Mechanism of Action of Donor-Specific Transfusion in Inducing Tolerance: Role of Donor MHC Molecules, Donor Co-stimulatory Molecules, and Indirect Antigen Presentation

KOJI KISHIMOTO,* XUELI YUAN,* HUGH AUCHINCLOSS, JR., †
ARLENE H. SHARPE, ‡ DIDIER A. MANDELBROT, § and MOHAMED H. SAYEGH*||

*Laboratory of Immunogenetics and Transplantation, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts; †Transplantation Unit, Surgical Services, Massachusetts General Hospital, Boston, Massachusetts; ¤Immunology Research Division, Department of Pathology, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts; ‡Immunology Research Division, Department of Pathology, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts; §Renal Division, University of Massachusetts Medical Center, Worcester, Massachusetts; and ||Nephrology Division, Children’s Hospital, Boston, Massachusetts.

Abstract. Donor-specific transfusion (DST) can synergize with T cell co-stimulatory blockade in inducing tolerance in several transplant models, but the mechanism of action of DST is poorly characterized. This study used genetically altered mice in an established model of cardiac transplantation to study the role of MHC and co-stimulatory molecule expression on DST cells in mediating the immunomodulatory effects of DST. In addition, to examine the role of indirect antigen presentation in the effect of DST, experiments used recipient mice that do not express MHC class II molecules on peripheral antigen-presenting cells, but do have functional CD4+ T cells (II4+). As previously reported, treatment with DST from wild-type donors in combination with CD154 blockade induced tolerance in wild-type recipients of cardiac allografts. Tolerance in this model is also induced despite the absence of MHC class I and II, CD40, or B7 molecules on transfused cells. In contrast, eliminating the indirect pathway using II4+ recipients blocked the induction of long-term cardiac allograft survival by DST. These results indicate that the indirect antigen recognition pathway mediates the immunomodulatory effect of DST in inducing transplantation tolerance in vivo.

Numerous studies have shown that transfusion of allogeneic donor cells (donor-specific transfusion [DST]) has the potential to induce tolerance to a specific allograft. In particular, transfusion of donor splenic leukocytes at the time of transplantation synergizes with B7/CD154 co-stimulatory signal blockade in promoting long-term allograft survival, inducing tolerance, and preventing chronic allograft rejection in some models (1–4). Postulated mechanisms of the immunomodulatory functions of DST include clonal deletion (5,6), induction of anergy (7), generation of regulatory cells (8,9), regulation of cytokine production (5,7,10), promotion of microchimerism (10), provision of soluble MHC antigens (11), or a combination of these mechanisms (5,7,10,12). However, the proximal mechanisms involving alloreactive T cell interactions with DST remain unknown. The aim of this study was to investigate the role of MHC and co-stimulatory molecule expression on the immunomodulatory functions of DST in a fully allogeneic cardiac transplant model using specific gene knockout mice as sources of DST. In addition, to investigate the role of indirect recognition of antigen provided by DST, we used as recipient transgenic mice that express MHC class II only on thymic epithelium and develop normal numbers of CD4+ T cells but express no MHC class II on peripheral antigen-presenting cells (APC) (13).

Materials and Methods

Mice

Wild-type (WT) C57BL/6 (H-2b), MHC class I–deficient (β2-microglobulin deficient) C57BL/6 (H-2b) mice, and CD40-deficient C57BL/6 (H-2b) mice aged 6 to 8 wk were purchased from the Jackson Laboratory (Bar Harbor, ME). BALB/c (H-2d) mice, MHC class II–deficient C57BL/6 (H-2b) mice, and MHC class I– and II–deficient C57BL/6 (H-2b) mice aged 6 to 8 wk were purchased from Taconic Farms (Germantown, NY). B7-1/B7-2–deficient mice (14) were backcrossed for 10 generations onto the C57BL/6 background. The II4+ mice (13) were generated on the B6 background and were provided by L. Glimcher (Harvard School of Public Health, Boston, MA).

Transplantation

Heterotopic vascularized cardiac transplants were performed using C57BL/6 mice as donors and WT BALB/c recipients. To study the
role of indirect antigen presentation, we performed transplants from WT BALB/c into WT or MHC class II−/− CD4+ (II−/−) C57BL/6 mice. The first cardiac allografts were placed in an intra-abdominal location (15), and second heart grafts were placed in the neck using a modification of Chen’s technique (16–18).

**Antibody**

The anti-CD154 hybridoma MR1 was a gift from Randy Noelle (Dartmouth Medical School, Hanover, NH), and the anti-CD25 hybridoma PC-61 was a gift of Dr. Lawrence Turka (University of Pennsylvania, Philadelphia, PA). Both mAb were manufactured from the hybridomas by Bioexpress Cell Culture Services (West Lebanon, NH) (19).

**Treatment**

Animals were treated with either a single dose of 0.25-mg intraperitoneal injection of anti-CD154 mAb (MR1) alone or a single dose of 0.25 mg of MR1 combined with intravenous injection of $5 \times 10^6$ donor strain splenocytes (DST) on the day of transplantation. Anti-CD25 mAb (PC-61) was administered as 500 μg intraperitoneally on day 0, plus 250 μg intraperitoneally on days 2, 4, 6, 8, and 10. WT, both B7-1 and B7-2 double knockout (B7-1/B7-2−/−), CD40−/−, MHC class I−deficient, MHC class II−deficient, or both MHC class I− and class II− (MHC class I/II) deficient mice were used as DST donors.

**ELISPOT**

ELISPOT assays were performed as described previously (16,18,19). Immunospot plates (Cellular Technology, Cleveland, OH) were coated with 4 μg/ml rat anti-mouse IFN capture mAb (R4-6A2), then blocked for 1 h with sterile PBS that contained 1% BSA. Splenocytes ($1 \times 10^6$ in 200 μl of HL-1 medium that containing 1% l-glutamine) were then placed in each well in the presence of $1 \times 10^6$ irradiated (3000 rad) syngeneic or allogeneic splenocytes and cultured for 24 h at 37°C in 5% CO₂. After washing with PBS, followed by PBS that contained 0.05% Tween (PBST), 2 μg/ml biotinylated rat anti-mouse IFN-γ detection mAb (XMG1.2) was added overnight. The plates were then washed and incubated with streptavidin-HRP (Dako, Carpinteria, CA) diluted at 1:2000 in PBS/1% BSA. All mAb were purchased from BD PharMingen (San Diego, CA). After washing three times with PBST followed by PBS, the plates were developed using 800 μl of 3-amino-9-ethylcarbazole (Sigma-Aldrich, St. Louis, MO; 10 μg dissolved in 1 ml of N,N-dimethylformamide) mixed in 24 ml of 0.1 M sodium acetate (pH 5.0) plus 12 μl of H₂O₂. The resulting spots were counted on a computer-assisted ELISPOT image analyzer (T Spot Image Analyzer; Cellular Technology).

**Statistical Analyses**

Kaplan-Meier survival graphs were constructed, and log-rank comparison of the groups was used to calculate $P$ values.

**Results**

**Graft Survival and Tolerance in Recipients Treated with MR1 and DST**

As we have previously reported (3), most heart grafts from B6 donors to Balb/c recipients were rejected by day 100 posttransplantation when animals were treated with a single injection of 0.25 mg of MR1 alone, but treatment with the same dose of MR1 combined with DST from WT animals resulted in long-term graft survival in the majority of animals (Figure 1). Tolerance was also demonstrated in this treatment group, because second heart grafts from the donor strain survived long-term without requiring any treatment (mean survival time [MST] >72 d; $n = 3$).

We then performed studies to address the mechanism of prolonged graft survival induced by MR1 plus DST. We performed ELISPOT assays to quantify IFN-γ-producing cells in

![Figure 1. The effect of co-administration of donor splenocytes plus a single injection of MR1 on cardiac allograft survival. Wild-type (WT) C57BL/6 hearts were transplanted into BALB/c recipients. Recipients were treated on the day of transplantation with MR1 (250 μg intraperitoneally) alone or with MR1 (250 μg intraperitoneally) in combination with $5 \times 10^6$ donor splenocytes intravenously (donor-specific transfusion [DST]). *$P = 0.0016$.](https://example.com/figure1.png)
controls and treated recipients. Balb/c allograft recipients of B6 hearts had significantly fewer IFN-γ-producing cells when treated with MR1 plus DST (151 ± 14.42 IFN-γ spots/million cells in controls versus 9.75 ± 3.4 IFN-γ spots/million cells in treated mice at day 7; \( P = 0.0001; n = 3/\)group). Even by day 30, when the effect of the MR1 antibody per se is gone, recipients that were treated with MR1 + DST had limited numbers of IFN-γ-producing cells (47.77 ± 55.86 IFN-γ spots/million; \( n = 3 \)), as compared with rejecting controls (151 ± 14.42 IFN-γ spots/million cells at day 7; \( P = 0.0331 \)). These data indicate that the administration of DST plus MR1 results in persistent reduction in the frequency of alloreactive Th1 cells in vivo.

Graft Survival and Tolerance in Recipients Treated with MR1 and DST: Role of MHC Molecules

To investigate the role of MHC molecules expressed on trans fused donor cells, we used MHC class I–deficient, MHC class II–deficient, or MHC class I/II–deficient B6 mice as sources of DST cells. Cardiac grafts were from WT B6 donors. As shown in Figure 2, allograft survival times in animals that were treated with MR1 in combination with DST from MHC class I–deficient, MHC class II–deficient, or MHC class I/II–deficient mice are comparable to that of recipients that were treated with DST from WT mice. Furthermore, recipients that were treated with MR1 + DST from each of the three MHC-deficient mice were shown to be tolerant, as evidenced by acceptance of second heart grafts (MST >91 d; \( n = 7 \)). Limited data that second grafts from a third party strain were rejected (MST < 20 d; \( n = 3 \)) further suggest that tolerance is antigen specific, as previously demonstrated by others (20). These findings indicate that surface expression of MHC molecules on donor cells is not necessary for the immunomodulatory functions of DST and suggest that the effect of DST is not mediated by direct antigen presentation of intact MHC molecules on donor cells.

The results above also suggest an active regulatory process mediating inhibition of alloimmune responses and induction/maintenance of tolerance. Therefore, we tested the role of CD25+ regulatory T cells (21) by administering the depleting PC-61 anti-CD25 mAb in addition to MR1 + DST from MHC class I/II–deficient mice. The addition of the anti-CD25 mAb abrogated the effect of MR1 + DST and prevented induction of long-term allograft survival in all recipients (MST = 23 ± 11 d). These data indicate that CD25+ regulatory T cells play an important role in the induction/maintenance of prolonged graft survival by MR1 + DST in our model.

Graft Survival and Tolerance in Recipients Treated with MR1 and DST: Role of Co-stimulatory Molecules

We have previously shown that co-stimulation in trans can mediate allograft rejection as well as co-stimulation in cis (22). Therefore, DST cells might provide co-stimulatory signals even in the absence of MHC molecule expression, the latter being provided by graft cells. T cell co-stimulation could potentially contribute to tolerance induction by two different mechanisms. One would be the reduction of alloreactive T cell clone size by activation induced cell death (AICD), in that T cells initially would be activated by CD154-CD40 and CD80–86 co-stimulation followed by AICD. The second mechanism would be negative regulatory co-stimulation via CTLA-4-B7 interactions that would suppress T cell alloreactivity (19). To examine these possibilities, we used CD40−/− and B7-1/B7-2−/− mice as sources of DST, with cardiac allografts from WT B6 mice. Treatment of recipients with MR1 combined with either CD40−/− DST or B7-1/B7-2−/− DST promoted long-term cardiac allograft survival (Figure 3) as effectively as DST from WT mice. These results demonstrate that the mechanism of action of DST does not involve expression of either CD40 or B7 co-stimulatory molecules on the DST cells.

![Figure 2](image.png)
Long-Term Cardiac Allograft Survival Is not Achieved in II⁺4⁻ Recipients Treated with MR1 in Combination with DST

Our results with DST cells that lack either MHC molecules or co-stimulatory molecules suggested that direct recognition of DST cells by recipient T cells is not essential for tolerance induction. Therefore, we examined the role of indirect allorecognition using II⁺4⁻ mice as recipients. II⁺4⁻ mice lack class II- expressing APC, so their CD4⁺ T cells cannot recognize donor antigens by indirect presentation. In these experiments, WT BALB/c hearts were placed into C57BL/6 recipients, because the II⁺4⁻ mice are available only on a B6 background. Similar to our data in BALB/c recipients, WT B6 recipients that were treated with MR1 + DST had better cardiac allograft survival than recipients that were treated with MR1 alone (Figure 4A). In contrast, treatment of II⁺4⁻ recipients with MR1 + DST from WT animals did not prolong graft survival time compared with those that were treated with MR1 alone (Figure 4B). These data indicate that direct allorecognition alone is not sufficient to allow tolerance induction by DST plus CD154 blockade and suggest that indirect recognition of DST cells by host CD4⁺ T cells is necessary for induction of long-term allograft survival and tolerance in our model. Prolongation of graft survival induced by MR1 alone is similar in WT and II⁺4⁻ mice (P = 0.93), demonstrating that the II⁺4⁻ mouse is an appropriate model in which to study the effect of DST in the absence of indirect antigen presentation.

To address the possibility that the II⁺4⁻ mice tend to be resistant to tolerance induction because of an increased frequency of directly alloreactive CD4⁺ T cells as compared with WT mice, we performed an adoptive transfer experiment. In this experiment, 2 × 10⁷ splenocytes from II⁺4⁻ mice were transferred to WT B6 recipients of BALB/c allografts to increase the frequency of directly alloreactive T cells in WT animals. The number of T cells used is based on published studies indicating that numbers smaller than what we used, when transferred into nude recipients, can mediate acute allograft rejection (23). Despite the presence of additional CD4 T cells from II⁺4⁻ mice, MR1 + DST induced long-term survival in three of four mice (>60 d).

Discussion

In this study, we investigated the proximal mechanisms mediating the immunomodulatory functions of DST when combined with CD154 T cell co-stimulatory blockade in inducing tolerance in vivo. Specifically, we investigated the roles of surface expression of MHC molecules, co-stimulatory molecules, and indirect allorecognition. Our data indicate that the immunomodulatory functions of DST are dependent on neither surface expression of allogeneic MHC molecules nor B7 or CD40 co-stimulatory molecules. Indeed, our results show that graft survival in recipients that are treated with DST from MHC and co-stimulatory molecule--deficient donors are comparable to those that are treated with DST from WT animals, suggesting that direct recognition of DST cells in cis or in trans (22) does not contribute to tolerance induction when combined with CD154 blockade. Although it was previously reported that the effect of DST + MR1 in regulating alloreactivity is dependent on an intact B7-CTLA4 pathway (24), our results using B7⁻1/B7⁻2⁻deficient DST cells indicate that this mechanism is not related to B7 expression on DST cells.

Our observations suggested an important role for indirect recognition in tolerance induction, and we addressed this possibility using II⁺4⁻ mice, in which indirect recognition by CD4⁺ T cells is absent. We did not observe any prolongation of allograft survival in II⁺4⁻ mice that were treated with DST and anti-CD154 mAb as compared with anti-CD154 alone, indicating that indirect recognition of DST cells, at least by CD4⁺ T cells, was necessary for long-term graft survival. This result is consistent with data from our group (25) and others...
Prolonged graft survival.

We previously showed that cardiac allografts in II–4– deficient mice induced long-term graft survival in our recipients does not prevent the induction of tolerance by MR1 against the possibility that II–4–deficient DST cells in the absence of MR1 (Ig control) fails to prolong graft survival (MST = 7, n = 4).

The intriguing finding that DST cells that lack surface expression of both class I and class II MHC molecules are tolerogenic to a fully mismatched graft suggests that recognition of processed alloantigen in the form of allopeptides may induce regulatory cells that can suppress directly and indirectly alloreactive T cells. Our finding that anti-CD25 antibody prevents induction of tolerance by MR1 + DST demonstrates the importance of regulatory T cells in our model. Other studies of these cells, including CD4+CD25+ T cells, have highlighted the role of co-stimulation in the development of regulatory cells (30). Because indirect allorecognition is critical in the induction of tolerance by co-stimulatory blockade (25), future studies should directly address the role of indirect allorecognition in the induction of regulatory cells.

Acknowledgments

This work was supported by National Institutes of Health Grants RO1-AI-34965 and PO1-AI-41521 to M.H.S., RO1-AI-34965 and PO1-AI-41521 to M.H.S., PO1-AI-56299 to A.H.S., and American Heart Association Grant 0050335N to D.A.M.

We thank Karla S. Stenger, Susan P. Shea, and Jean P. Kut for invaluable technical assistance.

References


Figure 4. The effect of DST on cardiac allograft survival in II–4– mice. WT BALB/c hearts were transplanted into either WT (A) or II–4– (B) C57BL/6 recipients. Recipients were treated on the day of transplantation with MR1 (250 μg intraperitoneally) in combination with 5 × 10⁶ donor splenocytes intravenously. *P = 0.0210; **P = 0.2866.

(26) emphasizing the importance of indirect antigen presentation in inducing tolerance. Several lines of evidence argue against the possibility that II–4– mice resist tolerance induction by MR1 + DST as a result of increased numbers or alloreactivity of their CD4 T cells. In this study, we demonstrated that the adoptive transfer of II–4– cells to WT recipients does not prevent the induction of tolerance by MR1 + DST. In addition, we previously showed that cardiac allografts in II–4– mice recipients are acutely rejected despite the administration of depleting antibody to CD4 (27). If rejection in II–4–/H9262 mice were mediated by increased numbers of CD4+ T cells, then depleting CD4+ T cells instead would have induced prolonged graft survival.

Although DST cells from MHC class I–deficient or class II–deficient mice induced long-term graft survival in our report, it does not necessarily rule out the possibility that donor MHC antigens contribute to tolerance induction. MHC class I– or MHC class II–deficient mice that we used have gene disruption of one of the polypeptide chains that constitute an MHC molecule. MHC class I–deficient mice, which are homozygous for a β2-microglobulin gene disruption, express little if any functional MHC class I antigens on the cell surface (28) yet are theoretically able to produce other polypeptide chains of MHC class I molecules. Similarly, MHC class II–deficient mice lack cell surface expression of class II molecules (29) yet are able to produce A-α and E-β. Therefore, our findings suggest that indirect recognition of MHC peptides, rather than direct recognition of surface-expressed molecules, is required for tolerance induction mediated by DST administration.


13. Steele DJ, Laufer TM, Smiley ST, Ando Y, Grusby MJ, Glim-

12. Brennan DC, Mohanakumar T, Flye MW: Donor-specific trans-

15. Corry RJ, Winn HJ, Russell PS: Primarily vascularized allografts

17. Chen ZH: A technique of cervical heterotopic heart transplantation


14. Bluestone JA, Abbas AK: Natural versus adaptive regulatory T


20. Zheng XX, Markees TG, Hancock WW, Li Y, Greiner DL, Li

21. Waldmann H, Graca L, Cobbold S, Adams E, Tone M, Tone Y:

22. Mandelbrot DA, Kishimoto K, Auchincloss H Jr, Sharpe AH,


24. Markees TG, Phillips NE, Gordon EJ, Noelle RJ, Shultz LD,


27. Zimmerman G, Bredt DS, Montecucco C: A role for neuronal

28. Zijlstra M, Bix M, Simister NE, Loring JM, Raulet DH, Jaenisch

29. Grusby MJ, Johnson RS, Papaioannou VE, Glimcher LH: De-

30. Bluestone JA, Abbas AK: Natural versus adaptive regulatory T


