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Cutting Edge: Recipient MHC Class II Expression Is Required to Achieve Long-Term Survival of Murine Cardiac Allografts After Costimulatory Blockade[1]

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To study the role of the direct and indirect pathways in achieving tolerance, we used genetically altered mouse strains in two ways: 1) MHC class II-deficient mice were used as donors of skin and cardiac grafts to eliminate the direct $CD4^+$ T cell response, and 2) B6 II^-4^+ mice, which are MHC class II-deficient mice expressing an MHC class II transgene only on thymic epithelium, were used as recipients of normal grafts. These mice cannot mount an indirect response. Eliminating the indirect pathway actually made it more difficult to achieve prolonged allograft survival when we used costimulatory blockade than when both pathways were available. Costimulatory blockade was ineffective even when $CD4^+$ T cells from normal animals were transferred into recipients that lacked MHC class II molecules. These results suggest that an active $CD4^+$ response through the indirect pathway is necessary for costimulatory blockade to be effective in prolonging allograft survival. ***The Journal of Immunology,*** **2001, 167: 5522–5526.**

Previous studies have shown that alloantigens can be recognized by host T cells through either the direct or indirect pathways (1–4) and that $CD4^+$ T cells activated through either the direct pathway alone (5) or the indirect pathway alone (6) can mediate skin graft rejection in the absence of immunosuppression. However, the relative strength of each of these pathways in the setting of immunosuppression, and their relative susceptibility to tolerance induction is uncertain. Therefore, we initiated a series of experiments to determine which pathway of graft rejection would be more easily suppressed.

The study of graft rejection in the absence of a direct pathway for $CD4^+$ T cell stimulation was made possible by the generation of class II-deficient mice. These mice can be used as donors, which leaves only the indirect pathway available for the activation of recipient $CD4^+$ T cells. More recently, the insertion of a class II transgene that is expressed only on thymic epithelium into the class II-deficient mice has generated animals with normal numbers of $CD4^+$ T cells but no class II molecules on their APCs. These mice can be used as recipients to study rejection when only the direct pathway is available for $CD4^+$ T cell activation. Using these genetically modified mouse strains, we tested the effectiveness of costimulatory blockade for prolonging allograft survival when only one or the other pathway was available.

Materials and Methods

Animals

Normal mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice lacking MHC Ags (II^-) and mice expressing MHC class II Ags only on their thymic epithelium (II^-, 4^+) were bred in our laboratories.

The generation and phenotype of mice lacking MHC class II Ags have been previously described (7). For these experiments, mice from the thirteenth generation backcross of $(B6 \times 129)F_1$ to B6 mice were used.

Mice expressing MHC class II Ags only on their thymic epithelium were generated by breeding class II-deficient mice with a B6-transgenic strain, 36.5 (the gift of Dr. D. Lo, The Scripps Research Institute, La Jolla, CA), which expresses the Eα transgene only on thymic epithelium (8). These mice are class II deficient on all cells other than thymic epithelium. They have normal numbers of peripheral $CD4^+$ cells, which proliferate in an allogeneic MLR in vitro (9).

Skin grafting

Trunk skin grafts were placed on the lateral thoracic area (10). Rejection was recorded when there was >90% destruction of the tissue.

Heterotopic heart transplantation

Vascularized heart grafts were transplanted using microsurgical techniques essentially as described by Corry et al. (11).

Reagents, Abs, and in vivo T cell depletion

Cyclosporin A (CsA)[3] (Sandimmune; Novartis Pharmaceuticals, East Hanover, NJ), 35 mg/kg/day, was administered subcutaneously in olive oil started on the day of transplantation for 14 days. MR1, a hamster mAb specific for murine CD154 (Bioexpress Cell Culture Services, West Lebanon, NH), and a control purified hamster Ig (ICN Pharmaceuticals, Aurora, OH), were administered i.p. (250 μg) at the time of transplantation (12). The fusion protein murine CTLA4Ig (a kind gift of Dr. R. Peach from

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[3] Abbreviations used in this paper: CsA, cyclosporin A.

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Bristol-Myers Squibb, Princeton, NJ) and a control Ig, murine mAb L6 (Oncogene, Seattle, WA), were administered i.p. (250 μg) 2 days after transplantation (13). Anti-CD4 (GK1.5; rat anti-mouse CD4) and anti-CD8 (2.43; rat anti-mouse CD8) ascites were prepared from hybridomas obtained from the American Type Culture Collection (Manassas, VA). $CD4^+$ T cells and $CD8^+$ T cells were depleted using the GK1.5 and 2.43, respectively. All treated mice received 0.1 ml i.p. of unpurified ascites (roughly equivalent to 100 μg of purified Ab) on -6, -3, and -1 days before and twice a week after transplantation until graft rejection (14, 15).

Statistics

Kaplan-Meier survival graphs were constructed, and the log-rank comparisons of the groups were used to calculate p values. Differences were considered to be significant at $p < 0.05$.

Results

Costimulatory blockade does not achieve prolonged cardiac allograft survival in recipients lacking MHC class II molecules

A series of murine cardiac allografts were transplanted into MHC-mismatched recipients using anti-CD40L Ab (MR1), CTLA4-Ig, or both reagents together for immunosuppression. Mice from normal strains were used as donors and recipients and, in addition, mice from genetically altered strains lacking either donor or recipient MHC class II molecules were used to eliminate either the direct or indirect pathway for $CD4^+$ T cell activation. In addition, some recipients were treated with anti-CD8 Ab so that any rejection observed would clearly depend on the $CD4^+$ T cell response.

As shown in Fig. 1, elimination of the indirect pathway made it more difficult to achieve prolonged graft survival using any of the costimulatory blocking combinations. Not only was graft survival shorter in the absence of the indirect pathway than in the absence of the direct pathway, but graft survival was also shorter in the absence of the indirect pathway than when both pathways were available. Thus, contrary to our initial expectation that eliminating one or the other pathway of alloreactivity might make it easier to prolong survival, eliminating the indirect pathway actually made it harder.

Costimulatory blockade was also less effective in the absence of the indirect pathway for prolonging skin graft survival

To determine whether the results obtained in Fig. 1 would also be obtained for a different type of transplant, skin grafts were performed using combined costimulatory blockade in the same strain combinations. All of the recipients were treated with anti-CD8 Ab to ensure that rejection depended on $CD4^+$ T cells. As shown in Fig. 2, survival was again shortest in recipients that lacked an indirect pathway, and it was significantly shorter than in the recipients that could generate both a direct and an indirect response.

There was no evidence for humoral immunity as the cause of the faster graft rejection in the mice lacking an indirect pathway

Although the previous experiments included treatment with anti-CD8 Ab, they did not eliminate the possibility of an unusual humoral mechanism of rejection in the recipients lacking MHC class II Ags. Therefore, alloantibody production was measured in the various types of recipients of cardiac allografts. No alloantibody response was detected in mice lacking the indirect pathway, whether or not costimulatory-blocking agents were administered (data not shown). High levels of donor alloantibody were detected in normal mice after cardiac allograft rejection, and these levels were diminished after treatment with CTLA-4Ig and eliminated by treatment with anti-CD40L. Thus there was no evidence for an unusual humoral mechanism to explain the rejection by recipients lacking MHC class II molecules after treatment with costimulatory-blocking agents.

The recipients lacking MHC class II molecules are not inherently more resistant to immunosuppression

Takeda et al. (16) and Kirberg et al. (17) have shown that $CD4^+$ cells do not survive for long periods of time in vivo in the absence of self class II molecules. Thus it is likely that $CD4^+$ cells in the II^-4^+ mice turn over rapidly and represent recent thymic emigrants. Therefore, it was possible that the $CD4^+$ cells in these mice are inherently more resistant to immunosuppression than the cells in normal animals. To address this possibility, skin grafts were performed in the same strain combinations, but the immunosuppression was changed to suboptimal doses of CsA coupled with anti-CD8 Ab treatment. As shown in Fig. 3, the trend in the susceptibility to this immunosuppressive protocol was the opposite of that shown with costimulatory blockade. Mice lacking an indirect pathway showed more prolonged graft survival than mice with both pathways, and it was easier to suppress mice lacking an indirect response than mice lacking a direct response. Thus there is no generalized resistance to immunosuppressive therapy on the part of $CD4^+$ cells in the II^-4^+ mice.

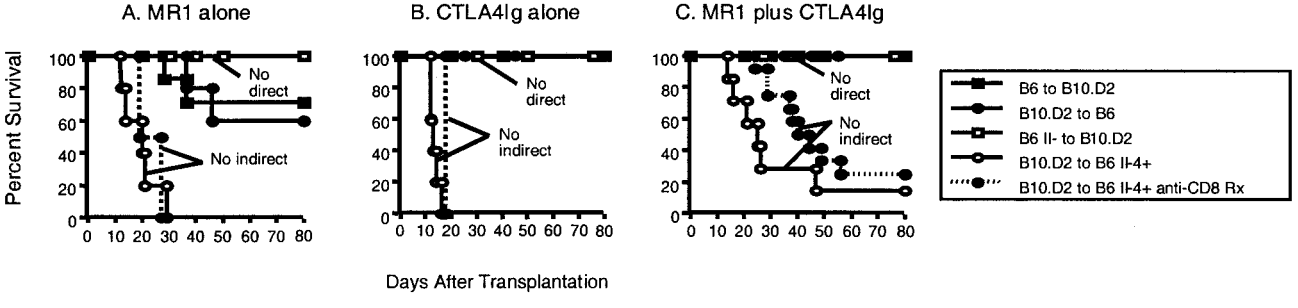

FIGURE 1. Costimulatory blockade is less effective when the indirect pathway is lacking for allogeneic cardiac transplantation. Costimulatory blockade was achieved with MR1 alone (*A*), CTLA4-Ig alone (*B*), and both reagents (*C*) according to the doses and schedule described in *Materials and Methods*. Groups included four to six mice except for those with anti-CD8 therapy, which included two mice per group (*A* and *B*) and seven mice per group (*C*). Cardiac allograft survival for recipients lacking an indirect pathway was significantly shorter than for other recipients in all cases ($p < 0.05$). The mice with long surviving cardiac allografts shown in Fig. 1, which did have an intact indirect pathway, did not turn out to demonstrate robust tolerance. Although their cardiac allografts survived for many more weeks, when the experiments were allowed to continue, subsequent donor skin grafts showed only moderate (about 1 wk) specific prolongation of survival. About half of the recipients that rejected donor skin grafts went on to reject their hearts over the next several weeks, while the remainder kept their cardiac allografts despite the skin graft rejection (data not shown).

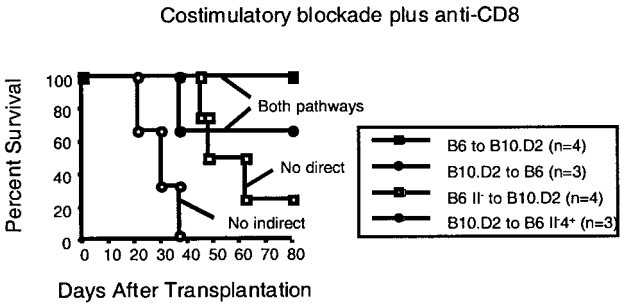

FIGURE 2. Costimulatory blockade is less effective when the indirect pathway is not available for allogeneic skin graft rejection. All of the recipients were treated with combined costimulatory blockade and anti-CD8 Ab according to the doses and schedule described in *Materials and Methods*. Skin graft survival on recipients without class II Ags was significantly shorter than when both pathways were available ($p < 0.05$).

$CD4^+$ cells from II^-4^+ mice are equally susceptible to costimulatory blockade in vitro and normal $CD4^+$ cells are resistant to costimulatory blockade in vivo in the absence of recipient MHC class II molecules

Another possibility for the findings shown above is that $CD4^+$ cells from II^-4^+ recipients are selectively resistant to costimulatory blocking agents compared with cells from normal mice. MLRs were therefore performed with both types of $CD4^+$ cells, and inhibition was attempted with anti-CD40L Ab and/or CTLA4-Ig. In these strain combinations, both types of T cell responses were equally susceptible in vitro to inhibition by CTLA4-Ig while neither was susceptible to inhibition by anti-CD40L Ab (data not shown). Thus there did not appear to be a selective resistance by $CD4^+$ cells from II^-4^+ mice to costimulatory blockade.

An additional experiment was performed in vivo to determine whether $CD4^+$ cells from normal animals would be susceptible to costimulatory blockade if they were transferred into recipients that lacked MHC class II molecules. Three types of hosts were used for these adoptive transfer experiments: normal mice, II^-4^+ mice, and ordinary class II knockout mice. In each case, the hosts were depleted of $CD4^+$ and $CD8^+$ cells by Ab treatment and then were given CD8-depleted T cells from normal mice and treatment with costimulatory blockade. As shown in Fig. 4, cardiac allografts survived indefinitely with costimulatory blockade on normal mice that received the adoptive transfer of normal T cells, but they were rejected by both types of recipients that lacked their own class II molecules. These results indicate that it is not the nature of the $CD4^+$ T cells in II^-4^+ mice that make them resistant to costimulatory blockade, but rather the absence of the recipient class II molecules (and thus the capacity to mount an indirect response) that allows graft rejection. Therefore, it appears that costimulatory blockade requires the induction of an active alloimmune indirect response in the recipient to achieve prolonged graft survival.

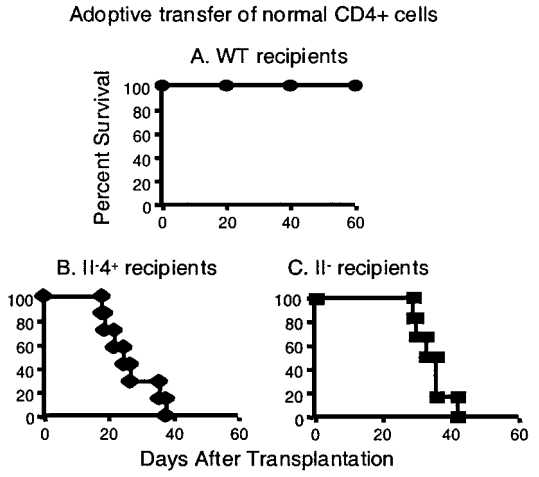

FIGURE 4. Costimulatory blockade is not effective when normal $CD4^+$ cells are transferred into recipients lacking MHC class II molecules. All recipients were treated with both anti-CD4 and anti-CD8 mAb 2 wk before the transplantation (0.1 ml i.p. of ascites for 3 days), and some of the recipients were thymectomized (two per group) 3 wk before the transplantation. CD8-depleted spleen cells (100×10^6) from wild-type B6 mice were transferred intravenously into B6 wild-type recipients ($n = 4$) (*A*), B6. II^-4^+ recipients ($n = 7$) (*B*), or B6. II^- recipients ($n = 6$) (*C*) on the day of cardiac transplantation from B10.D2 donors. All recipients were then treated with MR1 plus CTLA4-Ig combined with anti-CD8 mAb as described in *Materials and Methods*.

Discussion

The striking finding from these experiments is that removing one of the pathways of alloreactivity actually makes it more difficult, not easier, to achieve prolonged allograft survival using costimulatory blockade. This was not the result that we anticipated when these studies were initiated and thus we considered spurious explanations for our findings.

One possible explanation for the results was that some unusual feature of the II^-4^+ mice (the recipients lacking the indirect pathway) made them capable of rejecting heart grafts by alternative mechanisms that are resistant to costimulatory blockade. We examined this possibility by looking for B cell or CD8-dependent mechanisms to account for the rejection by these recipients. However, our experiments indicate that the resistance to costimulation does involve the $CD4^+$ cells in these animals. We also considered

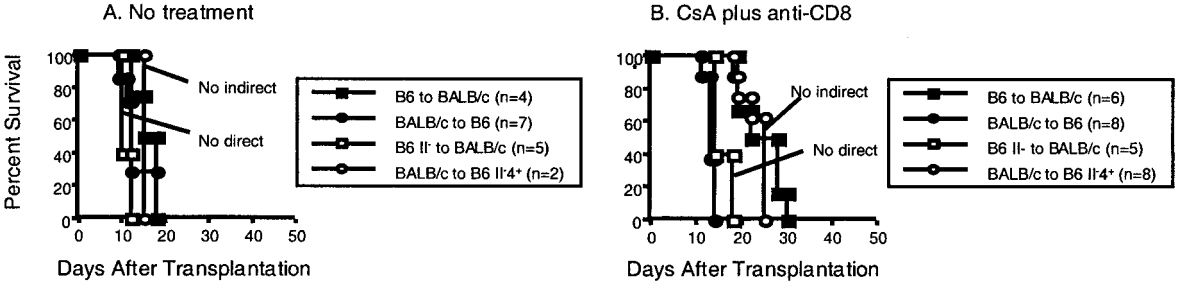

FIGURE 3. CsA immunosuppression is at least as effective in recipients lacking an indirect pathway as in other mice. Recipients were given no treatment (*A*) or treatment with CsA and anti-CD8 Ab (*B*) as described in *Materials and Methods*. Skin graft survival was significantly prolonged on II^-4^+ recipients receiving treatment compared with normal B6 recipients, with our without treatment ($p < 0.05$), while survival of skin grafts lacking MHC class II molecules on normal recipients was not prolonged by treatment.

the possibility that the $CD4^+$ cells in these mice are different from those in normal animals in some way that makes them resistant to immunosuppressive treatment. However we found that even normal $CD4^+$ cells transferred into recipients lacking MHC class II molecules are resistant to costimulatory blockade. When performing this experiment we were concerned that the $CD4^+$ cells that were adoptively transferred into the class II-negative recipients might not survive for long enough in this environment. However, the outcome was that rejection did occur after this transfer, and thus adequate survival was not the problem. Therefore, we concluded that the correct interpretation of our findings is that to achieve prolonged allograft survival using costimulatory blockade, $CD4^+$ T cells must be able to respond actively through the indirect pathway. It is possible that a select population of regulatory $CD4^+$ T cells fails to survive in the class II-deficient mice. This explanation for our findings is consistent with our interpretation that the indirect pathway is required to achieve prolonged survival using costimulatory blockade.

Although we did not anticipate the outcome of these experiments, our findings are consistent with those obtained by several other laboratories that have been studying transplantation tolerance. For example, earlier studies showing that prolonged allograft survival can be achieved after intrathymic injection of donor MHC peptides had already suggested that manipulations of the indirect pathway can alter the course of rejection when both the direct and indirect responses are available (18, 19). In addition, several laboratories have shown that active regulatory T cells are induced by various tolerance-inducing strategies, including the use of costimulatory blockade and of nondepleting T cell Abs (20–29). Thus it is not necessarily surprising that eliminating one of the pathways of T cell alloreactivity should turn out to eliminate the capacity to generate the active T cell regulation that allows graft survival to occur.

The important contribution of our experiments to the work that has been done by others is that our experiments address the issue of the specificity of the regulatory T cell population. Other groups have provided information about the phenotype of these cells, characterizing them as $CD4^+CD25^+$, at least in some cases. However, there is very limited information available about what determinants are recognized by these regulatory cells. Waldmann and colleagues (30) have reported that regulatory T cells can be specific for determinants presented by the indirect pathway. In contrast, the finding of linked suppression, which has often been associated with regulatory T cells, has suggested to others that the critical suppressor determinant (the one recognized by the regulatory T cell population) was likely to be one on donor APCs. This is because coexpression of the intact donor Ag and the linked determinant on a donor APC would be the simplest explanation for the linkage phenomenon. The results of our experiments agree with those of Waldmann's because they suggest that at least a component of the regulatory T cell response must involve recognition of peptides of donor Ags presented by recipient MHC molecules. They go further than the previous results because our findings suggest that regulation through the indirect pathway is not only possible, but it is required.

A limitation of our studies is that they suggest conclusions based on what does not occur in the class II-deficient mice. Therefore, it will be essential to perform experiments in normal mice treated with costimulatory blockade to confirm that a population of regulatory T cells specific for indirect determinants is induced by this treatment. Our preliminary studies in normal animals support this conclusion (data not shown).

A perplexing aspect of these and earlier findings is that regulatory T cells that are specific for determinants of the indirect pathway apparently have the capacity to affect the function of T cells that are specific for determinants of the direct response. Because these determinants are presented by two different sets of APCs (those of the recipient in the first case and those of the donor in the second), it is not clear what accounts for the linkage that is associated with regulatory T cell tolerance. This linkage does exist, because transplantation tolerance induced by costimulatory blockade does show specificity. However, the simple idea that linkage is achieved by coexpression of determinants on a single APC is not sufficient to explain our findings.

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