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Anergic cells induced by the blockade of CD40-CD154 and CD28-B7 costimulatory pathways act as potent immunoregulatory cells *in vitro* and *vivo*

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Background This study was to evaluate whether anergic cells induced by the blockade of CD40-CD154 and CD28-B7 costimulatory pathways can act as potent immunoregulatory cells *in vitro* and prolong cardiac allograft survival after adoptive transfer.

Methods Anergic cells were induced *in vitro* by the addition of anti-CD154 and anti-CD80 monoclonal antibodies (mAbs) to primary MLR (mixed lymphocyte reaction) consisting of BALB/c as responder and C3H as stimulator. Anergic cells were added to a newly formed MLR in assessing the regulatory capacity and antigen specificity of anergic cells. The ability of anergic cells to respond to antigen and/or exogenous recombinant mouse interleukin-2 (rmIL-2) was tested. For *in vivo* studies, anergic cells were intravenously injected into 3.0-Gy γ-irradiated BALB/c mice immediately after heterotopic abdominal cardiac transplantation. To prolong allograft survival, recipient mice injected with anergic cells received rapamycin therapy (1 mg · day^{-1} · kg^{-1}).

Results Anergic cells strongly suppressed the proliferation of naïve BALB/c splenocytes against the original (C3H) stimulator in a dose-dependent manner, but they failed to suppress the proliferation of naïve BALB/c splenocytes against the third-party (C57BL/6J) stimulator. The anergic state was reversed by both original (C3H) stimulator and additional exogenous IL-2. In *in vivo* studies, untreated irradiated BALB/c mice rejected C3H cardiac allografts with a mean survival time of (8.6 ± 1.1) days, whereas those injected with the anergic cells rejected the allografts with a mean survival time of (11.8 ± 1.9) days, which was slightly longer than that of the untreated mice. The protocol based on anergic cells injection plus rapamycin therapy could prolong allograft survival significantly [(29.6 ± 4.4) days].

Conclusions Anergic cells induced by the blockade of CD40-CD154 and CD28-B7 costimulatory pathways can act as potent immunoregulatory cells *in vitro*, and prolong cardiac allograft survival after adoptive transfer in the presence of rapamycin therapy. This procedure might be clinically useful for prolonging allograft survival if optimal protocols are developed.

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Induction of tolerance to allografts in an antigen-specific way is the ultimate goal for clinical organ transplantation. Although the mechanisms responsible for tolerance induction are not completely clear, multiple nonmutually exclusive phenomena have been indicated in the context of transplantation. These include immunological ignorance, induction of nonresponsiveness or anergy, clone deletion, immunological deviation and immunoregulation.[1] Immunoregulatory T cells have been proposed to act via intercellular interactions that are based on competition for antigen-presenting cells (APCs) surface antigens and/or locally produced cytokines. Maintenance of the tolerant state by immunoregulatory T cells might be of clinical importance for long-term graft survival.[2,3] Activation of mature T lymphocytes is a multi-step phenomenon requiring both antigen-specific triggering of the T-cell receptor (TCR) complex on the T cell and additional signaling via costimulation. A key costimulatory signal results from binding the CD28 receptor on T cells with CD80 (B7-1) and CD86 (B7-2) ligands on APCs.[4] More recently, the CD40-CD154 (CD40L) pathway has been shown to attribute to the

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regulation of T-cell activation, both by independently costimulating T cells and at least in part by up-regulating CD80/CD86 molecules on APCs. Combined inhibition of both B7 and CD40 pathways showed a synergistic effect on graft survival in both rodent and primate transplant models.[5,6]

The interaction between TCR and antigen-MHC complex in the absence of these two costimulatory signals induces a state of T-cell anergy *in vitro* as well as *in vivo*. Anergic T cells generated *in vitro* by blocking either of these two costimulatory signals exert immunoregulatory effect on other T cells and act as suppresser cells in *in vitro* systems.[7-9] Furthermore, anergic cells generated *in vitro* by blocking CD28-B7 costimulatory pathway suppress islet allograft rejection after adoptive transfer.[9,10] To our knowledge, however, the regulatory capacity of anergic cells induced by both anti-CD154 and anti-CD80 monoclonal antibodies (mAbs) to suppress alloreactive lymphocyte responses *in vitro* and rejection to solid organ *in vivo* has not been investigated.

In the present study, we elaborated whether anergic cells induced by anti-CD154 and anti-CD80 mAbs in primary mixed lymphocyte reaction (MLR) could suppress a newly formed allogeneic MLR of naïve lymphocytes *in vitro* and prolong cardiac allograft survival after adoptive transfer in the presence of rapamycin therapy.

METHODS

Animals

Ten-week-old BLAB/c ($H\text{-}2^d$), C3H ($H\text{-}2^k$) and C57BL/6J ($H\text{-}2^b$) male mice were purchased from the Experimental Animal Center of the Chinese Academy of Science, Shanghai and were kept in a specific pathogen-free mouse facility. For MLR, splenocytes from BLAB/c and C3H mice were used as responder or stimulator cells, respectively. Splenocytes from C57BL/6J mice were used as third-party stimulator cells. For cardiac transplantation, BLAB/c and C3H mice were used as recipients and donors, respectively.

Preparation of splenocytes

The spleen was aseptically removed and mechanically teased in 10 ml of RPMI1640 (GibcoBL Company, USA). Cell suspensions were filtered through 300 μm nylon mesh, and then treated with NH4Cl/Tris buffer to remove red blood cells. Thereafter, cells were washed three times and prepared for *in vitro* culture in complete medium of RPMI1640 containing 10% fetal bovine serum.

Effect of mAbs on MLR *in vitro*

Responder cells and stimulator cells (4×10^5 each) were placed into each well of 96-well round-bottom plates in 200 μl of complete medium in the presence of various concentrations of functional blocking anti-CD154 (clone MR1, BD PharMingen Company, USA) and/or anti-CD80 mAbs (clone 16-10A1, BD PharMingen Company, USA). After a 4-day incubation at 37℃ in a humidified 5% CO_2 atmosphere, the cells were pulsed with 1 μCi/well 3H-TdR (China Atomic Energy Research Institute), and proliferation was measured during the last 6 hours. The cells were harvested onto glass filter paper and analyzed by a scintillation counter (Model LS6500, Beckman Company, USA).

Generation of anergic cells

To obtain anergic cells, 4×10^6 BALB/c splenocytes were cocultured with 4×10^6 C3H splenocytes in the presence of anti-CD154 and anti-CD80 mAbs (10 μg/ml, optimal inhibitory concentration based on above MLR) in a 12-well flat-bottom plate containing 3 ml of complete medium. After a 5-day culture, the cells were harvested, washed and allowed to recuperate in fresh medium without mAbs for 2 days. Viable cells were obtained by discontinuous density gradient centrifugation using lymphocyte-M (Cedarlane Lab, Canada). Control cells, cultured without mAbs, were obtained from the same procedure.

Proliferation assays

The proliferative capacity of these cells was evaluated by various proliferation assays to assess the anergy state. In some experiments, anergic or control cells (2×10^5/well) were restimulated with 20-Gyγ-irradiated C3H splenocytes (2×10^5/well) for 4 days, in some, anergic or control cells (2×10^5/well) were cultured with recombinant mouse IL-2 (rmIL-2, R&D Company) for 2 days, and still in some experiments, anergic or control cells (2×10^5/well) were restimulated with 20-Gyγ-irradiated C3H splenocytes (2×10^5/well). After a 4-day culture, these cells were incubated with rmIL-2 for 2 days to observe the reversal of the anergy state. ^{3}H-TdR incorporation of all experiments was measured during the last 6 hours and all tests were performed in triplicate.

Immunoregulatory potential of anergic cells

The regulatory capacity of anergic cells was analyzee in a newly formed MLR. In 96-well round-bottom plates, different concentrations of anergic or control cells were added to a newly formed MLR consisting of both original BLAB/c responder and C3H stimulator splenocytes (2×10^5/well each) to determine the immunoregulatory potential. Antigen specificity of the regulatory

phenomenon was examined in cocultures performed with third-party C57BL/6J stimulator splenocytes. Different concentrations of anergic cells were added to a newly formed MLR consisting of naïve BALB/c responder splenocytes against original C3H or third-party C57BL/6J stimulator (2×10^5/well each). ^{3}H-TdR incorporation of all tests was measured during the last 6 hours and all tests were performed in triplicate.

Transplantation and treatment protocols

All recipient mice were irradiated at a dose of 3.0 Gy in order to accommodate the adoptively transferred cells. According to the operation method described by Hancock et al,[10] heterotopic abdominal cardiac allografting was done with the use of C3H donors and 3-Gyγ-irradiated BALB/c recipients. Anergic cells generated *in vitro* using the method described above were washed twice, and viable cells were obtained by discontinuous density gradient centrifugation using lympholyte-M (Cedarlane Laboratory, Canada). Anergic cells (4×10^7) were intravenously injected into the recipient mice immediately after transplantation. Some recipient mice received rapamycin therapy (1 mg · day^{-1} · kg^{-1}, beginning on 1 day after transplantation), and if the transplanted mice survived beyond 14 days, the therapy was stopped. Recipient mice were divided into the following groups: untreated irradiated mice; mice injected with anergic cells (4×10^7); mice given rapamycin (Wyeth Pharmaceutical Company, USA); mice injected with control cells (4×10^7) plus rapamycin; mice injected with anergic cells (4×10^7) plus rapamycin.

Statistical analysis

All *in vitro* experiments were repeated three times. Data were expressed as mean ± standard deviation. Graft survival in different experimental groups was compared using the log-rank test. Student's *t* test was used for comparison of paired and unpaired measurements. All statistical calculations were performed by SPSS10.0 software.

RESULTS

Combination of anti-CD154 and anti-CD80 mAbs inhibits lymphocyte proliferation in primary MLR

Monoclonal antibodies directed against CD154 and/or CD80 ligands were tested in the polyclonal primary MLR to study their applicability to the induction of anergy. In primary MLR, anti-CD154 mAb alone produced suppression in a dose-dependent fashion, whereas a combination of anti-CD154 and anti-CD80 mAbs inhibited lymphocyte proliferation more significantly, especially at a high concentration (Fig. 1). The proliferative response was strongly inhibited in the presence of 10 μg/ml each of two mAbs, so these concentrations of two mAbs were used to induce anergic cells.

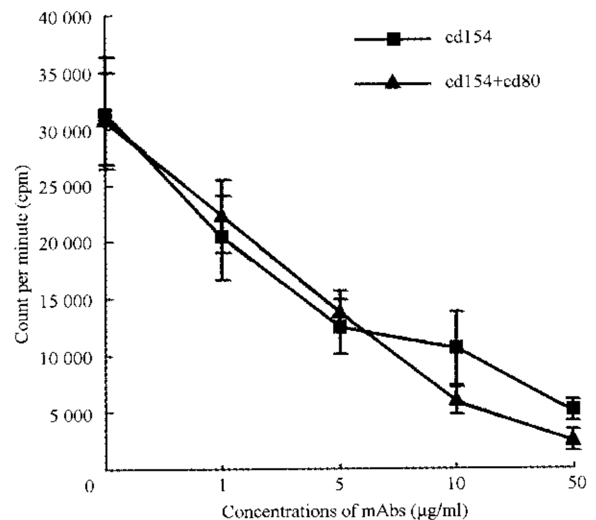

Fig. 1. Effect of anti-CD154 and/or anti-CD80 mAbs on primary MLR.

Induction of anergic cells in primary MLR by combinated anti-CD154 and anti-CD80 mAbs

Primary MLRs were performed in the absence (control cells) or presence of mAbs. Viable cells were harvested and restimulated with irradiated original stimulator cells (C3H). Control cells responded with secondary proliferative kinetics, whereas those from the mAbs-blocked MLR were hyporesponsive (Fig. 2). The failure to proliferate was accompanied by a seriously impaired IL-2 production which was detected in the culture supernatants by ELISA (R&D Company, (64.0 ± 15.3) vs (631.8 ± 108.0) pg/ml, $P<0.01$). The presence of mAbs in the primary MLR might explain the hyporesponsiveness. To exclude this possibility, culture cells were harvested after 5 days, and allowed to recuperate in fresh medium without mAbs for 2 days. Furthermore, hyporesponsiveness was not due to deletion because antigenic restimulation in the presence of exogenous IL-2 led to a response of anergic cells (Fig. 2). Thus, combined mAbs blocking of the costimulatory ligands in the primary polyclonal MLR induces genuine anergy, which is not the result of cell death.

Reversion of the anergic state by antigen restimulation and exogenous recombinant mouse interleukin-2 (rmIL-2)

After anergy induction, the original (C3H) antigen

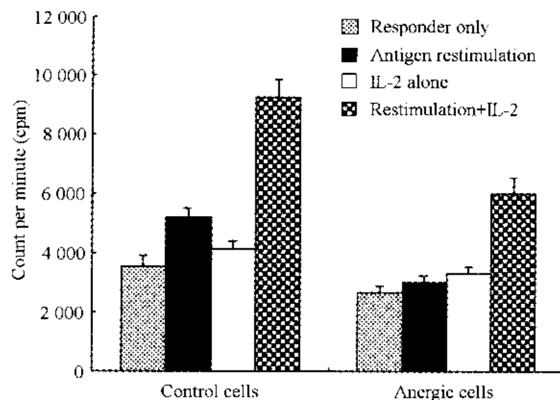

Fig. 2. Reversal of anergic state by antigen restimulation and exogenous IL-2. The anergic or control cells were restimulated with 20-Gyγ-irradiated C3H splenocytes for 4 days (group antigen restimulation) or without restimulation (group responder only). The anergic or control cells were cultured with recombinant mouse IL-2 for 2 days (group IL-2 alone). The anergic or control cells were restimulated with 20-Gyγ-irradiated C3H splenocytes. After a 4-day culture, these cells were incubated with rmIL-2 for 2 days to observe the reversal of the anergy state (group restimulation + IL-2).

restimulation in the presence of exogenously added IL-2 was consequently analyzed, showing the proliferation of anergic cells after antigenic restimulation either with or without of exogenously added IL-2 and their response to IL-2 alone (Fig. 2). The presence of both antigen and IL-2 resulted in reversal of the anergic state, while only a slight response was observed with either antigen or IL-2 alone. This indicates that anergic state can be reversed only if both antigen restimulation and exogenous IL-2 are present at the same time.

Anergic cells exert alloantigen-specific suppressive effect on primary MLR via infectious tolerance

To test whether these anergic cells had an immunoregulatory effect on proliferative responses of naïve responder cells to the original stimulator cells, anergic cells were added to a newly formed MLR in assessing the regulatory capacity. Proliferative response of naïve BALB/c splenocytes to C3H stimulator cells was inhibited by the addition of anergic cells in a dose-dependent manner. In contrast, no such suppressive effect was observed when control cells without mAbs treatment were added to the culture (Fig. 3). Antigen-specificity of this suppressive phenomenon was investigated in cocultures using original responder cells (BALB/c) and third-party stimulator cells (C57BL/6J). As shown in Fig. 4, the third-party MLR was hardly affected by anergic cells.

Effect of anergic cells on cardiac allograft survival

To test whether anergic cells generated *in vitro* could

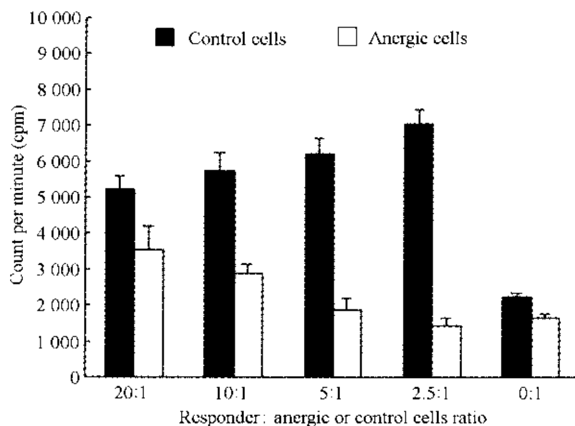

Fig. 3. Suppressive effect of anergic cells on a newly formed MLR. The anergic or control cells were added to the primary MLR, consisting of fresh C3H splenocytes as the responder and BALB/c splenocytes as stimulators, at the indicated responder/anergic or control cells ratio.

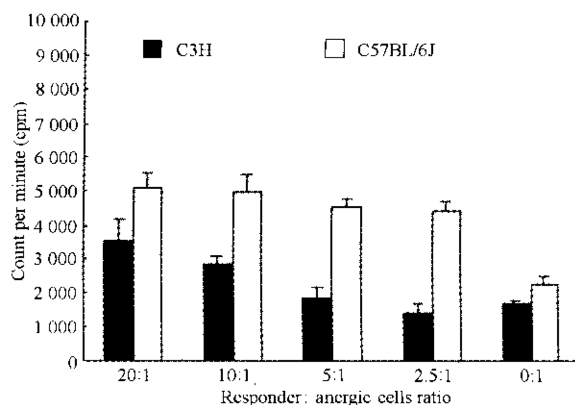

Fig. 4. Alloantigen-specific anergic cells. The anergic cells were added to the primary MLR, consisting of fresh C3H splenocytes as the responder and BALB/c or C57BL/6J splenocytes as stimulators, at the indicated responder/anergic cells ratio.

prevent allograft rejection *in vivo*, the anergic cells were injected intravenously into 3-Gy irradiated BALB/c mice immediately after transplantation of C3H cardiac allograft. Untreated irradiated animals showed acute rejection of the allografts with a mean survival time (MST) of (8.6 ± 1.1) days. Animals injected with anergic cells showed a MST of (11.8 ± 1.9) days, which was slightly longer than that of untreated animals. To prolong cardiac allograft survival, recipient mice injected with anergic cells received rapamycin therapy. It was found that injection of anergic cells plus rapamycin therapy could prolong allograft survival more significantly [(29.6 ± 4.4) days, $P < 0.01$], but they failed to lead to permanent survival of allograft. Interestingly, allograft

rejection in the mice injected with control cells plus rapamycin therapy [(9.6 ± 0.9) days] occurred earlier than in those mice with rapamycin therapy alone [17.2 ± 1.9) days, $P < 0.01$], indicating that control cells without antibody induction accelerated rejection (Fig. 5)

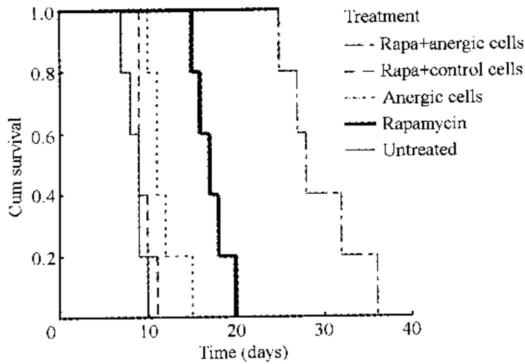

Fig. 5. Effect of anergic cells on cardiac allograft survival shown by the log-rank test; recipient mice received anergic cells injection + rapamycin therapy could prolong cardiac allograft survival ($P < 0.01$, versus group untreated or group anergic cells by the log-rank test). Allograft rejection in the mice injected with control cells + rapamycin therapy occurred earlier ($P < 0.01$, versus group rapamycin therapy by the log-rank test).

DISCUSSION

In the present study, proliferation of alloreactive lymphocytes in primary MLR was completely blocked by the combination of anti-CD154 and anti-CD80 mAbs. These anergic cells had an alloantigen-specific immunoregulatory function because they suppressed proliferation of naïve lymphocytes in response to the same stimulator cells in a dose-dependent manner. These anergic cells adoptively transferred to cardiac allograft recipient in the presence of rapamycin therapy were able to prolong graft survival. These results indicate that anergic cells induced by the blockade of CD40-CD154 and CD28-B7 costimulatory pathways can be successfully used as immunoregulatory cells to prolong allograft survival and might be a putative tool for alloantigen-specific adoptive immunotherapy in transplant medicine.

In this study, cells rendered anergic by the addition of anti-CD154 and anti-CD80 mAbs could suppress the proliferation of naïve lymphocytes when they were added to primary MLR against the same stimulator cells. Waldmann[11] initially proposed the idea of "infectious tolerance" by the experiments on the suppressive ability of anergic T-cell clones induced by anti-CD4 plus anti-CD8 mAbs. In some later experiments, it has been shown that alloantigen-stimulated PBL or PBMC, rendered anergic by blocking the CD28-B7 or CD154-CD40 costimulatory pathway, exhibited suppressor activity in subsequent MLR.[8,12,13] Although both the source of cells and the methods of anergy induction were different between their and our experiments, our present data were consistent with their findings. These results suggest that anergic cells are not inert, but may act as immunoregulatory cells via infectious tolerance.

Several hypotheses have been put forward to explain the mechanism by which anergic cells regulate immune responses. One hypothesis involves that anergic cells might simply compete for space or ligands on the antigen-presenting cells (APCs), so that any further interactions with T cells are compromised, resulting in a suboptimal activation and tolerance induction in naive or primed T cells. Another possibility is that anergic cells may make specific cytokines like IL-4 and IL-10 that interact directly with any non-tolerant T cells. A third alternative hypothesis is that anergic cells may express surface molecules that might modulate APCs function via RANK/RANKL or CTLA-4/B7 so that the APCs then present antigen to all further T cells in a tolerogenic context, for example without appropriate costimulation.[3,14]

Although the tolerance and associated suppression are dependent on specific antigen, they also interfere with responses to third-party antigens if they are present on the same MHC moleculea process known as linked suppression.[3] In our experiments, the anergic cells failed to suppress naive responder cells directed toward third-party stimulators, because third-party stimulators did not share the same MHC molecule expressed on the APCs with the original stimulator used for anergy induction. However, specificity of the response in this kind of system is not always easy to confirm because linked recognition might also lead to third-party tolerance via minor antigens.[14]

Recovery of the proliferative response of anergic cells by exogenously added IL-2 was previously demonstrated in distinct experimental settings. Either anergic T cells were first restimulated and subsequently left in culture medium with exogenously added IL-2,[8,12] as in our experiments, or alternatively, anergic cells were directly restimulated with IL-2.[9] We found that anergic cells were recovered by antigen and exogenous IL-2 restimulation. The mechanisms why in some experiment antigenic restimulation was unnecessary to reverse the anergic state remained unclear. Differences in the source of T cells (human vs. murine) and antigens might account for the different results. However, this suggests that combined

mAbs blocking of the costimulatory ligands induces anergy, which is not the result of cell death, and reveals the importance of the absence of costimulation in this type of anergy.

It has been shown that injection of anergic cells induced *in vitro* plus recipient irradiation (2.5 Gy) could suppress the rejection of islet allografts and lead to permanent survival *in vivo*.[9] Because of the different mechanisms between solid organ transplantation and cell transplantation, we observed anergic cells slightly prolonged cardiac allograft survival of irradiated recipient mice in our model. We consider that the function of anergic cells is a dynamic process and postoperative IL-2 produced at the transplant site might reestablish the proliferative capacity of the anergic cells as demonstrated *in vitro*. It is possible that anergic cells function gradually under the cover of peritransplant immunosuppressive therapy, which can suppress the production of IL-2 or block the function of IL-2. Cyclosporine A and FK506, which completely block TCR-triggered calcineurin activation, are totally incompatible with costimulation blockade in inducing anergic cells. But rapamycin, which blocks cell cycle progression and the cytokine-driven signaling pathways by the binding of IL-2 and IL-2R, facilitates tolerance induction.[15,16] So we tested whether anergic cells injection plus rapamycin therapy could prolong cardiac allograft survival of irradiated recipients. It was found that anergic cells injection plus rapamycin therapy had synergistic effects that could prolong allograft survival significantly. Although we need to develop optimal protocols for permanent engraftment, these results suggest that anergic cells play an active immunoregulatory role on specific tolerance induction after transplantation.

In conclusion, the present results indicate that anergic cells induced by the blockade of CD40-CD154 and CD28-B7 costimulatory pathways act as potent immunoregulatory cells *in vitro*, and prolong cardiac allograft survival after adoptive transfer in the presence of rapamycin therapy. The use of anergic cells might be useful as a new strategy for prolongation of allograft survival in clinical transplantation.

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