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*J Immunol* 1998; 161:1659-1663; http://www.jimmunol.org/content/161/4/1659

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Impaired Alloantigen-Mediated T Cell Apoptosis and Failure To Induce Long-Term Allograft Survival in IL-2-Deficient Mice

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We examined whether IL-2 regulates alloimmunity responses by studying allograft survival in wild-type (IL-2+/+) and IL-2 gene-knockout (IL-2−−/) mice. The acute rejection of vascularized, cardiac allografts and the generation of allospecific CTLs were not impaired in the absence of IL-2. In contrast, blocking the B7-CD28 T cell costimulation pathway with CTLA4Ig induced long-term allograft survival (>100 days) in IL-2+/+ recipients but failed to do so in IL-2−−/ mice or in wild-type mice that had been treated with IL-2-neutralizing Ab around the time of transplantation. Allografts rejected by IL-2−−/ recipients exhibited extensive mononuclear cell infiltrates despite CTLA4Ig administration. In vivo allostimulation in the absence of IL-2 led to exaggerated T lymphocyte proliferation and impaired apoptosis of activated T cells in untreated and CTLA4Ig-treated mice. These findings indicate that endogenous IL-2 is required for the induction of long-term allograft survival, and that IL-2 regulates alloimmunity responses by preparing activated T lymphocytes for alloantigen-induced apoptosis. The Journal of Immunology, 1998, 161: 1659–1663.

IL-2 regulates lymphocyte proliferation, differentiation, and function (1, 2). IL-2 drives the clonal expansion of Ag-activated CD4+ T cells and regulates their differentiation in culture into either IFN-γ- or IL-4-producing Th lymphocytes; it also promotes CTL precursor differentiation and activates NK cells, B lymphocytes, and macrophages.

IL-2 gene-knockout (IL-2−−/) mice have been used along with IL-2α (IL-2α−−/), IL-2β (IL-2β−−/), and IL-2Rα (IL-2Rα−−/) gene-knockout mice to investigate the role of IL-2 in context of the whole immune system (4, 5). Unexpectedly, mice that do not produce IL-2 (IL-2−−/) or those that lack functional high-affinity IL-2Rs (IL-2Rα−−/ and IL-2Rβ−−/) were found to be immunocompetent (6–9). When exposed to normal bacterial flora, these mice displayed lymphoid hyperplasia due to an increased proliferation of mature T and B cells and developed severe autoimmunity that was characterized by hemolytic anemia or inflammatory bowel disease (8–11). These findings suggest that IL-2 plays a dual role in the immune system. On one hand, it has a redundant mitogenic role that can be replaced by other lymphocyte growth factors such as IL-4, IL-7, IL-9, and IL-15. On the other hand, it is critical for limiting immune responses against foreign or self Ags (12–16).

The redundant role of IL-2 in allotolerance is underscored by data showing that IL-2−−/ mice acutely reject pancreatic islet allografts and generate an effective CTL response to allogeneic tumor cells (17). It is not known, however, whether IL-2 contributes to the induction of transplantation tolerance or long-term allograft acceptance by limiting alloimmune responses. To test this hypothesis, we compared vascularized cardiac allograft survival in wild-type (wt) (IL-2+/+) mice with that in IL-2−−/ mice that had been treated with CTLA4Ig, a recombinant fusion protein that blocks the B7-CD28 T cell costimulatory pathway and induces long-term allograft survival in rodents (18–21). We report that IL-2 is essential for the induction of long-term allograft survival. We also provide evidence that IL-2 is critical for limiting alloantigen-induced T cell proliferation in vivo, at least in part by promoting the apoptosis of activated T lymphocytes.

Materials and Methods

Mice
Male C3H/He (H-2k), wt BALB/c (H-2d), and IL-2−−/ BALB/c (H-2d) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) (6). All IL-2−−/ mice were bred at the Emory University/Veterans Affairs Medical Center animal facility in microisolators that had been supplied with sterile food and water (specific pathogen-free environment). Hematocrits of IL-2−−/ mice were checked periodically to ensure the absence of autoimmune hemolytic anemia. The inactivation of IL-2 gene function in these animals was confirmed by performing an IL-2-specific ELISA (Genzyme, Cambridge, MA) on splenocyte supernatants that were collected at 0, 24, 48, 72, and 96 h following Con A stimulation (3 μg/ml).

Reagents used in vivo
Human rCTLA4Ig was provided by Dr. Peter S. Linsley (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA). Neutralizing rat

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0022-1767/98/$02.00
anti-mouse IL-2 mAb was purchased from Genzyme. Reagents were di-
luted in endotoxin-free PBS (Life Technologies, New Haven, CT) and
sterile-filtered before use.

Transplantation procedures
Fully allogeneic (MHC class I- and class II-disparate), vascularized, het-
erotopic (intraabdominal) heart transplantation was performed as described
previously (22) using 8- to 10-wk-old male C3H/He donors and 6- to
8-wk-old male IL-2−/− and IL-2−/−/BALB/c recipients. Mice received 200
μg of human CTLA4Ig i.p. on the day 2 postransplantation or were left
untreated. A separate group of IL-2−/− recipients that had been treated
with CTLA4Ig also received 250 μg of neutralizing rat anti-mouse IL-2 mAb
(Genzyme) i.p. at 2 days before and 4 days after transplantation. Reaction
was identified by a loss of palpable cardiac contractions, at which time the
recipient was sacrificed and the allograft was removed for analysis. Cardiac
allograft survival of >100 days was considered long-term survival. To
determine whether CTLA4Ig induced alloanigen-specific unresponsiveness
(transplantation tolerance), C3H/He and third-party C57BL/6 (H-2b)
(The Jackson Laboratory) full-thickness torso skin was transplanted to IL-2
−/− recipients whose heart allografts were still functioning past the 100-
day mark. Skin rejection was defined as >90% necrosis of the allograft. All
procedures conformed to International Animal Care and Use Committee
standards. A statistical analysis of survival data was performed using the
Mann-Whitney U test.

CTL generation
We injected 6- to 8-wk-old male IL-2−/− and IL-2−/−/BALB/c (H-2b)
mice i.p. on days 0 and 9 with 1 × 107 C3H/He splenocytes suspended in
PBS. Mice were sacrificed on day 13; their splenocytes were enriched for
T lymphocytes by applying to nylon wool columns (Polyscience, War-
nington, PA) (23) and were assayed immediately for allospecific CTL ac-
tivity by incubating with either 1 × 105 Con A-activated C3H/He (H-2b)
splenocytes or P815 (H-2b) target cells (American Type Culture Collection,
Manassas, VA) for 2.5 h (24). Target cells were preloaded with cal-
cein-AM (Molecular Probes, Eugene, OR), and calcein release was mea-
sured in a LS50B luminescence spectrometer (Perkin Elmer, Foster City,
CA) (25). Experiments in which the spontaneous calcein release was
>30% of maximum release were rejected. Allospecific cytotoxic activity
was calculated according to the following formula: Percentage of specific
lysis = 100 × [(test release − spontaneous release)/maximum release −
spontaneous release].

Histopathology
Formalin-fixed, paraffin-embedded cardiac allograft tissue that had been
removed at the time of rejection was stained with hematoxylin and eosin or
periodic acid-Schiff and examined by a pathologist (F.K.B.) who was un-
aware of the identity of the recipients. Acute cellular rejection was graded
according to the criteria of the International Society for Heart Transplantation
(26).

Reverse transcription PCR
Cardiac allograft and spleen tissue that had been resected at time of rejec-
tion was snap-frozen in liquid nitrogen. RNA was extracted in guanidinium
salt solution and purified by the CsCl gradient method (27). A total of 5 μg
of total RNA was reverse-transcribed using oligo(dT) primers and Super-
script reverse transcriptase according to the manufacturer’s instructions
(Life Technologies). Next, 10% of cDNA was subjected to 30 cycles of
PCR amplification in a Perkin Elmer Thermocycler 480 using mouse IL-2,
IFN-γ, TNF-α, and IL-4-specific primer pairs (28). Fifteen percent of each
PCR reaction was electrophoresed on 2% SeaKem LE agarose gels
(American Bioanalytical, Natick, MA) and stained with ethidium bromide.
RT-PCR controls included “no RNA” (blank) and “no reverse transcrip-
tase” reactions.

5-bromo-2′-deoxyuridine (BrdU) labeling of T cells
IL-2−/− and IL-2−/−/BALB/c mice were injected in the footpads with 1 ×
107 C3H/He splenocytes suspended in PBS. Control mice were injected in
the footpads with 1 × 107 syngeneic splenocytes. After 4 days, mice were
pulsed i.p. with 0.8 mg of BrdU (Sigma, St. Louis, MO) and sacrificed after
24 h. Popliteal and inguinal lymph node (LN) cells were isolated and en-
riched for T cells by applying to nylon wool columns (Polysciences). Surface
staining for murine CD4 and CD8 was performed using phyco-
erothrin-conjugated Abs (PharMingen, San Diego, CA). Cells were then
fixed in 70% ethanol followed by 1% paraformaldehyde and incubated with
50 Kunitz/ml of DNase I (Sigma) for 10 min at room temperature
(29). Cells were subsequently stained with FITC-conjugated anti-BrdU Ab

Results
IL-2 is not essential for acute rejection of vascularized
allografts or for in vivo generation of allospecific CTL activity
Steiger et al. previously demonstrated that IL-2−/− mice reject
nonvascularized pancreatic islet cell transplants and generate a
specific CTL response to allogeneic tumor cells (17). To determine
whether IL-2 is essential for the acute rejection of vascularized
allografts, we transplanted C3H/He hearts to untreated IL-2−/−
and IL-2−/−/BALB/c recipients. As shown in Figure 1A, acute
cardiac allograft rejection was not delayed in the absence of IL-2;
the median survival times (MSTs) of hearts transplanted to un-
treated IL-2−/− and IL-2−/− recipients were 6 and 5 days, respec-
tively. IL-2−/−/ and IL-2−/− mice generated significant allospecific
CTL activity when immunized with allogeneic splenocytes; how-
ever, CTL activity was lower in the absence of IL-2 (Fig. 1B).

IL-2 is essential for induction of long-term allograft survival
CTLA4Ig administration on day 2 postransplantation resulted in
long-term cardiac allograft acceptance (>100 days) in 30% of IL-
2−/− recipients but in none of the IL-2−/− recipients (Fig. 2). The
MST of heart allografts was significantly longer in the IL-2−/−/group
(38 days; n = 10) than in the IL-2−/− group (13 days; n =
10) (p = 0.001; Mann-Whitney U test). This finding was con-
firmed by the administration of neutralizing IL-2 Abs to IL-2−/−/
recipients that had been treated with CTLA4Ig. As shown in Figure 2, in vivo neutralization of IL-2 around the time of transplantation blocked long-term allograft survival (MST = 18 days; n = 6) (p = 0.02 when compared with the IL-2+/+ group; Mann-Whitney U test). C3H/He skin transplants were acutely rejected by CTLA4Ig-treated IL-2+/+ recipients that had accepted primary heart allografts, suggesting that donor-specific tolerance was not achieved in this model. Cardiac grafts, however, continued to function despite the rejection of skin grafts, suggesting that the response to skin was directed against skin-specific minor histocompatibility Ags (30). Syngeneic hearts that had been transplanted to IL-2−/− recipients survived indefinitely (Fig. 2), indicating that alloantigen-independent factors did not contribute to early allograft loss in the IL-2−/− group. Moreover, hemolytic anemia was not observed in IL-2−/− recipients. The hematocrit (mean ± SD) of IL-2−/− recipients at the time of allograft rejection (44% ± 4%; n = 7) was comparable with that of IL-2+/+ recipients (43% ± 2%; n = 4).

Cardiac allograft pathology in IL-2−/− and IL-2+/+ recipients

Cardiac transplants were analyzed at the time of heart beat cessation. Allograft pathology in either untreated or CTLA4Ig-treated IL-2−/− mice was characterized by a diffuse and extensive cellular infiltrate consisting of lymphocytes, monocytes, neutrophils, and occasional eosinophils. There was also evidence of vasculitis, myocyte necrosis, interstitial edema, and hemorrhage. These findings were consistent with grade 3 to 4 acute cellular rejection, which was also observed in cardiac allografts that were rejected by untreated IL-2+/+ recipients. Long-term-accepted cardiac allografts removed from CTLA4Ig-treated IL-2+/+ mice revealed interstitial fibrosis and focal mononuclear cell infiltrates that occasionally involved vessel walls. The histopathology of the cardiac allografts rejected by CTLA4Ig-treated IL-2+/+ recipients was similar to that observed in the allografts rejected by untreated mice. An examination of functioning, syngeneic heart grafts that had been removed from IL-2−/− mice at 100 days posttransplantation showed normal myocardial architecture and rare lymphocytes.

Intragraft cytokine mRNA expression

Heart allografts that had been removed from IL-2+/+ and IL-2−/− mice at the time of rejection were analyzed for cytokine expression by nonquantitative RT-PCR. IL-2 mRNA was not detected in allografts that were rejected by either untreated or CTLA4Ig-treated IL-2−/− recipients. In contrast, the expression of other T cell-derived cytokines such as IFN-γ, TNF-α, and IL-4 was comparable with that seen in grafts rejected by IL-2+/+ mice. IL-2 mRNA was not detected in spleen tissue removed from IL-2−/− recipients. The latter finding confirms the absence of IL-2 expression by donor passenger leukocytes that could have migrated to the spleens of recipients.

Increased alloantigen-induced LN T cell proliferation in IL-2−/− mice

IL-2−/− mice display excessive lymphoid hyperplasia upon colonization with normal bacterial flora (10). Therefore, we investigated whether alloantigen exposure also leads to unregulated T lymphocyte proliferation in these mice. In vivo alloregenesis stimulation resulted in a significantly greater percentage of BrdU+ T lymphocyte-enriched LN cells in IL-2−/− mice compared with IL-2+/+ mice (Fig. 3). An exaggerated proliferation of IL-2−/− T cells was detected in both the CD4+ and CD8+ subpopulations (data not shown). CTLA4Ig, which was administered 2 days after alloregenesis stimulation, inhibited BrdU uptake by IL-2+/+ T lymphocytes to the level seen in mice primed with syngeneic cells (Fig. 3). In contrast, CTLA4Ig inhibited IL-2−/− T lymphocyte proliferation only partially (Fig. 3). These data indicate that alloantigen stimulation in the absence of IL-2 leads to deregulated in vivo T cell proliferation.
Impaired alloantigen-induced LN T cell apoptosis in IL-2−/− mice

To investigate the role of IL-2 in regulating the survival of activated T lymphocytes, we compared T cell apoptosis between IL-2+/+ and IL-2−/− mice following repeated, in vivo stimulation with allogeneic spleen cells. The population of TUNEL+ cells observed in the T lymphocyte-enriched LNs of allostimulated IL-2+/+ mice was significantly greater than that observed in allostimulated IL-2−/− mice (Fig. 4). CTLA4Ig treatment increased the relative number of TUNEL+ cells from 9 to 14% of gated cells in allostimulated IL-2+/+ mice (p < 0.05, ANOVA) and from 2 to 4% of gated cells in allostimulated IL-2−/− mice (p < 0.05, ANOVA) (Fig. 4). CTLA4Ig did not increase apoptotic T cells in the LNs of IL-2+/+ or IL-2−/− control mice that had been stimulated with syngeneic spleen cells. These findings indicate that CTLA4Ig enhances the apoptosis of activated but not resting T lymphocytes, and that IL-2-independent pathways also play a role in CTLA4Ig-induced apoptosis.

Discussion

In this study, we observed that the acute rejection of vascularized cardiac allografts and the generation of allospecific CTLs are not impaired in IL-2-deficient mice. In contrast, a postoperative blockade of the B7-CD28 T cell costimulation pathway induced long-term allograft survival in IL-2+/+ recipients but failed to do so in IL-2−/− mice or in wt mice that were treated with IL-2-neutralizing Ab around the time of transplantation. Our results confirm that IL-2 plays a redundant role in acute allograft rejection (17). On the other hand, our findings also suggest a novel and critical role for IL-2 in inducing long-term allograft survival.

Severe autoimmune hemolytic anemia has been described in IL-2−/− and IL-2R−/− BALB/c mice exposed to normal bacterial flora (8–10). Therefore, it can be argued that the early loss of cardiac allografts in CTLA4Ig-treated IL-2−/− mice resulted from alloantigen-independent factors such as generalized autoimmunity. We do not believe that this was the case in our experiments because: 1) IL-2−/− mice were kept in a specific pathogen-free environment and did not develop autoimmune manifestations, 2) the syngeneic heart grafts transplanted to IL-2−/− recipients survived indefinitely and did not exhibit significant lymphocytic infiltrates, and 3) the histopathology of the cardiac allografts removed from IL-2−/− mice was consistent with acute cellular rejection. We also demonstrated that the administration of IL-2-neutralizing Ab to wt recipients at the time of transplantation prevented the induction of long-term allograft survival. This observation strongly suggests that the failure to achieve allograft acceptance in IL-2−/− mice is due to IL-2 deficiency in the adult animal rather than to altered cytokine networks that could have developed in the gene-knockout embryo.

We also observed in this study that in vivo allostimulation in the absence of IL-2 leads to exaggerated LN T cell proliferation and impaired apoptosis of activated T cells. An unregulated expansion of LN T lymphocytes paralleled the extensive cellular infiltrates that were observed in the allografts rejected by IL-2−/− mice. These findings are consistent with previous studies (4, 5). Sadlack et al. observed that IL-2−/− mice that are exposed to normal bacterial flora develop lymphadenopathy, splenomegaly, and severe autoimmunity (10, 11). Enlarged lymphoid organs were characterized by high numbers of activated T and B lymphocytes. Similarly, excessive numbers of activated lymphocytes were found in IL-2Rα−/− and IL-2Rβ−/− mice (8, 9). Lenardo and coworkers demonstrated that IL-2 predisposes mature T lymphocytes to apoptosis following repeated stimulation with nominal Ag in vitro or in vivo (12–14). Others have confirmed these findings by demonstrating that superantigen-induced T cell deletion and Fas-mediated, activation-induced T cell death are impaired in IL-2−/− mice (8, 15, 16). In our study, the exaggerated proliferation of IL-2−/− LN T cells can be partially explained by the reduced apoptosis of these cells. It is possible that IL-2 limits the proliferation of alloreactive T lymphocytes by triggering additional feedback mechanisms. For example, T cell activation up-regulates CTLA4 expression on the cell membrane (31). Subsequently, high-affinity B7-CTLA4 interactions inhibit cell cycle progression (32, 33). Perez et al. demonstrated that CTLA4 engagement is crucial for the peripheral tolerance that is induced by blocking the CD28 T cell costimulation pathway (34).

TCR ligation in the absence of costimulation has been shown to induce T cell anergy in vitro (35). The anergic state is characterized by absent IL-2 production and, in some cases, is reversed by exogenous IL-2 (35). In contrast, we found that the long-term allograft survival induced by a B7-CD28 blockade in vivo depends upon endogenously produced IL-2. This paradox could have resulted from inherent differences between in vivo and in vitro studies. First, a B7-CD28 blockade does not abolish IL-2 production in vivo; however, it does lead to long-term allograft acceptance (30, 36). Second, in addition to T cell anergy, the deletion of alloantigen-specific T cells may constitute another mechanism by which the B7-CD28 blockade induces long-term allograft survival (37). In fact, we observed in this study that CTLA4Ig increases alloantigen-driven T lymphocyte apoptosis. These findings suggest that activation-induced cell death, which is Fas-mediated (38), may contribute to long-term allograft survival. However, we found that CTLA4Ig administration leads to long-term allograft acceptance in gld mice that lack functional Fas ligand molecules (our unpublished observations). Although these data indicate that the actions
of CTLA4Ig are not Fas-mediated, they do not rule out the participation of Fas-independent T cell apoptosis pathways in the induction of long-term allograft survival.

Larsen et al. proposed that the silencing of donor-specific T lymphocytes in mice treated with inhibitors of B7-CD28 and CD40-CD40 ligand interactions is an active process requiring signaling through the TCR, because concomitant cyclosporin A administration resulted in a premature rejection of skin grafts (36). Cyclosporin A suppresses the synthesis of cytokines such as IL-2 and IFN-γ, which are secreted by activated T cells (39). In a previous study, we found that IFN-γ is critical for the long-term allograft survival that is induced by blocking the CD28 and CD40 ligand costimulation pathways (40). In this study, we demonstrated that IL-2 is also critical for achieving long-term allograft survival, which further suggests that tolerance induction is an active process involving T lymphocyte stimulation by alloantigens. These observations are pertinent to testing CTLA4Ig in clinical transplantation, in which the majority of patients are treated with cyclosporin A.

Acknowledgments

We thank Dr. Peter S. Linsley (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA) for providing the rCTLA4g.

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