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CD4 Help-Independent Induction of Cytotoxic CD8 Cells to Allogeneic P815 Tumor Cells Is Absolutely Dependent on Costimulation

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Mice made transgenic (Tg) for a rat anti-mouse CD4 Ab (GK mice) represent a novel CD4-deficient model. They not only lack canonical CD4 cells in the periphery, but also lack the residual aberrant Th cells that are found in CD4<sup>-/-</sup> mice and MHC class II<sup>-/-</sup> mice. To analyze the role of CD4 help and costimulation for CTL induction against alloantigens, we have assessed the surface and functional phenotype of CD8 cells in vivo (e.g., clearance of allogeneic P815 cells) and in vitro. In our CD4-deficient GK mice, CTL responses to allogeneic P815 cells were induced, albeit delayed, and were sufficient to eliminate P815 cells. Induction of CTL and elimination of allogeneic P815 cells were inhibited both in the presence and absence of CD4 cells by temporary CD40 ligand blockade. This indicated that direct interaction of CD40/CD40L between APCs and CD8 cells may be an accessory signal in CTL induction (as well as the indirect pathway via APC/CD4 interaction). Furthermore, whereas in CTLA4Ig single Tg mice P815 cells were rejected promptly, in the double Tg GK/CTLA4Ig mice CTL were not induced and allogeneic P815 cells were not rejected. These findings suggest that CD40/CD40L is involved in both CD4-dependent and CD4-independent pathways, and that B7/CD28 and CD40 costimulatory pathways can act independently of each other (8) or influence each other. For example, modulation of CD40 can up-regulate B7 on APC (9–11); and CTLA4Ig, a negative regulator of B7/CD28 interaction, can block the ability of anti-CD40 Ab to stimulate CTL (3). Conversely, ligation of CD28 can induce CD40L expression (12). These findings not only illustrate a pathway whereby CD4 help for CD8 cell activation is educed via APCs, but also reinforce the importance of costimulation for activation of naive CD8 T cells. Nevertheless, costimulation blockade does not always prevent CD8 activation and allograft rejection. For example, in a TCR Tg/RAG<sup>-/-</sup> mouse, induction of CTL was not affected by CTLA4Ig treatment (7).

Independence of CD4 help and costimulation for CTL induction in TCR Tg mice could be simply due to the sheer abundance of CTL precursors with high intrinsic affinity to a single Ag. In such mice, costimulation-dependent population expansion that is critical for a normal immune response may not be necessary. Thus, we reassessed in this study the requirement of CD4 help and costimulation for CTL induction to allogeneic tumor P815 in wild-type mice (in which the T cell frequency is more representative of an allograft recipient) compared with a novel CD4-deficient mouse. To investigate the role of costimulation and CD4 help, we have generated a novel CD4-deficient mouse that is Tg for a rat anti-mouse CD4 Ab (GK1.5). These GK mice have a permanent absence of peripheral CD4 cells without the residual Th cells present in commonly used CD4<sup>-/-</sup> mice (13) and MHC class II<sup>-/-</sup> mice (14, 15). Requirement of B7/CD28 costimulation for CTL induction in the presence or absence of CD4 cells was evaluated in CTLA4Ig Tg mice and in double Tg GK/CTLA4Ig mice. The role of CD40/CD40L interaction in the induction of CTL in the presence and the absence of CD4 cells was evaluated with blocking anti-CD40L Ab.

We show in this study that although CTL responses to allogeneic P815 tumor cells were optimal and more rapid with the presence of CD4 cells, they can be induced in their absence. This CD4...
Materials and Methods

Mice

C57BL/6 mice with mutations in MHC class I.C.H-2<sup>bm1</sup> (termed bm1) were used as wild-type mice. All Tg mice were produced on this bm1 background. The light chain was derived from the rat mAb GK1.5, and the heavy chain was a fusion of the V<sub<H1> of GK1.5 to C region of mouse IgG2c (16) and shown to be functional by transfection (17). The genes were cloned behind the human CMV promoter to generate GK Tg mice. CTLA4<ig> Tg mice have previously been described (18). These mice respectively produce anti-CD4 Ab and CTLA4<ig> abundantly into the circulation (see below). Doubly Tg mice were produced by crossing between homozygous GK and homozygous CTLA4<ig> mice. BALB/c mice (H-2d) were used as donor and source of stimulator for MLR. These animals were bred under specific pathogen-free conditions at our Institute.

Cell lines and Abs

P815 (H-2d) is a mastocytoma-derived tumor line; EL-4 (H-2b) is a thymoma-derived cell line. Cell lines were maintained in DMEM supplemented with 10% FCS and 5 mM HEPES. The mAb 53-6.7 and YT169 (anti CD8), and MR1 (anti CD40L) for in vivo experiments were produced in our laboratory. YTA3.1.2 is an anti-CD4 Ab that recognizes a different epitope to GK1.5 (19) and was used to confirm depletion of CD4 cells. The conjugated Abs 53-6.7 (anti CD8) and H129 (anti CD4) were purchased from Sigma (St. Louis, MO); the conjugated Abs Mel1-14 (anti CD62L), IM7 (anti CD44), PC61 (anti CD25), H57-595 (anti-TCR<beta>), and 34-2-12 (anti-H-2<sup>b</sup>) were purchased from PharMingen (San Diego, CA).

Assessment of CTL function in vivo by clearance of target cells

Mice were injected i.p. with 5<sup>10</sup> P815 cells and killed at various times. Peritoneal cells were harvested by lavage and counted; numbers of surviving P815 cells were calculated from the percentage of H-2<sup>d</sup>-positive cells, as determined by FACS. Splenocytes were removed for cell culture and FACS analysis of CD8 cells. In some experiments, mice were treated with 0.5 mg of anti-CD40L Ab each on days 0 and 3 relative to P815 injection.

Cell culture

Splenocytes from P815-primed and unprimed animals were cultured at 5<sup>10</sup> cells/ml in 2 ml vol with 10<sup>6</sup> irradiated (200 Gy) P815 cells or 5<sup>6</sup> m<sup>1</sup>g/ml anti-CD3 (145-2C11). Culture supernatants were harvested at various times after culture, and cytokine levels in the supernatants were evaluated. In some cases, living cells were harvested from the bulk culture and their lytic activity was assayed.

Assessment of CTL activity in vitro

For testing direct killing of targets, spleen cells from in vivo primed animals were prepared and used as effectors in the standard 4-h <sup>51</sup>Cr release assay. As targets, P815 and control EL-4 cells were labeled with <sup>51</sup>Cr for 90 min at 37°C and 10<sup>4</sup> cells were incubated with various numbers of effector cells in 96-well round-bottom plates for 4 h. Supernatants after plate centrifugation were harvested and assayed for specific lysis. As a measurement of in vivo effector function, splenocytes were tested for direct killing immediately ex vivo in a chromium release assay (no in vitro culture). CTL effectors to P815 cells in bm1 mice were not detected at day 6 after P815 injection, but appeared at day 10. CTL effectors appeared later in GK mice: they were not detected at day 10, but appeared at day 14 (Fig. 3A). A standard CTL assay (6 days in vitro stimulation followed by chromium release assay) was used to detect CTL precursors. CTL precursors to P815 cells could be detected much earlier (6 days after injection) for both bm1 mice and GK mice. Whereas splenocytes harvested at that time showed no direct killing of P815 cells, CTL effectors did develop after in vitro stimulation (Fig. 3B).

Results

Mice Tg for anti-CD4 Ab are a novel model of CD4 cell deficiency

To examine whether CD8 CTL to P815 cells could be induced in the absence of CD4 cells, both wild-type bm1 mice and CD4-deficient GK mice were injected i.p. with 5<sup>10</sup> P815 cells. Allogeneic tumor growth was quantitatively monitored by peritoneal leucocytic responses. In bm1 mice, tumor cells were cleared 2 wk after injection. In contrast, tumor clearance was slower in GK mice compared with wild-type bm1 mice (Fig. 2). At 1 wk, P815 cells in GK mice were <b>7-fold</b> more abundant than in wild-type bm1 mice (Fig. 2A). P815 cells had been totally cleared in wild-type mice by 2 wk (Fig. 2A), whereas it required an additional 1–2 wk in GK mice. The rejection of P815 cells in GK mice was indeed mediated by CD8 cells, as depletion of CD8 cells by mAb treatment prevented the elimination of allogeneic tumor cells (Fig. 2B).

CTL induction and allogeneic tumor clearance were delayed in CD4-deficient mice

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Evidence of CD8 cell activation in CD4 cell-deficient GK mice was also found by FACS analysis. Activated CD8 cells down-regulate CD62L expression (21). The proportion of CD62L<sup>low</sup> CD8<sup>+</sup> cell population in spleen was increased after exposure to P815 cells in both wild-type mice and GK mice (Fig. 4B). It was noted that CD8 expression was also down-regulated on the CD62L<sup>low</sup> cells (Fig. 4B). This is not surprising, as activated CD8 cell-independent CTL induction, however, is absolutely dependent on direct costimulation of B7/CD28, whereas CD40/CD40L is involved in both CD4-dependent and CD4-independent pathways.

To test helper activity of GK mice, mice were immunized with 50 mg of alum-precipitated (4-hydroxy-3-nitrophenol) acetyl (NP) conjugated to keyhole limpet hemocyanin (KLH) (NP:KLH ratio 15:1). Serum Abs with high affinity for NP were detected with NP<sub>3</sub>-BSA-coated plates, as described previously (20).

Cell induction and allogeneic tumor clearance were delayed in CD4-deficient mice

To examine whether CD8 CTL to P815 cells could be induced in the absence of CD4 cells, both wild-type bm1 mice and CD4 cell-deficient GK mice were injected i.p. with 5<sup>10</sup> P815 cells. Allogeneic tumor growth was quantitatively monitored by peritoneal leucocytic responses. In bm1 mice, tumor cells were cleared 2 wk after injection. In contrast, tumor clearance was slower in GK mice compared with wild-type bm1 mice (Fig. 2). At 1 wk, P815 cells in GK mice were <b>7-fold</b> more abundant than in wild-type bm1 mice (Fig. 2A). P815 cells had been totally cleared in wild-type mice by 2 wk (Fig. 2A), whereas it required an additional 1–2 wk in GK mice. The rejection of P815 cells in GK mice was indeed mediated by CD8 cells, as depletion of CD8 cells by mAb treatment prevented the elimination of allogeneic tumor cells (Fig. 2B).

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cells have been found previously to down-regulate CD8 (22). Activated CD8 cells were high in CD44 and CD25 expression as well (data not shown).

The above results confirmed that CD8 can be primed into CTL effectors in the absence of CD4 cells, but that CD4 cells were important for optimal CTL induction. One way that CD4 cells can help CTL induction is via IL-2 production. Bulk culture (24 h) of splenocytes from P815-injected mice showed that unlike cells from wild-type bm1 mice that produced substantial amounts of IL-2 (80 pg/ml), cells from GK mice did not produce detectable amounts of IL-2 in vitro (<0.2 pg/ml). Likewise, Ag-specific IFN-γ production by splenocytes after 72-h culture was also only detected in bm1 mice (2.2 ng/ml), but not in GK mice (<0.1 ng/ml). In support of this role of IL-2, addition of exogenous IL-2 improved the response of CD8 cells from our CD4-deficient mice in vitro (see below).

Treatment with blocking anti-CD40L Ab suppressed CTL induction and clearance of allogeneic cells in wild-type and CD4-deficient mice

During cross-priming of CD8 cells, CD40L and CD40 interaction between CD4 cells and APC results in APC activation and then indirectly helps CD8 activation (2–4). In some cases, direct CD40L/CD40 interaction between CD8 cells and APC can occur (23, 24). To examine how CD40L/CD40 interaction contributes to CTL induction to allogeneic MHC Ag, both bm1 mice and GK mice were treated with anti-CD40L Ab, MR1. Two injections of 0.5 mg MR1 resulted in delayed tumor rejection in both bm1 mice and GK mice, although numbers of tumor cells in GK mice were significantly higher than in bm1 mice. At 2 wk after P815 injection, the numbers of P815 cells that accumulated in the peritoneal cavity of MR1-treated wild-type mice and GK mice were 22 and 50 million, respectively (Fig. 5A). As a measurement of in vivo effector function, splenocytes were tested for direct killing immediately ex vivo in a chromium release assay (no in vitro culture). CTL induction in MR1-treated mice was greatly inhibited for both wild-type and GK mice (Fig. 5B). The proportion of CD62Llow CD8 cells was also reduced (Fig. 4B). Furthermore, presumably because of reduced expansion after priming, the percentage of CD8 cells in MR1-treated P815-primed mice (whether wild-type or GK) was one-half that in corresponding untreated P815-primed mice (Fig. 4A). With no significant change in total numbers of splenocytes (all about 1.2 × 10^8), the decreased percentage reflects a decrease in absolute numbers of CD8 cells. We conclude that...
direct CD40L/CD40 interaction between CD8 cells and APC is critical for CTL induction in the absence or presence of CD4 help. Transient MR1 treatment did not induce tolerance in this study. Even in the absence of CD4 cells, in MR1-treated GK mice the tumor cells were cleared by 4 wk (data not shown). It may be that continuous blockade of CD40 and CD40L interaction is required for long-term suppression of CTL induction.

CTLA4Ig prevented CTL induction and clearance of allogeneic cells in CD4-deficient mice, but not wild-type mice

The B7/CD28 costimulation pathway has been studied extensively and represents one of the major pathways for T cell costimulation (25). B7 blockade with fusion protein CTLA4Ig has been shown to inhibit or prevent the rejection of allo- and xenografts (26, 27). However, graft rejection can still occur in the absence of B7/CD28 costimulation (7, 28). We examined in this study an allogeneic tumor model whether CD8 cell activation in the absence of CD4 help is sensitive to costimulation blockade by CTLA4Ig.

We have previously produced mice Tg for CTLA4Ig under the rat insulin promoter (18). Islets from these mice inhibited MLR in vitro (18). In these mice, CTLA4Ig secreted by the pancreatic islets led to circulating CTLA4Ig that could be detected by binding splenocytes, previously stimulated by LPS to up-regulate CD86 (B7-2) (Fig. 6A). Because the detection method could potentially underestimate levels of CTLA4Ig due to saturation of B7 epitopes, serum samples were serial diluted for testing their CTLA4Ig levels. Based on mean fluorescence intensity at sample dilution (1 in 10) that B7 binding was unsaturated, Fig. 6B showed that CTLA4Ig Tg mice and GK/CTLA4Ig doubly Tg mice had a comparably high level of circulating CTLA4Ig before and 2 wk after injection of tumor cells. The levels of CTLA4Ig in Tg mice correspond to 0.1 mg/ml serum.

When mice were challenged i.p. with \( 5 \times 10^6 \) P815 cells, both GK and CTLA4Ig single Tg mice rejected their tumor cells at 2 wk and CD8 cells appeared to be activated (high percentage of CD8 cells were CD62Llow) (Fig. 7). In contrast, GK/CTLA4Ig doubly Tg mice failed to reject the P815 cells; indeed, the P815 cells multiplied to be \( 2 \times 10^8 \) in the peritoneum (Fig. 7A). By about 4 wk, these mice succumbed to the tumor burden. This was in accord with the low numbers of activated CD8 cells (Fig. 7B) and the lack of CTL induction in GK/CTLA4Ig Tg mice (Fig. 8B).

CD8 cells from GK/CTLA4Ig doubly Tg mice can respond in vitro in the presence of IL-2

As shown above, splenocytes isolated from wild-type and GK mice early on (6 days post-P815 injection) contained very few mature CTL effectors, but they did contain CTL precursors that...
matured into effectors after in vitro culture. It is possible that splenocytes from doubly Tg mice were also primed to become CTL precursors, but did not mature into effectors in vivo. To investigate this possibility, splenocytes from primed doubly Tg mice were cultured with irradiated P815 cells and/or IL-2. Addition of exogenous IL-2 (1 ng/ml) was required for CD8 cells to expand, to become killers, and to secrete IFN-γ; there was no response when P815 cells alone were used (Fig. 8).

Discussion
Anti-CD4 Ab Tg mice were used in this study as the model of CD4 cell deficiency. The advantage of the model is that mice lack the aberrant T cell population that is found in MHC class II−/− mice and CD4−/− mice. MHC class II−/− mice have residual CD1-restricted CD4 population (15, 29), and CD4−/− mice have increased numbers of an abnormal population of CD4−CD8−TCRαβ+ cells (13, 30, 31). CD8 cells in CD4−/− mice can also be MHC class II restricted (32). These minor populations can function as helpers, and therefore may represent a problem in interpretation of results, especially in vivo, in which such minor populations can be readily expanded. This may also explain why allograft rejection is found to be delayed in mice that lack the aberrant T cell population.

FIGURE 4. FACS of splenocytes from mice injected i.p. with 5 × 10^6 P815 cells. Some groups of mice also had two i.p. injections of MR1 (0.5 mg each at day 0 and day 3 relative to P815 injection). Splenocytes from P815-primed and unprimed animals were double stained with PE anti-CD4 plus FITC anti-CD8 (A) and PE anti-CD8 plus FITC anti-CD62L (B). The annotated numbers indicate the percentages of a particular population. Activated cells are CD62L^-.

FIGURE 5. Tumor clearance and CTL induction in MR1-treated bm1 and GK mice. Experimental groups were the same as in Fig. 4. Peritoneal cells and spleens were harvested 2 wk after P815 injection. Tumor growth and CTL activity were assayed as in Figs. 2 and 3A. A, Tumor growth; B, direct killing of P815 cells (without in vitro stimulation of splenocytes).

FIGURE 6. A, Binding by Tg CTLA4Ig. Sera were prepared from wild-type bm1 mice (dotted line), CTLA4Ig Tg, or GK/CTLA4Ig doubly Tg mice. B7-binding activity in sera was evaluated by FACS with LPS-stimulated splenic B220^+ cells. B, Estimation of serum Tg CTLA4Ig. Sera from four mice in each group before or 14 days after injection of P815 cells were tested by FACS. Data represent the means and SD of mean fluorescence intensity at 1/10 dilution.
whose CD4 cells are depleted by Ab, but not in MHC class II-deficient mice and CD4-deficient mice (33).

Our results indicate that CD4 cells provide help in the induction of CTL against allogeneic P815 cells. In response to allogeneic cells, requirement of CD4 help for CTL induction may vary with precursor frequency. When there are abundant Ag-specific cells, e.g., in anti-Ld TCR/RAG Tg mice (2C), and thus expansion is less important, optimal CTL response and P815 tumor clearance are achieved without the involvement of CD4 cells (7). When there are fewer cells, e.g., in adoptive transfer experiments into syngeneic mice with only few (4 – 8) million of TCR Tg cells, alloreactive stimulated expansion of the transferred cells was critically dependent on cotransfer of CD4 cells (6). In our study in which H-2d P815 cells are injected into fully mismatched MHC wild-type bm1 mice, the abundance of T cells capable of recognizing alloantigen in recipient mice probably falls between the above two systems. An alloreactive CTL response develops in mice lacking peripheral CD4 cells, and the response is sufficient to clear the tumor cells. However, development of CTL response in the absence of CD4 cells is suboptimal and delayed when compared with wild-type mice, reflecting the need of CD4 help for expansion of activated CD8 cells and optimal CTL response. The delay in CTL induction may have been the reason that CTL were not detected in CD4 cell-depleted mice in an earlier study (5). The results from CD4 cell-deficient mice are reminiscent of CTL responses to some viral infections that are believed to be CD4 independent. For example, although both CD4 cell-deficient and wild-type mice are capable of mounting CTL to clear influenza infection, the numbers of responding CTL precursors were much lower in CD4 cell-deficient mice (34). Similarly, a primary CTL response to acute lymphocytic choriomeningitis virus infection was produced in CD4 cell-deficient mice, and this was sufficient to clear infection. However, in the absence of CD4 cells, the CTL response was not sustained and infection could persist (35, 36). One conclusion that befits all of these findings is that the major contribution of CD4 cell help for optimal CTL induction is facilitating population expansion.

One way that CD4 cells provide help is through the provision of cytokines such as IL-2 (37, 38). In the above 2C T cell adoptive transfer study, in vivo expansion of transferred cells was largely related to the ability of cotransferred CD4 cells to produce IL-2, as

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**FIGURE 7.** A, Tumor clearance and CTL induction in GK mice and GK/CTLA4Ig mice. Mice were i.p. injected with 5 × 10⁶ P815 cells. Two weeks after, cells from the peritoneal lavage cells were thoroughly flushed and harvested. P815 cells were calculated from the total number of peritoneal lavage and the percentage of H-2Dd-positive cells. Data represent the means and SD of three to four mice in each group. Two similar experiments were performed. B, FACS of CTL effectors. Splenocytes were double stained for CD8 and CD62L. Percentages of CD8 cells (inside frame) and CD62Lhi/CD8+ cells (arrow) were calculated and indicated at the bottom of the figure. There were no differences in CD62L expression on CD8 cells among three strains of unimmunized mice.

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**FIGURE 8.** In vitro stimulation of splenocytes from P815-injected GK/CTLA4Ig mice. GK/CTLA4Ig mice were i.p. injected with 5 × 10⁶ P815 cells for 2 wk. Splenocytes were cultured at 5 × 10⁶/ml in 2 ml vol with medium alone, 2.5 × 10⁶ irradiated (200 Gy) P815 cells, 1 ng/ml IL-2 (2.5–7.5 × 10⁶ U/mg; PharMingen, San Diego, LA), or both. Cells were cultured for 5 days. A, Numbers of recovered CD8 cells. Viable cells were harvested, enumerated, and stained for CD8. Data represent the numbers of CD8 cells recovered, calculated from total cell numbers and percentages of CD8 cells. The starting number of CD8 cells put into culture is indicated by an arrow. B, Cytotoxicity of cultivated cells. Viable cells recovered from 5-day cultures were assayed for cytotoxicity to P815 cells. Mean and SD of triplicates for each group are shown. Killing of control EL-4 cells was less than 5% (data not shown). C, IFN-γ production. Supernatants from 5-day cultures were assayed for their IFN-γ levels. The IFN-γ standards used were purchased from PharMingen (0.2–1 × 10⁸ U/mg). Mean and SD of triplicates for each group are shown. Detection limit = 0.3 ng/ml.
CD4 cells from IL-2\(^{-/-}\) mice did not result in the expansion of 2C cells (6). In our study, P815-pulsed bm1 splenocytes that contain both CD4 and CD8 cells produced much more IL-2 than cells from CD4 cell-deficient mice. It is plausible that IL-2 produced by CD4 cells may enhance CTL responses by expansion of activated precursors. Indeed, we have shown that CD8 cells, either naive or primed, proliferate and become effectors in vitro in response to stimulation by P815 allograft and IL-2 (Fig. 8), whereas naive CD8 cells did not respond to allografts from P815 without exogenous IL-2 (data not shown). Although IL-2 can also directly enhance cellular cytotoxicity of CD8 cells as well (39), the amount of IL-2 required for generation of killer activity is 10-fold lower than the amounts of IL-2 required for proliferation (40).

Another pathway in which CD4 cells provide help for CD8 cells is via activation of APCs. CD40/CD40L interaction between CD4 cells and APCs has been demonstrated to be critical for in vivo CD8 priming (2–4), although CD4-independent mechanisms may also exist (41). Ligation of CD40 by an agonist anti-CD40 Ab can restore CD8 CTL activities in CD4\(^{-}\) T cell-depleted mice (2–4). In our study, interruption of CD40/CD40L interaction by anti-CD40L Ab resulted in failure to clear tumor cells and to induce CTL response, notably both in the presence and the absence of CD4 cells. This suggested that in addition to the known role of CD40/CD40L in CTL induction via indirect interaction through APC and CD4 cells (2–4), there can be direct interaction of CD40/CD40L between APC and CD8 cells (23, 24), although in normal circumstances this direct pathway may be masked by the CD4 pathway. As for strong CTL inducers such as lymphocytic choriomeningitis virus, Pichinde virus, or vesicular stomatitis virus, circumstances this direct pathway may be masked by the CD4 pathway.

The development of CTL responses to allogeneic Ags in the absence of CD4 help poses the important question of whether the CD4 cell-independent CTL response is dependent on costimulation. Optimal T cell activation is believed to require two signals: ligation of TCR to MHC/peptide complex (signal 1) and costimulation (signal 2). Several reports have described activation of CD8 cells without the need for costimulation, but this requires high dose or persistent Ag exposure and the use of TCR Tg mice (7, 43–46). Others have also pointed to a critical role of costimulation in induction of CD8 CTL (47, 48). A cogent example is in a syngeneic tumor system, in which without CD4 help, induction of CTL responses was effective, only when the MHC class I\(^{+}\) and class II\(^{+}\) tumor cells were transfected with B7 (47) (although this may still require involvement of B7-expressing host APC (49, 50)). As discussed above for CD8 dependence, these results can be largely reconciled by the premise that lack of costimulation can be compensated for by a very strong signal 1 at the total population level (abundant specific T cells and high Ag dose). In our study, CTL induction to allogeneic tumor cells in the absence of CD4 cells was totally abrogated by B7 being blocked by circulating CTLA4Ig; thus, the allogeneic tumor cells persisted for a long time in our study and indeed mice were overcome by the tumor burden. The main contribution of CD28-mediated signaling is to promote the release of IL-2, which in turn expands activated CTL (51). As already mentioned above, the expansion process that may be less important when the cells are very abundant (preexpanded) such as in TCR Tg. We believe that without using TCR Tg models, our experimental setting is more representative of an allograft into a transplant recipient and that induction of CTL to allogeneic tumor cells in the absence of CD4 cell help is critically dependent on the B7/CD28 costimulation pathway.

Interestingly, CD8 activation is blocked by Tg CTLA4Ig, only when CD4 cells are absent. This is reminiscent of some studies that show that CTLA4Ig is only effective in blocking transplant rejection when host CD4 cells are depleted (52, 53). We do not believe that this is simply due to CD4 cells consuming CTLA4Ig, because we can easily detect free CTLA4Ig in non-CD4-deficient mice. One possible explanation is that in the absence of CD28, costimulation of CD4 cells can be mediated via CD40 (54, 55), and so the lack of B7/CD28 costimulation in CD8 cells is compensated for by this CD4 help.

The current study concludes that generation of allogeneic CD8 effectors can be independent of CD4 help, and that CD8 cell activation without CD4 cell help is more sensitive to costimulation blockade. In our allogeneic system, CD40/CD40L has a role in CTL priming for both CD4-dependent and CD4-independent responses, but B7/CD28 is critical for the CD4-independent pathway only. These influences should be considered in alloimmunity under different transplantation settings in the hope of more successful intervention.

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