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Dependency of Direct Pathway CD4\(^+\) T Cells on CD40-CD154 Costimulation Is Determined by Nature and Microenvironment of Primary Contact with Alloantigen\(^1\)

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Blockade of the CD40-CD154 costimulatory pathway can inhibit CD4\(^+\) T cell-mediated alloimmune responses. The aim of this study was to define the in vivo requirement for CD40-CD154 costimulation by CD4\(^+\) T cells that respond to alloantigen following direct recognition. We used TCR-transgenic CD4\(^+\) T cells that are reactive to the MHC class II alloantigen, H2A\(^\alpha\). An experimental in vivo model was established that allowed direct comparison of the fate of a trace population of H2A\(^\alpha\)-reactive CD4\(^+\) T cells when challenged with different forms of H2A\(^\alpha\) alloantigen under conditions of CD40-CD154 costimulation blockade. In this study, we demonstrate that an i.v. infusion of H2A\(^\alpha\) leukocytes in combination with anti-CD154 therapy rapidly deletes H2A\(^\alpha\)-reactive CD4\(^+\) T cells. In contrast, following transplantation of an H2A\(^\alpha\) cardiac allograft, H2A\(^\alpha\)-reactive CD4\(^+\) T cell responses were unaffected by blocking CD40-CD154 interactions. Consistent with these findings, combined treatment with donor leukocytes and anti-CD154 therapy was found to be more effective in prolonging the survival of cardiac allografts compared with CD154 mAb treatment alone. The dominant mechanism by which donor leukocyte infusion and anti-CD154 therapy facilitate allograft acceptance is deletion of donor-reactive direct pathway T cells. No evidence for the generation of regulatory cells by this combined therapy was found. Taken together, these results clearly demonstrate that naive alloreactive CD4\(^+\) T cells have distinct requirements for CD40-CD154 costimulation depending on the form and microenvironment of primary alloantigen contact. *The Journal of Immunology, 2004, 172: 2163–2170.

For the generation of a productive T cell response, T cells need both Ag-specific signals through the TCR and costimulatory signals through accessory cell surface molecules (1–3). Provision of Ag-specific signals to naive T cells in the absence of costimulatory signals is generally believed to result in incomplete T cell activation (4–6). These findings have formed the basis for developing strategies to induce T cell unresponsiveness to transplanted organs by specifically blocking costimulatory signals while leaving Ag-specific signals through TCR/CD3 complex intact. It is now widely accepted that one of the critical costimulatory pathways required for producing effective CD4\(^+\) T cell responses is that of CD40 and its ligand CD154 (7, 8).

Several rodent and primate studies have clearly demonstrated that blockade of the CD40-CD154 costimulatory pathway can prevent acute allograft rejection. However, indefinite graft acceptance is rarely achieved with this monotherapy (9–13). Recently, CD8\(^+\) T cells capable of mediating allograft rejection, despite blockade of CD40-CD154 interactions, have been identified (14–18). However, even in the absence of CD8\(^+\) T cells, blockade of CD40-CD154 interactions can under some circumstances fail to prevent allograft rejection and/or the development of transplant arteriosclerosis (14, 19–22). These findings suggest that alloreactive CD4\(^+\) T cells can also contribute to destructive alloimmune responses without a requirement for CD40-CD154 costimulation.

The fact that CD4\(^+\) T cells can recognize alloantigen via two distinct routes, the so-called direct and indirect pathways of allorecognition, has led to the hypothesis that CD4\(^+\) T cells might have a different requirement for costimulation depending on the route of alloantigen recognition (21). Indeed, recently it has been shown that costimulation blockade was extremely effective in abrogating alloresponses mediated by CD4\(^+\) T cells that recognize alloantigen via the indirect pathway (21). In contrast, graft rejection mediated by CD4\(^+\) T cells that recognize alloantigen exclusively by the direct pathway could not be prevented by blocking CD28/B7 and/or CD40/CD154 interactions (21, 23).

Together these studies suggest that following transplantation, stimulation of both CD4\(^+\) and CD8\(^+\) T cells via direct interaction with allo-MHC/peptide does not require CD40-CD154 costimulation. In clear contrast to alloimmune responses elicited following transplantation, it has been reported that alloreactive CD8\(^+\) T cells become critically dependent on CD40-CD154 interactions when the initial alloantigen challenge is in the form of a cell infusion (21). To date, it is unclear whether CD4\(^+\) T cells that recognize alloantigen directly also manifest different requirements for CD40-CD154 costimulation depending on the alloantigen-presenting cell. In this study, we demonstrate that in the absence of CD40-CD154 costimulation, alloreactive direct pathway CD4\(^+\) T cells rapidly delete when challenged with alloantigen in the form of a leukocyte infusion, but continue to respond normally to a transplanted allograft.

Materials and Methods

**Mice**

CBA.Ca (CBA; H\(^2\)k), C57BL/10 (B10; H\(^2\)b), BALB/c (H\(^2\)d), and B10.BR (H\(^2\)b) mice were purchased from Harlan (Bicester, U.K.). CBA.Ca RAG-1

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were considered statistically significant.

respectively.

William Dunn School of Pathology, Oxford, U.K.). Values of statistical application developed and kindly provided by S. Cobbold (Sir

CD4/H11001

A total of 60%–90% of peripheral CD4 T cells coexpress the transgenic TCR: Vα11, Vβ3. All mice were bred and housed in the Biomedical Services Unit, John Radcliffe Hospital (Oxford, U.K.) in accordance with the Animals (Scientific Procedure) Act 1986 of the United Kingdom. All experiments were performed with mice aged between 6 and 12 wk old.

**Heterotopic heart transplantation**

Abdominal vascularized heterotopic heart transplants were performed essentially as documented by Corry et al. (27). Rejection was defined as a complete cessation of palpable cardiac contraction and was confirmed by direct visualization after laparotomy.

**Skin transplantation**

Full-thickness B10 tail skin was transplanted to graft beds prepared on the left lateral thorax of anesthetized CBA RAG −/− recipient mice. Graft rejection was defined by complete destruction of the skin.

*Graft survival data were analyzed by the log rank method (28) using a statistical application developed and kindly provided by S. Cobbold (Sir William Dunn School of Pathology, Oxford, U.K.). Values of \( p < 0.05 \) were considered statistically significant.

**Ab treatment**

CBA mice were treated with either anti-CD154 mAb (MR1; American Type Culture Collection, Manassas, VA) or hamster control Ab (hamster Ig(Hlg); Jackson ImmunoResearch Laboratories, West Grove, PA) at 500 µg/day i.p. on days 0, 2, and 4 posttransplantation.

For the depletion of CD4+ and CD8+ T cells, B10.BR recipients were thymectomized 14 days before transplantation, followed 2 days later by i.p. injection of depleting mAbs against CD4 (YTA3.1; 100 µg/dose) and CD8 (YTS169; 100 µg/dose) on days −12 and −11. Hybridomas YTA3.1 and YTS169 were a generous gift from H. Waldmann, Sir William Dunn School of Pathology.

**Pretreatment protocol**

Mice received 200 µg of the anti-CD4 mAb YTS 177.9 (hybridoma YTS177.9 was kindly provided by H. Waldmann) i.v. on days −28 and −27. On day −27, they also received \( 10^6 \) CFSE-labeled spleen cells i.v. Spleens were harvested on day 0 for cell isolation.

**Adoptive transfer (AT) protocols**

Lymph nodes and spleens were harvested from TCR-transgenic mice, and leukocytes were prepared by passing the tissue through a stainless steel mesh. Spleen cells were depleted of erythrocytes by osmotic shock. An aliquot of cells was stained with anti-CD8 and anti-clonotypic TCR-biotin mAb (T98; kindly provided by A. Mellor) or anti-CD4, anti-Vα11, and anti-Vβ3, and analyzed by flow cytometry to determine the percentage of H2A s -reactive CD4 + T cells and H2A + -reactive CD4 T cells, respectively.

H2A-reactive cells were labeled with CFSE (Molecular Probes, Leiden, The Netherlands). An equivalent of \( 6 \times 10^6 \) H2A-reactive CD4 T cells was injected i.v. into T cell-depleted thymectomized B10.BR mice. One day after AT, i.e., day 0, recipients either were left untreated or received a challenge with H2A s splenocytes i.v. or were transplanted with a B10.S(7R) cardiac allotransplant. Mice were then treated with MR1 or the control hamster Ab, as described previously.

CD8 T cells from BM3 TCR-transgenic CBA RAG −/− mice and CD4 CD25+ cells were purified using MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) (29). All syngeneic CBA RAG −/− mice received \( 1 \times 10^5 \) purified H2A 𝑘-reactive CD8+ T cells alone or in combination with \( 5 \times 10^5 \) purified CD4 CD25+ or CD4 CD25− cells.

**Results**

**In vivo proliferation of adoptively transferred alloreactive CD4 T cells**

To evaluate the in vivo response of a physiological number of naive alloreactive CD4 T cells, we established an experimental approach in which a trace population of TCR-transgenic CD4 T cells (-D), reactive to the MHC class II alloantigen, H2A +, can be followed. Briefly, T cell-depleted syngeneic B10.BR mice received \( 6 \times 10^6 \) CFSE-labeled TCR-transgenic CD4 + T cells and either were left untreated or received an infusion of \( 10 \times 10^6 \) B10.S(7R) splenocytes 1 day after AT. Three days after in vivo stimulation with H2A + splenocytes, 85.4 ± 3.0% of the TCR-transgenic CD4 + T cells found in the spleen had divided more than once, as judged by loss of CFSE fluorescence intensity (Fig. 1). Alloreactive CD4 T cells that were not challenged with donor splenocytes did not proliferate (0.6 ± 0.2% divided >1; Fig. 1).

**Isolation of graft-infiltrating cells (GIC)**

GICs were prepared by collagenase digestion of cardiac grafts, as described by Wagoner et al. (30). Grafts were minced and incubated with 1 mg/ml type VII collagenase solution (Sigma-Aldrich, Poole, Dorset, U.K.) for 30 min at 37°C. GICs were isolated by Ficoll separation (Histopaque-1083, 6.41 g/dl; Sigma-Aldrich), according to manufacturer’s instructions.

**Flow cytometric analysis**

H2A-reactive CD4 T cells were identified by positive staining for Vα11 and Vβ3 chains of the transgenic TCR using biotinylated mAbs (BD PharMingen, Oxford, U.K.). The biotin-labeled anti-Vα11 and anti-Vβ3 mAb were developed with streptavidin-conjugated CyChrome (BD PharMingen) and streptavidin-conjugated allophycocyanin (BD PharMingen), respectively. To determine the phenotype of H2A-reactive CD4 T cells, samples were finally stained with PE-labeled mAbs (BD PharMingen). All of the following mAbs were purchased from BD PharMingen: anti-CD8 allophycocyanin (53-7.6), anti-CD4 PE (RM4-5), anti-CD25 PE (3C7), and anti-CD44 PE (IM7.8.1). The forward scatter (FSC) characteristics of H2A-reactive CD4 T cells were quantified and used as an index of cell size.

**FIGURE 1**

TCR-transgenic CD4 T cells proliferate following in vivo challenge with H2A splenocytes. A total of \( 6 \times 10^6 \) CFSE-labeled monospecific TCR-transgenic CD4 T cells (-D) was adoptively transferred into T cell-depleted syngeneic B10.BR mice. The following day, mice were challenged i.v. with \( 10 \times 10^6 \) H2A 𝑘(B10.S(7R)) or H2A + (BALB/c) splenocytes or were left untreated. Three days later, TCR-transgenic CD4 T cells in the spleen were identified by staining for CD4 and the transgenic TCR chains Vα11 and Vβ3. The CFSE fluorescence intensity of gated TCR-transgenic CD4 T cells was analyzed by four-color flow cytometry. The results represent the mean percentage of TCR-transgenic CD4 T cells that have divided more than once ± SD; \( n = 2 \) or 3 mice per group.

Abbreviations used in this paper: Hlg, hamster Ig; AT, adoptive transfer; DC, dendritic cell; FSC, forward scatter; GIC, graft-infiltrating cell; MST, median survival time.
Furthermore, TCR-transgenic CD4⁺ T cells proliferated specifically to H2A⁺ alloantigen, as infusion of 10 × 10⁶ splenocytes from third-party control BALB/c mice (H2A⁺) did not induce multiple rounds of cell division (3.8 ± 2.0% divided >1; Fig. 1). Therefore, this novel experimental in vivo approach allowed direct analysis of a trace population of H2A⁺-reactive CD4⁺ T cells following direct recognition of alloantigen.

H2A⁺-reactive CD4⁺ T cells are deleted when stimulated in vivo with H2A⁺ splenocytes and in the presence of anti-CD154 mAb

Although it has recently been shown that a combined therapy consisting of anti-CD154 mAb and a single infusion of donor splenocytes leads to rapid activation followed by deletion of alloreactive T cells (24, 31), the exact fate of CD4⁺ T cells that recognize alloantigen exclusively via the direct pathway under these conditions is unknown. To address this issue, we determined the absolute number of H2A⁺-reactive CD4⁺ T cells in the spleen of -D AT mice that received an infusion of 10 × 10⁶ H2A⁺ splenocytes on day 0 and anti-CD154 therapy (MR1; 500 μg on days 0 and 2), or hamster control Ab (HIg; 500 μg on days 0 and 2). No significant difference in absolute number of TCR-transgenic CD4⁺ cells was observed in the spleen of mice that were either untreated or treated with anti-CD154 mAb only (AT only (n = 2); 9.2 ± 0.3 × 10⁶ vs AT + MR1 (n = 3); 11.0 ± 2.0 × 10⁶ H2A⁺-reactive CD4⁺ T cells; day 3) (Fig. 2). As expected, mice that were challenged i.v. with H2A⁺ alloantigen contained substantially higher numbers of H2A⁺-reactive CD4⁺ T cells in the spleen compared with control mice (AT + splenocytes + HIg (n = 3); 2.1 ± 0.5 × 10⁷ H2A⁺-reactive CD4⁺ T cells; day 3). In clear contrast, administration of H2A⁺ alloantigen while blocking CD40-CD154 interactions resulted in a 100-fold decrease in the number of H2A⁺-reactive CD4⁺ T cells in the spleen compared with mice that did not receive the anti-CD154 mAb (AT + splenocytes + MR1 (n = 3); 2.0 ± 1.0 × 10⁴ H2A⁺-reactive CD4⁺ T cells; Fig. 2).

H2A⁺-reactive CD4⁺ T cells rapidly acquire an activated phenotype before their deletion following donor leukocyte infusion and CD154 blockade

Next, we determined whether H2A⁺-reactive CD4⁺ T cells had proliferated and acquired an activated phenotype before their deletion.

CFSE analysis revealed that 90.0 ± 4.7% of gated alloreactive CD4⁺ T cells present in the spleen had divided at least once by 2 days after challenge with a H2A⁺ splenocyte infusion in combination with HIg control Ab (AT + splenocytes + HIg; Fig. 3A). Furthermore, these cells had undergone blast formation (FSC; 74.7 ± 5.1% FSChigh; Fig. 3B) and expressed increased levels of CD25 and CD44. As expected, H2A⁺-reactive CD4⁺ T cells that were not exposed to H2A⁺ splenocytes showed little proliferation (4.8 ± 3.2%) and remained naive, as evidenced by their small size and low expression of both CD25 and CD44. Interestingly, H2A⁺-reactive CD4⁺ T cells stimulated with H2A⁺ splenocytes in combination with anti-CD154 mAb had also proliferated (74.2 ± 6.3%) and exhibited an activated phenotype similar to the group that received H2A⁺ splenocytes + HIg treatment (Fig. 3). These findings clearly demonstrate that H2A⁺-reactive CD4⁺ T cells become activated after encountering allologeneic leukocytes in vivo.

FIGURE 2. H2A⁺-reactive CD4⁺ T cells are deleted following in vivo challenge with H2A⁺ splenocytes, despite CD154 blockade. A total of 6 × 10⁶ CFSE-labeled H2A⁺-reactive CD4⁺ T cells was adoptively transferred into T cell-depleted syngeneic B10.BR mice. Some mice were challenged i.v. with a donor leukocyte infusion (10 × 10⁶ H2A⁺ splenocytes). One group received anti-CD154 treatment (MR1; 500 μg), while another group received the hamster control Ab (HIg; 500 μg) at the time of splenocyte infusion. Two days later, gated H2A⁺-reactive CD4⁺ T cells in the spleen were analyzed for their proliferative response, as judged by loss of CFSE fluorescence. The mean percentages (± SD) of H2A⁺-reactive CD4⁺ T cells that had divided at least once were determined; n = 2 or 3 mice per group (A). Gated H2A⁺-reactive CD4⁺ T cells were analyzed by four-color flow cytometry. The mean percentages (± SD) of H2A⁺-reactive CD4⁺ T cells are shown (B), based on an increase in size, as judged by FSC characteristics or expression of the activation markers CD25 and CD44. The results are representative of two such experiments.

FIGURE 3. H2A⁺-reactive CD4⁺ T cells are activated following in vivo challenge with H2A⁺ splenocytes, despite CD154 blockade. A total of 6 × 10⁶ CFSE-labeled H2A⁺-reactive CD4⁺ T cells was adoptively transferred into T cell-depleted syngeneic B10.BR mice. Some mice were challenged i.v. with a donor leukocyte infusion (10 × 10⁶ H2A⁺ splenocytes). One group received anti-CD154 treatment (MR1; 500 μg), while another group received the hamster control Ab (HIg; 500 μg) at the time of splenocyte infusion. Two days later, gated H2A⁺-reactive CD4⁺ T cells in the spleen were analyzed for their proliferative response, as judged by loss of CFSE fluorescence. The mean percentages (± SD) of H2A⁺-reactive CD4⁺ T cells that had divided at least once were determined; n = 2 or 3 mice per group (A). Gated H2A⁺-reactive CD4⁺ T cells were analyzed by four-color flow cytometry. The mean percentages (± SD) of H2A⁺-reactive CD4⁺ T cells are shown (B), based on an increase in size, as judged by FSC characteristics or expression of the activation markers CD25 and CD44. The results are representative of two such experiments.
and that blockade of the CD40-CD154 costimulation pathway does not prevent initial proliferation and activation of CD4⁺ T cells that recognize alloantigen via the direct pathway of allore cognition.

**Blockade of CD40-CD154 costimulatory interactions does not affect the response of direct pathway H2A⁺-reactive CD4⁺ T cells to a cardiac allograft**

Having established that H2A⁺-reactive CD4⁺ T cells become activated, but are rapidly deleted following i.v. challenge with H2A⁺ splenocytes under conditions of CD40-CD154 blockade, we next sought to determine the fate of alloreactive CD4⁺ T cells in response to a H2A⁺ cardiac allograft under similar conditions.

Briefly, we adoptively transferred 6 × 10⁶ CFSE-labeled H2A⁺-reactive CD4⁺ T cells into T cell-depleted athymic syngeneic B10.BR mice (-D AT). The following day, -D AT recipients were either transplanted with a H2A⁺ cardiac allograft or left untransplanted. Seven days after transplantation, CFSE analysis revealed that 22.0 ± 3.4% of gated alloreactive CD4⁺ T cells had divided at least once when a H2A⁺ cardiac allograft was transplanted (n = 3; Fig. 4A). Furthermore, H2A⁺-reactive CD4⁺ T cells showed a similar proliferative response after H2A⁺ cardiac allograft transplantation, despite blockade of CD40-CD154 interactions (19.1 ± 3.6% alloreactive CD4⁺ T cells have divided; n = 4). H2A⁺-reactive CD4⁺ T cells obtained from mice that were left untransplanted showed little proliferation (4.6 ± 2.3% alloreactive CD4⁺ T cells have divided; n = 3; Fig. 4A). H2A⁺-reactive CD4⁺ T cells that have divided following challenge with a H2A⁺ cardiac allograft showed an activated phenotype, as judged by high level expression of CD44 (Fig. 4B). Similar levels of CD44 expression were also found on H2A⁺-reactive CD4⁺ T cells that have divided in response to a transplanted cardiac allograft, despite blockade of CD40-CD154 interactions (Fig. 4B).

We next determined whether H2A⁺-reactive CD4⁺ T cells that have been stimulated by a transplanted H2A⁺ cardiac allograft were deleted following blockade of the CD40-CD154 costimulation pathway. Seven days after transplantation, the absolute numbers of donor H2A⁺-reactive CD4⁺ T cells were found to be similar in the spleen of transplanted B10.BR mice irrespective of whether CD40-CD154 interactions were blocked or not (MR1 or Hlg treatment respectively; Fig. 4C).

Although we could not find any evidence for deletion of alloreactive T cells in the spleen, it was possible that deletion was occurring in the graft itself. To establish whether this was the case, GICs were analyzed. However, similar numbers of H2A⁺-reactive CD4⁺ T cells were found in transplanted cardiac allograft from mice that received either control Ab, Hlg (2.3 ± 1.9 × 10³; n = 2 GIC preparations), or anti-CD154 therapy (1.6 ± 1.0 × 10³; n = 2 GIC preparations). H2A⁺-reactive CD4⁺ T cells that had homed to the graft in both sets of mice had strongly proliferated, as judged by loss of CFSE (Fig. 4D).

**Anti-CD154 therapy failed to inhibit direct alloreactive CD4⁺ T cell responses**

Having demonstrated that CD154 blockade had no effect on H2A⁺-reactive CD4⁺ T cells that had responded to a H2A⁺ cardiac allograft when analyzed 7 days after transplantation, we investigated whether CD154 blockade had any effect on H2A⁺-reactive CD4⁺ T cells at a time when the majority of systemic cells had responded to the cardiac allograft. We found that 40 days after transplantation, 57.0 ± 6.8% (n = 3) of H2A⁺-reactive CD4⁺ T cells had divided once or more in response to the H2A⁺ cardiac allograft (Table I). However, no significant inhibition of proliferation of H2A⁺-reactive CD4⁺ T cells responding via the direct pathway of allore cognition was found following CD154 blockade (48.9 ± 5.9% of H2A⁺-reactive CD4⁺ T cells had divided; n = 3; Table I). Moreover, there was no significant difference in the number of H2A⁺-reactive CD4⁺ T cells in the spleens of transplanted mice that received either control Ab (Hlg; 2.7 ± 1.1 × 10⁵ H2A⁺-reactive CD4⁺ T cells) or anti-CD154 treatment (MR1; 2.6 ± 0.1 × 10⁵ H2A⁺-reactive CD4⁺ T cells).
Additionally, CD154 blockade did not prevent the activation of H2A<sup>+</sup>-reactive CD4<sup>+</sup> T cells by the graft as determined by the FSC characteristics and cell surface expression of CD44 (Table I).

Together these findings show that blockade of the CD40-CD154 costimulatory pathway failed to inhibit H2A<sup>+</sup>-reactive CD4<sup>+</sup> T cell responses to a cardiac allograft. More interestingly, in clear contrast to the finding after infusion of allogeneic leukocytes (Fig. 2), alloreactive CD4<sup>+</sup> T cells were not deleted when challenged with an H2A<sup>+</sup> cardiac transplant during CD154 blockade.

**Combined treatment with donor leukocytes and anti-CD154 treatment improves the survival of B10.S(7R) cardiac allografts in B10.BR recipients**

Having established that a single i.v. infusion of H2A<sup>+</sup> splenocytes, in contrast to a transplant, plus a brief course of anti-CD154 therapy dramatically reduced the clone size of donor-reactive CD4<sup>+</sup> T cells, we wanted to investigate whether this combined treatment might be more effective than anti-CD154 mAb monotherapy in prolonging the survival of cardiac allografts in a relevant nontransgenic mouse model.

B10.BR mice were pretreated with 10<sup>6</sup> B10.S(7R) splenocytes in combination with either MR1 or Hlg (days −7, −5, and −3; 500 µg/dose) 7 days before transplantation of a fully MHC-mismatched B10.S(7R) cardiac allograft. Combined treatment of donor splenocyte infusion and MR1 was found to be more effective in prolonging the survival of B10.S(7R) cardiac allografts in B10.BR recipients (median survival time (MST) 94 days; n = 12) compared with MR1 treatment alone (MST 25 days; n = 7; p < 0.05 compared with splenocyte + MR1 group; Fig. 5). In contrast, B10.BR recipients that received donor splenocytes + Hlg rejected B10.S(7R) cardiac allografts acutely, MST of 6 days (n = 2). Therefore, alloantigen pretreatment at the time of CD154 blockade is of greater benefit than solely blocking CD40-CD154 interactions at the time of transplantation.

**Donor leukocyte infusion and anti-CD154 treatment do not generate regulatory cells before transplantation**

Having clearly established the potent effect of alloantigen pretreatment and CD154 blockade on allograft survival, we investigated whether in addition to the deletion of T cells recognizing alloantigen via the direct pathway, donor alloantigen infusion + anti-CD154 mAb had other potential benefits on graft survival in terms of the generation of regulatory T cells before transplantation.

To address this question directly, spleens from CBA recipients that had been pretreated with donor leukocytes (10 × 10<sup>6</sup> B10 splenocytes; D−28) and three doses of MR1 (500 µg/dose; days −28, −26, and −24) were harvested on day 0, and leukocytes were prepared. In this experiment, mice were left 28 days instead of the 7 days, as we have previously demonstrated that 28 days after pretreatment with leukocyte infusion + anti-CD4 mAb was optimal for the generation of regulatory cells before transplantation (32). A total of 50 × 10<sup>6</sup> or 100 × 10<sup>6</sup> unsplenocytes was adoptively transferred into naive secondary syngeneic CBA recipients 1 day before transplantation of a donor-type B10 cardiac allograft. We found that these cells were unable to delay or prevent B10 allograft rejection (50 × 10<sup>6</sup> and 100 × 10<sup>6</sup> splenocytes; MST 7 days; n = 3 and n = 4, respectively), in contrast to our previous findings with donor splenocytes + anti-CD4 (33). Naive CBA mice that were left untreated rejected B10 cardiac allografts with similar kinetics (MST 7 days; n = 6; Fig. 6).

As a positive control within this study, 50 × 10<sup>6</sup> unsplenocytes from CBA recipients that had accepted B10 cardiac allografts for over 100 days following treatment with anti-CD154 at the time of transplantation (500 µg MR1 on days 0, 2, and 4) prolonged the survival of all B10 cardiac allografts (MST >100 days; n = 9; Fig. 6).
CD4+ T cells that have escaped deletion following donor leukocyte infusion + anti-CD154 blockade failed to prevent allograft rejection

Although these data suggest that mice treated with donor leukocytes + MR1 do not possess the capacity to regulate the immune response of a complete naïve T cell repertoire, it is possible that the frequency of donor alloantigen-specific regulatory cells was too low to find evidence for their existence in this relatively difficult test. Therefore, we enriched CD4+ T cells expressing CD25 (IL-2R α-chain) and studied, in an AT model, whether these cells exhibit suppressive capacity. We have recently described an AT model in which skin allograft rejection mediated by a monospecific population of TCR-transgenic CD8+ T cells, reactive to MHC class I alloantigen H2Kb, can be used to evaluate the potential of cotransferred cells to suppress rejection (29).

Briefly, 5 x 10^5 purified CD4+CD25+ or CD4+CD25− T cells were cotransferred with 1 x 10^6 H2Kb-reactive CD8+ T cells into CBA RAG−/− mice. The following day, all mice were transplanted with an H2Kb+ skin graft. Consistent with our previous findings (29), 1 x 10^6 H2Kb-reactive CD8+ T cells acutely rejected all H2Kb+ skin grafts (MST 18 days; n = 4; Fig. 7), whereas cotransfer of 5 x 10^5 purified CD4+CD25+ T cells from MR1-pretreated CBA mice with long-term surviving B10 cardiac allografts had the capacity to suppress H2Kb-reactive CD8+ T cell-mediated skin allograft rejection (MST >50 days; n = 5; Fig. 7). In clear contrast, cotransfer of 5 x 10^5 purified CD4+CD25− or 5 x 10^5 purified CD4+CD25− T cells, isolated from the spleen of CBA mice 28 days after pretreatment with donor leukocyte infusion and MR1, failed to prevent B10 skin allograft rejection mediated by H2Kb-reactive CD8+ T cells (MST 20 days, n = 5 and MST 10 days, n = 3, respectively; Fig. 7). Value of p > 0.05.

CD4+CD25+ T cells compared with H2Kb-reactive T cells-only group; p > 0.05. CD4+CD25− T cells compared with H2Kb-reactive T cells-only group.

As a control group, we isolated CD4+CD25+ cells from CBA mice that had been pretreated with a donor leukocyte infusion (10 x 10^6 B10 splenocytes) under the cover of a modulating anti-CD4 mAb (YTS177). Studies from our laboratory have previously demonstrated that this donor leukocyte infusion-based induction strategy enables immunoregulatory T cells to develop within 28 days (32, 33). Indeed, cotransfer of CD4+CD25+ T cells, isolated from CBA mice pretreated with a donor leukocyte infusion and YTS177, significantly delayed H2Kb-reactive CD8+ T cell-mediated B10 skin graft rejection (MST 35 days; n = 2; Fig. 7).

Taken together, these findings demonstrate that therapy with a combination of donor leukocytes + anti-CD154 does not result in the generation of regulatory cells before transplantation, but exerts its effect via deletion of alloreactive T cell clones.

Discussion

The mechanisms directly responsible for preventing CD4+ T cell-mediated rejection following anti-CD154 therapy have not yet been identified, but have been proposed to involve costimulation blockade (9, 11), deletion (31, 34), immune deviation (11, 35), and immunoregulation (15, 29, 36, 37). A detailed evaluation of the effect of CD154 blockade on CD4+ T cells responding to alloantigen has been complicated by the fact that these cells can recognize alloantigen via two distinct routes of Ag presentation, namely the direct and indirect pathways of allorecognition, the latter requiring reprocessing of alloantigen and presentation by recipient APCs. Recently, Yamada et al. (21) reported that in the absence of the indirect pathway of alloantigen recognition, perioperative CD154 blockade failed to achieve long-term allograft acceptance. This study suggests that the requirement for CD40-CD154 costimulation by CD4+ T cells may be dependent on the form of alloantigen recognized.

To define the requirement for CD40-CD154 costimulation by CD4+ T cells that recognize alloantigen exclusively via the direct pathway, we developed an experimental in vivo model, which allowed us to follow the fate of a trace population of CD4+ T cells with known alloreactivity. We demonstrate that CD4+ T cells that recognize alloantigen, H2A, by the direct pathway initially acquired an activated phenotype (Fig. 3), but then were rapidly deleted when stimulated in vivo with H2A+ splenocytes in the absence of the CD40-CD154 costimulation (Fig. 2). The finding that blockade of costimulation pathways can result in the deletion of alloreactive T cells has also been shown by others (24, 31, 34, 38). However, this is the first report that provides clear evidence that CD4+ T cells, reactive to alloantigen following direct recognition, respond by deletion.

CD8+ T cells are also rapidly eliminated from the periphery by combined alloantigen + anti-CD154 treatment (24). Costimulation blockade has been shown to enhance the induction apoptosis in the responding T cells (34) through IL-2-dependent, but FAS/FAS ligand-independent interactions (39). More recently, a critical role for complement has also been implicated in reducing the clone size of alloreactive T cells and facilitating long-term graft survival (40).

In stark contrast to the data obtained with alloantigen infusion (Fig. 2), our studies clearly demonstrate that CD4+ T cells responding to donor alloantigen via the direct pathway of allorecognition are not deleted following transplantation of a cardiac allograft under conditions of CD40-CD154 costimulation blockade (Fig. 4). Indeed, we have also reported that donor-reactive CD8+ T cells also failed to undergo deletion after transplantation in the presence of anti-CD154 therapy (16). Taken together, these data clearly show that both CD4+ and CD8+ direct pathway T cells are critically dependent on CD40-CD154 interactions when the first encounter with alloantigen is in the form of a leukocyte infusion, but that both T cell subsets are able to generate effective responses when the transplanted organ is the initial source of alloantigen.

We believe that possible explanations for this observation may lie in: 1) differences in the context in which alloantigen is presented; 2) the type of cells that present alloantigen; and 3) differences in alloantigen load.
Following transplantation, donor APC resident in the transplanted tissue may become activated due to the ischemia caused by the surgery and/or uptake or interaction with necrotic cells. Certainly, such passenger cells have been shown to migrate to the draining lymphoid organs following transplantation, suggesting that they have received activation signals and are actively differentiating. As a consequence, these activated APCs may be able to stimulate naive alloreactive T cells in a CD40-CD154-independent manner due to the expression of additional costimulatory molecules (e.g., receptor activator of NFκB (41)) and/or increased expression of adhesion or MHC molecules. In contrast, when the initial alloantigen challenge is delivered in the form of a leukocyte infusion, APCs would presumably remain quiescent, and therefore the response of direct pathway alloreactive CD4+ T cells would still be critically dependent on CD40-CD154 interactions.

In support of this hypothesis, it has been shown that i.v. infusion of immature donor-derived dendritic cells (DC), while blocking CD40-CD154 interactions, not only facilitated cardiac allograft survival, but also enhanced apoptosis of donor-reactive T cells in both the spleen and the transplanted heart. In contrast, mice pre-treated with mature DC under the cover of anti-CD154 mAb rejected their cardiac allografts with faster kinetics compared with the untreated control group, presumably due to the CD40-CD154-independent expansion/sensitization of alloreactive T cells (42).

Dependence of T cells on CD40-CD154 interactions may also be dictated by the type of APC involved in addition to its activation status. The predominant type of APC that migrates out of transplanted tissue is the DC (43, 44), which will therefore be the principal cell in the secondary lymphoid organs to present alloantigen via the direct pathway. In contrast, when a splenocyte preparation is used to stimulate alloreactive T cells, presentation of MHC class II alloantigen is also likely to be influenced by non-professional APC such as B cells. It has been well described that DC, in contrast to resting B cells, can activate naive CD4+ T cells in vitro in the absence of CD40-CD154 interactions (8, 45–48). It is possible, therefore, that the response to a splenocyte preparation will be more dependent on the CD40-CD154 costimulation pathway for alloantigen stimulation than the response to an APC population migrating out of an organ.

In addition to potential differences in the type and activation status of APCs that migrate from the graft or that are present in a splenocyte preparation, differences in alloantigen load might also impact the dependency of direct pathway T cells on CD40-CD154 costimulation. We believe that in comparison with a donor leukocyte infusion, APCs would presumably remain quiescent, and therefore the response of direct pathway alloreactive CD4+ T cells would still be critically dependent on CD40-CD154 interactions.

Although both direct and indirect recognition of MHC alloantigens by CD4+ T cells have been demonstrated to operate in cardiac allograft rejection, the relative contribution of these two different pathways to graft rejection remains controversial (51). It has been proposed that CD40-CD154 costimulation may play a more significant role in the interaction between cells in the recipient immune system following indirect presentation of alloantigen.

In support of this hypothesis, CD40−/− recipient mice were found incapable of rejecting wild-type cardiac allografts (20). This finding suggested that expression of CD40 molecules by recipient, rather than donor cells, provided the critical costimulatory signals. Similar findings were reported using CD80/CD86−/− double-knockout recipient mice (52). The importance of the CD40-CD154 costimulatory pathway for indirect CD4+ T cell alloreactivity can also be seen when alloantibody responses are examined. For the generation of alloantibodies, recipient B cells present allogeneic peptides by the indirect pathway to CD4+ T cells, a process that is dependent on CD40-CD154 interactions (53, 54). Taken together, these results are consistent with the idea that blockade of CD40-CD154 interactions in the absence of a donor leukocyte infusion prevents CD4+ T cell-mediated rejection by affecting CD4+ T cells that recognize alloantigen by the indirect pathway.

Although we have clearly demonstrated that CD4+ T cells that respond to alloantigen via the direct pathway of allorecognition are rapidly deleted following pretreatment with alloantigen and anti-CD154, it is not known how this treatment affects CD4+ T cells that recognize alloantigen by the indirect pathway. To examine this question, we used a nontransgenic model in which both the direct and indirect pathways of alloantigen recognition were intact. Using AT experiments, we were unable to demonstrate the generation of regulatory cells by donor leukocyte infusion + anti-CD154 therapy. Therefore, it is likely that recognition of alloantigen during blockade of CD40-CD154 interactions does not generate regulatory cells, but rather enables regulatory cells to develop or expand following interactions with the allograft itself.

In conclusion, we believe that results presented in this work, together with our previous findings (16), indicate that both CD4+ and CD8+ T cells that recognize alloantigen via the direct pathway of allorecognition do not require CD40-CD154 costimulation when responding to a transplanted allograft. However, these cells are critically dependent on CD40-CD154 interactions when stimulated by infusion of donor alloantigen, as evidenced by their rapid deletion from the T cell repertoire under these conditions. Therefore, these data suggest that the inclusion of alloantigen in any potential clinical therapy involving costimulatory molecule blockade would be of significant benefit, enabling manipulation of both the direct and the indirect pathways of alloantigen recognition.

References

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