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Anti-LFA-1 Induces CD8 T-cell Dependent Allograft Tolerance and Augments Suppressor Phenotype CD8 cells

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Abstract

The induction of tolerance to transplanted organs is a major objective in transplantation immunology research. Lymphocyte function-associated antigen-1 (LFA-1) interactions have been identified as a key component of the T-cell activation process that may be interrupted to lead to allograft tolerance. In mice, α LFA-1 mAb is a potent monotherapy that leads to the induction of donor-specific transferable tolerance. By interrogating important adaptive and innate immunity pathways, we demonstrate that the induction of tolerance relies on CD8⁺ T-cells. We further demonstrate that α LFA-1 induced tolerance is associated with CD8⁺CD28⁻ T-cells with a suppressor phenotype, and that while CD8 cells are present, the effector T-cell response is abrogated. A recent publication has shown that CD8⁺CD28⁻ cells are not diminished by cyclosporine or rapamycin, therefore CD8⁺CD28⁻ cells represent a clinically relevant population. To our knowledge, this is the first time that a mechanism for α LFA-1 induced tolerance has been described.

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Keywords

Rejection; tolerance; suppressor cells

1. Introduction

Clinical immunosuppression for organ transplantation has gradually improved allograft survival, but at the cost of a multitude of side effects. The induction of donor-specific tolerance to transplanted organs would greatly reduce patient reliance on immunosuppressants and potentially enhance long-term graft survival.

Popular targets for inducing tolerance are the leukocyte function-associated antigen-1 (LFA-1)/intercellular adhesion molecule-1 (ICAM-1) and CD40/CD154 activation axes of mono- and dual-therapies, although most studies to date have concentrated on combinational therapies [1–6]. While these studies are informative, the use of multiple therapies can confound our ability to define the mechanisms of action for each agent. Here we concentrated on an α LFA-1 mAb to induce donor-specific tolerance and to explore adaptive and innate immunity requirements of that induction process.

Previous studies identified a requirement for host MHC class I molecules in the induction of LFA-1 tolerance to islet allografts [7, 8]. Conversely, our group demonstrated that host MHC class I molecules were dispensable for acute rejection of murine cardiac allografts, but rejection depended on the *direct* (donor MHC-restricted) pathway of donor antigen presentation by donor MHC class II on APCs to host CD4⁺ T-cells to the point that *direct* CD4 T-cells are required and sufficient [9]. Therefore, there appears to be differential MHC class/T-cell phenotype requirements for tolerance and for rejection. In this study, we demonstrate that α LFA-1 monotherapy induces tolerance to cardiac allografts and we identify cell populations important in the tolerance induction process.

2. Materials and Methods

2.1 Animals:

Inbred female BALB/cByJ (BALB/c H-2^d), C57Bl/6J (B6, H-2^b), C3H/HeJ (C3H, H-2^k), β -2 microglobulin deficient (MHC class I deficient) B6.129P2-B2m^{tm1Unc}/J (B6 β 2M^{-/-}, H-2^b), C57Bl/6-rag^{tm1/mom} (B6 rag1^{-/-}, H-2^b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Female C57Bl/6 CD1d^{-/-} (CD1d, H-2^b) mice were obtained from L. van Kaer, Vanderbilt, and bred in-house. BALB/c-C3H F1 (H-2^{d/k}) mice were bred in-house. 4C TCR transgenic B6 mice (specific for an unknown peptide presented by I-A^d) were obtained from Dr. S.M. Kang of UCSF and bred in-house. They were subsequently crossed with the CD45.1 congenic strain and the FoxP3 GFP reporter mouse and bred in-house. All mice were housed under pathogen-free conditions and all procedures were performed in accordance with a University of Colorado Denver IACUC approved protocol and cared for in an AAALAC-accredited facility according to the guidelines established by the National Institutes of Health.

2.2 Heterotopic Cardiac Transplantation:

For tolerance induction experiments, hearts from BALB/c mice were transplanted into B6, B6 $\beta 2M^{-/-}$ or CD1d $^{-/-}$ mice. For adoptive transfer experiments, hearts from BALB/c, C3H or BALB/c-C3H F1 mice were transplanted into B6rag $^{-/-}$ mice or syngeneic (B6-B6) grafts were performed. To explore the role of *indirect* (host MHC-restricted) antigen presentation, BALB/c hearts were transplanted into B6 $\beta 2M^{-/-}$ recipients. Because we did not have access to BALB/c $\beta 2M^{-/-}$ mice to interrogate the *direct* pathway we reversed our standard strain combinations and transplanted B6 $\beta 2M^{-/-}$ hearts into BALB/c recipients.

Vascularized grafts were transplanted according to standard microsurgical techniques [10, 11]. Briefly, the harvested donor heart was placed in 4°C saline until transplantation. An end to side anastomosis of the donor aorta to the recipient aorta and an end to side anastomosis of the donor pulmonary artery to the recipient IVC were made using running 10–0 nylon sutures. Heart graft survival was monitored daily by palpation with completion of rejection defined as cessation of detectable beat and confirmed by laparotomy under anesthesia.

2.3 mAb therapy:

Antibody therapies followed the previously used protocol [12] with rat anti-mouse α LFA-1 mAb (KBA; rat IgG2a, cell line generously provided by Dr. Ihara, Charlestown, MA), 200 μ g i.p. on days 0, 1, 7 and 14 post-transplant. Control Ab therapy was rat IgG at the same doses and time points as the therapy antibody. CD8 T-cells were depleted with rat anti-mouse α CD8 mAb (2.43; rat IgG2b), 250 μ g i.p., on days –1, 0, 1 and 2 for the induction phase, and days 27, 28, 29 and 30 for the maintenance phase. NK1.1+ cells were depleted with a single dose (500 μ g) of NK1.1-specific antibody (PK136; mouse IgG2a; HB191 ATCC) on day –1 relative to transplant. Anti-PD-1 (J43; hamster IgG) was administered at 500 μ g i.p. on day 0, and then 250 μ g on days 2, 4, 6, and 8 post-transplant. Anti-CD154 (MR-1; hamster IgG), 250 μ g i.p., was administered on day –1 and twice a week for 5 weeks, 10 doses total. Anti-CD25 antibody (PC61; rat IgG1) was administered i.p. at 500 μ g on days –1 and +2 relative to transplant.

KBA, 2.43, GK1.5 and NK1.1 were generated by ascites production and quantitated by isotype-specific ELIS. Control rat IgG was obtained from Sigma-Aldrich. MR-1, J43 and PC61 were purchased from Bioxcell. The activity of α CD8, α CD25, α NK1.1 and α PD-1 mAbs is depicted in supplementary figure 1.

2.4 Adoptive cell transfers:

Naïve or “tolerant” leukocytes: Spleens were harvested from naïve B6 mice or long term surviving recipients of α LFA-1 mAb treated allografts according to previously published methods [13]. On POD3–7 heart-grafted recipients were injected with 3×10^7 unfractionated splenocytes i.p. For the dominant tolerance experiment 3×10^7 naïve and 3×10^7 tolerized splenocytes were co-injected into recipients.

4C cells, in vivo proliferation experiment: Spleens and lymph nodes were harvested from 4C CD45.1 FoxP3 GFP B6 mice and the tissues dissociated into a single cell suspension. This suspension was labeled with a cell proliferation dye, eFluor 670 (eBioscience, San Diego,

CA) according to the manufacturer's instructions. Briefly, 5 μ M eFluor 670 were incubated with 10 $\times$ 10⁶ cells/ml for 10 min at 37°C and washed 2–3 times with cold media. 2.5 $\times$ 10⁶ cells were injected i.v. on POD5.

2.5 Flow cytometry:

Freshly isolated lymphocytes and splenocytes were labeled with PerCP-Cy5.5 CD4 (eBioscience, clone RM4–5), PE V β 13 (MR12–4, Biolegend), PE-Cy7 CD45.1 (A20, eBioscience), BV650 CD8 (53–6.7, BD Biosciences), PE-Cy7 CD11c (HL3, BD Biosciences), PE CD28 (37.51, eBioscience), PE H-2K^d (SF1–1.1, BD Biosciences), PerCP-Cy5.5 CD3 (eBioscience, 45-0031-82), PE-Cy7 CD25 (eBioscience, 552880), PE FoxP3 (eBioscience, 12-5773-80B), APC NK1.1 (eBioscience, 17-5941-82), PE PD-1 (eBioscience, 12-9985-81), Alexa 647 CD85k, for ILT3 staining (H1.1, Biolegend), UV455 viability dye (eBioscience, ref. 65-0868-14), eFluor 506 viability dye (eBioscience, 65-0866-14), eFluor 670 (eBioscience, 65-0840-85).

Approx. 5 $\times$ 10⁵ cells were labeled for 20 min at 4°C with the indicated Abs.

Data was acquired on a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software version 9.9.9 (TreeStar, Ashland, OR).

Gating strategies. For proliferation study: Lymphocytes were gated from SSC-A/FSC-A, live cells from SSC-A/UV455, single cells from FSC-H/FSC-A, CD45.1⁺ from SSC-A/CD45.1, proliferation of donor specific T-cells from upper right quadrant of CD4⁺V β 13⁺. For cell populations: Lymphocytes were gated from SSC-A/FSC-A, live dead cells from SSC-A/UV455, single cells from FSC-H/FSC-A, then cells of interest as described in the Results section.

2.6 Statistical analysis:

Kaplan-Meier U test, Student T-test and Fisher Exact test using commercially available software were used to determine significance of graft survival data and to compare independent population variables. Quoted survival times are mean days $\pm$ standard deviation. A p value of less than 0.05 was considered significant.

3. Results

3.1 α LFA-1 monotherapy induces long-term survival of murine cardiac allografts.

In our model of fully vascularized solid organ transplantation, BALB/c mouse cardiac allografts were acutely rejected in untreated wild-type B6 mice, while a course of α LFA-1 mAb extended graft survival to >100 days 23/24 mice (Fig. 1a). In contrast, recipients treated with control rat IgG were acutely rejected their grafts in almost identical tempo to untreated recipients (Fig. 1a).

3.2 α LFA-1 treatment confers donor specific transferable tolerance, but not linked suppression or dominant tolerance.

To determine whether α LFA-1 therapy induced transferable tolerance, 3×10^7 splenocytes from recipients of long-term (>100 days) surviving cardiac allografts were adoptively transferred into B6rag^{-/-} recipients of BALB/c heart grafts. These transferred cells did not reject the allografts, thereby demonstrating transferable tolerance, whereas cells transferred from naïve B6 mice were capable of rejecting the grafts (Fig. 1b). Additionally, B6rag^{-/-} recipients of third party hearts (C3H) acutely rejected their grafts following adoptive transfer of splenocytes tolerized to BALB/c, demonstrating donor-specificity (Fig. 1b).

To explore the existence of linked suppression we transferred 3×10^7 BALB/c tolerant splenocytes into B6rag^{-/-} recipients of BALB/c-C3H F1 donor hearts. The transferred cells rejected these mixed MHC grafts, indicating the absence of linked suppression (Fig 1c). To determine the property of dominant tolerance, 3×10^7 tolerized B6 splenocytes were co-injected with 3×10^7 naïve B6 splenocytes into B6rag^{-/-} mice bearing BALB/c grafts. These allografts were acutely rejected, indicating a lack of dominant tolerance in this model (Fig. 1c)

3.3 Adoptively transferred cells must experience donor-type antigen and do not transfer global hyporesponsiveness.

As proof of principal that tolerized cells must experience donor-type antigen in order to transfer tolerance, and to demonstrate that we are not transferring a state of α LFA-1 induced hyporesponsiveness, recipients with B6-B6 syngeneic grafts were treated with α LFA-1 and at the completion of 100 days post-transplant, splenocytes from these animals were adoptively transferred into B6rag^{-/-} recipients of BALB/c hearts. In contrast to tolerized splenocytes, the transferred cells from these mice acutely rejected BALB/c allografts. (13.7 ± 1.5 days, $n=3$ DNS).

3.4 α LFA-1 tolerance induction requires host MHC class I.

A primary goal of this study was to explore the requirement for MHC class I expression based on previous findings for islet allo- and xenograft transplantation [14, 15]. We transplanted control BALB/c cardiac allografts into untreated MHC class I deficient B6 $\beta 2M^{-/-}$ recipients and these grafts were acutely rejected (Fig. 1d). Anti-LFA-1 resulted in prolonged allograft survival in wild-type B6 mice, but, in stark contrast, cardiac allografts were rejected acutely in α LFA-1 treated B6 $\beta 2M^{-/-}$ recipients with kinetics similar to untreated recipients. To explore if this requirement for the MHC class I pathway was solely an *indirect* one, B6 $\beta 2M^{-/-}$ hearts were transplanted into α LFA-1 treated BALB/c recipients. We found that the majority of grafts survived >100 days (Fig. 1e), demonstrating that host but not donor MHC class I expression, is required for α LFA-1 induced tolerance. As this experiment represented a reversal of our usual strain combinations, the relevant control experiments are included in Table I.

3.5 α LFA-1 tolerance induction is independent of NK-cells and CD1-restricted NKT-cells.

The B6 $\beta 2M^{-/-}$ mouse is deficient in NKT-cells and possesses hyporesponsive NK-cells [14, 16]. Since NK-cells have been implicated in some forms of tolerance induction [7, 17], we

turned our attention to interrogating these innate immune populations. When BALB/c heart bearing B6 recipients were treated with α LFA-1 and depleting α NK1.1 mAb, grafts survived >100 days (DNS).

NKT-cells restricted by the non-classical class I MHC molecule CD1 have also been implicated in tolerance induction [18, 19]. Therefore, the loss of tolerance observed in CD1^{-/-} mice may be attributable to either NKT or NK-cells. To ascertain any involvement of these cell populations, B6 CD1^{-/-} recipients were untreated, treated with α LFA-1 alone or in combination with α NK1.1. While untreated recipients acutely rejected grafts, α LFA-1 resulted in dramatically prolonged survival, in stark contrast to α LFA-1 treated B6 β 2M^{-/-} recipients. The addition of α NK1.1 was insufficient to break tolerance (DNS). This finding indicates that neither NKT-cells or NK-cells are required for α LFA-1 induced tolerance. As a control experiment, when B6 recipients of BALB/c hearts were treated with both α LFA-1 and α NK1.1 mAb the grafts persisted for >100 days (n=5). (DNS).

3.6 PD-1 is not required for α LFA-1 tolerance induction.

Programmed cell death-1 (PD-1) has also been implicated as permissive for tolerance induction [20–23]. To investigate if this pathway is required for α LFA-1 induced tolerance, we treated one group of mice with α LFA-1 alone and another group with α LFA-1 and anti-PD-1 mAb. We were unable to break tolerance with administration of the PD-1 antibody (DNS).

3.7 CD8⁺ T-cells are required for the induction, but not the maintenance of α LFA-1 tolerance.

Since B6 β 2M^{-/-} recipients are also deficient in CD8⁺ T-cells [14, 16], it was important to investigate if these cells were required in this model. We transplanted BALB/c hearts into wild-type B6 recipients and depleted CD8⁺ T-cells either 1) within the peri-transplant period (induction phase) or delayed 28 days after transplantation (maintenance phase). In both cases we maintained our standard α LFA-1 treatment. We found that the induction of tolerance to cardiac allografts depended on the initial presence of CD8⁺ T-cells with 6/7 grafts rejecting by day 26. Surprisingly, when α CD8 mAb treatment was delayed the grafts persisted >100 days demonstrating that CD8⁺ T-cells are not required for the maintenance of tolerance once it is established (Fig. 2a).

3.8 The requirement for CD8⁺ T-cells is therapy specific.

Another widely used tolerizing mAb is α CD154, so to expand our understanding of the mechanisms of tolerance induction we explored whether the requirement for CD8⁺ T-cells in the induction of tolerance is specific to α LFA-1 treatment. B6 recipients of BALB/c cardiac grafts received α CD154 with or without α CD8. As a monotherapy, α CD154 prolonged allograft survival and concurrent CD8⁺ T-cell depletion at the time of transplant did not affect survival (Fig. 2b), highlighting the probability that different mechanisms are involved in different tolerance-induction regimens.

3.9 CD4⁺ regulatory cells are not required for α LFA-1 tolerance induction.

Utilizing flow cytometry, we interrogated CD4⁺FoxP3⁺ expression in graft recipients on POD10 and found no significant differences in the CD4⁺FoxP3⁺ populations when we compared untreated recipients with α LFA-1 treated and α LFA-1 plus α CD8 treated recipients at this time point (Fig. 3a).

To further investigate a requirement for CD4⁺ regulatory cells in α LFA-1 induced tolerance we treated B6 recipients of BALB/c allografts with α LFA-1 and α CD25 at the time of transplantation. With this treatment grafts survived >100 days while treatment with α CD25 in otherwise untreated recipients resulted in acutely rejected grafts, which closely mirrors published data [24] (Fig. 3b).

Taken together, these data are highly suggestive that CD4⁺FoxP3⁺ T-regulatory cells are not required for α LFA-1 induced tolerance.

3.10 The alloreactive CD4 response is restrained by α LFA-1 treatment.

With a demonstrable lack of a CD4 regulatory mechanism, it seemed possible that CD8⁺ T-cells may affect graft destructive, direct (i.e. donor MHC-reactive) CD4⁺ T-cells, which we showed mediate acute cardiac allograft rejection [9]. To investigate, we performed an *in vivo* proliferation assay utilizing a 4C CD45.1 FoxP3 GFP reporter mouse, a CD4⁺ TCR-transgenic with specific reactivity to BALB/c MHC class II. The congenic markers permitted us to distinguish adoptively transferred cells from the endogenous CD45.2⁺ cells of B6 mice, and the FoxP3 GFP reporter let us visualize 4C FoxP3⁺ T-regulatory cells. Transplants were performed according to the scheme in Table II.

When recipients received α LFA-1, total lymphocytes in the mediastinal lymph nodes (the draining lymph nodes for the heterotopic heart model [25]) were reduced in both the proportion (73% reduction) and number (90% reduction), relative to untreated controls (Fig. 4a). When CD8⁺ T-cells were depleted the proportion and numbers of cells were increased compared to α LFA-1 treatment alone, and given that most of these remaining cells are CD4⁺, this represents an increase in the proportion and number of cells of over 200% compared to those animals which were CD8⁺ T-cell replete.

We next analyzed the adoptively transferred, directly BALB/c-reactive 4C (CD45.1⁺ CD4⁺V α 13⁺) TCR transgenic T-cells. Relative to untreated transplant recipients, there was a minor but significant decrease in the frequency, and a large decrease in the number of anti-BALB/c CD4⁺V β 13⁺ cells under α LFA-1 administration, which was reversed when CD8 cells were depleted (Fig 4b). There was also a significant decrease in *in vivo* proliferation of these cells with α LFA-1 administration that was partially reversed by depletion of CD8 cells (Fig. 4c). It is unclear from this result if the reduction in proliferation of graft reactive cells was due to the action of the α LFA-1 mAb directly on the CD4⁺ T-cell population, or the presence of a potentially suppressive effect of CD8⁺ T-cells, or both. Therefore, we expanded our investigation into the role of CD8 T-cells in this model.

3.11 The proportion of CD8⁺CD28⁻ T-cells is increased in αLFA-1 treated recipients.

Since CD8⁺ T-cells are required in this model, we turned our attention to the possible involvement of CD8⁺CD28⁻ T-suppressor (Ts) cells. These cells have been shown to be antigen specific [26], and capable of inducing tolerogenic changes in donor-antigen presenting APCs which then suppress the activity of donor-antigen specific CD4⁺ T-cells [27, 28]. It has also been shown that this is a contact-dependent system rather than relying on the secretion of cytokines [29]. We used flow cytometry to investigate the incidence of antigen experienced (CD44^{hi}) CD8⁺CD28⁻ T-cells on post-operative day 7 (POD7) in naïve mice, untreated transplant recipients and those treated with αLFA-1. CD44 was used as a marker of antigen experience within the CD8⁺ population. Anti-LFA-1 treated B6 recipients demonstrated significant increases in CD8⁺CD44^{hi}CD28⁻ cells in the draining mediastinal lymph nodes compared to untreated transplanted controls, with similar increases in other compartments (Fig. 5a). While there was a reduction in the numbers of these cells in the draining lymph nodes, this reduction was only half that observed for all cells in the nodes, as shown in Fig. 4a, right panel. Additionally, we found statistically significant phenotypic shifts in CD8 and CD4 T-cells under αLFA-1 administration in each of the three compartments we observed (Table III). When comparing cells of an effector CD4 type (CD4⁺CD44^{hi}CD28⁺) with those of a suppressive CD8 type (CD8⁺CD44^{hi}CD28⁻), the balance of cell types shifted in favor of the potentially suppressive phenotype by a factor of between 2.7 and 4.1 (Table IIIa). Similar phenotype shifts were also observed when comparing effector type CD8 cells and suppressor type CD8 cells, by a factor of between 3.8 and 5.7, again in favor of the suppressive type (Table IIIb).

3.12 αLFA-1 increases the expression of ILT3 on host-derived CD11c cells in draining lymph nodes.

When donor antigen-presenting APCs are engaged by T-suppressor cells, APCs upregulate inhibitory receptor immunoglobulin like transcript 3 (ILT3) and are then directed toward suppressive phenotype [30–32]. As this represents a mechanism of CD8 suppressor T-cells, we assessed host-derived (H-2K^b) CD11c⁺ cells (APCs) in the draining lymph nodes for co-expression of ILT3 in αLFA-1 treated graft recipients and found a significant increase in ILT3 expression compared to naïve mice, transplanted untreated controls, and αCD8 treated recipients (Fig. 5b).

4. Discussion

We define tolerance as an antigen-specific non-destructive response resulting in immune regulation that leads to long-term graft survival. The search for donor-specific tolerance induction is a long-held goal of the transplant immunology community and would likely correlate with reductions in immunosuppression for patients. This study demonstrates robust donor-specific tolerance by blocking the LFA-1 (CD11a) molecule.

LFA-1 blockade with efalizumab has been used successfully to treat psoriasis, an autoimmune disease. Guttman-Yassky et al [33] investigated T-cell subsets in treated patients and reported T-cell hyporesponsiveness and a decrease in CD28⁺ expression, which were very important findings in the context of the current study. Efalizumab has also been used to treat

patients who received islet transplants [34, 35] and renal transplants [36] and Nicolls and Gill provide compelling arguments that LFA-1 blockade may have further clinical roles [37]. Theoretically, this is achieved by a phenotypic shift in the T-cell/APC activation process by down-regulation of the costimulatory LFA-1/ICAM-1 synapse. However, it may be oversimplistic to think of α LFA-1 treatment as merely costimulation blockade. When LFA-1 and ICAM-1 molecules engage each other, T-cell receptors on T lymphocytes and MHC molecules on APCs have markedly increased avidity [38, 39]. By allowing antigen presentation by APCs to T-cells, albeit with only moderate avidity, and blocking costimulation, the T-cells can be said to be antigen experienced but are not fully activated and may be directed down an anergic/hyporesponsive pathway that may lead to donor-antigen specific tolerance. Because costimulation is blocked, there is no induction of the α chain of the IL-2 receptor (CD25) and the T-cells retain only the low affinity β and χ chains of the IL-2 receptor resulting in functionally altered effector T-cells. Intact LFA-1 also facilitates endothelial transmigration of T-cells to sites of injury and for general immune surveillance [40, 41]. Li reported that peri-transplant blockade of LFA-1 resulted in a paucity of infiltrating cells within the graft [42] and Setoguchi [43] showed that post-transplant treatment with α LFA-1 mAb resulted in CD8 T-cells of a memory profile within the cardiac grafts, but that these cells were incapable of injurious activity.

In the current study we interrogated distinct cell populations from both the innate and adaptive arms of the immune system and found a role for CD8⁺ T-cells in the induction of tolerance. Surprisingly, this requirement for CD8⁺ T-cells was not vital for the maintenance of tolerance and was therapy specific. When CD8⁺ T-cells were depleted from BALB/c-B6 grafts treated with α LFA-1 grafts survived slightly longer than controls, but tolerance was not established. The requirement for CD8⁺ T-cells for tolerance induction was in stark contrast to the requirement for *direct* CD4⁺ T-cells for acute rejection in this cardiac allograft model system [9].

While not performed as a part of this study, our group has previously reported the results of *in vitro* MLR using α LFA-1 and demonstrated a clear suppression of both the CD4⁺ and CD8⁺ T-cell responses [44]. In the current study, we observed a reduction in proliferation, proportion and number of donor-reactive CD4⁺V β 13⁺ TCR-transgenic cells from adoptively transferred lymphocytes in the draining lymph nodes 10 days post-transplant (5 days post-adoptive transfer) when α LFA-1 was administered. In each case, these reductions were reversed when CD8⁺ T-cells were depleted. Allied to this, we found a significant reduction in total lymphocytes under α LFA-1 administration, which was partly recovered in the absence of CD8⁺ T-cells. In a separate experiment, on POD7 we saw very consistent phenotypic shifts away from effector CD4 and CD8 type cells towards CD8 suppressor type cells in all the observed compartments.

We found a significant increase in CD8⁺CD28⁻ T-cells, a potentially suppressive population. An important part of the CD8⁺CD28⁻ T-suppressor cell-based cascade is the up-regulation of ILT3 on APCs [30–32], and indeed, we observed this up-regulation in the draining lymph node when LFA-1 was blocked. This increase was reversed when CD8⁺ T-cells were depleted, adding weight to the possible requirement for CD8⁺CD28⁻ T-suppressor cells in this model. While others have found an increase in CD4⁺CD25⁺FoxP3⁺ T-regulatory cells

when using LFA-1 blockade in a skin graft model [2], we were unable to find similar increases in the cardiac allograft model. In fact, depleting CD25⁺ cells had no effect, which is consistent with a role for CD8⁺CD28⁻ suppressor cells as they are CD25⁻ [45–48].

The increase in CD8⁺CD28⁻ cells in association with α LFA-1 may help explain why α LFA-1 and CTLA4-Ig have been reported to be synergistic in transplant models [2, 49, 50]. CTLA4-Ig competes for T-cell CD28 engagement with CD80/CD86 on APCs, thereby blocking any potential alloreactivity by CD28⁺ cells. It is also possible that α LFA-1 is highly effective as a monotherapy because it has been shown that when T-suppressor cells engage APCs, ICAM-1 is down-regulated [27], ICAM-1 being the ligand of LFA-1. CD80/86 molecules are also down-regulated in this scenario.

Clinically, the presence of CD8⁺CD28⁻ T-suppressor cells has been correlated with improved transplant outcomes [51–53] and it has also been shown that the suppressive effects of CD8⁺CD28⁻ T-cells are not diminished by cyclosporine or rapamycin administration [54]. Therefore, the generation of a population of donor-antigen specific CD8⁺CD28⁻ T-suppressor cells may have future clinical relevance.

5.0 Conclusion

In conclusion we have shown that the administration of α LFA-1 mAb constitutes a potent monotherapy that induces tolerance in a fully mismatched cardiac allograft model. We have also shown that induction of tolerance depends on CD8⁺ T-cells, that there is a significant increase in the proportion of CD8⁺CD28⁻ T-cells and ILT3⁺ on APCs, and there is a phenotypic shift away from effector cells toward suppressive cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

β2M^{-/-}	beta-2 microglobulin-deficient
DNS	data not shown
ILT3	immunoglobulin like transcript 3
POD	post-operative day
SPL	spleen

6.0 References:

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Highlights

- α LFA-1 monotherapy induces long-term survival of murine cardiac allografts and confers donor specific transferable tolerance, but not linked suppression or dominant tolerance.
- Adoptively transferred cells must experience donor-type antigen and do not transfer global hyporesponsiveness.
- α LFA-1 tolerance induction requires host MHC class I.
- CD8⁺ T-cells are required for the induction, but not the maintenance of α LFA-1 tolerance.
- The proportion of CD8⁺CD28⁻ T-cells is increased in α LFA-1 treated recipients.

Figure. 1: (a). α LFA-1 treatment induces long-term survival of murine cardiac allografts. BALB/c mouse cardiac allografts acutely rejected in untreated wild-type B6 ($\diamond$, $n=16$), while in the same strain combination rejection was abrogated when treated with α LFA-1 mAb, with 23/24 surviving greater than 100 days ($\blacktriangle$, $n=24$). Grafts treated with the control rat IgG acutely rejected in tempo similar to untreated grafts ($\blacksquare$, $n=5$). Controls vs. α LFA-1 treated, $p<0.001$.

Figure. 1: (b). α LFA-1 treatment confers donor specific cardiac allograft tolerance.

Splenocytes from long-term surviving α LFA-1 treated allografted animals were adoptively transferred into B6rag^{-/-} recipients of BALB/c heart grafts and the transferred cells did not reject those allografts (■, n=6). When transferred into B6rag^{-/-} recipients of C3H heart grafts (▲, n=4), those grafts were robustly rejected. When reconstituted with 3×10^7 naïve B6 splenocytes, BALB/c hearts were rejected (×, n=5). Untreated B6rag^{-/-} recipients of BALB/c hearts were not rejected (◆, n=5).

Figure. 1: (c). α LFA-1 treatment does not confer linked suppression or dominant tolerance. For linked suppression experiments, BALB/c tolerant splenocytes were transferred into B6rag^{-/-} recipients of BALB/c-C3H F1 donor hearts. The transferred cells were able to reject these mixed MHC grafts (■, n=5). Dominant tolerance was also not observed by the transfer of the combination of 3×10^7 tolerized splenocytes co-injected with 3×10^7 naïve B6 splenocytes (◆, n=4).

Figure 1: (d, e). α LFA-1 cardiac allograft tolerance induction requires host but not donor MHC class I expression.

(d) BALB/c mouse cardiac allografts rejected acutely in B6 $\beta 2M^{-/-}$ recipients (■, n=4). Cardiac allografts were rejected acutely in α LFA-1 treated B6 $\beta 2M^{-/-}$ recipients with similar kinetics to untreated recipients (▲, n=5).

(e) To explore if this requirement for the MHC class I pathway was solely an *indirect* one, we transplanted B6 $\beta 2M^{-/-}$ hearts into α LFA-1 treated BALB/c recipients and found prolonged survival of grafts (▲, n=5) while the untreated cohort acutely rejected (■, n=4, p=0.01).

a

Figure 2: CD8⁺ T-cells are required for the induction, but not the maintenance of αLFA-1 cardiac allograft tolerance and the induction requirement for CD8⁺ cells is therapy specific. (a) Since B6 β2M^{-/-} recipients are also deficient in CD8 T-cells we investigated if these cells were required in this tolerance model. We found that the induction of tolerance observed in wild type vascularized cardiac grafts was dependent on the presence of CD8⁺ T-cells (▲, n=7). When αCD8 mAb treatment was delayed for 28 days the grafts persisted >100 days (◆, n=5, p<0.01) demonstrating that CD8⁺ T-cells are not required for the maintenance of tolerance once established. ↑ indicates timing of “late” administration of the αCD8 mAb.

(b) To enquire if this induction requirement was therapy specific recipients were treated with α CD154 mAb with (◆, n=5) or without α CD8 mAb (▲, n=8) and the results between these two groups were indistinguishable from each other.

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a

b

Figure 3: CD4 regulatory cells are not required for αLFA-1 cardiac allograft tolerance induction.

(a) On POD 10 CD4⁺FoxP3⁺ cell populations were not significantly different. n=3. (b) To investigate the potential role of CD25⁺ T-cells in the observed tolerance induction, B6 recipients of BALB/c hearts were treated with PC-61 mAb with or without αLFA-1 mAb. B6 mice treated with PC-61 alone were acutely rejected (■, n=5) while those also treated with αLFA-1 survived long-term (▲, n=5, $p < 0.002$).

Figure 4: α LFA-1 administration causes a reduction in lymphocytes and a subdued response by graft reactive CD4 T-cells in the draining lymph nodes.

(a) Mediastinal lymph nodes from naïve mice and untreated graft recipients contained similar proportions of lymphocytes, whereas those from α LFA-1 and α LFA-1 & α CD8 treated recipients contained significantly reduced lymphocyte proportions ($p < 0.00001$ in both cases). Cell numbers were reduced ten-fold from controls when treated with α LFA-1 ($p < 0.05$) and with α LFA-1 and α CD8 ($p < 0.05$), which were both slightly lower than those in naïve animals. $n=3$.

(b) *In vivo* proliferation of graft reactive CD4⁺V β 13⁺ cells in the mediastinal lymph nodes; using a gating strategy of lymphocytes/live–dead cells/single cells/CD45.1⁺ cells, we gated

on CD4⁺Vβ13⁺ double-positive cells and looked at proliferation histograms. The proportion and number of CD4⁺Vβ13⁺ cells within adoptively transferred, eFluor 670 labeled CD45.1⁺ cells were reduced in the presence of αLFA-1 mAb, which was reversed when CD8 cells were depleted. A reduction in proliferation of these cells was also observed, and this reduction was partially reversed by the depletion of CD8 T-cells (* p<0.05). Similar differences were present in the mesenteric lymph nodes, but not in the spleen. n=3.

(c) Representative flow cytometry histograms of proliferation of CD4⁺Vβ13⁺ cells.

a

b

Figure 5: αLFA-1 mAb treatment increases the proportion of CD8⁺ CD28⁻ T-cells and increases ILT3 expression on host APCs in cardiac allograft recipients.

(a) The proportion of CD8⁺CD44^{hi} cells that are CD28⁻ was significantly increased in αLFA-1 treated recipients compared to controls in all observed compartments. Cell numbers were also significantly reduced in lymph nodes but not in the spleen. (* p<0.05, ** p<0.01) As would be expected, of the few CD8⁺CD44^{hi} cells present in naïve mice, almost all of them are CD28⁻. n=3.

(b) Recipient derived APCs (CD11c⁺ cells) taken from the draining lymph nodes demonstrate a significant increase in ILT3 expression in the αLFA-1 treated group compared to naïve, transplanted untreated, and αLFA-1 mAb/αCD8 mAb treated graft recipients.

Increased ILT3 expression is an indicator that the APCs have been engaged by T-suppressor cells. (* $p<0.05$) $n=3$.

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Table I:
Comparing strain combinations for antigen pathway experiments.

Because we did not have access to BALB/c $\beta 2M^{-/-}$ mice for donor hearts to test the requirement for the *direct* pathway of MHC class I antigen presentation we used B6 $\beta 2M^{-/-}$ as heart donors and BALB/c mice as recipients. The table of results reveals no significant differences when the strain combinations are reversed.

Donor	Recipient	Treatment	n	Mean $\pm$ S.D.	Survival Data
BALB/c	B6 $\beta 2M^{-/-}$	-	4	15.8 ± 3.3	12, 15, 16, 20
B6 $\beta 2M^{-/-}$	BALB/c	-	4	15.5 ± 3.1	13, 14, 15, 24

Table II:
Scheme of transplants performed for in vivo proliferation experiments.

Group #	4C cell transfer	Cardiac allograft	α LFA-1	α LFA-1 & α CD8
1	+	+	–	–
2	+	+	+	–
3	+	+	+	+

Table III:
Phenotypic shift in effector and suppressor cell types.

(a) On POD7 there was a shift in cell type from a CD4 effector type toward a CD8 suppressor type.

CD8s = CD8⁺CD44^{hi}CD28⁻ and CD4e = CD4⁺CD44^{hi}CD28⁺

CD8s:CD4e	Untreated	α LFA-1 treated	Fold increase	p value *
Med LN	0.29:1	1.18:1	4.1	<0.0001
Mes LN	0.43:1	1.54:1	3.6	<0.0001
SPL	0.50:1	1.35:1	2.7	<0.0001

(b) This shift was also seen when comparing CD8 effector and suppressor type cells.

CD8s = CD8⁺CD44^{hi}CD28⁻ and CD8e = CD8⁺CD44^{hi}CD28⁺

CD8s:CD8e	Untreated	α LFA-1 treated	Fold increase	p value *
Med LN	0.35:1	2.00:1	5.7	<0.0001
Mes LN	0.62:1	3.23:1	5.2	<0.0001
SPL	0.85:1	3.23:1	3.8	<0.0001

*
by Fisher Exact Test