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Trafficking of T Lymphocytes after Allo-Transplantation: A New Experimental Model

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Abstract: Leukocytes are believed to access sites of inflammation via cell adhesion molecule (CAM) mediated adhesion, activation and transmigration through the vascular endothelium. However, little is known about the mechanisms by which immune effector cells gain access to transplanted organ. The objective of this work was to evaluate a simple experimental model to characterize the migration of T lymphocytes to vascular tissue following transplantation. Brown Norway aortic allografts were transplanted orthotopically into Lewis recipients (n = 10). T cells were isolated from naïve or allo-exposed (activated) Lewis spleens using nylon wool columns. Cr^{51} labeled naïve T cells and In^{111} labeled activated T cells ($15x10^6$ of each) were injected into animals, 9 days after transplantation with or without anti-α4/β2_monoclonal antibody (n = 5/group). All animals were then sacrificed on day 10 and evaluated by histology and γ counts. All allografts had significant infiltration of the adventitia by mononuclear cells at day 10 as compared to control aorta. There was an average of 220+33 Cr^{51} cpm and 224 + 30. In^{111} cpm as compared to control aorta ($p<0.0001$). Anti α4/β2 mAb treatment resulted in significant reduction in labeled T cell infiltration with a 89% reduction in Cr^{51} cpm and 73% reduction in In^{111} cpm both approaching control levels. We have demonstrated that T lymphocytes infiltrate vascular tissue early after transplantation and appears to be mediated via α4/β2 integrin mechanism. This represents direct evidence of the role of CAM in trafficking of T cells in transplanted allografts and the characterization of a model to evaluate this trafficking.

Key words: Transplantation, inflammation, cell adhesion, T lymphocytes, integrin

INTRODUCTION

The presence of infiltrating T lymphocytes in acute and chronic rejection of transplanted organs has been well established[1,2]. However, the exact mechanisms by which these immune effector cells gain access to the transplanted organ remains to be characterized. To date most of the available information in transplant models has been derived from immunohistochemical studies demonstrating predominant mononuclear cell infiltration with both CD8+ and CD4+ T lymphocytes[2] .

Based on inflammatory models, Leukocytes are believed to access sites of inflammation via Cell Adhesion Molecule (CAM) mediated adhesion, activation and transmigration through the vascular endothelium[3]. Similarly, in experimental models of solid organ transplantation, vascular endothelial cells have been proposed to mediate selective recruitment of T cell subsets[4]. The expression of many CAMs has been shown to become up-regulated on vascular endothelial and parenchymal cells of transplanted organs early in the rejection process[5,6]. The importance of CAMs in facilitating solid organ allograft rejection is also supported by studies that have attempted to block the interactions between CAMs and their ligands with monoclonal antibodies or with soluble ligands which have resulted in variable prolongation of graft survival[7-11]. However from these studies significant differences were observed between treatment regiments and the degree of responses between species suggesting that yet to be identified additional molecules may play a role in heart allograft rejection[12].

Despite evidence that CAM's play a critical role in leukocyte trafficking following solid organ transplantation, their remains significant controversy as to the relative contributions, the dynamics and the kinetics of these interactions. The objective of the present study was to evaluate a simple experimental model to characterize leukocyte trafficking in the context of solid organ transplantation.

MATERIALS AND METHODS

Animals: Inbred male Brown Norway ($RT1.A^n$) and Lewis ($RT1.A^1$) rats weighing 300 to 350 grams were purchased from Harlan Sprague Dawley (Indianapolis,USA) and housed in the Medical Sciences Animal Care Facility with food and water ad libitum for 1 week prior to experimentation in accordance with the guidelines of the Canadian Council of Animal Care[13]. Infrarenal abdominal

aortic interposition graft transplantation was performed as described by Mennander *et al.*,[14]. Briefly donor Brown Norway (BN) rats were anaesthetized with pentobarbital (50mg/kg) and a midline abdominal incision was made. The infrarenal abdominal aorta was exposed and harvested for transplantation. The donor aorta was implanted orthotopically into a Lewis recipient by an end-to-end microsurgical anastomotic technique using interrupted 9-0 nylon (Sharpoint, Reading, PA, USA) sutures.

All Lewis animals (n = 10) received a BN aortic interposition graft on day 0 and were allowed to recover. At day 9, $30x10^6$ radiolabelled T cells were injected intra-venously (i.v.) into each animals, with or without anti-α4/β2_antibody (n = 5 in each group). The skin on the back of the animal was then shaved and 0.05 mL of test sample containing 300 U INF-γ, 100 ng of LPS were injected intradermally, using 30-gauge needles, into duplicate skin sites. Each of these stimuli has been previously shown to recruit T lymphocytes to dermal sites over 6-20 h (15,16). Diluent (RPMI 1640, 0.1% pyrogen free human serum albumin) was injected in control skin sites. All animals were then sacrificed on day 10 (24 h migration assay) and evaluated by histology and γ counts. At the time of sacrifice, samples of blood, spleen, liver, cervical nodes, injected skin sites (standard 13 mm punch size), native control aorta in addition to the transplanted segment of aorta. All samples were analyzed for Cr^{51} or In^{111} content in a Wallac Wizard III gamma radioisotope counter (Wallac, Gaithersburg, MD). Results were expressed as cpm of accumulated Cr^{51} or In^{111} per 24 h migration period per 10^6 cpm injected on labeled cells.

T cell isolation and labeling: Donor T lymphocytes were obtained from 2 different groups of animals: naïve Lewis rats and allo-transplanted Lewis rats. Allo-transplanted Lewis rats consisted of recovered Lewis animals 6 weeks after having received a BN aortic interposition graft while naïve animals had no prior exposure to BN antigens. Spleen T lymphocytes were isolated from donor animals as previously described[17,18]. Briefly, the spleen was minced to obtain a cell suspension and RBC's lysed with 0.84% NH_4Cl. T cells were purified by passage through a nylon wool column. The recovered cells from naïve spleen (no previous exposure to BN antigen) were labeled with Cr^{51} while the allo-exposed cells (from allo-transplanted animals) were labeled with In^{111}-labelled oxine. $15x10^6$ Cr^{51} T cells and $15x10^6$ In^{111} T cells, were washed and re-suspended for i.v. injection. Where indicated, monoclonal antibodies 3 mg of TA-2 (anti-α4, IgG^1) and 3 mg WT-3 (anti-β2, IgG^1) were also administered i.v.. Dosage of the mAb was based on previous work[9,18].

Tissue processing: Following gamma counts all tissue harvested was processed for histology. Briefly all tissue was fixed in 10% formalin for 24 h paraffin embedded, serially sectioned (5μm) and stained for histology (Hematoxylin and eosin). Important cell subsets infiltrating the aortic grafts were evaluated using immunocytochemical techniques on paraffin embedded tissue. Briefly, slides will be deparaffinized; endogenous peroxidase quenched (HOOH/MeOH); non-specific staining blocked with normal horse serum and subsequently incubated with primary antibody specific for T lymphocytes (OX-8, IgG^1 specific for CD8 expressing cells) and monocyte macrophage lineage cells (ED-1, IgG^1). Sections are then incubated with biotinylated secondary antibody and labelled with peroxidase avidin/biotin complex using 3,3' diaminobenzidine as the chromogen (Vectorlab, Burlingame, CA).

Statistics: Means were obtained from 5 animals in each group. Data was reported as mean and standard error of the mean. Analysis of variance (ANOVA) was used to evaluate groups and Post hoc Fishers test of least significant difference (PLSD) was used to assess statistical significance, with $p<0.05$ being the limit of significance.

RESULTS

A total of 10 Lewis rats received an orthotopic allograft aorta. One animal dyed prior to harvesting resulting in a 90% survival. All animals received labeled naïve ($15x10^6$ Cr^{51} T lymphocytes) and allo-stimulated ($15x10^6$ In^{111} T lymphocytes) T lymphocytes. Two groups of animals were created based on weather monoclonal antibodies again α4/β2 integrins were given concomitantly with the labeled cells (n = 5 per group).

At 10 days after transplantation, there was significant mononuclear cell infiltration principally of the adventicia of the transplanted aorta as compared to control aorta (Fig. 1 a,b.). Using immunocytochemical techniques on paraffin embedded tissue a significant number of these infiltrating cells were shown to be T lymphocytes (CD8+) or monocyte/macrophages lineage cells (ED1+) (Fig. 1 c,d.). There was minimal intimal proliferation observed but there was some evidence of early destruction of the vascular media manifested by loss of elastic laminae (Fig. 1 a,b).

The intensity of naïve T lymphocyte (Cr^{51} labelled T lymphocytes) accumulation into allo-aortic tissue

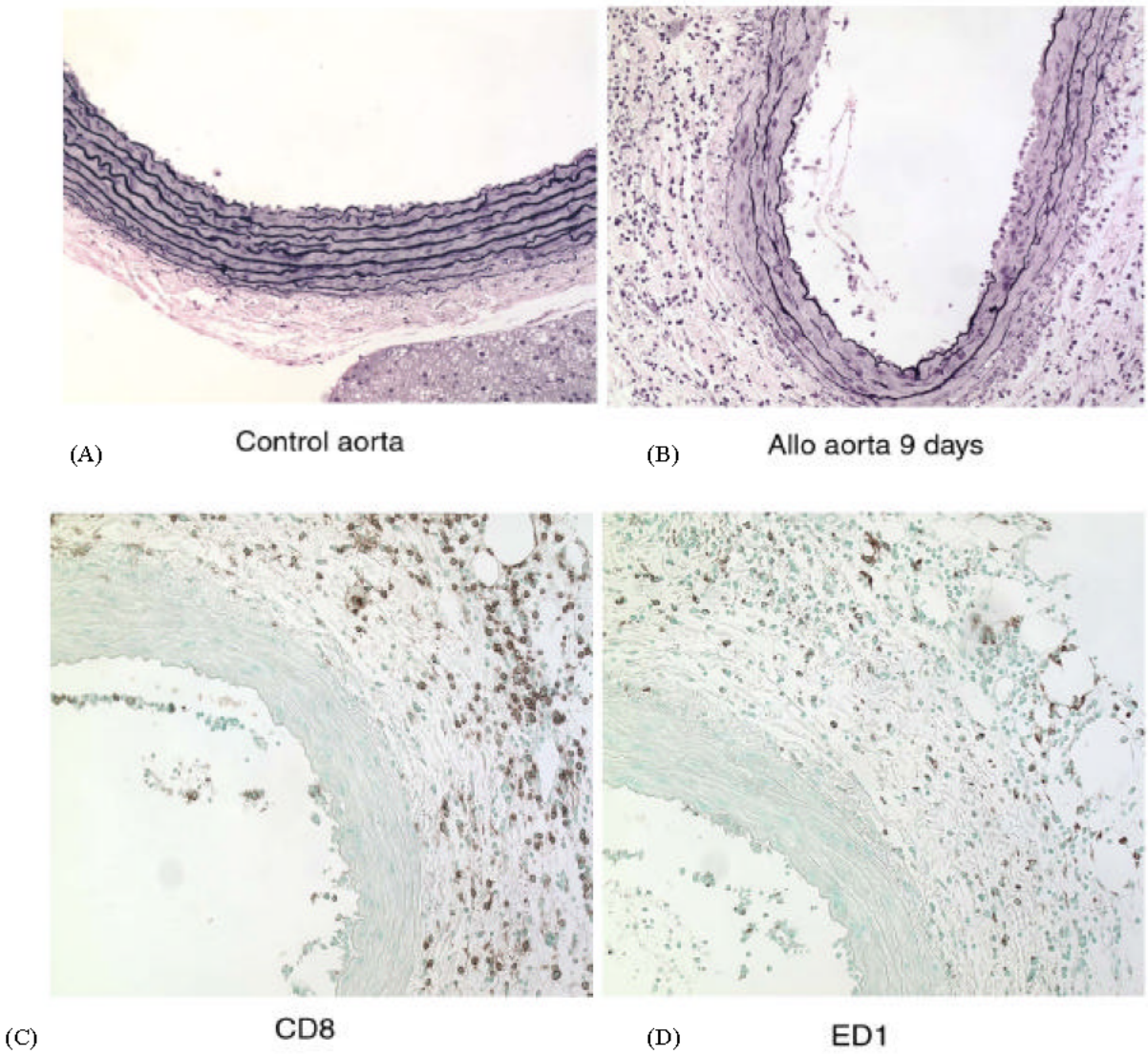

Fig. 1: Representative photomicrograph of rat aorta 10 days after transplantation. a) control thoracic aorta, b) allotransplantaed aorta, c) anti-CD8 staining in allotransplanted aorta, d) anti-ED1 staining in allotransplanted aorta. Adv=adventitia, Arrow=infiltrating leukocyte, L=lumen

(BN to Lewis transplanted) was 220 + 33 cpm as compared to 77 + 23 cpm in animals treated with anti $\alpha 4/\beta 2$ integrin mAb ($p = 0.0008$) and 59 + 12 cpm in control aorta ($p<0.0001$) (Fig. 2). No significant differences in intensity of naïve Tlymphocyte accumulation in the transplanted aorta could be demonstrated between mAb treated animals and control aorta ($p = 0.57$). Therefore, anti $\alpha 4/\beta 2$ integrin therapy resulted in 89% reduction in infiltration by naïve T lymphocytes using a 24 h migration assay.

The intensity of activated Tlymphocyte (In^{111} labelled T lymphocytes) accumulation into allo-aortic tissue (BN to Lewis transplanted) was 224 + 30 cpm as compared to 75 + 30 cpm in animals treated with anti $\alpha 4/\beta 2$ integrin mAb ($p = 0.0003$) and 21 + 9 cpm in control aorta ($p<0.0001$) (Fig. 3). No significant differences in intensity of naïve Tlymphocyte accumulation into the transplanted aorta could be demonstrated between mAb treated animals and control aorta ($p = 0.08$). Therefore, anti $\alpha 4/\beta 2$ integrin therapy resulted in 73% reduction in infiltration by activated T lymphocytes using a 24 h migration assay.

Control skin injection sites confirmed the validity of the migration assay and are illustrated in Fig. 4[16,17]. Briefly both LPS (100 ng) and INFγ (300 U) resulted in significant accumulation of naïve (Cr^{51}) and activated (In^{111}) labeled

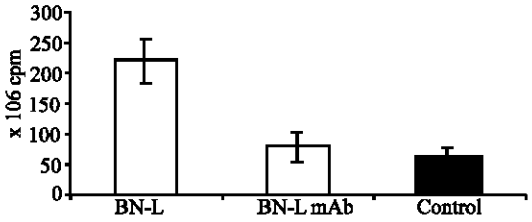

Fig. 2: Histogram illustrating counts per million from labeled naïve T lymphocytes (Cr^{51}) in transplanted aorta. BN-L = Brown Norway to Lewis allotransplanted aorta, BN-L mAb=allotransplanted aorta in animal receiving anti-α4β2 antibody, control=control aorta

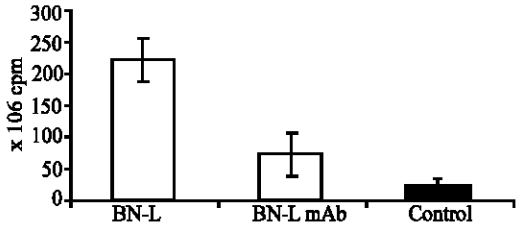

Fig. 3: Histogram illustrating counts per million from activated T lymphocytes (In^{11}) in transplanted aorta. BN-L= brown Norway to Lewis allotransplanted aorta, BN-L mAb=allotransplanted aorta in animal receiving anti-α4β2 antibody, control=control aorta

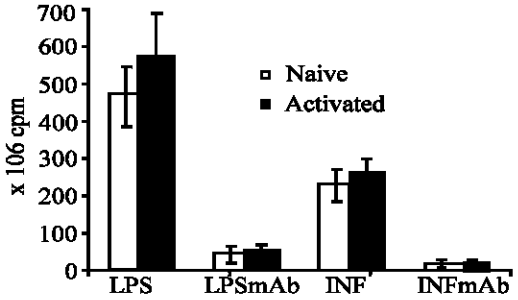

Fig. 4: Histogram illustrating counts per million from labeled naïve T lymphocytes (Cr^{51}) and activated T lymphocytes (In^{111}) in LPS or INFγ injected skin sites. Animals were grouped into untreated or treated with anti-α4β2 antibody (mAb)

T lymphocytes. Anti-α4/β2 mAb therapy resulted in a 92% reduction in naïve T lymphocyte infiltration to LPS skin site and 94% reduction to INFγ skin site. Similarly anti-α4/β2 mAb therapy resulted in a 92% reduction in activated Tlymphocyte infiltration to LPS skin site and 95% reduction to INFγ skin site.

DISCUSSION

The molecular process that promotes the extravasation of leukocytes to sites of inflammation is believed to be via cell adhesion molecule (CAM) mediated adhesion, activation and transmigration through the vascular endothelium[19,20]. The sequence of events requires circulating leukocytes to first bind selectins expressed by activated endothelium and by the leukocytes themselves making the leukocyte decelerate by rolling on the endothelium [19]. The rolling leukocytes can then become activated by chemoatractants (chemokines) thereby increasing the affinity of their integrin molecules for ligands on activated endothelium. Finally the chemokines found at sites of inflammation help mediate the final steps allowing for the leukocyte to squeeze between endothelial cells into the site of inflammation a process called extravasation[19,21].

Because integrin molecules control firm leukocyte adhesion a necessary step prior to infiltration, we hypothesized these molecules to be central to controlling leukocyte trafficking into transplanted organs. The integrin family of CAM includes heterodimeric proteins that are composed of non-covalently bound α and β subunits. There are currently 15 α and 8 β-chains known to date[20]. However, the relative role of individual integrin molecules in leukocyte migration varies with the tissue site and clinical situation supporting the need for their characterization[20]. In established models of adjuvant arthritis for example, blocking antibody to α4 and β2 integrins can significantly reduce the severity of joint inflammation and inhibit neutrophil and T lymphocyte migration into joints[22,23]. On the other hand migration of neutrophils into intestinal model of inflammation has been shown to be largely β2 integrin (CD18) dependent[24,25].

In the present study we have described a simple model of solid organ transplantation which can be used to characterize the molecular steps involved in Tlymphocyte infiltration. We have shown that T lymphocytes infiltrate vascular tissue early after transplantation and was mediated primarily by α4_and or_β2 integrin mechanism. This represents direct evidence of the role of CAM in trafficking of T cells in transplanted allografts. The novelty of the model lies in its application to study any solid organ transplantation models and as such precisely characterize the mechanisms involved in leukocyte infiltration and the relative roles of various CAM's. Intravital microscopy using a skin allograft model has been described with findings suggesting a central role of L-selectin in regulating T cell infiltration[26]. However, the skin graft model used may not have accurately replicated

vascularized organ transplantation limiting the generalizability of the findings.

To date the interaction of β2 integrins and its ligand ICAM-1found to be on the surface of endothelial cells have been shown to be Important in organ transplantation [12]. Several attempts have been made in animal models to block the interactions between these CAM with monoclonal antibodies, soluble ligands, or anti-sense oligonucleotides to prevent leukocyte infiltration following transplantation and have resulted in variable prolonged graft survival[7-11]. ICAM-I has also been found to be up-regulated in vascular endothelium of transplanted organs supporting its important role in leukocyte trafficking[5,6]. However, early randomized clinical trial using anti-ICAM-1 mAb (enlimomab) in renal transplantation have not resulted in lower incidences of adverse events, improved patient survival or improved graft survival[27]. Furthermore, adding to the complexity and need for characterization, CAM have also been shown to be important In the initiation of allogeneic T-cell responses working as accessory molecules to the T-cell receptor[4,28].

The present study was not designed to evaluate the relative contribution of α4 or β2 integrins or to identify other CAM responsible for Tlymphocyte trafficking but rather establishing a reliable model to evaluate cellular trafficking. Our findings were also unable to demonstrate a significant difference in the intensity of infiltration between Cr^{51} labeled naïve and In^{111} labeled activated T cells. This observation is likely due to the normally small proportion of splenic T lymphocytes that are activated effector cells or memory cells[29]. In characterizing this experimental model we did not verify the expression of marker of activation, which are known to identify T cells more likely to express the appropriate integrins and chemokine receptors necessary for migration into inflammatory sites[18,30].

In summary we propose a simple model to evaluate important steps in the inflammatory cascade involved with organ transplantation. Because the inflammatory cascade is based on a complex network of molecular steps, which are dictated by numerous cellular, spatial and temporal variables, a complete understanding is necessary to allow appropriate therapy to be designed and applied. Ultimately one must be able to differentiate events that lead to organ injury and destruction versus necessary events which regulate and control tissue repair.

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