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The Role of CD40-CD154 Interaction in Antiviral T Cell-Independent IgG Responses

Eva Szomolanyi-Tsuda, James D. Brien, Jill E. Dorgan, Raymond M. Welsh, and Robert L. Garcea

Polyomavirus (PyV) infection elicits protective T cell-independent (TI) IgG responses in T cell-deficient mice. The question addressed in this report is whether CD40 signaling plays a role in this TI antiviral IgG response. Because CD40 ligand (CD40L) can be expressed on numerous cell types in addition to activated T cells, it is possible that cells other than T cells provide CD40L to signal through CD40 on B cells and hence positively influence the antiviral TI IgG responses. In this study, we show, by blocking CD40-CD40L interactions in vivo with anti-CD40L Ab treatment in TCR \( \beta \times \delta \)−/− mice and by using SCID mice reconstituted with CD40−/− B cells, that the lack of CD40 signaling in B cells results in a 50% decrease in TI IgG secreted in response to PyV. SCID mice reconstituted with CD40L−/− B cells also responded to PyV infection with diminished IgG secretion compared with that of SCID mice reconstituted with wild-type B cells. This finding suggests that B cells may provide the CD40L for CD40 signaling in the absence of T cell help during acute virus infection. Our studies demonstrate that, although about half of the TI IgG responses to PyV are independent of CD40-CD40L interactions, these interactions occur in T cell-deficient mice and enhance antiviral TI Ab responses. *The Journal of Immunology*, 2000, 164: 5871–5877.

Recently we reported that polyomavirus (PyV) infection elicits protective TI IgM and IgG responses in T cell-deficient mice (9, 10). In contrast to infection with PyV, immunization with the viral capsid protein 1 (VP1) or with virus-like particles assembled from VP1 into highly organized, repetitive structures almost identical with those of the intact virions does not lead to TI IgG synthesis (11). These findings suggest that signals generated by live virus infection, such as the secretion of cytokines and the induction of membrane determinants on certain cell types, may be essential for generating efficient isotype-switched humoral responses in the absence of T cells.

The studies reported here were initiated to test whether CD40-CD40L interaction plays any role in the antiviral TI IgG responses to PyV. Although it was long believed that CD40L was expressed only on activated CD4+ Th cells, CD40L recently has been detected on the surface of CD8+ T cells, NK cells, mast cells, basophils, eosinophils, dendritic cells, and human B cells (12–20). Therefore, CD40L expressed on cells other than T cells may provide costimulatory signals that have a positive effect on TI Ab responses to viruses. In this report, we show by blocking CD40-CD40L interactions in vivo with anti-CD40L Ab treatment in TCR \( \beta \times \delta \)−/− mice and by using SCID mice reconstituted with CD40−/− B cells that the lack of CD40 signaling in B cells results in a decrease in the TI IgG secreted in response to PyV. Our data also suggest that B cells themselves may provide functional CD40L for this interaction, because SCID mice reconstituted with CD40L−/− B cells also responded to PyV infection with diminished IgG secretion.

**Materials and Methods**

**Mice and virus infections**

C57BL/6 and C57BL/CD40L−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/SCID and C57BL/TCR \( \beta \times \delta \)−/− mice were bred and housed under specific pathogen-free conditions in the Department of Animal Medicine of the University of Massachusetts Medical School. C57BL/CD40−/− mice were kindly provided by Dr. Hitoshi Kikutani (Osaka University, Osaka, Japan). Age-matched groups of 6- to 19-wk-old mice were used for the experiments. The mice were infected i.p.
with $2 \times 10^7$ PFU PyV strain A2, which was originally a gift from Dr. Michel Fluck (Michigan State University, East Lansing, MI).

In vivo CD40L blocking

The mAb MR1 (21) (produced as ascites and kindly provided by Dr. Tom Markes, University of Massachusetts Medical School, Worcester, MA) was used to block CD40L in vivo. Mice were injected i.p. with 0.25 mg of MR1 on days 1, 2, 6, and 9 and were infected with PyV on day 0.

Adoptive transfers

Adoptive transfer of B cell-containing splenocyte populations of wild-type and mutant (CD40$^{-/-}$ and CD40L$^{-/-}$) mice into C57BL/SCID mice was performed as described previously (9). Briefly, spleen cell suspensions were obtained by homogenizing spleens between frosted glass microscope slides, and the erythrocytes were lysed by treatment with 0.83% ammonium chloride. After in vitro T cell depletion (see below), the cells were pooled, and the number of viable cells was counted. Aliquots of the T cell-depleted splenocytes were stained with anti-B220-peridinin chlorophyl protein and anti-CD19-FITC or with anti-CD4-FITC and anti-CD8-PE (PharMingen, San Diego, CA) and were analyzed by FACS to determine the number of B cells and to test the quality of T cell depletion (see next paragraph). Spleens of C57BL/6 and CD40L$^{-/-}$ mice were obtained by homogenizing spleens between frosted glass microscope slides, and the erythrocytes were lysed by treatment with 0.83% ammonium chloride. After in vitro T cell depletion (see below), the cells were pooled, and the number of viable cells was counted. Aliquots of the T cell-depleted splenocytes were stained with anti-B220-peridinin chlorophyl protein and anti-CD19-FITC or with anti-CD4-FITC and anti-CD8-PE (PharMingen, San Diego, CA) and were analyzed by FACS to determine the number of B cells and to test the quality of T cell depletion (see next paragraph).

In vitro T cell depletions

Spleen cell suspensions were incubated with rat anti-mouse Thy1.2 Ab (PharMingen) for 45 min on ice, washed with RPMI 1640 containing 10% FCS, and then incubated with a 1:10 dilution of rabbit complement (Pel-Freeze Clinical Systems, Brown Deer, WI) at 37°C for 45 min. The cell suspensions were washed repeatedly with media, subjected to one more round of Thy1.2 depletion, counted with trypan blue to obtain the viable cell count, suspended in PBS, and used in adoptive transfer experiments. The efficiency of T cell depletion was tested by FACS analysis employing anti-CD4-FITC and anti-CD8-PE Abs (PharMingen), and the samples were found to be 99% free of CD4$^+$ and CD8$^+$ T cells.

ELISA assays

Virus Ag-specific ELISA assays were done using purified VP1 PyV capsid Ag (50 ng/well) that was produced by recombinant baculovirus expression vectors in Sf9 insect cells and purified as described (22). The serum samples were tested in duplicates using biotinylated goat anti-mouse IgM or IgG and streptavidin-HRP (Vector Laboratories, Burlingame, CA) to detect IgM or IgG, respectively. A Southern Biotechnology Associates (Birmingham, AL) isotyping kit was used to measure IgG isotypes.

Results

Ab responses to PyV in CD40$^{-/-}$ mice

Ab responses to PyV infection were tested in mice that had a targeted mutation in the gene encoding CD40 (CD40$^{-/-}$ mice). Early IgM responses specific for the PyV major capsid Ag VP1 were observed in CD40$^{-/-}$ mice and were not significantly different from those seen in C57BL/6 and TCR $\beta$×$\delta$$^{-/-}$ mice (Fig. 1A). On day 14 postinfection, PyV-specific IgG was detectable in serum samples obtained from CD40$^{-/-}$ mice (Fig. 1, B and C). Comparison of the magnitude of this IgG response indicated that the absence of CD40 signaling led to a 10-fold decrease in virus-specific IgG titers, and this virus-specific IgG titer was approximately the same magnitude as that detected in TCR $\beta$×$\delta$$^{-/-}$ mice (Fig. 1C). Thus, about 10% of the antiviral IgG synthesis is independent of CD40 signaling in PyV-infected mice.

FIGURE 1. PyV-specific Ab responses in CD40$^{-/-}$ mice. A, VP1-specific IgM on day 5 postinfection in sera of CD40$^{-/-}$ (○; n = 3), TCR $\beta$×$\delta$$^{-/-}$ (●; n = 4), and C57BL/6 (▲; n = 3) mice, determined by ELISA assays. Mean OD ± SE shown; the OD values obtained in the assay with uninfected mouse serum were below 0.09. C57BL/6 (▶; n = 3) mice on day 14 postinfection. IgG titers are expressed as the reciprocal of the highest serum dilutions that give an OD $> 0.1$ in the ELISA assay.

Blocking of CD40L in TCR $\beta$×$\delta$ decreases antiviral TI IgG responses

Mice lacking T cells respond to PyV infection with the generation of protective IgM and IgG Abs (10). Assuming that Th cells are the only cell type expressing significant levels of CD40L, the TI Ab...
response should be completely independent of CD40 signaling. However, recent findings that CD40L can be expressed on cells other than T cells suggest the possibility that CD40L expressed on non-T cells might enhance TI Ab responses by interacting with CD40 on B cells.

The first approach to test whether CD40L signaling has an influence on the TI IgG responses to PyV was to block in vivo CD40-CD40L interactions in TCR \(\beta\times\delta^{-/-}\) mice using the CD40L-specific mAb MR1. This Ab was administered to TCR \(\beta\times\delta^{-/-}\) mice following the protocol and dosage previously shown to successfully inhibit CD40-CD40L interactions in immunocompetent C57BL/6 mice (see Materials and Methods and Ref. 23). Serum samples taken on day 14 after PyV infection from T cell-deficient anti-CD40L Ab-treated mice consistently had 2-fold lower PyV-specific IgG levels than TCR \(\beta\times\delta^{-/-}\) mice that did not receive treatment to block CD40L (Fig. 2, A and B). Treatment of C57BL/6 mice with the anti-CD40L Ab resulted in the expected decrease in VP1-specific IgG, indicating the efficient blocking of CD40L achieved by the in vivo administration of the MR1 Ab in this experiment (Fig. 2A).

On day 14 after PyV infection, TCR \(\beta\times\delta^{-/-}\) mice treated with anti-CD40L Ab, similarly to the TCR \(\beta\times\delta\) mice that received no such treatment, showed no symptoms of PyV-induced disease. DNA samples prepared from kidneys of PyV-infected TCR \(\beta\times\delta^{-/-}\) mice treated or untreated with anti-CD40L Abs were analyzed by Southern blot to test whether the diminished IgG responses in the former group were associated with differences of viral clearance. In these experiments no viral DNA was detected in the kidney DNA samples of either group of mice, whereas DNA from SCID mice kidneys had a strong PyV-specific band (data not shown). Thus, the TI Ab responses generated in the absence of CD40-CD40L interactions were sufficient for protection and viral clearance.

TI IgG responses to PyV are predominantly IgG2a and IgG2b with a variable amount of IgG3 also synthesized, whereas the IgG1 response is strictly TD (11). It is of interest that anti-CD40L Ab treatment of TCR \(\beta\times\delta^{-/-}\) mice did not lead to significant changes of this isotype composition.

**CD40^{-/-} B cells respond to PyV infection with diminished TI IgG synthesis in vivo**

The other approach to test whether CD40 signaling in B cells facilitates virus-specific TI IgG responses was to ask how a CD40^{-/-} B cell population will respond in the absence of T cells to PyV in vivo, compared with the response of wild-type B cells. To address this question, we performed adoptive transfer experiments. Spleen cell suspensions prepared from CD40^{-/-} or C57BL/6 mice, respectively, were depleted of T cells in vitro by treatment with anti-Thy1 Ab and complement. These splenocyte populations devoid of T cells but containing B cells were then transferred into SCID mice, and the reconstituted mice were infected with PyV on the day after the cell transfer. Virus-specific serum IgG levels were lower in mice that received CD40^{-/-} Thy-depleted spleen cells than in mice reconstituted with wild-type cells at all three time points tested: on day 14 (data not shown), day 21 (Fig. 3A), and day 37 (Fig. 3B) postinfection. These results confirmed the findings obtained in the in vivo CD40L blocking experiments, suggesting that CD40 signaling in B cells may enhance TI IgG responses to PyV. In addition, these experiments raised an important question: in the absence of T cells, which cell types provide CD40L for stimulating CD40 on B cells?

**The lack of CD40L expression on B cells results in a decrease in TI IgG responses to PyV**

We next tested whether B lymphocytes could stimulate each other by expressing CD40L in the absence of T cell help. Spleen cells from CD40L^{-/-} mice were prepared and subjected to T cell depletion in vitro. The resulting cell suspension was transferred into B6/SCID mice, generating T cell-deficient mice with intact CD40L gene in all cell types except in B cells. These mice, as well as SCID mice reconstituted with T cell-depleted splenocytes from wild-type (CD40L^{-/-/-}) mice, were infected with PyV, and their virus-specific Ab responses were measured. Remarkably, SCID mice reconstituted with CD40L^{-/-} B cells had significantly lower VP1-specific IgG levels in their sera on day 21 (Fig. 4) as well as on day 56 postinfection (data not shown) than did SCID mice that had received wild-type B cells. This difference in IgG production was not due to a generalized defect of CD40L^{-/-} B cell function or to a lack of survival of CD40L^{-/-} B cells during adoptive transfer, because serum levels of virus-specific IgM tested on day...
5 were the same or higher in SCID mice with CD40L
2
2
B cells than they were in the control group of SCID mice reconstituted
with wild-type B cells (Fig. 4 A). The presence of functional Th
cells in the reconstituted mice was also excluded because their
serum samples lacked detectable VP1-specific IgG1 (day 21 serum
tests; tested in 1:100 dilutions), and this isotype is strictly TD (6, 11).
Importantly, our results showing an ~50% drop in antiviral IgG responses in SCID mice reconstituted with CD40L
2
B cells, respectively, compared with the ones with wild-type B cells suggested that B cells may provide functional CD40L
to each other, thus enhancing the antiviral TI IgG responses.

Discussion
In this paper we provide evidence that CD40-CD40L interactions
contribute to the efficiency of antiviral TI IgG responses. This
conclusion is based on two sets of experiments. First, we showed
that blocking CD40L in vivo by treatment with the MR1 mAb led to a 2-fold decrease in the level of PyV-specific IgG synthesized.

Then we demonstrated that SCID mice reconstituted with a
CD40L
2
B cell population mounted a significantly lower antiviral
IgG response compared with the responses measured in mice re-
constituted with wild-type B cells. Furthermore, because SCID
mice reconstituted with CD40L
2
B cells have a similar decrease
in PyV-specific IgG responses, our data suggest that the CD40L
participating in this interaction may be expressed on B cells.

The immune system evolved to provide protection against in-
vading pathogens, and it reacts differently to infectious viruses
than to inert Ags. Inert proteins are TD Ags, and the generation of
isotype-switched Abs in response to these Ags requires T cell help
(24). Accordingly, immunization with the PyV VP1 capsid protein
or with its repetitively structured form assembled into virus-like
particles does not induce VP1-specific IgG secretion in the absence
of T cell help in contrast, infection with PyV elicits protective TI
IgG responses in T cell-deficient mice (11). Because infection with
live viruses activates several cell types and induces numerous cy-
tokines produced by cells other than T cells, these signals acting on
B cells may enable them to secrete isotype-switched TI Abs.
The virus-induced signals and pathways involved in TI IgG synthesis in response to viruses are unknown. It is possible that in the absence of T cells, the “usual” T cell-derived helper signals are supplied by non-T cells. Alternatively, in the course of TI IgG responses, a different set of signals may activate distinct TI pathways of B cell activation, differentiation, isotype switching, and Ab secretion. Interaction of CD40 on B cells with CD40L on activated T cells is an essential component of T cell help, and it is required for several steps of TD Ab responses to noninfectious protein Ags (e.g., OVA or SRBC) (25). CD40L−/−or CD40−/−mice immunized with these Ags lack germinal center formation and isotype switching and do not establish B cell memory (2–5). In this study, we demonstrated that PyV infection leads to readily detectable virus-specific IgG synthesis in CD40−/−mice, although the antiviral IgG titer in the serum of CD40−/−mice is 10-fold lower than in wild-type C57BL/6 mice. This finding is consistent with observations that other viruses, such as LCMV (6, 7) and vaccinia virus (7), induce virus-specific IgG responses in CD40−/−mice that are 1–10% of the responses measured in immunocompetent wild-type mice. Thus, IgG responses to live, infectious viruses have a CD40-independent component.

Next, we questioned whether TI IgG responses to PyV previously observed in TCR βΔδ−/−mice are completely independent of CD40 signaling. Assuming that CD40L is expressed only on Th cells, TI responses would be necessarily CD40-independent. However, if cells other than T cells express CD40L in virus-infected mice, these cells then may stimulate TI IgG responses. Our experiments suggest that indeed this is the case in that TI IgG responses are reduced in the absence of CD40 signaling by ~50%. This means that although a fraction (half) of the TI IgG responses are independent of CD40, the induction of TI IgG responses is enhanced by CD40-CD40L interactions.

Which cell types provide CD40L for these interactions? Importantly, the finding that B cells with a disrupted CD40L gene also produced decreased amounts of antiviral IgG when transferred into SCID mice may indicate indirectly that CD40L expression is induced on murine B cells in vivo by the virus infection. This result also suggests that B cells are able to provide “help” for each other when they encounter infectious Ags in the absence of Th cells. Alternatively, the lower IgG response to PyV in mice reconstituted with CD40L−/−B cells could indicate that B cells evolved in an environment entirely free of CD40L have altered effector functions. We feel that this interpretation of the data is less likely. The synthesis of same or slightly elevated levels of virus-specific IgM in mice carrying CD40L−/−B cells in comparison with mice that received normal B cells indicates that CD40L−/−B cells are able to respond to Ags with Ab secretion. Moreover, humans with the hyper IgM syndrome who carry a mutation in the gene coding for CD40L and therefore do not express functional CD40L have B cells that are fully functional in vitro (26).

Previous reports described the induction of CD40L in human peripheral B cells in vitro by ionomycin and phorbol ester stimulation and on human B cell lymphomas (20, 27). Moreover, most recently a study demonstrated CD40L expression on human tonsillar B cells ex vivo and in situ (28). In the mouse, hyperreactive B cells from autoimmune male BXSB mice have been shown to express CD40L (29). The induction of CD40L expression on murine B cells in vitro by anti-IgM or anti-CD38 Ab treatment has also been reported (30). Our data suggest that B cells may express functionally active CD40L, which could enhance antiviral TI responses in mice. It is an important task for future investigations to define what are the signals inducing CD40L expression on B cells under these circumstances. Thus far we have been unable to demonstrate the induction of CD40L on splenic B cells of PyV-infected TCR β×δ−/−mice directly by FACS staining ex vivo. A possible reason for this result is that only a very small fraction of the B cells in the spleen may be activated by the virus.

The CD40-CD40L interaction provides essential costimulatory signals for the activation of T and B cell responses to “inert” or noninfectious Ags (27). However, some infectious agents (such as LCMV, vaccinia virus, and B. burgdorferi) have the ability to elicit isotype-switched Ab responses even in the absence of CD40 signaling (6–8). IgG responses to PyV are 10-fold lower in CD40−/−mice than in their wild-type C57BL/6 controls, and TI IgG titers (which amount to ~10% of the IgG responses seen in normal immunocompetent mice) have an additional 2-fold reduction in the absence of CD40 signaling. Remarkably, these greatly diminished Ab responses are still sufficient to ensure protection from PyV infection, in that TCR β×δ−/−mice treated with Abs against CD40L survive the acute phase of PyV infection without out visible signs of PyV-induced disease.

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