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Research paper

Anti-LFA-1 induces CD8 *T*-cell dependent allograft tolerance and augments suppressor phenotype CD8 cells

Robert J. Plenter[a,b,*], Todd J. Grazia[b,c], Marilyne G. Coulombe[a], Michelle K. Nelsen[a,c], Christine M. Lin[d], K. Scott Beard[a], Tinalyn M. Kupfer[a], Martin R. Zamora[b,1], Ronald G. Gill[a,1], Biagio A. Pietra[e,f,1]

[a] Dept. of Surgery, Colorado Center for Transplantation Care, Research and Education, University of Colorado Denver, Aurora, CO, United States
[b] Dept. of Medicine, Division of Pulmonary Sciences and Critical Care Medicine, University of Colorado Denver, Aurora, CO, United States
[c] Dept. of Immunology and Microbiology, University of Colorado Denver, Aurora, CO, United States
[d] Division of Pulmonary, Critical Care & Sleep Medicine, University Florida, Gainesville, FL, United States
[e] Dept. of Pediatrics, Division of Cardiology, University of Colorado Denver, Aurora, CO, United States
[f] Div. of Pediatric Cardiology, University of Florida, Gainesville, FL, United States

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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, αLFA-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 αLFA-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 αLFA-1 induced tolerance has been described.

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 αLFA-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 αLFA-1 monotherapy induces tolerance to cardiac allografts and we identify cell populations important in the tolerance induction process.

Abbreviations: $\beta 2M^{-/-}$, beta-2 microglobulin-deficient; DNS, data not shown; ILT3, immunoglobulin like transcript 3; POD, post-operative day; SPL, spleen

* Corresponding author at: Colorado Center for Transplantation Care, Research and Education, University of Colorado Denver, 1775 Aurora Ct., Box B-140, Aurora, CO 80045, United States.
E-mail address: Robert.Plenter@ucdenver.edu (R.J. Plenter).

[1] Co-senior authors.

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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 β2M$^{-/-}$, 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 β2M$^{-/-}$ 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 β2M$^{-/-}$ recipients. Because we did not have access to BALB/c β2M$^{-/-}$ mice to interrogate the *direct* pathway we reversed our standard strain combinations and transplanted B6 β2M$^{-/-}$ 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 αLFA-1 mAb (KBA; rat IgG2a, cell line generously provided by Dr. Ihara, Charlestown, MA), 200 μg 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 αCD8 mAb (2.43; rat IgG2b), 250 μg 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 μg) 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 μg i.p. on day 0, and then 250 μg on days 2, 4, 6, and 8 post-transplant. Anti-CD154 (MR-1; hamster IgG), 250 μg 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 μg 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 αCD8, αCD25, αNK1.1 and αPD-1 mAbs is depicted in Supplementary Fig. 1.

2.4. Adoptive cell transfers

Naïve or "tolerant" leukocytes: Spleens were harvested from naïve B6 mice or long term surviving recipients of αLFA-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 μM eFluor 670 were incubated with 10×10^6cells/ml for 10 min at 37 °C and washed 2–3 times with cold media. 2.5×10^6 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 Vβ13 (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. 5×10^5 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$^+$Vβ13$^+$. 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 ± standard deviation. A p value of < 0.05 was considered significant.

3. Results

3.1. αLFA-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 αLFA-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. αLFA-1 treatment confers donor specific transferable tolerance, but not linked suppression or dominant tolerance

To determine whether αLFA-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