCR$\beta\delta$ KO mice at 1 week postinfection, but the IgG responses to PyV were significantly lower in TCR$\beta\delta$ KO/MyD88$^{-/-}$ mice at 2 and 4 weeks postinfection (~40-fold lower on week 2 and ~100-fold lower on week 4). TCR$\beta\delta$ KO/MyD88$^{-/-}$ mice generated on average slightly higher but not statistically significantly different levels of VP1-specific IgM (Fig. 6). These results suggest that MyD88 signaling is required to generate high levels of sustained PyV-specific IgG responses in T cell-deficient mice. It is important to note that on week 6 postinfection, the virus loads in TCR$\beta\delta$ KO and TCR$\beta\delta$ KO/MyD88$^{-/-}$ mice were not significantly different (data not shown).

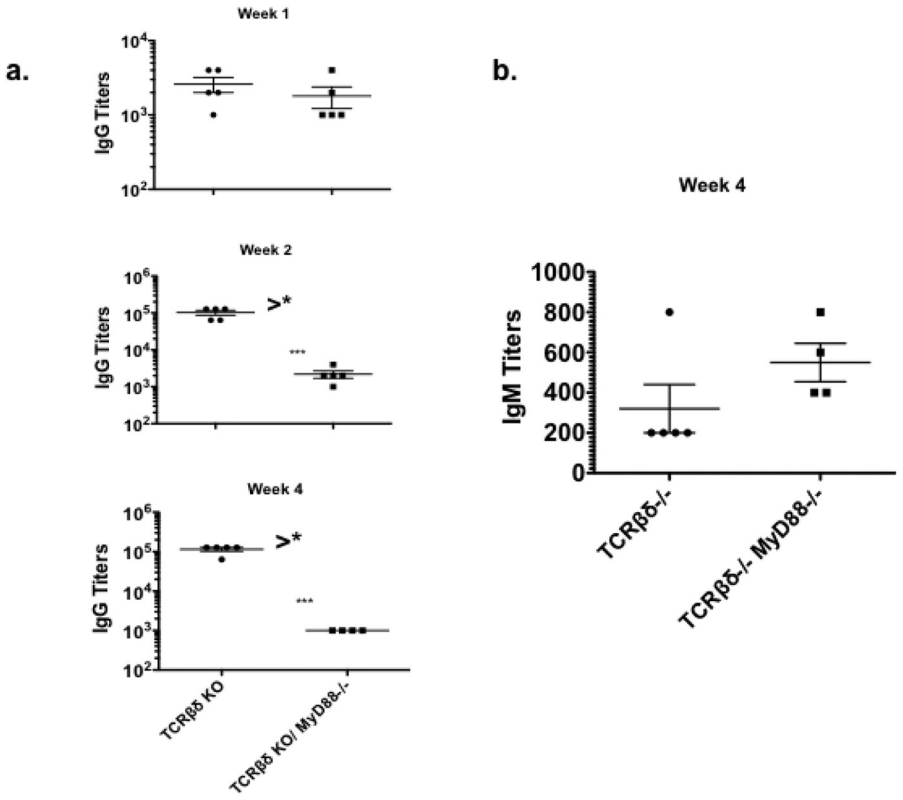


FIG 6 Antiviral serum IgG is diminished in T cell-deficient MyD88$^{-/-}$ mice. (a) VP1-specific serum IgG titers of TCR$\beta\delta$ KO (MyD88$^{+/+}$) and TCR$\beta\delta$ KO/MyD88$^{-/-}$ mice 1, 2, and 4 weeks postinfection shown (on a log scale). >*, IgG titers equal or larger than 10^5. (b) VP1-specific serum IgM titers of the same mice 4 weeks postinfection (note that the *y* axis is linear).

DISCUSSION

In this study, we show that mice have long-lasting serological memory after PyV infection and that high levels of virus-specific serum IgG are maintained even in the absence of T cells. This is achieved without the formation of GC, LL PCs in bone marrow, or B_{MEM}. The maintenance of this "TI serological memory," however, requires high virus load and intact MyD88-mediated pathways in the host. PyV is a TI Ag, which differs in many ways from some well-characterized and frequently used TI Ags such as bacterial polysaccharides, 4-hydroxy-3-nitrophenylacetyl (NP)-lipopolysaccharide (LPS) or NP-Ficoll. The chemical nature of the viral capsid is protein, not polysaccharide. Moreover, PyV is not inert, but a live, replicating virus that can elicit innate, inflammatory, or danger signals. Therefore, it was of interest to systematically investigate the mechanisms that allow the maintenance of potentially life-saving long-term IgG levels in virus-infected TCR$\beta\delta$ KO mice.

The formation of GC is a prerequisite for B_{MEM} and long-lived PC formation in TD responses. GC are normally not generated in the absence of T cells, but under unusual circumstances, such as in BCR transgenic mice with a high frequency of high-affinity antigen-specific B cells, or after immunization with very high doses of TI Ags (NP-Ficoll), short-lived GC, defined by clusters of PNA-positive and GL-7-positive B cells, can be induced in a TI manner (12, 13). These TI GC, however, do not appear to be functional, as no somatic hypermutation was detected in the TI GC B cells (12), and these GC did not persist past day 5 following immunization. In this study, we showed that TCR$\beta\delta$ KO mice infected with PyV did not show an increase in GC B cell frequencies in the spleen above background levels on day 7 and later. Therefore, the long-lasting TI serum IgG responses to PyV are not products of an unusual, pathogen-induced TI GC reaction.

The nonsecreting form of B cell memory resides in antigen-experienced quiescent B_{MEM}, which can be rapidly activated in recall responses, giving faster and more robust responses to rechallenge than naive B cells. B_{MEM} are usually formed in TD responses, but there are some reports of TI B_{MEM}. One of the most cited examples of "TI B cell memory" is a report by Alugupalli et al. (8). This report showed that TI IgM generated by B1b cells is crucial for the clearance of *Borrelia hermsii*, a pathogen causing relapsing fever in mice. Moreover, the B1b cell population that expands during *B. hermsii* infection can survive for months in a pathogen-free host and provides a long-lasting protective effect, as it can respond to *Borrelia* with IgM secretion. The "TI B cell memory" in this case is not manifested by continuously secreting LL PCs or by B_{MEM} giving fast IgG recall responses. Instead, the heightened humoral immunity is due to the increased number of B1b cells that are in a nonsecreting silent state but are capable of secreting IgM again if they are stimulated (8).

Immunization with the TI type II Ag, NP-Ficoll, can also lead to B cell responses that persist for months. These IgM and IgG3 responses are generated by extrafollicular B1b plasmablasts, not by the conventional memory B cell populations (23). Whether the relative longevity of the extrafollicular B1b cells compared to ex-

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trafollicular B2 TD plasmablasts is due to self-renewal of the antigen-activated B1b cells or to other mechanisms has not been elucidated. Relatively long-lived bone marrow PC responses to NP-LPS were reported recently, but these were unswitched IgM-secreting PCs (24).

Using the definition of Ag-experienced resting, nonsecreting B cells for B_{MEM}, Obukhanych et al. (9) reported the formation of such memory B cells, some of which were isotype switched to IgG, in response to the TI Ag NP-Ficoll. The B cells in this study were labeled with a bromodeoxyuridine (BrdU) pulse when they were dividing upon the first encounter with the Ag, so that the antigen-experienced cells could be distinguished from the naive B cells. The TI B_{MEM} described in this report, however, were fundamentally different from the "conventional" B_{MEM} that usually form in a TD response, because they did not give an enhanced "recall" IgG response to secondary challenge (9).

In our studies, we tested whether the spleen cells of PyV-immune T cell-deficient mice can mount a fast and enhanced recall IgG response to PyV rechallenge in a functional adoptive transfer assay. Using this assay, we found that recall IgG responses were given only by the splenic B cells of PyV-immune mice that had $\alpha\beta$ T cells, such as B6 and TCRδ KO mice, but not by TCRβ KO or TCR$\beta\delta$ KO mice. Therefore, $\alpha\beta$ T cell help is essential for the formation of recall B_{MEM} even when immunized with PyV, an infectious viral TI Ag.

LL PCs can maintain serological memory even in the absence of any other B cells, as shown by experiments using depletions of B cells with Ab to CD20 (25). The CD20-specific depleting Ab eliminates both naive B cells and B_{MEM} but spares fully differentiated PCs. We found that mice without T cell help, including both the $CD4^+$ T cell-defective I-$A^{b-/-}$ mice (18) and the $\alpha\beta$ T cell-defective TCRβ KO or TCR$\beta\delta$ KO mice, could not accumulate IgG-secreting PCs in their bone marrow, the site of LL PC accumulation following acute virus infections (15). Therefore, LL PCs could not be responsible for the maintenance of TI antiviral serum IgG.

This report demonstrates the existence of high antiviral serum IgG for as long as 6 months after infection (the length of our study) in TCRβ KO and TCR$\beta\delta$ KO mice. Previously we had observed that the TI IgG responses to PyV in I-$A^{b-/-}$ mice were short-lived and that they greatly decreased after day 23 postinfection (18). How could we reconcile these results that seem to be contradictory? A major difference between PyV-infected I-$A^{b-/-}$ and TCRβ KO or TCR$\beta\delta$ KO mice is the amount of persisting virus they harbor. Consistent with the antiviral role of $CD8^+$ T cells, I-$A^{b-/-}$ mice, which have $CD8^+$ cytotoxic T cell responses, have a low level of PyV persistence that is undistinguishable from that of B6 mice. On the other hand, TCRβ KO or TCR$\beta\delta$ KO mice lack $CD8^+$ T cell responses and have a >10-fold-higher persisting PyV load compared to that of the B6 mice. We propose that the high virus load continuously activates naive B cells, maintaining the serum Ab levels by IgG secretion by short-lived PCs. In the $CD4^+$ T cell-deficient I-$A^{b-/-}$ mice, the virus load may not be sufficiently high to ensure continuous B cell activation and sustained IgG responses. This scenario is supported by our data showing that transfer of $CD8^+$ T cells into T cell-deficient mice decreases virus load and that it is also associated with a reduction of antiviral IgG responses with time, similar to what was seen in I-$A^{b-/-}$ mice.

TCRβ KO or TCR$\beta\delta$ KO mice had high virus load in bone marrow, where B cells develop (Fig. 4C). This finding raises the question of how can B cells that develop in the presence of PyV Ag maintain their reactivity and not become tolerized to PyV? Most viral infections activate innate immune responses and induce multiple inflammatory signals, which may be important for the early control of the pathogens, but they also shape the adaptive immune responses. We have found previously that PyV infection activates MyD88-mediated pathways, and these pathways play a crucial role in the maintenance of long-term IgG responses to PyV infection in immunocompetent (T cell-sufficient) mice (22). MyD88 mediates signaling from most TLRs (except TLR3) and the cytokine receptors IL-1R (interleukin-1 receptor) and IL-18R. For the induction of long-lasting IgG responses to PyV, the cytokine receptors were dispensable. These experiments show that PyV as an Ag can simultaneously engage the B cell receptors and TLRs. Remarkably, in transgenic B cell models, the presence of an Ag with this "dual engagement capacity" was shown to lead to a breach of anergy. Self-Ags that could bind the B cell receptor and engage TLR7 or TLR9 generated autoantibodies (26). In bone marrow, PyV can engage the BCR and be endocytosed by B cells and then may be recognized by endosomal TLR7/TLR9-MyD88 mediated pathways. We speculate that the ability of PyV to initiate signaling by both the BCR and the endosomal TLRs could be a key step in allowing continuous PyV-specific Ab production. In agreement with this hypothesis, we found that the mice that were deficient in both T cells and MyD88 signaling had diminished IgG responses to PyV, in contrast to the long-lasting high-titer TI serum IgG in MyD88-sufficient T cell-deficient mice. Therefore, the continuous activation of naive B cells emerging from bone marrow by the high virus load may be successful only if innate immune pathways are also activated by the Ag. This hypothetical mechanism would allow the newly differentiated B cells to develop anergy to harmless Ags they encounter in bone marrow, but Ags sensed by innate immunity would still trigger Ab secretion.

We have reported previously that long-term humoral immunity to PyV was defective in mice that lacked MyD88 even in the presence of T cells, as the MyD88 KO mice failed to form LL PCs. The results of this current study taken together with the previous findings suggest that important mechanisms may ensure that long-lived Ab responses, both TI and TD, are generated and maintained only to those Ags that activate innate immunity by their pathogen-associated molecular patterns. This safeguard would ensure protective humoral immunity against a viral pathogen even under the conditions of partial immunodeficiency (lack of T cells) but would greatly reduce the long-term production of irrelevant or autoreactive Abs and the occurrence of pathogenic autoantibody-induced conditions.

MATERIALS AND METHODS

Mice and infection. All the mice used in the studies were on the C57BL/6 (B6) background. TCRβ KO, TCR$\beta\delta$ KO, TCRδ KO, and SCID mice were originally obtained from the Jackson Laboratory (Bar Harbor, ME), and colonies of these mice were maintained in the Department of Animal Medicine of the University of Massachusetts Medical School under specific-pathogen-free conditions. TCR$\beta\delta$ KO/MyD88$^{-/-}$ mice were bred by crossing TCR$\beta\delta$ KO mice and MyD88 KO mice, and the mice were maintained at the animal facilities at the University of Massachusetts Medical School. Mice were used when they were between 8 and 12 weeks of age, virus infections were done intranasally (i.n.) or intraperitoneally (i.p.) with ~10^6 PFU/ mouse of PyV strain A2. All the procedures using

animals were done according to protocols approved by the University of Massachusetts Medical School Animal Care and Use Committee.

VP1-specific ELISA. VP1-specific enzyme-linked immunosorbent assays (ELISAs) were conducted as previously described (27). Briefly, purified recombinant VP1 protein was used to coat the wells of 96-well plates (using 50 ng/well). Bound Ab was detected using biotin-conjugated goat Abs specific for mouse IgG and streptavidin-conjugated horseradish peroxidase (HRP) (Vector Laboratories). ELISA plates were developed using BD OptEIA 3,3′,5,5′-tetramethylbenzidine (TMB) substrate set (BD Pharmingen), and the reactions were stopped with 2 N sulfuric acid. Optical densities were read at 450 nm by $THERMO_{MAX}$ microplate reader and SoftMax software. Data are represented as optical density versus serum dilution (raw data) or are shown as IgG concentration expressed in arbitrary units, which were calculated using a reference positive serum dilution curve. IgG endpoint titers are defined as the highest serum dilutions showing optical density in the ELISA above the values of negative-control serum (mean ± standard deviation [SD]).

VP1-specific ELISPOT assay. To determine the number of VP1-specific Ab-secreting cells (ASCs), multiscreen HTS filter plates (Millipore) were coated with purified VP1 protein (0.1 μg/ml in carbonate buffer, 50 μl/well). Wells coated with unlabeled goat anti-mouse Ig(H+L) (Southern Biotech) were used for determining total IgG ACSs, and some wells were coated with carbonate buffer only (no-coat controls). The plates were incubated overnight at 4°C and then blocked for 30 min at 37°C with RPMI 1640 plus 10% fetal bovine serum (FBS). The cells were plated in duplicate wells in 0.2 ml in each well at a concentration of 10^7 cells/ml, followed by 2-fold dilutions. The plates with cells were incubated for 4 h at 37°C, then the cells were discarded, and the plates were washed and blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min at 37°C. Bound Ab was detected using biotinylated goat Abs specific for mouse IgG and streptavidin-conjugated HRP. The spots were developed with ELISPOT 3-amino-9-ethylcarbazole (AEC) substrate set (BD Pharmingen) according to the manufacturer's protocol and counted using the CTL ImmunoSpot (Cellular Technology Ltd.).

qPCR to measure viral DNA genome copy number. DNA was prepared from organ homogenates and used for quantitative PCR (qPCR) assays to measure PyV copy numbers, as described previously (4).

Flow cytometry. Flow cytometry was performed as described previously (22). Abs used in these studies included fluorescein isothiocyanate (FITC)-labeled rat anti-mouse T and B cell-activating Ag GL7 (clone GL7; BD Pharmingen), phycoerythrin and Cy7 (PECy7)-labeled rat anti-mouse CD19 (clone 1D3; BD Pharmingen), PE-labeled hamster anti-mouse CD95 (BD Pharmingen), Pacific Blue-labeled rat anti-mouse B220 (clone RA3-6B2; BD Pharmingen). At least 100,000 events were counted using an LSRII (BD Biosciences) and analyzed using FlowJo software (Tree Star).

B cell memory assay. When the mice were between 8 and 12 weeks of age, they were infected intraperitoneally with ~10^6 PFU/mouse of PyV strain A2. Spleens were harvested from uninfected or PyV-infected (for 3 to 4 months) TCRβ KO, TCRδ KO, TCRβδ KO, and B6 mice, and 10^7 spleen cells were transferred intravenously into uninfected SCID mice. One day after the cell transfer, some of the SCID mice of each group were infected with PyV (~10^6 PFU/mouse i.p.), others were left uninfected. Five to 7 days postinfection, recall PyV-specific responses from the sera of SCID mice were measured by ELISA.

Transfer of $CD8^+$ T cells into T cell-deficient mice. Spleen cells harvested from Ly5.1 B6 mice were pooled and treated with 0.84% NH_4Cl to lyse red blood cells (RBC) and then resuspended in magnetic cell separation (MACS) buffer as instructed by the manufacturer's protocols (Miltenyi Biotec MACS kit). CD8 T cell isolation kit (Miltenyi Biotec) for negative selection of CD8 T cells was used to purify CD8 T cells from the wild-type splenocytes. Cell purity was checked by flow cytometry. Cells were resuspended in Hanks balanced salt solution (HBSS) and intravenously transferred into recipient mice. For CD8 T cell transfer experiments, 7×10^6 wild-type CD8 T cells were intravenously (i.v.) transferred into each of 5 TCRβδ KO mice.

Statistical methods. For statistical comparisons, unpaired Student's *t* test was used, and data with $P < 0.05$ were considered statistically significant.

ACKNOWLEDGMENTS

This work was supported by grant RO1 CA 66644 (to E.S.-T.), training grant T32 AI007349 (F.M.R.), and RO1 AI073651 (to R.M.W.). We thank Jill Dorgan Le and Ben Lamothe for technical assistance.

This work was supported by NIH training grant T32 AI-007349 (to F.M.R.) and NIH research grants RO1 CA066644, ARRA fund CA066644-S1 (to E.S.-T.), and RO1 AI073651 (to R.M.W.).

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