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T cell-independent and T cell-dependent immunoglobulin G responses to polyomavirus infection are impaired in complement receptor 2-deficient mice

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Abstract

Polyomavirus (PyV) infection induces protective T cell-independent (TI) IgM and IgG antibody responses in T cell-deficient mice, but these responses are not generated by immunization with viral proteins or virus like particles. We hypothesized that innate signals contribute to the generation of isotype-switched antiviral antibody responses. We studied the role of complement receptor (CR2) engagement in TI and T cell-dependent (TD) antibody responses to PyV using CR2-deficient mice. Antiviral IgG responses were reduced by 80–40% in CR2−/− mice compared to wild type. Adoptive transfer experiments demonstrated the need for CR2 not only in TD, but also in TI IgG responses to PyV. Transfer of CR2−/− B lymphocytes to SCID mice resulted in TI antiviral IgG responses that corresponded to 10% of that seen in wild-type B cell-reconstituted mice. Thus, our studies revealed a profound dependence of TI and TD antiviral antibody responses on CR2-mediated signals in PyV-infected mice, where the viral antigen is abundant and persistent.

Keywords

Complement receptor; TI antiviral antibody responses; Polyomavirus infection

Complement activation through the classical or the alternative pathways is an important step in the innate immune responses to pathogens, including viruses. Covalent attachment of C3 fragments to virus particles may indicate the infectious origin of the antigen and may lead to enhanced efficiency of B cell responses via co-ligation of the B cell receptor (BCR) and the complement receptor signaling complex CD21/CD19/CD81 on B lymphocytes (Carroll, 2004).

The role of the binding of active complement components (e.g., C3) to the complement receptors CD35 and CD21 in antibody responses to TD protein antigens is well documented.

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The two complement receptors CD35 and CD21 are differentially spliced products encoded by the *Cr2* gene in mice, and they both are expressed on B lymphocytes and on follicular dendritic cells (FDC). CD35/CD21 (CR2) signaling on B cells decreases the activation threshold of the BCR, thus enhancing TD B cell responses to limiting amounts of antigens (Carter and Fearon, 1992; Dempsey et al., 1996). In addition, CR2 signaling on B cells promotes B cell survival in TD responses, probably by enhancing B cell survival in the germinal centers (Fischer et al., 1998). Finally, CR2 expression on FDCs is thought to promote secondary and memory B cell responses by prolonging antigen persistence (Chen et al., 2000; Wu et al., 2000).

The effect of complement receptor signaling on antibody responses to TI antigens is less clear. Early studies done by Pepys et al. with animals treated with complement-depleting cobra venom factor (CVF) suggested that complement is not necessary for the induction of TI antibody responses to the synthetic antigen polyvinylpyrrolidone (Pepys, 1974; Pepys, 1972). Subsequently, however, other studies demonstrated impaired antibody responses in C3-deficient dogs, and in CVF-treated mice immunized with low dose of the TI antigens DNP-Ficoll and DNP-polyacrylamide (Matsuda et al., 1978; O'Neil et al., 1988). Treatment of mice with a monoclonal antibody directed against the C3d-binding site of CR2 also led to greatly reduced antibody responses in nude mice and decreased antibody responses in normal mice to the type 2 TI antigen dextran (Wiersma et al., 1991), suggesting that CR2 signaling may enhance antibody responses to TI antigens. The CR2 dependence of TI responses to polysaccharide bacterial cell wall components (prototypical TI type 2 antigens) was recently re-examined in *Cr2*−/− mice. Decreased primary IgM and IgG responses to group B Streptococcal polysaccharides were found in the absence of CR2 expression, and this effect was thought to be related to impaired localization of this antigen to the marginal zone and to FDC in the spleen (Pozdnyakova et al., 2003). Marginal zone (MZ) B cells, which express CD21 at a higher level than follicular B cells, are believed to play a major role in IgM and IgG3 antibody secretion to TI type 2 antigens. This idea is supported by observations in both men and mice. The onset of B cell responsiveness to bacterial polysaccharides and the maturation of MZ B cells coincides in childhood, and in irradiated mice the reconstitution of MZ B cells is associated with the reappearance of antibody responses to TI type 2 antigens (Lane et al., 1986; Lee et al., 1991).

Do CR2-mediated mechanisms operate to enhance antiviral antibody responses in vivo? Although several viral models have been studied, the role of CR2 signaling in antiviral antibody responses in general is still not well understood. In many virus infections, the amount of antigen is not limiting, and thus antiviral TD antibody responses would not be expected to depend on CR2 signaling. Consistent with this idea, some studies have found that antibody (IgG) responses to infection with live, replicating viruses, such as LCMVand VSV, were not affected by a deficiency in C3 or CR2, whereas antibody responses to non-replicating forms (mutant or killed) of these viruses were C3 and CR2 dependent (Ochsenbein et al., 1999). Induction of IgG responses by the HSV strain KOS1.1 that can replicate at the periphery, however, was greatly reduced in the absence of CR2 (Da Costa et al., 1999). Antigen persists in many virus infections, and in these situations the CR2 on FDCs may not be essential for the maintenance of memory B cells or long-living plasma

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cells. In VSV infection in mice, neutralizing serum IgG levels several months post-infection are normal in C3-deficient mice, slightly reduced in C4- and CR2-deficient mice, and greatly reduced in CD19 KO mice compared to wild type. These changes in serum IgG levels are accompanied with similar decreases in antibody forming cell frequencies in the bone marrow (Ochsenbein et al., 1999), suggesting a role for CR2 in the maintenance of long-term humoral immune responses in this virus infection.

Effects of C3 on TI antibody responses to viruses have also been observed. Ochsenbein et al. (1999) reported that TI neutralizing IgM responses to VSV, polio virus, and to recombinant vaccinia virus carrying VSV G protein were delayed and decreased in C3− mice, whereas the TD IgG responses of the C3− mice to these viruses seemed to be normal (Ochsenbein et al., 1999).

The goal of the studies described here is to better understand how TI and TD IgM and IgG responses are affected by CR2 signaling in infections with rapidly replicating viruses, using PyV infection in mice as a unique model of TI and TD antiviral antibody responses. PyV, although can be presented in the form of peptide antigens on MHC classes I and II, thus it is able to activate T cells, can also elicit biologically significant, protective, isotype-switched TI antibody responses in the absence of functional T cells in the host (Szomolanyi-Tsuda et al., 1998). The TI IgG responses are directed against the VP1 capsid protein, and they are generated only by live virus infection, not by immunization by virus-like particles. We have postulated a role for innate immune signals activated by the virus in the induction of these VP1-specific IgG responses. This report demonstrates that CR2 has a significant role in enhancing both TD and TI antiviral IgG responses in mice infected with PyV, despite the abundance and persistence of the virus antigen. Thus, CR2-mediated signals activated during infection with PyV in T cell-deficient hosts may be important innate helper signals enhancing humoral immunity.

Results

Reduced antiviral IgG responses in PyV-infected CR2 KO mice

To test the effect of CR2 expression on humoral immune responses to PyV, we compared virus-specific IgM and IgG levels in the serum of Cr2−/− mice and normal mice at different time points following PyV infection, using polyoma major capsid protein (VP1)-specific ELISA assays. IgM titers, which peak on days 5–7, were not significantly different between the two groups on day 7 (Fig. 1A) or on day 20 (data not shown). VP1-specific IgG levels of serum samples taken on day 14, however, were significantly reduced in Cr2−/− mice compared to their wild-type counterparts. In the representative experiment shown in Fig. 1B, this reduction was more than 2-fold (below 50% of the IgG titers of wild type), and the antiviral IgG titers, although continued to increase with time in both groups, remained significantly diminished in Cr2−/− mice at the subsequent time points tested (days 21 and 28) (Figs. 1C and D). Reinfection with PyV on day 28 led to the doubling of VP1-specific IgG titers by day 33 (5 days post-re-infection) in both wild-type and Cr2−/− mice. This experiment demonstrated that although the significant differences in the antiviral IgG levels were maintained in the two groups of mice after repeated infections, Cr2−/− mice could also give good responses to PyV re-infection (Fig. 1E).

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We then determined whether the reduction in virus-specific IgG titers in CR2-deficient mice reflected impaired switching to a particular IgG isotype. Isotype-specific ELISA assays showed that there was a reduction in all virus-induced isotypes tested (IgG1, IgG2a, IgG2b, and IgG3; Fig. 2), suggesting that the diminished VP1-specific IgG responses did not arise as a consequence of the impairment of switching per se to a specific isotype, rather they may result from defects in survival and/or differentiation of the IgG-secreting B cells.

The virus-specific antibody-secreting cells (ASC or plasma cells) are mostly in the spleen during acute systemic virus infection, but after the resolution of the acute infection a large number of the ASCs migrate to the bone marrow. The plasma cells residing in the bone marrow are thought to be long lived, and together with the memory B cells they are responsible for the maintenance of long-term humoral immune responses (Slifka et al., 1998). VP1-specific ASC numbers in the spleen and bone marrow tested by ELISPOT assays on day 20 post-infection were lower in CR2 KO mice than in their wild-type counterparts (Fig. 3). As the decrease was ~50% at both of these locations, the diminished antibody responses in the CR2 KO mice are probably not due to a specific defect in the formation of the bone marrow-residing plasma cells.

Differences in virus clearance in Cr2−/− and wild-type mice

To assess the importance of CR2-mediated mechanisms in the clearance of PyV infection in the host, we determined the viral load in the spleens and kidneys of Cr2−/− and wild-type mice using real-time PCR. DNA samples were prepared from these organs harvested at 4 weeks post-infection and were analyzed using PyV sequence-specific primers to determine the number of the PyV genome copies in the sample, then these values were normalized for genomic DNA copy numbers (determined by mouse β actin-specific primers). The kidneys of CR2 KO mice had 40–700 copies of PyV DNA per 10^3 cells, whereas the viral DNA was very low but detectable in the samples from wild-type kidneys on day 28 post-infection (Fig. 4) (the limit of detection is approximately one copy of PyV genome per 10^3 cells). Kidneys are well-known sites of persistence in PyV-infected mice (Rochford et al., 1992). Remarkably, viral DNA levels in the spleens of all of the CR2 KO mice were below the limit of detection of our assays, although PyV was readily detectable in wild-type spleens (Fig. 4). These differences in virus clearance in the CR2 KO mice compared to wild-type in the kidneys may be the consequences of the lower antiviral IgG levels, or lack of other complement-dependent mechanisms involved in the control of the virus. In the spleens, the lack of CR2 on the FDCs may decrease the level of the persistence of virus–antibody complexes.

TD IgG responses rapidly decline in SCID mice reconstituted with Cr2−/− B cells

The magnitude and longevity of T cell-dependent antibody responses that were generated by Cr2−/− B cells in the absence of a newly emerging B cell population from the bone marrow was tested by performing adoptive transfer experiments. Spleen cells from naive Cr2−/− mice as well as from wild-type controls were transferred into SCID mice and then tested for their ability to secrete IgM and IgG in response to PyV. Similar to the responses of PyV-infected Cr2−/− mice described above, there were no appreciable differences in early (day 5) IgM levels in mice reconstituted with Cr2−/− or normal spleen cell populations (data not

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shown). IgG levels tested on day 14, however, were significantly decreased in mice that carried Cr2−/− B cells (Fig. 5A), corresponding to approximately 20% of the responses in mice reconstituted with wild-type splenocytes ($P = 0.02$). Moreover, unlike in the Cr2−/− mice, these IgG responses decreased to undetectable levels by days 21–28 (Figs. 5B–C), suggesting that the transferred B cells, which were activated by PyV and differentiated into IgG-secreting plasma cells, were short-lived. Indeed, an increased percentage of GC phenotype B cells (CD43−/B220+/CD19+/Gl7+) isolated from the spleens of PyV-infected CR2-deficient mice stained positive with annexin V (74% on day 3 and 73–75% on day 14), an early marker of apoptotic cells death, compared to GC B cells from PyV-infected wild-type mice (59–64% on day 3 and 59–68% on day 14, respectively).

TI responses to PyV are impaired in the absence of CR2

PyV is an effective inducer of TI IgG responses in T cell-deficient mice. The antibodies generated in the absence of T cell help have neutralizing activity and are able to clear the virus partially (Szomolanyi-Tsuda and Welsh, 1996, and EST unpublished results), thus protecting T cell-deficient mice from the acute, lethal effect of PyV infection observed in SCID mice (Szomolanyi-Tsuda et al., 1994). A major goal of these studies with CR2-deficient mice was to determine whether TI IgG responses are enhanced by CR2 signaling in PyV-infected hosts. To answer this question, we transferred T cell-depleted spleen cells from uninfected Cr2−/− mice or wild-type mice into SCID mouse recipients, and following PyV infection we evaluated antiviral IgM and IgG responses of the reconstituted mice. Although IgM levels on day 5 post-infection of the mice with CR2-deficient B cells were on average lower than those of their wild-type counterparts, these differences were not statistically significant (Fig. 6A). Days 21 and 28 IgG responses of the mice with T cell-depleted CR2 KO splenocytes were significantly diminished, on day 21 approximately 20% ($P < 0.04$), and on day 28 about 13% ($P = 0.01$) compared to wild-type (Figs. 6B and C). CR2 KO mice depleted in vivo of CD4 T cells by treatment with GK1.5 monoclonal antibody also showed decreased IgG responses to PyV compared to CD4-depleted wild-type mice in day 16 post-infection (Fig. 6D), suggesting that the lack of CR2 has an influence on TI antibody responses. It is important to consider, however, that the CD4-depleted mice had CD8 T cells that could effectively reduce viral antigen levels (Byers et al., 2003), and CD4-negative T cells might have also affected B cell responses (Sha and Compans, 2000); whereas using SCID mice reconstituted with T cell-depleted spleen cells, we studied B cell responses without the influence of any T cell. Our conclusion from these experiments taken together is that for normal TI IgG responses CR2 seem to be required, supporting the hypothesis that innate immune-derived signals are involved in the generation of these responses.

Discussion

We found that both TI and TD IgG responses to PyV are impaired in mice deficient in CR2 expression. Previously, it was demonstrated with inert protein antigens that at low antigen doses TD antibody responses depend largely on CR2 signaling (Ahearn et al., 1996; Heyman et al., 1990; Molina et al., 1996; Ochsenbein et al., 1999). For example, linkage of 2–3 copies of C3d to hen egg lysozyme, a TD antigen, enhanced the immunogenicity of this

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antigen 10^3- to 10^4-fold (Dempsey et al., 1996). Complement proteins are important players of the innate immune system, and the activation of the complement cascade is initiated by many pathogens, including viruses (Cooper et al., 1976). As a result of complement activation, C3d complement fragments are covalently attached to the antigen, perhaps "marking" its infectious origin. This modification may serve to enhance the ability of viral antigens, particularly when present at low amounts, to generate vigorous humoral immune responses, demonstrating the collaboration of innate and adaptive immunity to orchestrate the fight against invading pathogens. Reports showing the CR2 dependence of IgG responses when mice are immunized with non-replicating (inactivated or mutant) viruses, or viruses with limited replicative potential in the periphery, but not in live systemic virus infections support this hypothesis (Da Costa et al., 1999; Ochsenbein et al., 1999). PyV, however, replicates rapidly and extensively in mice. Thus, our findings suggest that CR2 may have a positive effect on TD responses even when the antigen is not limiting but abundant.

The antiviral IgG responses in CR2-deficient mice were only slightly reduced compared to the responses in wild type, and the difference was the same from day 14 and thereafter (Fig. 1). In adoptive transfer experiments, however, when the responses of transferred splenocytes were tested in the absence of replenishment of naive B cells from the bone marrow, the IgG levels rapidly fell below the level of detection (Fig. 5). This finding reveals a more profound defect of antiviral antibody responses, which may be masked in persistently PyV-infected mice by continuous activation of newly emerging B cells from the bone marrow.

In mice CR2 is expressed on B cells and on FDC, and two hypothetical mechanisms for the enhancement of humoral immune responses by C3d–CR2 interactions have been postulated. First, antigen-bound complement may act directly on B cells by binding to the CD21/CD19/CD81 BCR co-receptor complex (Carter and Fearon, 1992). In this case, simultaneous co-ligation of the BCR and CR2 is thought to deliver a strong positive signal for B cells promoting the secretion of antibodies. Alternatively, entrapment of antigen–antibody–complement complexes by CR2 expressed on FDC may ensure long-term antigen retention thus sustaining antibody responses. Experimental observations support both of these models (Carter and Fearon, 1992; Croix et al., 1996; Fischer et al., 1998; Qin et al., 1998). Our experiments did not exclude the possibility that CR2 expressed on FDC is playing a role in the enhancement of TI and TD IgG responses to PyV, but the adoptive transfer experiments make it likely that CR2 signaling on B cells is mostly responsible for this effect. In SCID mice reconstituted with CR2 KO spleen cells, the host can generate FDC (Pihlgren et al., 2003) that express wild-type CR2, although the B lymphocytes derived from the donor are CR2-deficient, and these mice show seriously impaired VP1-specific IgG responses.

Consistent with previous observations, the effect of complement–CR2 interactions on TD B cell responses may be, at least partially, caused by survival signals mediated by CR2 during the germinal center reaction (Chen et al., 2000; Fischer et al., 1998). This idea is supported by the increased fraction of annexin-V-positive germinal center B cells undergoing apoptosis in CR2-deficient mice compared to wild-type, and also by the rapidly declining IgG responses in SCID mice reconstituted with CR2-deficient splenocytes. Provision of survival signals is a major way of control in the immune system. CR2 signaling may

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function in this manner so that responses to non-infectious antigens that do not activate the complement system are not strong and sustained.

We found that TI antibody responses to PyV infection were also negatively affected in Cr2−/− mice (Fig. 6). Strong signals delivered to the BCR are thought to be very important in the induction of TI antibodies. In fact, B cell epitopes on TI type 2 antigens are usually arranged in a highly repetitive manner so that they can crosslink the BCR, which is presumed to be critical for the induction of antibody responses in the absence of T cell help. PyV is TI type 2 antigen by the definition that Xid B cells do not respond to it in the absence of T cells (Szomolanyi-Tsuda et al., 2001). Although PyV, similar to other TI type 2 antigens, highly repetitively organized, this repetitiveness does not entirely account for its ability to induce TI IgG responses. We have previously reported that virus-like particles assembled in baculovirus from recombinant VP1 capsid proteins cannot elicit TI IgG responses, only live PyV can (Szomolanyi-Tsuda et al., 1998). Thus, other signals induced by the live virus infection seem to be essential for the generation of isotype-switched antibody responses in T cell-deficient host. This study identifies CR2–C3d interaction as one of the innate signals enhancing TI IgG secretion to live virus infection.

Impaired antibody responses to group B streptococcal capsular polysaccharide in CR2-deficient mice were reported. These TI type 2 responses were mainly generated in the marginal zone area of the spleen, where the marginal sinuses delivered blood borne pathogens, and where MZ B cells, dendritic cells, and MZ macrophages were localized. One of the distinguishing features of the MZ B cells that produce antibodies to bacterial polysaccharides is their high expression of CR2. It has been shown that defective complement-mediated uptake and localization of the capsular bacterial polysaccharides to the MZ B cells is mainly responsible for the reduced antibody responses in CR2-deficient mice (Pozdnyakova et al., 2003). The TI antibody responses to PyV, however, are not generated predominantly by MZ B cells (Heath Guay, unpublished); therefore, the same scenario is not envisioned for VP1-specific TI antibody responses.

In our studies with PyV-infected CR2 KO mice or SCID mice reconstituted with CR2-deficient spleen cells, we found decreased IgG responses in the absence of CR2, but the antiviral IgM responses were not significantly affected, although in some experiments on average they were slightly lower in mice that had CR2 deficiency compared to controls. This finding is in contrast to the report on VSV infection by Ochsenbein et al. (1999) showing diminished TI IgM responses but normal IgG responses in C3−/− mice. Thus, the effect of CR2 deficiency seems to be quite different in various viral models. The factors that may be influencing the role of CR2-mediated mechanisms in antiviral antibody responses include the route of infection and the ability of the virus to replicate, both affecting local antigen dose. In addition, the starting germline affinity of BCR to viral B cell epitopes may also have some effect on the CR2 dependence of the B cell responses. Experiments with transgenic B cells on CR2 KO or wild-type background transferred into mice immunized with ligands with different affinities for the transgenic BCR demonstrated that CR2-mediated signals were more important for survival of B cells in the splenic follicles when the BCR had low affinity for the immunogen (Barrington et al., 2001).

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CD21 is part of a complex formed with CD19 and CD81. CD21 has a short intracellular domain and is thought to be incapable of transducing signals on it own. Thus, when CD21 is engaged, its ligand signals are transduced by CD19. Mice deficient in CD19, however, seem to have a more profound defect in antibody responses than CR2-deficient mice. Whereas CR2 KO mice have normal serum immunoglobulin levels, serum IgM is approximately 75%, and serum IgG1 is about 90% reduced in CD19 KO mice (Ahearn et al., 1996; Engel et al., 1995; Molina et al., 1996; Rickert et al., 1995). Moreover, VSV-infected C3−/−, C4−/−, and Cr2−/− mice have long-lasting virus-specific neutralizing IgG responses and maintain high frequencies of antibody-secreting cells in the spleen and bone marrow, but in CD19 KO mice the antiviral antibody titers in the serum and the number of antibody-secreting cells greatly decline with time, these mice have impaired B cell memory (Fehr et al., 1998; Ochsenbein et al., 1999). These observations all suggest that CD19 may also function in association with other receptors and may respond to other ligands that influence humoral immune responses, in addition to C3d–CD21. In fact, recently it was reported that CD19 is associated with RP105, a member of the TLR family expressed on B lymphocytes, forming the signaling component of the complex. So far, LPS is the only known ligand that activates the RP105/CD19 pathway.

The unique in vivo model of PyV infection in mice allowed us to study the effect of innate immune signals on TD and on TI antiviral IgM and IgG responses. We found that in the absence of CD21/CD35 in Cr2−/− mice the antiviral IgG responses were reduced, and in mice lacking newly recruited B cells, these responses were severely compromised. In addition, TI IgG responses to PyV generated in the absence of T cells were also greatly reduced; thus, CR2-mediated pathways can make important contribution to both TD and TI antiviral humoral immunity in this mouse model of natural virus infection.

Materials and methods

Mice, virus, infections

B6x129/CR2 KO mice and wild-type control B6X129 mice were originally obtained from the Center for Blood Research, Harvard University, Boston, MA. These mice, as well as B6/SCID mice, were bred and kept until infection in the Department of Animal Medicine at the UMMS under specific pathogen-free conditions. For the experiments 8–20 weeks old, age- and sex-matched groups of mice were used. Mice were infected intraperitoneally (ip) with 2×10^6 plaque forming units (pfu) of PyV strain A2, originally provided by Dr. Michelle Fluck (University of Michigan, Ann Arbor), propagated on NIH 3T3 cells, and quantified by plaque assays on UC1b cells as described (Staneloni et al., 1977).

Adoptive transfer experiments, in vitro and in vivo T cell depletion

Adoptive transfer of B cell-containing spleen cell populations from CR2−/− or wild-type mice into SCID mice was performed as described previously (Szomolanyi-Tsuda and Welsh, 1996). Briefly, spleens were homogenized between glass frosted microscope slides, and the resulting spleen cell suspensions were treated with 0.83% ammonium chloride to lyse erythrocytes. The resulting cells were washed and counted. In some experiments, T cells were depleted by negative selection on anti-mouse Thy1.2 AutoMacs separator

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columns (Miltenyi Biotech, Auburn, CA) following protocols given by the manufacturer. Aliquots of the cell suspensions were stained with antibodies specific for CD4, CD8, and CD19 and analyzed by FACS to determine the completeness of T cell depletion and the percentage of B cells. Forty million spleen cells of either wild-type or CR2 KO mice were given intravenously to SCID mice by tail vein injection, and the mice were infected 1 day later intraperitoneally with PyV. In the experiments transferring T cell-depleted spleen cells, $2–3 \times 10^7$ cells per mouse were given. CD4+ cells were depleted in vivo by ip injection with 0.5 mg GK1.5 monoclonal antibody given 1 day before and on days 9 and 14 post-infection. The depletion was confirmed by FACS analysis.

VP1-specific ELISA assays

Recombinant VP1 capsid protein (50 ng/well) that was produced in *Escherichia coli* (Leavitt et al., 1985) was used to coat 96-well plates, and the VP1-specific ELISA assays were done as previously described (Szomolanyi-Tsuda et al., 1998).

VP1-specific ELISPOT assays

A modification of the method described for detecting influenza virus-specific ASC (Guay et al., 2004) was used. Multiscreen HA plates (Millipore) were coated with 50 ng/well VP1 overnight at 4 °C and blocked the next day for 30 min at 37 °C with RPMI/10% FBS. Cells were plated in duplicate in 0.2 ml at 1×10^6, 2.5×10^5, 6.25×10^4, and 1.25×10^4 cells/well and incubated for 4 h at 37 °C. Bound antibody was detected using 100 μl biotin-conjugated goat antibodies specific for mouse IgG (Vector Laboratories) and Streptavidin-conjugated HRP (Vector Laboratories). Plates were developed with AEC substrate (Sigma) according to the manufacturer's protocol. Spots were counted using a dissection microscope.

FACS staining

Staining of spleen cell populations and FACS analysis was performed following standard protocols. Anti-CD4-FITC and anti-CD8-PE (BD Pharmingen) were used to test the efficiency of T cell depletions. The CD19, GL7, and annexin V-specific antibodies were all purchased from BD Pharmingen.

Real-time PCR assays to measure viral DNA in organs

Real-time PCR assay quantifying PyVA2 VP1 coding region and mouse genomic β actin sequence-specific DNA was used to measure virus load. The DNA samples used in the real-time PCR assays were prepared from mouse organs as previously described (Szomolanyi-Tsuda and Welsh, 1996) and predigested with *Bam*HI for 1 h at 37 °C to fragment the cellular DNA. *Bam*HI cuts the PyV genome once, linearizing it. PCR amplification was carried out in 50-μl reactions consisting of 50 mM Tris pH 8.0, 0.5 μg/ml BSA, 3 mM $MgCl_2$, 0.25 mM of each deoxynucleoside triphosphate, 0.5 U of Promega Taq Polymerase, 0.66 U SYBR-Green I (Molecular Probes), 0.1 mM forward primer, 0.1 mM reverse primer (Invitrogen, Carlsbad, CA), 5 nM Fluorescein (Bio-Rad, Hercules, CA), and 1 μg of appropriate DNA sample. The following primers were used: β actin forward primer: CGA GGC CCA GAG CAA GAG AG; β actin reverse primer: CGG TTG GCC TTA GGG TTC AG; PyV forward primer: CCC CCG GTA CAG GTT TCA GTC CCA TCAT; PyV reverse

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primer: GGC ACA ACA GCT CCA CCC GTC CTG CAG. PCR amplification using the PyV primers was carried out starting with one cycle at 95 °C for 3 min, 37 cycles of 95 °C for 30 s, 65 °C for 20 s, 72 °C for 45 s, with a measurement of fluorescence taken at 72 °C at the end of each cycle. A melting curve was generated after the completion of the 37 cycles. PCR amplification using the β actin primers was performed, starting with one cycle at 95 °C for 150 s, followed by 40 cycles of 95 °C for 30 s, 62 °C for 25 s, 72 °C for 25 s with a measurement of fluorescence taken at 72 °C at the end of each cycle, a melting curve was then established as described above for the PyV amplification. Negative controls included reaction with no DNA substrate, for the PyV amplification also DNA from uninfected mouse tissues. Twofold serial dilutions from 1 μg mouse DNA to 31 ng (from uninfected mouse) were used to generate a standard curve for the β actin amplifications. To determine PyV copy numbers, we used a recombinant plasmid containing PyV VP1 coding sequences in serial dilutions from 2×10^8 copies to 20 copies, and we mixed each of these standard plasmid dilutions with 1 μg of DNA from uninfected mouse tissues. All samples were run in duplicates. The obtained PyV copy number data were normalized for the amount of genomic DNA substrate in the reaction determined by the β actin gene-specific reaction. The results were first expressed as PyV copies/μg DNA. This value was then converted to PyV copies/cell. Assuming the DNA content of 2×10^5 mammalian cells to be 1 μg DNA, 100 copies of PyV/μg DNA (the approximate limit of detection) are equivalent to 0.0005 copy/cell or 1 copy of PyV genome in 2000 cells. The data were used for analysis only if the melting curve of the amplification product was characteristic for the PyV fragment (or the β actin sequence when β actin-specific primers were used).

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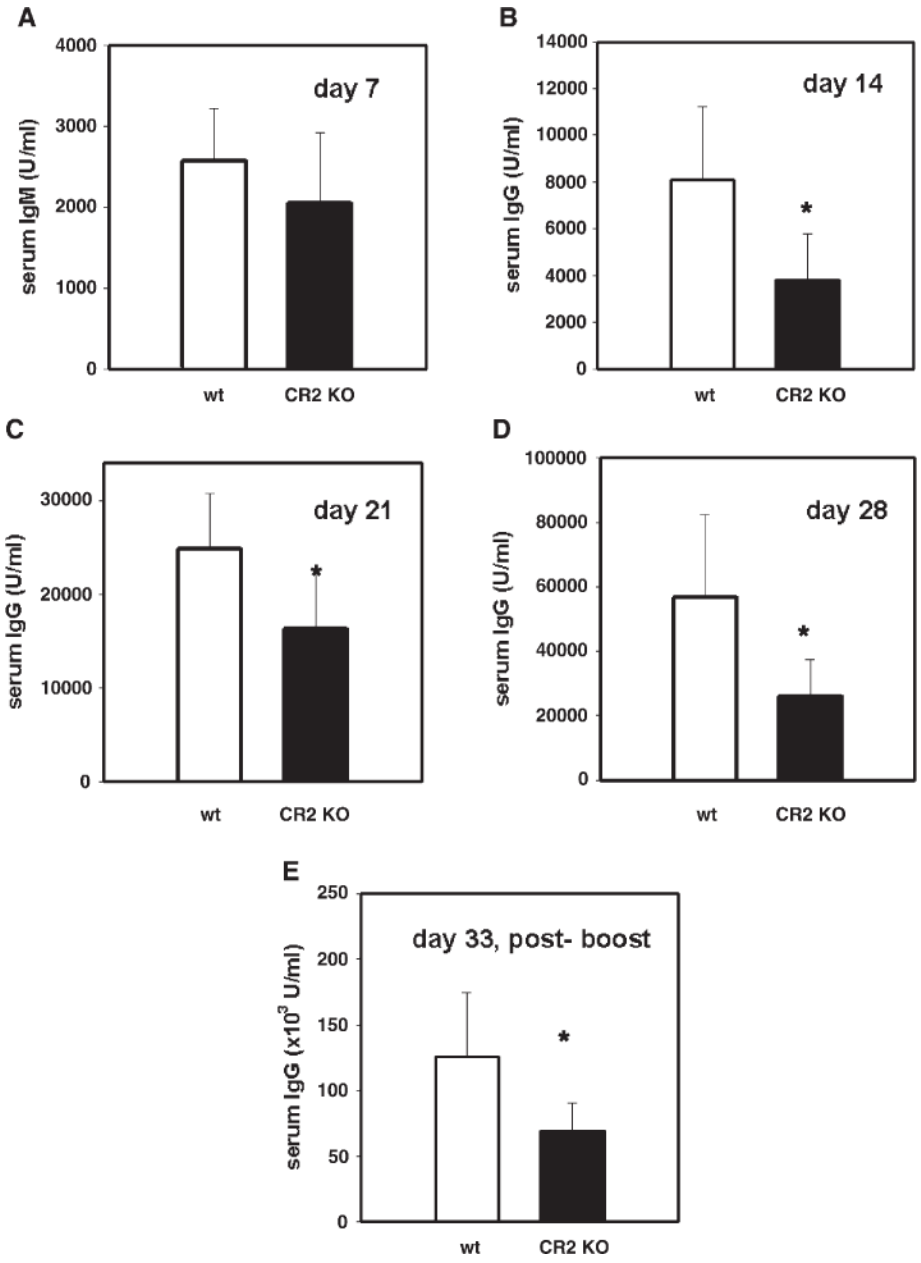

Fig. 1.

Virus-specific antibody responses of PyV-infected wild-type and CR2 KO mice. (A) VP1-specific IgM concentrations in serum samples taken from wild-type and CR2 KO mice on day 7 post-infection, expressed in arbitrary U/ml. Six mice per group were tested. (B) VP1-specific IgG responses on day 14 ($P < 0.02$), (C) on day 21 ($P = 0.03$), (D) on day 28 ($P = 0.02$), and (E) on day 33, 5 days after re-infection on day 28 ($P = 0.025$); $n = 6$ per group. The asterisks indicate statistically significant differences between the groups, the error bars show standard deviations. The experiment shown is a representative of three similar experiments.

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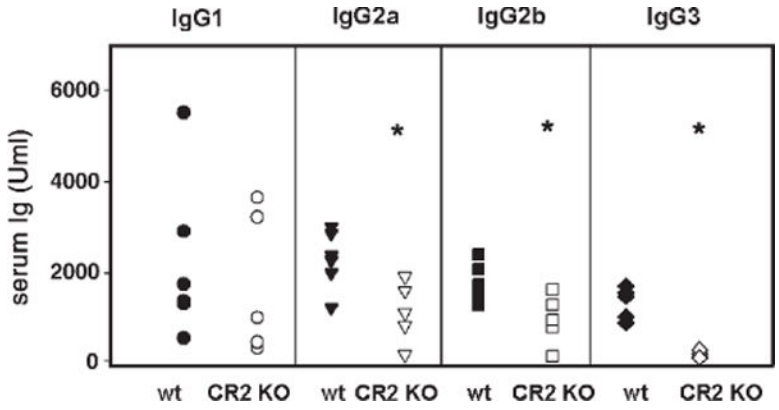

Fig. 2.

IgG isotypes of VP1-specific serum antibodies of wild-type and Cr2−/− mice. The isotype-specific ELISA assays were done on serum samples obtained on day 21 following PyV infection. The IgG1, IgG2a, IgG2b, and IgG3 concentrations are expressed in arbitrary units. The differences in IgG2a, IgG2b, and IgG3 are statistically significant ($P = 0.017$, $P = 0.02$, and $P = 0.0004$ for IgG2a, IgG2b, and IgG3, respectively); $n = 5–6$ per group.

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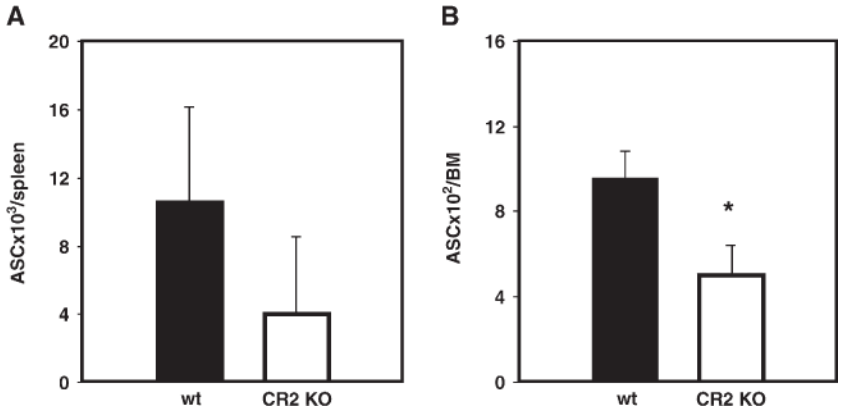

Fig. 3.

ASC numbers in the spleen (A) and in the bone marrow (B) of wild-type and CR2 KO mice on day 20 post-infection. The asterisk indicates statistically significant differences between the groups, the error bars show standard deviation, 3–4 mice per group were tested.

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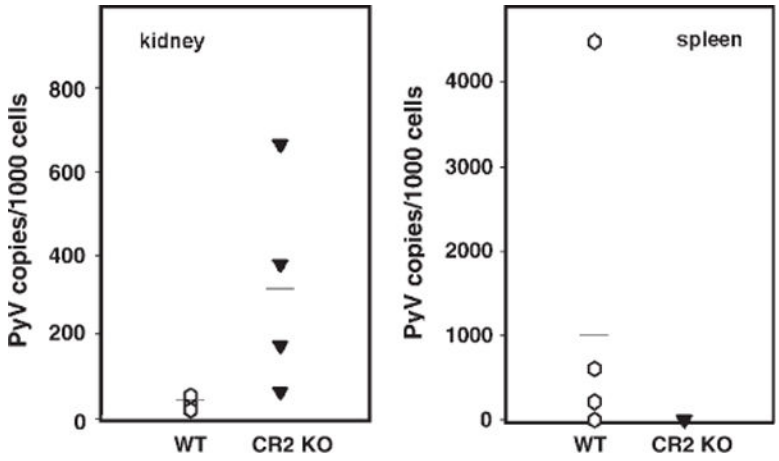

Fig. 4.

Viral load in organs of wild-type and CR2-deficient mice on day 28 post-infection, expressed as viral copy number/1000 cells, determined by real-time PCR. Four mice per group were tested.

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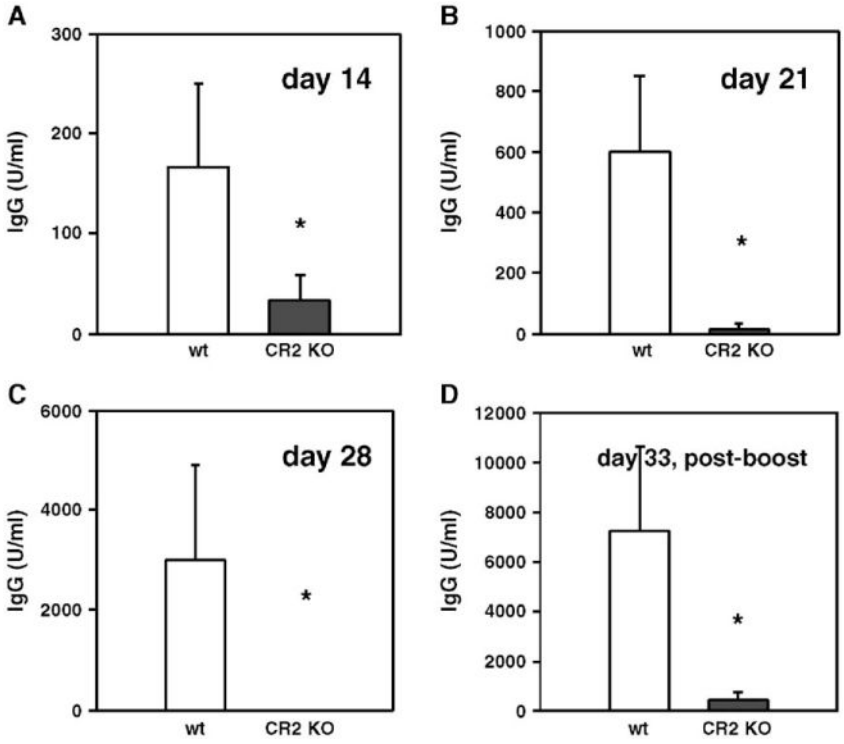

Fig. 5.

VP1-specific IgG titers in serum samples of SCID mice reconstituted with spleen cells of Cr2−/− and wild-type mice. (A) Day 14 ($P = 0.02$), (B) day 21 ($P = 0.003$), (C) day 28 ($P = 0.018$), and (D) day 33 ($P = 0.007$), 5 days following re-infection with PyV. Asterisks indicate statistical significance, the error bars show standard deviations. The experiment is a representative of three similar experiments; $n = 4$ per group.

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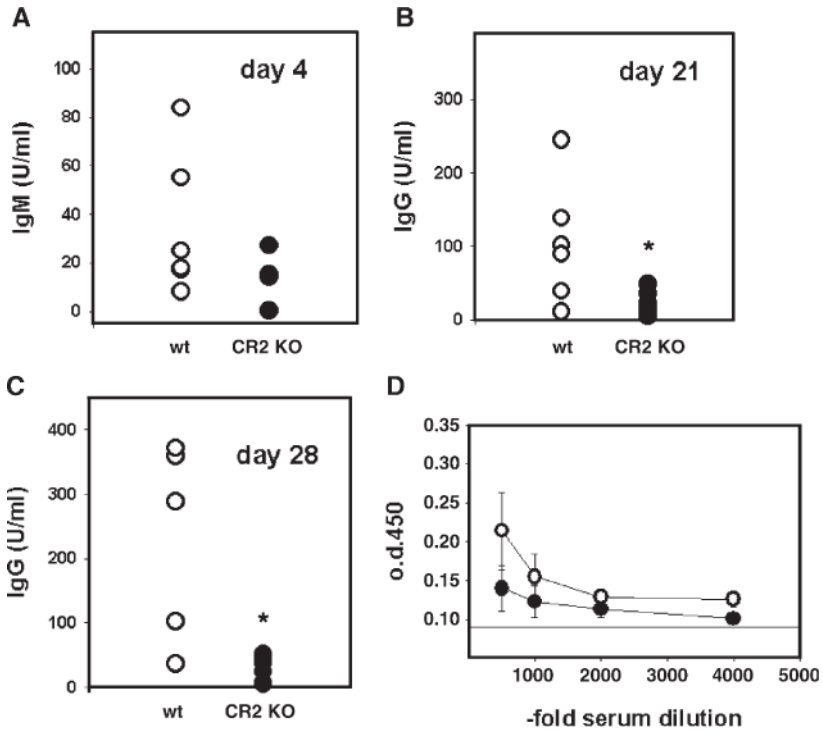

Fig. 6.

VP1-specific IgM and IgG titers in serum samples of SCID mice reconstituted with T cell-depleted spleen cells of Cr2−/− and wild-type mice. (A) Day 5 IgM responses ($P = 0.077$), (B) day 21 serum IgG ($P < 0.04$), and (C) day 28 IgG ($P = 0.01$). The asterisks indicate statistical significance. The experiment is a representative of three similar experiments; $n = 6$ per group. (D) VP1-specific serum IgG of PyV-infected, CD4 T cell-depleted CR2 KO (closed circles) and wild-type (open circles) mice on day 16 post-infection. Three to four mice per group were tested, $P = 0.058$ for 1:100 dilution.