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The Role of B7-1 and B7-2 Costimulation for the Generation of CTL Responses In Vivo

Luis J. Sigal,* Hans Reiser,† and Kenneth L. Rock2*

The role of B7-1 and B7-2 costimulatory molecules in the generation of Ag-specific CD8+ CTLs is not well understood. In this paper, we analyze the role of both B7-1 and B7-2 in the generation of CTLs to nonliving, exogenous Ag and to live virus. To analyze the role of B7 costimulation in the induction of CTLs, we blocked B7-1 and/or B7-2 in vivo by injecting C57BL/6 mice with anti-B7-1 and/or anti-B7-2 mAbs; the mice were subsequently immunized with either chicken OVA that had been cross-linked to beads as a model of exogenous Ags or with wild-type and recombinant vaccinia virus expressing different forms of chicken OVA as models of viral Ags. Our results indicate that B7 costimulation is necessary in the generation of CTLs for all of these Ags. Since the B7 molecules could be costimulating CD8+ and/or CD4+ T cells in wild-type animals, we also examined the role of costimulation in the generation of CTLs to exogenous and viral Ag in MHC class II-deficient mice lacking most CD4+ T cells. In these animals, a combination of both mAbs also blocked all CTL responses, indicating that the Th cell-independent activation of CTLs is dependent upon the B7-costimulatory signals supplied to the CD8+ cell. These findings contribute to the understanding of the role of costimulation for the generation of CTLs. We also discuss the implications of these findings on the role of professional APCs in the initiation of CTL responses. The Journal of Immunology, 1998, 161: 2740–2745.

Most T lymphocytes recognize Ag as a complex of an antigenic peptide bound to an MHC molecule present at the surface of an APC. Recognition is mediated through the TCR that is specific for a certain peptide/MHC combination. Two major subsets of T lymphocytes exist that can be distinguished by their expression of CD4 and CD8 molecules.

T cells bearing the CD4 marker (CD4+ T cells) recognize peptides bound to MHC class II molecules. These lymphocytes generally function as Th cells. By secreting cytokines and expressing bioactive cell surface molecules, they modulate the activity of B cells, other T lymphocytes, and macrophages (Mφ)3. MHC class II molecules are constitutively expressed by only a limited number of bone marrow (BM)-derived cell populations, such as Mφ, dendritic cells (DCs), and B cells. These cells, particularly DCs and Mφ, are able to induce immune responses upon the presentation of Ag to Th cells and, accordingly, are referred to as “professional APCs”. MHC class II molecules present peptides that are generated in endocytic compartments. Most of these peptides are derived from exogenous proteins that are internalized by the APC.

T lymphocytes expressing the CD8 marker (CD8+ T cells) recognize peptides bound to MHC class I molecules. Since MHC class I molecules are expressed by all nucleated cells in the body, CD8+ T cells are able to recognize Ags that are expressed not only by professional APCs but also by other cells. MHC class I molecules can present peptides to CD8+ T cells through two pathways. Typically, MHC class I molecules present peptides from endogenously synthesized proteins. Since the T cells that are reactive with MHC class I molecules are generally CTLs, this pathway of Ag presentation allows the immune system to recognize and eliminate cells that synthesize proteins to which the immune system has not been tolerized, such as certain tumor and viral Ags (1).

During the last few years, we and others have been investigating an alternative pathway of MHC class I presentation in which peptides from exogenous proteins are presented to CTLs (2). This pathway of MHC class I presentation has been demonstrated to function both in vitro and in vivo. The exogenous pathway may subserve multiple functions in vivo, including the generation of CTLs that are directed toward vacuolar pathogens, and for those diseases that exclusively affect somatic tissues without involving professional APCs. This pathway is also of interest in that it could be used to allow protein-based vaccines to elicit CTL immunity. Although it is clear that exogenous Ag can be presented on MHC class I molecules in vivo, little is known with regard to how this pathway stimulates CTL responses. For example, the requirements of accessory molecules for the initiation of CTL responses to exogenous Ag are unknown. This issue is important for understanding how this pathway operates and for the development of strategies to exploit it.

It is now accepted that the engagement of the TCR by peptide/MHC complexes (signal 1) is generally not sufficient to trigger a T cell response, and that ligation of a costimulatory receptor (signal 2) is required for T cell activation (3–5). Indeed, signaling through the TCR alone can induce a state of anergy in naive CD4+ T cells in certain situations (3, 6). The most studied costimulatory molecules are B7-1 (CD80) and B7-2 (CD86), which bind to the counterreceptors CD28 and CTLA-4 at the T cell surface. Like MHC class II molecules, B7-1 and B7-2 are expressed by professional APCs. However, while B7-2 is constitutively expressed in resting APCs, B7-1 is induced during APC activation. Both B7 molecules...
are considered essential for the induction of MHC class II-restricted T cell responses (3). In contrast, the role of B7 costimulation and professional APCs for the generation of MHC class I-restricted CTLs and the ability of these cells to stimulate effector CTLs are less clear. Due to the widespread expression of MHC class I in the organism, virtually all cell types can process Ag and be killed by CTLs (7, 8). However, most somatic cells lack costimulatory molecules (3, 9) and are consequently unable to provide the second signal that may be required to initiate a CD8\(^+\) T cell response (10). Second signals could be provided to CTLs by cytokines such as IL-2 (11, 12). However, although many CTL responses are dependent upon help from CD4\(^+\) T cells (13–15), some other CTL responses can be obtained in the absence of Th cells (16–19), including those responses that are generated with high doses of exogenous Ag (20). Moreover, CD28 and CTLA-4 are provided by Dr. Richard Hodes (National Cancer Institute, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD).

### Preparation of OVA cross-linked beads (OVA-beads)

Iron oxide beads (Biomag, Perceptive Diagnostics, Cambridge, MA) were covalently conjugated to chicken egg OVA (Sigma) according to the manufacturer’s instructions. The amount of OVA that bound to the beads was calculated according to the change of absorption at 230 nm of the starting OVA solution and of the supernatant following conjugation.

### Recombinant vaccinia viruses

A vaccinia virus expressing the full-length chicken OVA (Vac FL-OVA) and a vaccinia recombinant expressing the OVA peptide SIINFEKL preceded by the signal sequence of the E3/19K glycoprotein of adenovirus 2 (Vac ES-OVA) (31) were a kind gift of Dr. Jonathan Yewdell (National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD).

### Results and Discussion

To better understand the role of B7 in the initiation of CTL responses, mice were aseptically collected; single-cell suspensions were prepared by homogenization between two frosted glass slides. A total of 40 \(\times\) \(10^6\) spleen cells were cultured for 5 days in upright T25 tissue culture flasks (Becton Dickinson, Lincoln Park, NJ) in 10 ml of cDMEM in the presence of 1.5 \(\times\) \(10^5\) irradiated (4000 rad) EG7 cells. Effector cells, consisting of spleen cells from restimulation cultures, were cultured with hybridoma cells. The anti-B7-1 mAb was purified on a protein A-Sepharose column, and anti-B7-2 mAb was purified on a protein G-Sepharose column. The purified hamster and rat IgG that were used as controls were supplied by Cappel (West Chester, PA).

### Restimulation and CTL assays

Spleens from sacrificed mice were freshly explanted spleen cells, the incubation with the target cells occurred for 5 h; all other conditions remained the same. Each experiment was repeated at least twice, and two or three mice were used per experimental group for each experiment. The values represent the averages for each group, and variations between animals within a group were consistently <10%.

### Materials and Methods

#### Media

Cells were maintained either in cRPMI in an atmosphere of 5% CO\(_2\) in an %CO\(_2\), cRPMI consisted of RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) that was supplemented with 10% FCS (Atlanta Biologicals, Norcross, GA), 5 \(\times\) \(10^{-5}\) M 2-ME (Sigma, St. Louis, MO), 2 mM l-glutamine, antibiotics (Fungizone, Irvine Scientific), 0.01 M HEPES buffer (Irvine Scientific), and nonessential amino acids (Irvine Scientific). Where indicated, 0.5 mg/ml of G418 (Life Technologies, Grand Island, NY) was added to the culture medium (cRPMI/G418). cDMEM consisted of DMEM (Irvine Scientific) that was supplemented as described for cRPMI and with an additional 1 mM of sodium pyruvate (Irvine Scientific).

#### Cell lines and Abs

EL4 cells were obtained from American Type Culture Collection (Manas- sas, VA) and grown in cRPMI. EG7, an EL4 subclone that had been trans- fected with OVA (28), was a gift of Dr. Michael Bevan (University of Washington, Seattle, WA) and was grown in cRPMI/G418. MC57G cells were a kind gift of Dr. Peter Doherty (St. Jude Children’s Research Hospital Memphis, TN).

The 16-10A1 hybridoma producing anti-B7-1 mAb has been described previously (29). GL-1 cells producing anti-B7-2 mAb (30) were kindly provided by Dr. Richard Hodes (National Cancer Institute, National Institutes of Health (NIH), Bethesda, MD). Both Abs were affinity purified from ascites fluid that was obtained from nude mice that had been injected i.p. with hybridoma cells. The anti-B7-1 mAb was purified on a protein A-Sepharose column, and anti-B7-2 mAb was purified on a protein G-Sepharose column. The purified hamster and rat IgG that were used as controls were supplied by Cappel (West Chester, PA).

### Mice and inoculations

C57BL/6 female mice were purchased from Taconic (Germantown, NY) and were used between 6 and 8 wk of age. MHC class II-deficient mice were bred at the Dana Farber Cancer Institute and the University of Massachusetts Medical Center animal facilities and were used between 2 and 6 mo of age. For immunization with exogenous Ag, mice were injected s.c. with the indicated amounts of OVA-beads in 100 \(\mu\)l of PBS that was split between both flanks. Animals were sacrificed at 7 days postimmunization. For viral immunization, mice were inoculated i.p. with the indicated amount of virus in 0.5 ml of PBS and sacrificed at 6 days postinfection. A blockade of B7-1 and B7-2 costimulatory molecules was performed by an i.p. injection of the indicated Abs in 0.5 ml of PBS at the dosages and times indicated in the figures. The infusion schedule that was employed in this study has been shown previously to maintain saturation levels of anti-B7 mAbs in serum (32).

Spleens from sacrificed mice were aseptically collected; single-cell suspensions were prepared by homogenization between two frosted glass slides. A total of 40 \(\times\) \(10^6\) spleen cells were cultured for 5 days in upright T25 tissue culture flasks (Becton Dickinson, Lincoln Park, NJ) in 10 ml of cDMEM in the presence of 1.5 \(\times\) \(10^5\) irradiated (4000 rad) EG7 cells. Effector cells, consisting of spleen cells from restimulation cultures, were cultured with 6,000 or 20,000 Na\(^2\)Cr\(_2\)O\(_4\)-labeled target cells for 4 h at the indicated E:T ratios in 150 \(\mu\)l of cDMEM. The percentage of killing was calculated using the following formula: (experimental release – spontaneous release)/[full release – spontaneous release]) \(\times\) 100, where spontaneous release represents the counts obtained when the target cells were cultured in media in the absence of splenocytes and full release represents the counts obtained when the target cells were lysed with 1% Triton X-100. For CTL assays involving freshly explanted spleen cells, the incubation with the target cells occurred for 5 h; all other conditions remained the same. Each experiment was repeated at least twice, and two or three mice were used per experimental group for each experiment. The values represent the averages for each group, and variations between animals within a group were consistently <10%.

#### Restimulation and CTL assays

To better understand the role of B7 in the initiation of CTL responses (priming) and in the generation of effector CTLs, we decided to examine responses to well-characterized Ags. An s.c. injection of OVA-beads in mice induces a strong CTL response through the MHC class I exogenous pathway (33, 34). To determine the role of B7 costimulation in this response, C57BL/6 mice were injected with 60 \(\mu\)g of OVA-beads in the presence or absence of anti-B7 Abs. Mice were sacrificed 1 wk later, and their spleen cells were cultured for 5 days in the presence of EG7 cells, a transfectant of EL4 that expresses OVA. At the end of the 5-day culture period, the ability of the restimulated spleen cells to lyse EG7 and EL4 cells was analyzed in standard \(^{51}\)Cr release assays.

As shown in Figure 1, an infusion of either anti-B7-1 or anti-B7-2 mAb did not significantly inhibit the CTL response to OVA when
The induction of CTLs to exogenous Ag in vivo can be blocked by an infusion of anti-B7-1 and anti-B7-2 mAbs. C57BL/6 mice were injected s.c. with 60 μg of OVA-beads. Mice were infected i.p. on days −1 and 3 with 100 μg of the indicated Abs in 0.5 ml of PBS. Mice were sacrificed on day 7, and spleen cells were restimulated in vitro with EG7 cells (EL4 transfected with OVA). The data depict CTL activity as measured by a 51Cr release of restimulated cells against EG7 cells (■) or EL4 cells (○).

To further determine the role of B7 costimulation in the generation of CTL effectors, we compared the requirements for the induction of CTLs with exogenous Ag and with replicating virus. For this purpose, mice were treated with Abs to B7 and immunized with Vac FL-OVA. Mice were then sacrificed at 6 days postinfection. The induction of CTL responses to OVA was measured after 5 days of in vitro restimulation, and the in vivo generation of effector CTLs to vaccinia viral Ags was determined using freshly explanted cells without in vitro restimulation. Figure 2 demonstrates that the response to OVA was markedly decreased in mice that received a combination of anti-B7-1 and anti-B7-2 Abs compared with mice that were injected with control IgG. Furthermore, the response to native vaccinia epitopes was also almost completely abolished by the combination of anti-B7-1 and anti-B7-2 mAbs (Fig. 3). Therefore, the requirement for B7 costimulation is seen for several Ags in the generation of CTL effectors in vivo (vaccinia response) or for the induction of CTLs requiring restimulation in vitro (OVA response).

It has been proposed that the requirements of T cells for costimulation in vitro depend in part upon the level of TCR occupancy, which in turn depends upon the density of the peptide/MHC class I complexes at the surface of the APC (23, 35, 36). Recently, it has been reported that infecting cells with Vac FL-OVA produces the expression of ~3600 complexes/cell of SIINFEKL (the dominant OVA epitope) bound to H2-Kb (the restricting MHC molecule for SIINFEKL). The number of complexes was increased ~20-fold when cells were infected with Vac ES-OVA, which is a recombinant vaccinia construct that encodes only for SIINFEKL, or with SIINFEKL preceding by a signal sequence that translocates the peptide directly into the endoplasmic reticulum (37). To test whether increasing the number of Kb/SIINFEKL complexes per cell could override the need for costimulation, we treated C57BL/6 mice with anti-B7 Abs or control IgG and challenged them with Vac ES-OVA. Figure 4 demonstrates that even in this case, in which high levels of Ag density at the surface of the APC are expected, the CTL response in vivo is still dependent upon costimulation, since the combination of anti-B7 Abs effectively blocked the CTL response.

The experiments described thus far indicate an important role for B7 costimulation in the generation of CTL responses. However, the data do not indicate whether there is a direct interaction of the CTL with the costimulatory molecule and/or whether B7 is required to costimulate CD4+ T cells that help in the generation of CTLs. Earlier work in this laboratory has shown that MHC class II-deficient mice, which are devoid of CD4+ T cell responses, can mount strong CTL responses to exogenous particulate Ags (20). As a means of determining the respective role of B7-1 and B7-2 in the CD4+ T cell-independent generation of CTLs, MHC class II-deficient mice were treated with anti-B7 Abs and immunized with OVA-beads. As shown in Figure 5, treatment with anti-B7-1 alone did not block the generation of CTLs; however, treatment with anti-B7-2 (or a combination of anti-B7-1 and anti-B7-2) did abrogate responses. This result suggests that the Th cell-independent generation of CTLs requires a direct interaction of the MHC class I-restricted T cell with B7-2 molecules on a professional APC. This finding was not necessarily expected, since B7-1-transfected
tumors are superior to B7-2-transfected tumor cells at eliciting antitumor immunity in some experimental systems (38, 39). It is interesting that the results with MHC class II-deficient mice differ from those seen with wt mice, in which B7-1 and B7-2 are both important. Presumably, both B7-1 and B7-2 may also function by costimulating help from CD4+ T cells in the wt mice. In this context, it is noteworthy that recent studies have suggested that the uptake and presentation of tumor Ags by bone-marrow derived APCs is a dominant mechanism of CTL induction in response to B7-1-transfected tumor cells (26).

To determine whether a direct interaction between the CD8+ cell and a professional APC expressing B7 molecules is also necessary in the response to a replicating virus, MHC class II-deficient mice were immunized with a vaccinia recombinant and injected with either anti-B7-1, anti-B7-2, or a combination of both mAbs. A combination of both Abs completely blocked the generation of effector CTLs to vaccinia virus epitopes (Fig. 6) or to OVA in Vacc FL-OVA (data not shown). However, in contrast to the results obtained with OVA-beads, anti-B7-2 alone was not as effective in blocking the CTL response to virus. The reason for this difference between the response to viral and exogenous Ag is unclear. Conceivably, B7-1 might be more effective when larger amounts of Ag are presented (as may occur in a viral infection) or when it is expressed at higher levels (e.g., if induced by the viral infection or if presented on different APCs). Alternatively, the timing of the the interaction of CTLs with APCs could differ in the two situations, with one occurring before B7-1 induction and the other occurring after this molecule has been expressed. However, regardless of the mechanism, the data clearly show that CTL responses to both replicating viruses and exogenous Ag are dependent upon the direct interaction of the CTL precursors with B7 molecules in the absence of CD4+ T cells.

To date, the most direct investigation of the role of B7/CD28 costimulation in the generation of CTLs has used CD28– mice. These mice fail to respond to vesicular stomatitis virus and thymidine kinase (TK)-deficient (TK–) vaccinia virus (40). Therefore, our experiments of B7 blockade during infection with vaccinia FL-OVA that are TK– are in agreement with this earlier report. However, these authors reported that CD28– mice mount CTL responses to wt vaccinia virus (TK+) that are equivalent to the responses seen for wt mice and suggested that infection with wt vaccinia virus results in a B7-independent generation of CTLs. To address this issue in our system, we treated C57BL/6 mice (Fig. 7A) and MHC class II-deficient mice (Fig. 7B) with anti-B7 Abs as in previous experiments; however, in this case we infected them with wt vaccinia virus and measured the generation of effector CTLs from freshly explanted spleen cells. Our results indicate that a combination of anti-B7-1 and anti-B7-2 mAbs is effective at blocking the generation of CTL effectors to wt TK+ vaccinia in the presence or absence of T cell help. These findings suggest that B7 costimulation is necessary in the response to TK+ vaccinia, and that this necessity is due in part to a direct interaction between B7 and the CTL. The reasons accounting for the difference between our results and those of Kündig et al. are not clear. One possibility is that there are compensatory effects in the generation of the CD8+ repertoire in the absence of CD28 that allow CD28– mice to respond to wt vaccinia. However, other explanations are possible, and further investigation is necessary to clarify this issue.

Taken together, the results presented in this paper demonstrate a role for B7 costimulation in the generation of CTL responses. In addition, the results of experiments using MHC class II-deficient mice suggest that there is a direct interaction of the CD8+ T cell with the cell displaying the B7 molecule; these results also suggest that, in the absence of help, such an interaction is required for CTL generation. In this context, it is noteworthy that Mφ and DCs (both of which are BM-derived professional APCs) can process and present exogenous Ag on MHC class I molecules following phagocytosis in vitro (33, 41–44). Furthermore, reconstitution with Mφ has been found to restore CTL responses in mice treated with carrageenan or silica, which are known to deplete Mφ in vivo (45). As professional APCs, Mφ and DCs are also among the few cells that express B7 molecules (3). Therefore, although the identity of the APCs in vivo remains unknown, we think it is likely that Mφ and/or DCs are responsible for the presentation of exogenous Ag to CTLs in vivo, providing both the antigenic and the costimulatory stimuli.

Similarly, the need for direct B7 costimulation in the CTL response to vaccinia seems to implicate the participation of professional APCs in the CTL response to live viruses. In this case, the presentation of viral Ags could occur either through direct

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**FIGURE 4.** CTL induction is dependent upon costimulation even at high levels of epitope density. C57BL/6 mice were injected i.p. with 2 × 10⁷ PFU of Vac ES-OVA. Mice were injected i.p. on days −1 and 3 with 100 μg of the indicated Abs in 0.5 ml of PBS. Mice were sacrificed on day 6, spleen cells were restimulated in vitro with EG7 cells, and CTL activity was measured 5 days later against EG7 (■) or EL4 cells (○).

**FIGURE 5.** CTL induction by exogenous Ag in MHC class II-deficient mice is dependent upon B7-2 but not on B7-1. MHC class II-deficient mice (B16 background, H-2h) were injected s.c. with 60 μg of OVA-BM. Mice were injected i.p. on days −1 and 3 with 100 μg of the indicated Abs in 0.5 ml of PBS. Mice were sacrificed on day 7, and spleen cells were restimulated in vitro with EG7 cells. The data depict the CTL activity of restimulated cells against EG7 (■) or EL4 cells (○).
infection of the APCs or by a re-presentation of exogenous Ag released from other infected cells. Our data do not resolve whether CD8+ T cells need to interact with professional APCs and receive B7 costimulation in the presence of CD4+ help. However, these interactions may very well occur in CD4+-dependent T cell responses. Indeed, CD8+ T cells lack MHC class II molecules and cannot present Ag in a cognate manner to CD4+ cells. Therefore, a professional APC, by virtue of being capable of presenting on both MHC class I and MHC class II molecules, could facilitate the interaction between CD4+ and CD8+ T cells by bringing them together. As part of this process, it is likely that the CD8+ T cell would also interact with B7 molecules.

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FIGURE 6. The CTL response to replicating recombinant vaccinia in MHC class II-deficient mice is inhibited only by a combination of anti-B7-1 and anti-B7-2 Abs. MHC class II-deficient mice were injected i.p. with 2 × 10^7 PFU of recombinant vaccinia (TK–). Mice were injected i.p. on days –1 and 3 with 100 μg of the indicated Abs in 0.5 ml of PBS. Mice were sacrificed on day 6, and the CTL activity of fresh spleen cells was measured against vaccinia-infected (●) or uninfected (○) MC57G cells.

References


FIGURE 7. Anti-B7 Abs inhibit the CTL response to replicating wt vaccinia in wt and MHC class II-deficient mice. Both C57BL/6 mice (A) and MHC class II-deficient mice (B) were injected i.p. with 2 × 10^7 PFU of wt vaccinia virus. Mice were injected i.p. on days –1 and 3 with 100 μg of the indicated Abs in 0.5 ml of PBS. Mice were sacrificed on day 6, and fresh spleen cells were used in a 51Cr release assay with vaccinia-infected (●) or uninfected (○) MC57G cells.