The Journal of Immunology

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J Immunol 2000; 165:3088-3093; ;
doi: 10.4049/jimmunol.165.6.3088
http://www.jimmunol.org/content/165/6/3088

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Either B7 Costimulation or IL-2 Can Elicit Generation of Primary Alloreactive CTL[1]

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B7-1 and B7-2 are important costimulatory molecules in the activation of T cell immunity. We have used mice made genetically deficient in either or both B7 molecules to determine the role of B7 molecules in activation of primary alloreactive CTL. The absence of either B7-1 or B7-2 did not alter generation of CTL from unfractionated lymphocytes, but the absence of B7-2 greatly decreased CTL generation from purified $CD8^+$ responder cells. However, if B7-1 was induced on the stimulating cells then CTL generation was restored to wild-type levels. Absence of both B7-1 and B7-2 from MLR using whole splenocytes resulted in a profound reduction in generation of CTL. This could completely be reversed by the addition of IL-2. B7 molecules could directly costimulate $CD8^+$ cells, as purified $CD8^+$ cells developed into mature CTL when stimulated with wild-type APC, but not with B7-deficient APC. Again, IL-2 could drive CTL generation from purified $CD8^+$ cells, even in the absence of B7 molecules. Taken together, these results demonstrate an important role for B7 costimulation in CTL generation. ***The Journal of Immunology*****, 2000, 165: 3088–3093.**

Optimum T cell responses require costimulatory signals in addition to stimulation of the TCR. Costimulatory molecules B7-1 (CD80) and B7-2 (CD86) are expressed on APC and can costimulate T cell activation by binding to CD28 on T cells (1). CD28-B7 interactions enhance proliferation and cytokine production by $CD4^+$ cells, particularly production of Th2 cytokines such as IL-4 (2, 3). CTLA-4 (CD152) is another receptor for B7 molecules and is up-regulated by T cells following activation by the TCR (4). CTLA-4 has an important role in attenuation of the T cell response and immune tolerance (5, 6).

The role of B7 costimulation in generation of effector CTL has been controversial. While there is general agreement that B7-1 can enhance development of $CD8^+$ and $CD4^+$ CTL (7, 8), the role of B7-2 has been less clear. Some reports have indicated that B7-2 costimulation increases $CD8^+$ CTL generation (9), while other authors have been unable to detect any such effect (10). Recently, it was shown that B7-1 is more potent than B7-2 at costimulating naive $CD8^+$ cells (11). Previous studies have used cell lines transfected with B7-1 or B7-2 as APCs (7, 10, 11). As the B7 molecules are expressed at different levels and with different kinetics in vivo, it is also important to determine the role of these molecules when present on normal APC. Finally, it has been suggested that B7 costimulation is actually a strict requirement for generation of mature CTL from CTL precursors (CTLp)[3] (12, 13). These papers have suggested that direct B7 costimulation of CTLp is needed for their differentiation to mature effectors and that cytokines are not sufficient to overcome this requirement.

We have evaluated the role of B7 molecules in CTL generation using mice made genetically deficient in one or both of the B7 molecules (knockout, or $^{-/-}$, mice). This allows us to use naive CTLp stimulated with otherwise normal splenic APC. Using primary allogeneic MLR mismatched for both class I and class II, we found that the absence of either B7-1 or B7-2 did not alter CTL generation from whole spleen. However, if purified $CD8^+$ cells were stimulated using APC lacking B7-1 or B7-2, an important role for B7-2 was revealed. If both B7-1 and B7-2 were missing from the MLR, the CTL response was nearly abrogated, even in the presence of $CD4^+$ cells. Addition of IL-2 was able to completely restore generation of mature effector CTL despite the absence of B7 molecules. Together, these data demonstrate an important role for B7 costimulators in development of mature CTL.

Materials and Methods

Abs and Cytokines

Hamster anti-mouse-CD28 Ab is from the 37.51 hybridoma and was prepared as a concentrated supernatant (14). Control hamster IgG was obtained from Jackson ImmunoResearch (West Grove, PA). Recombinant mouse IL-2 was obtained from Collaborative Research (Bedford, MA). Anti-CD40 Ab was made from the 3/23 hybridoma, which was the kind gift of Dr. Gerold Klaus (15).

Mice

B7-$1^{-/-}$, B7-$2^{-/-}$, and B7-$1/2^{-/-}$ mice have been described previously (16, 17). The animals used in this study were inbred 129S4/SvJae or backcrossed from 129S4/SvJae onto the BALB/c background and then interbred to generate knockout mice. B7-$1^{-/-}$ BALB/c mice were backcross generation 10, and B7-$2^{-/-}$ BALB/c mice were backcross generation 6. The B7-$1/2^{-/-}$ BALB/c mice were backcross generation 3. In the generation of these BALB/c B7-$1/2^{-/-}$ mice, the second generation of mice was typed for the MHC by flow cytometry and only mice with the BALB/c MHC ($H\text{-}2^d$) were used for the next cross to wild-type BALB/c. Thus, the third generation BALB/c B7-$1/2^{-/-}$ mice were $H\text{-}2^d$. When the third generation backcross mice heterozygous for the B7-$1/2^{-/-}$ allele were made, we also selected wild-type mice to use as breeders to generate wild-type backcross generation 3 controls. Wild-type controls for the BALB/c B7-1 or B7-$2^{-/-}$ mice were commercial BALB/c mice obtained from Taconic. 129S4/SvJae wild-type mice were bred within our animal facility. Brigham and Women's Hospital and Harvard Medical School are American Association for

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Received for publication April 19, 2000. Accepted for publication July 5, 2000.

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[1] This work was supported by a Howard Hughes Graduate Fellowship to B.E.G. and National Institutes of Health Grants AI09709 to A.J.M. and AI38310 and AI1GF41584 to A.H.S.

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[3] Abbreviation used in this paper: CTLp, CTL precursor.

0022-1767/00/$02.00

the Accreditation of Laboratory Animal Care-accredited institutions, and the mice were cared for in accordance with institutional guidelines.

Mixed lymphocyte reactions

Primary one-way MLR were performed by mixing splenocytes from 129S4/SvJae responder mice with irradiated (2000 rad) stimulator splenocytes from BALB/c mice. A total of 5×10^6 responder cells and 2.5×10^6 stimulator cells were mixed in 2 ml of RPMI 1640 supplemented with HEPES buffer, 2-ME, and L-glutamine (Life Technologies, Gaithersburg, MD) and 10% FCS (Sigma, St. Louis, MO) in 24-well tissue culture plates. $CD8^+$ cells were enriched using modified IsoCell $CD4^+$ Isolation kits (Pierce, Rockford, IL), which have columns containing glass beads coated with a mixture of goat anti-mouse IgG and goat anti-rat IgG. Whole splenocytes were incubated with a mixture of rat anti-mouse $CD4^+$ (clone GK1.5, Ref. 18) and mouse anti-mouse class II MHC (clone 34-5-3, from PharMingen, San Diego, CA) washed twice, and run through the columns as described by the manufacturer. The resulting cells were generally 85–90% CD8. Less than 1% of the cells expressed $CD4^+$ (stained using the RM4-4 Ab (PharMingen, San Diego, CA), which is not blocked by GK1.5), B220, sIgM, Mac-1, or CD11c. When purified $CD8^+$ cells were used as responders, 1.5×10^6 responder cells were mixed with 5×10^6 stimulators as above. Some experiments included addition of recombinant mouse IL-2 (concentrations indicated in figure legends), anti-CD28 (at 1/1000 dilution, determined to be the optimum concentration), or control hamster IgG (at 1 μg/ml).

Cytotoxicity assays

Cells from 5-day MLR were used as effectors in conventional 4-h cytotoxicity assays. P815 (H-2^d) and EL4 (H-2^b) cells were labeled with 200 μCi of ^{51}Cr (DuPont, Boston, MA) in 200 μl at 37°C for 90 min, washed twice, and used as target cells. Five thousand target cells in 100 μl were added to 100 μl of effector cells or media only (for spontaneous ^{51}Cr release) or 2% Triton X-100 (for total ^{51}Cr release). Each group was tested in triplicate in 96-well V-bottom plates. Plates were incubated at 37°C for 4 h, centrifuged at 1000 rpm for 1 min, and 100 μl of media was removed and counted in a gamma counter. The percent specific lysis was calculated as (counts with effector − spontaneous counts)/(total counts − spontaneous counts) × 100.

Results

Analysis of primary CTL in the absence of either B7-1 or B7-2

We examined the role of B7 costimulation in generation of primary CTL. Five-day MLR were performed with unfractionated splenocytes from 129S4/SvJae mice (H-2^b) and unfractionated splenocytes from BALB/c (H-2^d) mice, using each as either the stimulator or responder population. For these studies, the relevant B7 molecule was absent from both the stimulating and the responding cells. The resulting effector cells were tested in 4-h cytotoxicity assays. There was no significant change in CTL generation in the absence of either B7-1 or B7-2 (Fig. 1). We have also determined the kinetics of CTL generation over the 5-day MLR. The absence of B7-1 or B7-2 did not reproducibly alter CTL generation.

Because whole spleen cells were used as the responding cells in these experiments, both Th cells and CTL could be stimulated. To assess the effect of the absence of each of the B7 molecules on $CD8^+$ CTL precursors, we purified $CD8^+$ cells from spleens and stimulated these with allogeneic splenocytes. The responder and stimulator cells were matched for the presence or absence of B7 molecules. Of note, the absence of B7-2 markedly reduced the generation of CTL from purified $CD8^+$ cells, while the absence of B7-1 had no significant effect (Fig. 2*A*). We next wanted to determine whether induction of B7-1 on the stimulating population of cells could drive CTL generation in the absence of B7-2. We induced B7-1 expression in the stimulating cells by treating the cells with anti-CD40 for 3 days before the MLR. Indeed, induction of B7-1 on the stimulating population elicited CTL (Fig. 2*B*). Of note, induction of B7-1 was required for the effect of anti-CD40 stimulation. If anti-CD40-treated B7-1/$2^{-/-}$ APC were used to stimulate CTL, CTL generation was reduced at least 10-fold compared

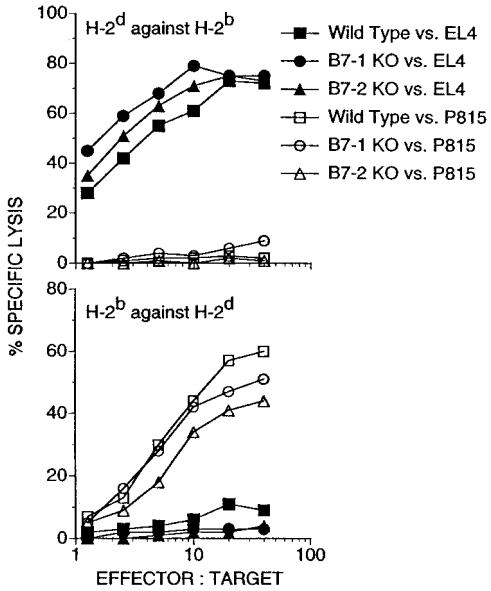

FIGURE 1. The absence of either B7-1 or B7-2 does not significantly alter CTL generation. 129S4/SvJae splenocytes were stimulated with irradiated BALB/c splenocytes for 5 days and tested for cytotoxicity in a 4-h cytotoxicity assay. The MLR were performed with wild-type cells, B7-1-deficient cells, or B7-2-deficient cells as responders and stimulators. Targets tested were P815 and EL4.

with either wild-type or B7-$2^{-/-}$ stimulators (data not shown). Therefore, either B7-1 or B7-2 can drive alloreactive CTL generation if the B7 molecule is expressed at an adequate level.

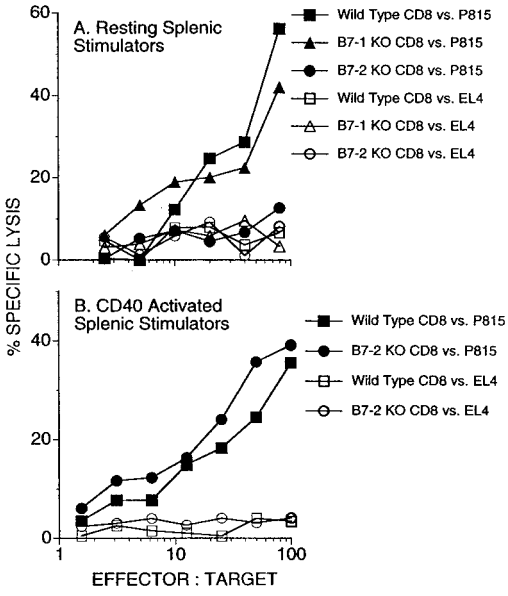

FIGURE 2. B7-2 is important in costimulation of purified $CD8^+$ cells. *A*, Purified $CD8^+$ cells from 129S4/SvJae mice were stimulated with irradiated BALB/c splenocytes for 5 days and tested for cytotoxicity in a 4-h cytotoxicity assay. The MLR were performed using wild-type cells, B7-$1^{-/-}$ cells, or B7-$2^{-/-}$ cells. Targets tested were the relevant target P815 and a negative control target EL4. *B*, BALB/c splenocytes were stimulated with anti-CD40 Ab 3/23 for 3 days to induce B7 expression, irradiated, and then used to stimulate purified $CD8^+$ cells from 129S4/SvJae mice for 5 days and tested for cytotoxicity in a 4-h cytotoxicity assay. The MLR were performed using wild-type cells or B7-$2^{-/-}$ cells. Targets tested were the relevant target P815 and a negative control target EL4.

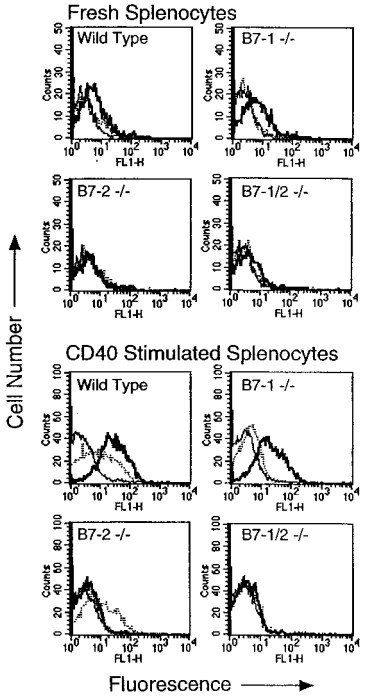

FIGURE 3. The remaining B7 molecule is expressed at normal levels in the B7-1$^{-/-}$ and B7-2$^{-/-}$ mice. Splenocytes from BALB/c mice of the indicated genotype were used fresh or after stimulation with anti-CD40 Ab 3/23 for 3 days. Cells were stained with anti-B7-1 (dotted line), anti-B7-2 (thick line), or a control rat-Ig (thin line) conjugated to RPE and analyzed by flow cytometry.

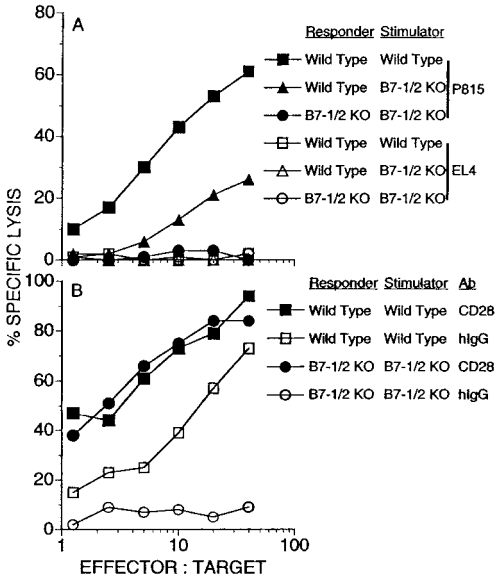

FIGURE 4. The absence of both B7-1 and B7-2 abrogates development of CTL, but this is reversed by CD28 stimulation. *A*, 129S4/SvJae splenocytes were stimulated with irradiated BALB/c splenocytes for 5 days and tested for cytotoxicity in a 4-h cytotoxicity assay. The MLR were performed with wild-type stimulators and responders, wild-type responders and B7-1/2-deficient stimulators, or B7-1/2 deficient responders and stimulators. Targets tested were the relevant target P815 and a negative control target EL4. *B*, 129S4/SvJae splenocytes were stimulated with irradiated BALB/c splenocytes for 5 days and tested for cytotoxicity in a 4-h cytotoxicity assay. The MLR were performed with wild-type splenocytes in the presence of anti-CD28 or control hamster IgG or B7-1/2-deficient splenocytes in the presence of anti-CD28 or control hamster IgG. Targets tested were the relevant target P815 and a negative control target EL4.

To ensure that the expression level of the remaining B7 molecules was similar to wild-type mice in the genetically altered mice, we performed flow cytometry for expression of B7-1 and B7-2 in wild-type, B7-1$^{-/-}$, B7-2$^{-/-}$, or B7-1/2$^{-/-}$ splenocytes (Fig. 3). We also determined the level of B7-1 and B7-2 on these cells after activation with anti-CD40 to demonstrate that B7-1 is induced on the B7-2$^{-/-}$ cells (Fig. 3). Freshly isolated splenocytes from wild-type or B7-1$^{-/-}$ mice have a detectable level of B7-2. B7-1 expression is not detectable on any of the fresh splenocytes. Activation of splenocytes with anti-CD40 Ab elicits increased B7-2 and B7-1 expression in the expected mouse strains. The level of expression is very similar in wild-type and genetically altered cells. Of note, B7-1 is induced on both wild-type and B7-2$^{-/-}$ splenocytes at comparable levels, and with similar kinetics.

B7 costimulation is important in generation of primary CTL

To determine whether the absence of both B7 molecules would alter the generation of primary CTL, MLR were performed using whole splenocytes from mice deficient in both B7 molecules. MLR using wild-type stimulators and responders, B7-1/2$^{-/-}$ stimulators and responders, or B7-1/2$^{-/-}$ stimulators and wild-type responders were compared. The absence of B7 molecules from the MLR resulted in a profound decrease in CTL generation (Fig. 4*A*). This experiment has been performed six times. In four of six experiments, the results shown in Fig. 4*A* were obtained. However, two experiments showed some cytotoxicity from the B7-deficient MLR, but the cytotoxicity was always significantly less than that of the MLR using wild-type cells (reductions of 3.8- and 4.8-fold in lytic cells). Much of the costimulatory activity is supplied by the stimulating population of cells, as wild-type cells stimulated with B7-1/2-deficient cells had a reduced CTL response.

The B7 molecules bind to two ligands, CTLA-4 and CD28. To test whether stimulation of CD28 alone could stimulate CTL generation in the absence of B7 molecules, stimulatory anti-CD28 was added to MLR performed with wild-type or B7$^{-/-}$ cells. Addition of the anti-CD28 Ab elicited CTL in the absence of B7 molecules, while no detectable CTL were generated with the control IgG (Fig. 4*B*). These data show that B7 molecules have an important role in CTL generation, and that CD28 stimulation alone is able to replace the effect of B7 costimulation in these conditions.

The effect of IL-2 on CTL generation in the absence of B7 costimulation

It has been suggested that B7 costimulation is required for development of CTL, and that IL-2 cannot overcome this need (12, 13). To determine whether IL-2 could elicit CTL in the absence of B7-1 and B7-2, we added IL-2 to MLR performed with B7-deficient or wild-type splenocytes. IL-2 was able to completely reverse the effect of the absence of B7 molecules on generation of mature CTL (Fig. 5). As little as 5 U/ml of IL-2 generated detectable CTL in the absence of B7 costimulation. Higher levels of IL-2 (10–25 U/ml) generated CTL at levels similar to or greater than those of wild-type cells in the absence of added IL-2. Addition of IL-2 to the wild-type MLR led to slightly higher levels of cytolysis than the same dose of IL-2 with the B7-deficient cells. Thus, in these culture conditions, there is no absolute requirement for B7-1 or B7-2 to generate CTL; IL-2 can elicit CTL even in the absence of these B7 costimulation.

B7 molecules can directly costimulate CD8$^+$ cells.

The lack of CTL generation in the absence of B7 molecules could be due to lack of costimulation to CD4$^+$ cells or to CD8$^+$ cells. To

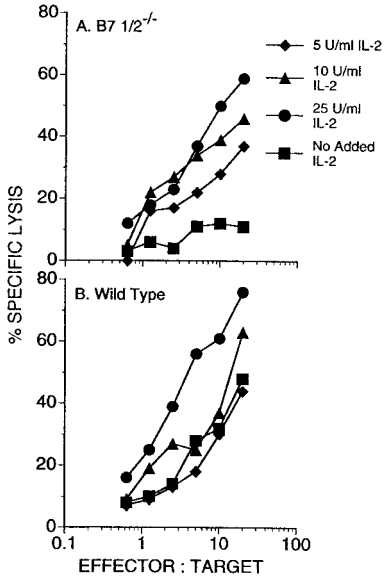

FIGURE 5. IL-2 elicits CTL in the absence of B7 costimulatory molecules. 129S4/SvJae splenocytes were stimulated with irradiated BALB/c splenocytes for 5 days and tested for cytotoxicity in a 4-h cytotoxicity assay. The MLR were performed with wild-type stimulators and responders (*A*) or B7-1/2-deficient responders and stimulators (*B*). IL-2 was added to the MLR at 25 U/ml, 10 U/ml, 5 U/ml, or none. Targets tested were the relevant target P815 (shown) and a negative control target EL4 (not shown; all had <10% specific lysis).

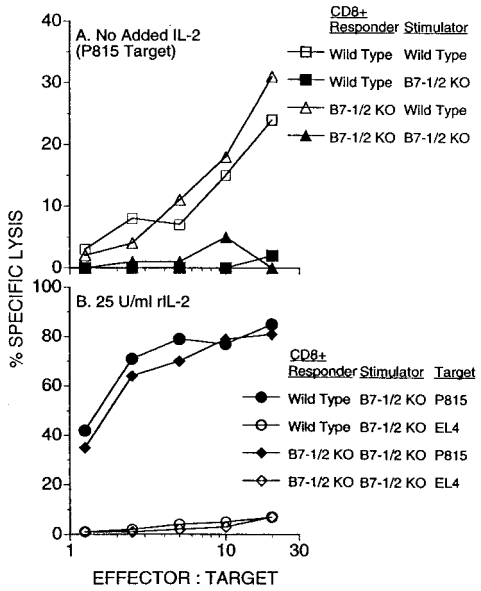

FIGURE 6. $CD8^+$ cells respond directly to B7 costimulation. *A*, Purified $CD8^+$ cells from 129S4/SvJae were stimulated with irradiated BALB/c splenocytes for 5 days and tested for cytotoxicity in a 4-h cytotoxicity assay. The MLR were performed using wild-type stimulators and responders, wild-type responders and B7-1/2-deficient stimulators, B7-1/2-deficient responders and wild-type stimulators, or B7-1/2-deficient stimulators and responders. Targets tested were the relevant target P815 (shown) and a negative control target EL4 (not shown; all had <10% specific lysis). *B*, Purified $CD8^+$ cells from 129S4/SvJae were stimulated with irradiated BALB/c splenocytes for 5 days and tested for cytotoxicity in a 4-h cytotoxicity assay. All MLR were performed with B7-1/2-deficient stimulator splenocytes. Responders were either wild-type or B7-$1/2^{-/-}$ $CD8^+$ cells with 25 U/ml of IL-2 added to the MLR. Targets tested were the relevant target P815 and a negative control target EL4.

determine whether B7 costimulation could enhance CTL generation by directly costimulating $CD8^+$ cells, we purified $CD8^+$ cells and stimulated these with irradiated wild-type or B7-deficient splenocytes. $CD8^+$ cells, whether purified from the B7-$1/2^{-/-}$ or the wild-type mice, developed into effector CTL when stimulated with wild-type splenocytes, but not when stimulated with the B7-deficient spleen cells (Fig. 6*A*). If IL-2 is added to the cultures, a very potent CTL response is generated even if the $CD8^+$ cells are stimulated with splenocytes lacking B7 molecules (Fig. 6*B*), confirming that the addition of IL-2 can overcome the effect of the lack of B7 molecules on CTL generation.

The role of CD28 in CTL generation

Our data are in contrast to a previous report that suggested that the absence of CD28 did not alter CTL generation (19). Because B7 molecules are recognized by both CD28 and CTLA-4, this raises the unlikely possibility that the lack of CTL generation in the absence of B7 molecules is due to a lack of B7-CTLA-4 interaction. However, the strains of mice used in our study and that of Kawai et al. are different. To determine whether various strain combinations differ in the need for costimulation in CTL generation, we obtained $CD28^{-/-}$ mice of the C57BL/6 and BALB/c backgrounds (Fig. 7). MLR were performed done as before, using wild-type cells to stimulate either wild-type or $CD28^{-/-}$ cells. Indeed, there is some difference between the strain combinations. The BALB/c responders generated essentially no detectable CTL in the absence of CD28, while the C57BL/6 cells had a decreased but easily detectable response despite the absence of CD28. In both cases, addition of 25 U/ml of IL-2 increased the CTL response of the $CD28^{-/-}$ cells to levels similar to the wild-type responder cells. These data, along with the data in Fig. 4, suggest that CD28 is indeed a critical costimulatory receptor in allogeneic CTL generation.

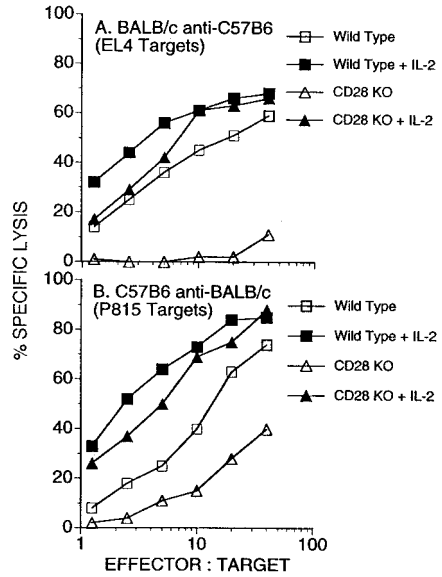

FIGURE 7. The role of CD28 stimulation varies with mouse strain. Splenocytes from BALB/c and C57BL/6 mice were used as stimulators and responders, in the indicated combination, in 5-day MLR. The stimulator cells were always wild type, while the responders were either wild type or $CD28^{-/-}$, in the presence or absence of 25 U/ml of IL-2. Targets tested were the P815 and EL4. For each strain combination, the allogeneic target is shown (that is, EL4 for the BALB/c effectors and P815 for the C57BL/6 effectors). The negative control targets had <10% specific lysis.

Discussion

The relative contributions of B7-1 and B7-2 to CTL generation have been unclear. Several reports have demonstrated that expression of B7-1 on stimulating cells can enhance generation of CTL both in vitro and in vivo. However, the ability of B7-2 to enhance CTL generation is less clear. Gajewski has shown that B7-1, but not B7-2 can elicit CTL generation when mouse $CD8^+$ cells are stimulated with B7-transfected P815 tumor cells (10). In contrast, Lanier et al. have found that either B7-1 or B7-2 are able to costimulate CTL generation from human $CD8^+$ cells (9). A recent study has demonstrated that B7-1 provides a quantitatively stronger costimuli for proliferation and IL-2 production of naive $CD8^+$ cells (11). All of these studies used either cell lines transfected with B7 molecules or B7 fusion proteins, each of which have the advantage of allowing comparison of controlled levels of the B7 molecules. Our studies used splenocytes from B7-deficient animals, allowing comparison of the signals delivered by the physiological levels of the remaining costimulatory molecules. Of note, the B7-$1^{-/-}$ and B7-$2^{-/-}$ mice have wild-type levels of the remaining B7 molecule as determined by flow cytometry, and CD40 stimulation induces the remaining B7 molecule with normal kinetics (Fig. 3).

That the absence of B7-2 abrogates CTL generation from purified $CD8^+$ cells, while lack of B7-1 has no effect, initially appears to conflict with the greater costimulatory strength of B7-1 than B7-2 for $CD8^+$ cells (11). The greater importance of B7-2 in our system is probably due to the higher level of B7-2 than B7-1 on resting splenocytes (Fig. 3 and Ref. 20). Others have also found that B7-2 can have a greater role than B7-1 in CTL generation in an in vivo system, again probably because of the expression levels of the two B7 proteins (21). Together, these data demonstrate that both the relative strength of the B7 costimulators and the level and kinetics of expression of the molecules must be considered in assessing the importance of the B7 molecules in CTL generation.

The absence of either of the B7 molecules does not alter CTL generation in MLR with whole spleen. However, the absence of B7-2 decreased CTL generation when purified $CD8^+$ cells were used as the responders. This was not because B7-1 is not able to stimulate CTLp; induction of B7-1 on APC led to CTL generation by purified $CD8^+$ cells. How can the difference between CTL generation from whole splenocytes and purified $CD8^+$ cells be explained? One possibility is that B7-1 expression is induced in the MLR when whole splenocytes are used as responders, but not induced with purified $CD8^+$ responder cells. B7-1 expression has been shown to be increased by ligation of CD40 on APC by CD40 ligand (CD154) expression on activated $CD4^+$ cells (22, 23). As $CD8^+$ cells express little or no CD40 ligand, purified $CD8^+$ cells may be unable to induce expression of B7-1 by APC in the MLR. Also, the paucity of APC in the responding population of the MLR using purified $CD8^+$ cells may contribute to reduced B7-1 costimulation. Thus, interactions between the CD40-CD40 ligand pathway and the B7-CD28/CTLA-4 pathway could explain the absence of CTL generation in the B7-2-deficient MLR with purified $CD8^+$ responders.

Our data suggest that the absence of B7 molecules in the stimulatory (allogeneic) population leads to marked reduction of CTL generation, despite the presence of B7 costimlators on the APC of the responding population (Fig. 4*A*). In the allogeneic response, CTL can be stimulated either by foreign MHC and peptide on the allogeneic cells or by self MHC with an allogeneic peptide. Because the CTL activity that we measured was against fully allogeneic targets, lysis would only reflect CTL stimulated by the allogeneic MHC. When wild-type splenocytes are stimulated with B7-$1/2^{-/-}$ cells, the CTL would have to get TCR stimulation and B7 costimulation from separate populations of cells. This suggests that costimulation and TCR stimulation may be more potent when given to the T cell by the same APC.

Some work has suggested that B7 costimulation is actually required for generation of cytolytic function. From studies using both TCR transgenic cells and alloreactive cells, Guerder et al. suggest that B7 costimulation is needed for the generation of mature effector CTL (13). In their system, the addition of IL-2 caused proliferation of $CD8^+$ cells but not generation of mature killers when B7 costimulation was blocked with CTLA-4-Ig. Similar conclusions were reached by Liu et al., who found that generation of anti-influenza virus CTL was abrogated in CD28-deficient mice and that a combination of Abs against B7-1 and B7-2 could block generation of effector CTL from CTLp (12). The authors conclude that B7 costimulation is required to generate mature CTL. Further, they suggest that the B7 costimulation is directly required by $CD8^+$ cells, as generation of influenza-reactive CTL is not dependent on $CD4^+$ cells.

In contrast, our data show that there is not an absolute requirement for B7 costimulation in CTL generation. Instead, either IL-2 or B7 costimulation can drive the generation of primary CTL. This is similar to the older demonstration that induction of anergy with paraformaldehyde-fixed APC in $CD8^+$ T cell clones can be overcome by the addition of IL-2 (24). Furthermore, it is clear that some, but not all, viral infections require CD28 for the generation of CTL (25, 26). This suggests that there may be two paths for CTL development. First, stimulation of CTLp by B7-expressing APC could drive CTL generation, even in the absence of significant IL-2 from a Th cell response. Second, stimulation of CTLp by virally infected cells that do not express B7 could drive CTL generation if activated Th cells are present to produce IL-2. Because IL-2 production by $CD4^+$ cells is largely dependent on B7 costimulation of the $CD4^+$ cell, this mechanism of CTL generation would also require B7 for optimum CTL activation (3, 27). These pathways are not mutually exclusive, and it may be that higher levels of IL-2 (from $CD4^+$ T cells) may make the CTL response less dependent on direct costimulation in vivo (28). The nature of the infectious process may determine whether one or the other process is dominant, or if both play important roles. For example, subtypes of influenza virus differ in the requirement for Th cells in CTL generation, and this correlates with induction of B7 costimulators (29).

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