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CD4⁺ LYMPHOCYTES IMPROVE VENOUS BLOOD FLOW IN EXPERIMENTAL ARTERIOVENOUS FISTULAE

Juan C. Duque, MD¹, Laisel Martinez, MS¹, Annia Mesa, PhD¹, Yuntao Wei, MD¹, Marwan Tabbara, MD¹, Loay H. Salman, MD², and Roberto I. Vazquez-Padron, PhD^{1,*}

¹DeWitt Daughtry Family Department of Surgery, Leonard M. Miller School of Medicine, University of Miami, Miami, FL

²Section of Interventional Nephrology, University of Miami Miller School of Medicine, Miami, FL

Abstract

Background—The role of immune cells in arteriovenous fistulae (AVF) maturation is poorly understood and has received, until quite recently, little attention. This study examines the role of T lymphocytes in AVF vascular remodeling.

Methods—Experimental fistulae were created in athymic *rmu* nude rats lacking mature T lymphocytes and euthymic control animals by anastomosing the left superior epigastric vein to the nearby femoral artery. Blood flow rates, wall morphology and histological changes were assessed in AVF 21 days after creation. The effect of CD4⁺ lymphocytes on AVF maturation in athymic animals was analyzed by adoptive transfer of cells after fistula creation.

Results—The absence of T lymphocytes compromised blood flow in experimental fistulae. Histopathological inspection of AVF from athymic rats revealed that T cell immunodeficiency negatively affected venous vascular remodeling, as evidenced by a reduced lumen, a thick muscular layer and a low number of inflammatory cells compared to control animals. Adoptive transfer of CD4⁺ lymphocytes from euthymic rats into athymic animals before and after fistula creation improved blood flow and reduced intima-media thickness.

Conclusion—These results point at the protective role of CD4⁺ lymphocytes in the remodeling of the AVF vascular wall.

*Correspondence: Roberto I. Vazquez-Padron, PhD, Division of Vascular Surgery, University of Miami Miller School of Medicine, 1600 NW 10th Avenue, RMSB 1009D, Miami, FL 33136, Telephone: 305-243-1154, Fax: 305-243-5636, rvazquez@med.miami.edu.

AUTHOR CONTRIBUTION

RIV-P and JCD designed the study; JCD and YW performed experiments; JCD, LM, AM, MT, LHS, and RIV-P analyzed data and interpreted results; JCD, LM and RIV-P prepared figures; LM and RIV-P drafted manuscript; JCD, LM and AM edited and revised manuscript; and RIV-P approved the final version.

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Keywords

Arteriovenous fistula; hemodialysis; T lymphocytes; inflammatory cells; vascular remodeling; maturation

INTRODUCTION

Creation of arteriovenous fistulae (AVF) for hemodialysis is one of the most common vascular surgical procedures in the United States due to the high prevalence of end-stage renal disease (ESRD) in the American population (United States Renal Data System). The AVF is the preferred type of vascular access, given its better performance in terms of survival and complications compared to grafts and central venous catheters.¹ However, fistulae frequently fail to mature due to stenosis secondary to neointimal hyperplasia (NIH) and inward (constrictive) vascular remodeling.²⁻⁴ Factors associated with AVF failure include vein diameter and hemodynamics,⁴⁻⁶ diabetes^{3, 7} and inflammation.⁸ The latter factor implicates cellular and systemic components of the immune system that are activated upon vascular trauma and can directly modify the remodeling of the outflow vein and stimulate neointima formation. Thus, it comes as no surprise that elevated levels of cytokines, acute phase reactants and inflammatory cells have been associated with negative outcomes in hemodialysis patients.⁸⁻¹¹

The mechanism by which cellular inflammation influences wall remodeling and development of neointimal hyperplasia in AVF has remained elusive and understudied so far. Macrophages, mast cells, neutrophils and lymphocytes infiltrate the AVF wall early after creation as part of the rapid healing response intended to maintain vascular integrity and hemostasis. Macrophages and leukocytes have been detected among proliferating cells in mature human AVF.¹² In addition, it is believed that inflammatory cells represent a source of matrix metalloproteinases (MMP) and cytokines, which allow the re-structuring of the venous wall while stimulating myofibroblastic growth and neointimal thickness.¹³⁻¹⁶ Immune cells also help with the clearance of pro-inflammatory and apoptotic material in the vascular wall.^{17, 18} Thus, a balanced immune response is necessary for positive vascular remodeling and the prevention of occlusive neointima formation and AVF stenosis.

While macrophages seem to intervene in AVF maturation, it is still unclear whether lymphocytes, and specifically T cells, play any relevant role during the adaptive response of the outflow vein to hemodynamic stress. Of note, T lymphocytes are active players in vascular wall processes like atherosclerosis and restenosis. Research indicates that T cells are associated with exacerbation of atherosclerotic lesions,¹⁹⁻²¹ while showing a protective effect against NIH after arterial injury.^{22, 23} Interestingly, in the setting of arterial injury, further characterization revealed that this protective effect was mediated by CD8⁺ cells,²⁴⁻²⁶ while their CD4⁺ counterparts in fact promoted neointima development.²⁷

In this study, we demonstrate for the first time that T lymphocytes participate in the remodeling of experimental AVF. We show that T cell deficiency exacerbates pathological remodeling in the venous segment and reduces blood flow rates after fistula creation. In addition, we demonstrate that peri-procedural CD4⁺ cell transfer reduces adverse

remodeling and improves experimental fistula function in immunodeficient animals. This work lays the foundation for future studies on the role of immune regulation for the proper maturation of AVF.

MATERIALS AND METHODS

Animals

Arteriovenous fistulae (AVF) and sham surgeries were performed in athymic *rmu* nude rats and Sprague Dawley (SD) control animals (Harlan, Indianapolis, IN). Experimental animals were male, 2-4 months old and weighed 280-320 grams. All studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Miami.

Arteriovenous fistula model

Experimental fistulae consisted of an end-to-side anastomosis of the femoral artery to the superficial epigastric vein, as previously described.²⁸ Briefly, athymic rats and euthymic control animals were anesthetized by vaporized isoflurane. The femoral and superficial epigastric vessels were exposed through a 2-cm incision at the right groin. The branching vessels from the femoral artery and epigastric vein were ligated with sterile 6-0 silk suture. Vessels were clamped and rinsed with heparinized saline solution. Then, the end of the epigastric vein and the side of the femoral artery were anastomosed with 10-0 monofilament interrupted sutures (Ethicon, Somerville, NJ). Experimental controls consisted of the contralateral non-operated veins as well as epigastric veins that were ligated at one end. Non-operated veins, ligated veins and AVF from athymic rats and euthymic controls were collected 21 days after surgery and formalin-fixed and paraffin-embedded for histopathology and morphometric analysis.

Measurement of AVF blood flow

Blood flow in the rat experimental AVF was assessed using a 0.7 mm perivascular flow probe (Transonic Systems Inc., Ithaca, NY) before euthanasia at 21 days post-surgery. Briefly, the inguinal incision in the right groin was re-opened to allow AVF dissection from the surrounding vessels. The probe was placed on the AVF and acoustic couplant gel (Transonic Systems Inc.) was applied to ensure proper signal capture. Flow measurements were recorded via a T200 flow meter (Transonic Systems Inc.).

Morphometric analysis

Wall thickness (i.e., intima-media thickness) and lumen area were measured on hematoxylin and eosin (H&E) stained sections taken at the juxta-anastomotic segment at approximately 3 mm from the anastomosis site. Measurements were taken by an expert blind to the source animal and treatment applied. Three sections were measured per AVF or vein, with six thickness values obtained and averaged from each section, which were taken clockwise every 10°. Measurements of lumen area were normalized against the average wall thickness of each vein. All morphometric measurements were performed on digital images using the Image Pro Plus (Media Cybernetics, Inc., Bethesda, MD) computer software.

Immunohistochemistry analysis

Epitope retrieval was performed in deparaffinized and re-hydrated sections that were previously treated with 3% hydrogen peroxide to quench endogenous peroxidases. Non-specific binding was minimized with blocking solution (DAKO, Carpinteria, CA), and primary antibodies (mouse anti-rat CD4 and CD68, AbD Serotec) were added at a 1:50 dilution for 1 hour at room temperature. Bound antibodies were detected using the DAKO Universal link kit (DAKO). Color was developed with a DAB chromogenic solution (DAKO). For detection of apoptosis, paraffin sections were processed using the ApopTag® Peroxidase In Situ Oligo Ligation (ISOL) Kit (Millipore, Billerica, MA) according to the manufacturer's guidelines. Images were taken with an Olympus 1X71 camera fitted to an Olympus BX 40 microscope (Olympus America Inc, Center Valley, PA). Quantification of labeled cells in IHC slides was performed by an expert blind to the source animal and treatment applied. Three sections were analyzed per AVF and four equidistant regions per section.

T cell adoptive transfer

Spleens from SD rats were collected at the time of euthanasia and immediately placed in ice-cold RPMI 1640 medium. Spleens were minced with glass slides in RPMI 1640 medium supplemented with 10% fetal bovine serum, and cells were filtered through a 70- μ m strainer to obtain single cell suspensions. Splenic cells were collected by centrifugation, and red blood cells were removed by lysis in NH_4Cl for 5 minutes. CD4^+ lymphocytes were isolated by positive selection from splenic cell suspensions via magnetic separation using CD4^+ microbeads (Miltenyi Biotec, Germany) and following the manufacturer's protocol. The percentage and purity of CD4^+ cells were assessed by flow cytometry before and after magnetic separation. Approximately 5.26×10^6 CD4^+ T cells/ml were injected into the tail vein of athymic and euthymic rats immediately after AVF creation and a booster of 2.29×10^6 CD4^+ T cells/ml 5 days after the surgery.

Statistical analysis

Results are expressed as means $\pm$ SE. Two-group comparisons were conducted using two-tailed *t*-tests for independent samples with unequal variances. Statistics were calculated with Prism 5 (GraphPad Software, La Jolla, CA).

RESULTS

Fistulae of athymic rats have lower flow rates and thicker walls than those of euthymic animals

To examine the role of lymphocytes in AVF maturation and remodeling, we created AVF in T-cell deficient athymic *rmu* nude rats and SD control animals ($n=7/\text{group}$). We assessed blood flow rates and morphometric wall measurements in the outflow vein at 21 days after AVF creation as primary and secondary endpoints. The mean flow rate in AVF of athymic rats is 3.53 ± 0.81 ml/min, compared to 16.44 ± 4.10 ml/min in euthymic animals ($p=0.009$); Figure 1A). These results correlate with the morphometric measurements of the AVF walls (Figure 1B-E). The H&E stained cross-sections demonstrated decreased luminal area and

thicker walls in AVF from athymic rats compared to euthymic controls (0.19 ± 0.05 vs. 0.47 ± 0.09 mm², $p=0.030$ and 0.29 ± 0.03 vs. 0.10 ± 0.02 mm, $p=0.002$, respectively; Figure 1B-C). In contrast, wall thickness and luminal area in both the contralateral non-operated veins and ligated veins (example in Figure 1F) were the same between euthymic and athymic animals (results not shown). These results demonstrate the key role of mature T lymphocytes in the remodeling of the AVF vascular wall during maturation.

The absence of T cells shows a trend toward reduction in macrophage recruitment and protects from apoptosis

Immunohistochemistry analysis was used to identify CD4⁺ T cells and CD68⁺ macrophages in AVF of athymic (n=5) and euthymic rats (n=5) at day 21 post surgery. As expected, no CD4⁺ lymphocytes were detected in the fistula walls of athymic nude rats whereas a prominent number infiltrated those of euthymic animals (27.29 ± 5.55 cells per 400x high power field [HPF], $p=0.002$; Figure 2A-C). CD4⁺ cells were observed throughout the wall of SD rats, including in the sub-endothelial space (Figure 2B). Similarly, a lower number of macrophages was observed in the AVF wall of athymic rats compared to control animals, although the comparison was not statistically significant (60.63 ± 5.26 vs. 87.20 ± 17.18 cells per HPF, $p=0.100$; Figure 2D-F). The location of macrophages in the athymic vascular wall was confined to the border with the adventitia, whereas they were dispersed throughout the wall of euthymic controls (Figure 2D-E). As in Figure 2A-B, vascular smooth muscle cells (VSMC) in the athymic nude wall display a normal morphology and organization, in contrast to the one seen in the SD controls. Because T lymphocytes are involved in cell apoptosis, the number of apoptotic cells in AVF from athymic nude and control SD rats was determined using the In Situ Oligo Ligation (ISOL) assay. As predicted by the lower number of inflammatory cells observed in the wall and the morphology of medial VSMC, athymic rats show a significantly lower number of apoptotic cells when compared to control SD rats (24.50 ± 8.50 vs. 70.67 ± 8.95 cells per HPF, $p=0.039$; Figure 3A-C). These results suggest that T lymphocytes play an active role as modulators of macrophage infiltration and VSMC survival during the remodeling of the AVF vascular wall.

Adoptive transfer of CD4⁺ cells improves AVF remodeling and blood flow rates in athymic rats

To confirm the protective role of CD4⁺ cells in AVF remodeling and maturation, athymic nude rats received adoptive transfer of CD4⁺ cells from euthymic SD animals (n=5) or a vehicle injection (n=5) right after AVF creation, with a booster at 5 days post-surgery. Two animals in the CD4⁺ transfer group died prior to the booster injection. Adoptive transfer of CD4⁺ cells into athymic rats significantly improved the blood flow rate in the fistula compared to the vehicle control (6.00 ± 1.00 vs. 2.80 ± 0.56 ml/min, $p=0.019$; Figure 4A). As expected, this positive effect correlated with a significant reduction in intima-media thickness measurements in AVF of animals receiving CD4⁺ lymphocytes versus vehicle control (0.11 ± 0.01 vs. 0.29 ± 0.04 mm, $p=0.017$; Figure 4B). Immunohistochemistry analysis of AVF from athymic cell recipients could not detect the transferred CD4⁺ cells at day 21 (results not shown), but did show a positive signal for macrophages (Figure 4C-F). Interestingly, in contrast to their location mostly in the adventitia of vehicle control walls (Figure 4C and E), macrophages were found in the media, intima and sub-endothelial line of

AVF from recipients of CD4⁺ cell transfer (Figure 4D and F). Moreover, despite the reduction in AVF wall thickness after T cell transfer, no significant differences in apoptotic cell numbers were found at 21 days between recipients of CD4⁺ cells or vehicle (24.50 ± 8.50 vs. 23.50 ± 2.50 cell per field, $p=0.9204$).

DISCUSSION

Proper remodeling and maturation of the fistula should lead to increased luminal area to accommodate the elevated blood flow required for hemodialysis. However, this is frequently compromised by stenosis of the outflow vein. In this study, we present evidence that supports the role of T lymphocytes in the remodeling of the AVF wall during maturation. We show for the first time that the absence of mature T cells leads to vascular constriction in the venous segment of the AVF. In addition, we demonstrate that CD4⁺ lymphocytes act as positive regulators of outward remodeling in rat experimental AVF. These results not only increase our knowledge regarding the cellular mechanisms that determine AVF maturation, but also identify a new therapeutic target to increase AVF success in hemodialysis patients.

The protective effects of T lymphocytes in AVF underscore the importance of a controlled cytotoxic, proliferative and inflammatory response in the vascular wall. Our results reveal that the presence of CD4⁺ lymphocytes coincide with the presence of macrophages and apoptotic cells in the remodeled AVF. Interestingly, the absence of mature T lymphocytes reduced macrophage infiltration, although not significantly, and diminished apoptosis in the athymic AVF wall. CD8⁺ T cells are better known for their cytotoxic effects than their CD4⁺ counterparts, but the latter are also able to induce apoptosis in the vascular wall.^{29, 30} VSMC death mediated by CD4⁺ cells contributes to plaque instability in atherosclerosis,³⁰ while CD8⁺ T cell-induced apoptosis limits neointima formation after arterial injury.^{24, 25} CD4⁺ cells can trigger VSMC apoptosis through the formation of immunologic synapses,³⁰ and the combination of Th1 and macrophage cytokines is synergistic in inducing VSMC death in vitro.²⁹

In the case of AVF remodeling, we have shown that CD4⁺ lymphocytes are beneficial to the maturation of the fistula, specifically by reducing venous wall thickness and improving blood flow rates in a model of T cell deficiency. The protective role of CD4⁺ cells in the remodeling of AVF contrasts with their exacerbating effect in atherosclerotic lesions²¹ and neointima development after arterial injury.²⁷ It is possible that these differences are caused by inflammatory triggers and/or compensatory factors that are specific to each type of remodeling process. For example, endothelial denudation, which is absent in AVF, is known to promote inflammation and NIH in injured arteries.³¹

This work also presents interesting findings that warrant further research. Namely, transfer of CD4⁺ cells into nude rats improves vascular remodeling and increases blood flow without elevating the number of apoptotic cells at day 21. In addition, the transferred CD4⁺ cells were not visible in the fistula at the time of sacrificing, with migration of macrophages to the inner layers of the wall being the main histological signature of the treatment. Future mechanistic and temporal analyses are needed to clarify whether VSMC apoptosis occurred at an earlier time point after adoptive transfer and/or if reduced wall thickness is due to the

inhibition of VSMC proliferation by cytokines and other local factors. Another interesting finding was that the adoptive transfer of CD4⁺ cells increased blood flow rates in recipient athymic animals but not to the level of control SD rats. This is likely due to a short-lived effect of the therapy or because CD8 cells may be needed to optimize it. It is possible that additional cell transfers prior to fistula creation and/or further down the remodeling period would enhance vein adaptation in athymic animals. Of note, in our hands, the rat epigastric-femoral fistula was found to be an excellent model for AVF wall remodeling with only moderate neointima development. A recent study indicated that there is no direct association between NIH and stenosis after fistula creation, as the latter occurs with equal frequency in the presence or absence of neointima formation.³²

The main limitation of this study resides in the lack of chronic kidney disease (CKD) conditions in our model. The physiological factors that are commonly associated with CKD are modulators of the immune function, with lasting effects on the activity of lymphocytes and on vascular remodeling. Several studies coincide in that CKD conditions result in lower numbers of circulating naïve CD4⁺ and CD8⁺ lymphocytes³³⁻³⁵ and enhanced apoptosis of T regulatory cells.³⁶ Based on our results, these changes might contribute significantly to adverse AVF maturation outcomes. Another limitation is the genetic differences between the nude *rnu* and SD rats, given that both are outbred strains. Unfortunately, nude *rnu* rats are not commercially available in an inbred background. Immunological differences between the strains used in this work could have contributed to a shortened viability of adoptively transferred cells, or to a heightened state of immunity in recipient animals that could have benefited the trafficking of leukocytes to the fistula. Further studies will be needed to clarify this confounding factor. Regardless of these limitations, we show for the first time that CD4⁺ cells help reduce intima-media thickness and improve blood flow rates in experimental AVF in the absence of CKD conditions. These results support the notion that the immune system plays an important role in AVF maturation. We therefore advocate that understanding CKD-induced changes in lymphocyte function and how these affect remodeling is a crucial step in preventing AVF stenosis.

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Figure 1. Arteriovenous fistulae in athymic nude rats exhibit lower blood flow rates and thicker walls than those in euthymic SD animals

A) Blood flow rates in athymic nude (black dots) and euthymic SD rats (gray dots) at 21 days post-surgery (n=7 per group). The horizontal line marks the mean of each group measurement. **B-C)** Lumen area (B) and venous wall thickness (C) in AVF from athymic nude (black) and euthymic rats (gray; n=6 per group). Bars represent the mean ± SEM. * $p < 0.05$, ** $p < 0.01$. **D-F)** Representative hematoxylin and eosin (H&E)-stained cross-sections of AVF from athymic nude (D) and euthymic rats (E), as well as a representative ligated vein from both types of animals (F). Scale bar = 250 μm .

Figure 2. The absence of T lymphocytes shows a trend toward reduction in macrophage infiltration during remodeling of the AVF wall

A-C) Immunohistochemistry staining (A-B) and cell count (C) of infiltrated CD4⁺ lymphocytes (brown spots) in AVF of athymic (A) and euthymic rats (B; n=5 per group). **D-F)** Immunohistochemistry staining (D-E) and cell count (F) of CD68⁺ macrophages in AVF of athymic (D) and euthymic rats (E). Arrows point to representative cells positive for either CD4 or CD68. Cell count is expressed as number of cells per 400x high power field (HPF). Scale Bar = 100 μm. Bars represent the mean ± SEM. ** p < 0.01.

Figure 3. The absence of T lymphocytes protects cells from apoptosis in the AVF wall
A-B) Representative microphotographs of ISOL-stained sections from AVF in athymic nude (A) and euthymic control rats (B; n=5 per group). Arrows point to representative apoptotic nuclei (brown) at 21 days post-surgery. Scale Bar = 50 μ m. **C)** Total number of apoptotic cells per 400x high power field (HPF). Bars represent the mean $\pm$ SEM. * $p < 0.05$.

Figure 4. Adoptive transfer of CD4⁺ lymphocytes improves remodeling and blood flow in AVF of athymic rats

A-B) Blood flow rate (A) and media thickness (B) in AVF of athymic animals after receiving CD4⁺ T cell adoptive transfer (n=3) from euthymic rats or vehicle injection (n=5). Bars represent the mean $\pm$ SEM. * $p < 0.05$. **C-F)** Immunohistochemistry staining for CD68⁺ macrophages in AVF of vehicle controls (C and E) or CD4⁺ T cell recipients (D and F). E and F are 400x high power field (HPF) images of boxed areas in C and D, respectively.