An Immunological Fingerprint Differentiates Muscular Lymphatics from Arteries and Veins

Eric A. Bridenbaugh, Wei Wang, Maya Srimushnam, Walter E. Cromer, Scott D. Zawieja, Susan E. Schmidt, Daniel C. Jupiter, Hung-Chung Huang, Vincent Van Buren, and David C. Zawieja

Abstract

The principal function of the lymphatic system is to transport lymph from the interstitium to the nodes and then from the nodes to the blood. In doing so lymphatics play important roles in fluid homeostasis, macromolecular/antigen transport and immune cell trafficking. To better understand the genes that contribute to their unique physiology, we compared the transcriptional profile of muscular lymphatics (prenodal mesenteric micro-lymphatics and large, postnodal thoracic duct) to axillary and mesenteric arteries and veins isolated from rats. Clustering of the differentially expressed genes demonstrated that the lymph versus blood vessel differences were more profound than between blood vessels, particularly the microvessels. Gene ontology functional category analysis indicated that microlymphatics were enriched in antigen processing/presentation, IgE receptor signaling, catabolic processes, translation and ribosome; while they were diminished in oxygen transport, regulation of cell proliferation, glycolysis and inhibition of adenylate cyclase activity by G-proteins. We evaluated the differentially expressed microarray genes/products by qPCR and/or immunofluorescence. Immunofluorescence documented that multiple MHC class II antigen presentation proteins were highly expressed by an antigen-presenting cell (APC) type found resident within the lymphatic wall. These APCs also expressed CD86, a co-stimulatory protein necessary for T-cell activation. We evaluated the distribution and phenotype of APCs within the pre and postnodal lymphatic network. This study documents a novel population of APCs resident within the walls of muscular, prenodal lymphatics that indicates novel roles in antigen sampling and immune responses. In conclusion, these prenodal lymphatics exhibit a unique profile that distinguishes them from blood vessels and highlights the role of the lymphatic system as an immunovascular system linking the parenchymal interstitium, lymph nodes and the blood.

Introduction

HISTORICALLY, MOST HAVE TREATED the lymphatic vascular system as part of the cardiovascular system. Anatomically, the lymphatic system is composed of a network of vascular structures that carry lymph that are linked to the specialized lymphoid tissues where critical immune functions occur, the lymph nodes. The network of lymphatic vessels starts as blind ended lymphatic capillaries or initial lymphatics that are the predominant site of lymph formation. Once formed, lymph moves through the network of lymphatic vessels (precollectors, collecting lymphatics, afferent prenodal lymphatics) going to the lymph node. At the node, multiple prenodal afferent lymphatics merge into the nodal subcapsular space, which connects to the lymph sinuses that interact with the different immune cell regions within the

node, as well as the high-endothelial venules. Before exiting the node, lymph enters the medullary sinus, which becomes the efferent postnodal lymphatic duct. The efferent lymphatic ducts connects to other post and prenodal lymphatic structures of the complex lymphatic network to eventually become the thoracic duct (or right cervical duct in the upper right quadrant of the human body) en route to connect with the venous blood in the neck. From a cardiovascular-centric perspective, the fundamental physiological function of the lymphatic system is to transport fluid and other constituents of lymph from the interstitium through the lymph nodes and back to the venous side of the circulatory system. But in fulfilling this task, the lymphatic system also plays significant roles in tissue fluid volume homeostasis, macromolecular/antigen transport, immune cell trafficking and lipid transport.^{15,42,49} From a clinical perspective, disruption of lymph

flow is primarily characterized by a profound accumulation of interstitial fluid distal to the site of lymphatic dysfunction. But chronic impaired lymph transport in the tissue is also usually accompanied by the buildup of inflammatory/immune cells and an increase in the adiposity.^{5,38} The resulting pathology, referred to as lymphedema, is most often chronic and debilitating. It is complicated by impaired immune function, the accumulation of inflammatory cells, recurrent infections and, in rare cases, the development of malignant tumors.⁵² Longstanding lymphedema usually leads to tissue fibrosis and chronic lipidemia, where lipid-filled adipocytes accumulate in the interstitial space. Cumulatively, lymphedema affects millions in the United States and hundreds of millions worldwide, and few efficacious therapies currently exist.^{39,52}

The constituents that make up lymph (water, ions, macromolecules, lipids, immune cells, particulate matter and other formed elements) are principally thought to be determined at the initial lymphatics. Lymph normally moves elements in a one-way fashion from the parenchymal tissue through the complex network of lymphatics through the node into the blood. Unlike the blood vascular system in which the fluid pressure gradient favors an essentially continuous fluid flow from the arterial to venous side of the network and back to the heart, the average pressures within the lymphatic network increases as lymph moves from the initial lymphatics to the great veins of the neck.^{51,63} Thus to transport lymph against this net hydrostatic pressure gradient, collecting lymphatics have a luminal valve system and an extensive investiture of lymphatic muscle cells (LMCs). These LMCs are capable of producing both slow, tonic and rapid, phasic contractions that respectively regulate lymphatic diameter/outflow resistance and generate the hydrostatic pressure gradients that propel lymph.^{8,15,63} In the absence of external driving forces, this intrinsic lymphatic "pumping" is necessary and sufficient to both generate and regulate lymph flow. Secondary lymphedemas (e.g., breast cancer treatment-related lymphedema or lymphatic filariasis) develop following disruption of the collecting lymphatic network, and deficiencies in the intrinsic lymphatic contractility are correlated with an increasing severity of peripheral secondary lymphedema.^{26,29,30} Lymphedema results in the accumulation of tissue fluids, macromolecules and immune cells that produce a state of chronic inflammation in the edematous tissues. Despite the importance of collecting lymphatic physiology to the biology and pathology of the lymphatic system, relatively little is known about the unique structural, contractile, regulatory, metabolic and immune composition of these vessels.

Recent studies have demonstrated that collecting lymphatics acquire a distinct molecular phenotype during development that distinguishes them from initial lymphatics. The lymphatic hyaluronan receptor LYVE1, a commonly used marker of lymphatic endothelial cells (LECs),³ is rapidly downregulated in LECs upon recruitment of LMCs,^{23,55} followed by the concomitant establishment of N-cadherin junctions between the LECs and LMCs.⁵⁵ EphrinB2 is restricted to collecting LECs within the mature lymphatic network and may be specifically expressed by those LECs in contact with LMCs.²³ Collagen IV expression is upregulated in the collecting LECs, and the transcription factor FOXC2 is found within the luminal valves of collecting lymphatic vessels.³³ Collecting LMCs respond to PDGF-B signaling in a fashion

similar to vascular smooth muscle cells (SMCs),⁵⁵ and LMCs likely express PDGF receptor- β .³³ LMCs express a unique mixture of smooth and striated contractile and regulatory proteins that are used to produce the phasic and tonic contractions, which generate and regulate lymph flow.²⁷ Because of the critical role the collecting lymphatics have in completing the transport of lymph from the tissue beds to the lymph nodes and venous system, the continued investigation of the collecting lymphatic phenotype and a better understanding of the mechanisms underlying its physiological functioning are important to the therapeutic treatment of lymphatic dysfunction and lymph-related pathologies. Additionally, the roles collecting lymphatics play in functions other than fluid transport (i.e., macromolecular exchange, lipid metabolism and immune cell trafficking) have not been carefully and systematically evaluated. An important step in this process is the determination of the unique genetic expression patterns that the muscularized lymphatics exhibit.

DNA microarray methodologies^{32,41} provide expression analysis of thousands of genes simultaneously, thus facilitating the identification of gene expression patterns that may provide clues to the cellular mechanisms underlying a tissue's biological activity. Microarray techniques have been successfully utilized to identify transcriptional diversity within organs or organ systems such as the individual gene expression patterns of the four chambers of the heart,⁵⁴ the organs of the gastrointestinal system,⁴ or the structures of the central nervous system.⁶¹ These observations collectively demonstrate the power of microarray technology for gene expression profiling and provide a model for the application of this technique to the transcriptional examination of the lymphatic system. Indeed, gene microarrays have been successfully used to identify the fundamental transcriptional differences between LECs and vascular endothelial cells^{16,35} as well as describe the changes in gene expression observed in LECs in response to growth factor treatment.^{45,58} Unfortunately, those studies focused solely on LECs with no examination of LMCs or any other cell types that may reside within the lymphatic wall. The LECs in those studies were derived from the human dermal microvasculature using antibody-binding techniques. The tissue source and the techniques employed both lead to a LEC population principally derived from initial lymphatic structures,⁴² thus limiting the application of this data to collecting lymphatic vessels. A single report has utilized microarray techniques to identify important differences between initial and collecting LECs²⁰; however, there are presently no reports on the transcriptional profile of lymphatic collecting vessels and their associated cells despite the relative importance of these structures to lymphatic and immune systems function.

To gain a better understanding of the genes that may contribute to the unique biology of the collecting lymphatic vessel, we designed mRNA expression microarray experiments to examine the transcriptional profile of rat muscular lymphatics. The rat is the only animal model for which there is currently a relative abundance of collecting lymphatic physiological data as well as sufficient characterization of the organism's genome. Because muscular lymphatics display regional variability in their contractile behavior¹² and contractile protein expression and have somewhat different roles in the lymphatic transport network, we examined the gene expression patterns of both the large, post nodal thoracic duct

and the smaller, prenodal mesenteric collecting lymphatics. Given the structural similarities and extensive knowledge base of the blood vasculature, the transcriptional profiles of these lymphatics were compared to those of the anatomically parallel axillary and mesenteric arteries and veins, respectively. We then went on to study some of the unique immunologically-related cells found within the structure of the lymphatic vessel walls, how these cells are differentially found in different parts of the lymphatic network and how these cells may be related to important immunological functions. We demonstrate that muscular prenodal lymphatics exhibit a unique profile (transcriptionally, protein expression and cell presence) of immune functions that distinguishes them from their associated blood vessels and we present a diverse array of genes that may be responsible for the unique biology of the collecting lymphatic vessels.

Materials and Methods

Animals and surgery

The animal facilities used for these studies have been accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC International) and adhere to the regulations, policies and principles detailed in the Public Health Service Policy for the Humane Care and Use of Laboratory Animals (PHS Policy, 1996) and the United States Department of Agriculture's Animal Welfare Regulations (Animal Welfare Act, AWA, 9CFR, 1985, 1992). The animals were exposed to a 12 hr light/dark cycle and given open access to food and water. All animal procedures performed for this study were reviewed and approved by the institutional animal care and use committee of Texas A&M University, College Station, TX.

Male Sprague-Dawley rats (300–400 g) were anesthetized with fentanyl–droperidol solution (droperidol 20 mg/ml, fentanyl 0.4 mg/ml; 0.3 ml/kg bodyweight im) and diazepam (2.5 mg/kg bodyweight im) prior to isolation of blood and lymph vessels. To isolate mesenteric arteries (MA), mesenteric veins (MV), and mesenteric lymphatics (ML), a short loop of small intestine was exteriorized through a midline abdominal incision. A window of mesentery (2–2.5 cm in diameter) containing the 2° and 3° arteries and veins derived from a single 1° mesenteric artery and vein as well as their associated lymphatics was excised and transferred to a small Petri dish containing RNAlater (Ambion, Austin, TX) to deactivate RNases except for those used for immunofluorescence. The thoracic duct (TD) from just above the diaphragm to approximately the level of the atria (~3–4 cm *in situ*) was rapidly and grossly excised through a midline incision of the ventral chest wall and transferred to RNAlater. The axillary artery (AA) and axillary vein (AV) just distal to the chest wall (~1.5 cm in length) were rapidly and grossly excised and transferred to RNAlater. The grossly isolated lymph and blood vessels were allowed to equilibrate in room temperature RNAlater for >30 min before being carefully cleaned of all extravascular tissue. For the mesenteric samples, all collecting lymphatics, arteries, and veins within the mesenteric windows were isolated and each pooled into a single ML, MA, or MV sample, respectively. Care was taken to ensure that the vessels remained submerged in RNAlater throughout the cleaning process and until immediately prior to tissue homogenization to prevent RNase activity. Samples acquired

from the same donor animal were handled in parallel for all experimental steps.

RNA isolation

To isolate total RNA, blood or lymph vessels were rapidly transferred from RNAlater to a 2 ml microcentrifuge tube containing 500 μ l chilled TRIzol Reagent (Invitrogen, Carlsbad, CA) and immediately homogenized for 20–30 sec with a rotor-stator homogenizer at 30,000 rpm before being placed on ice. Care was taken to position the vessels immediately adjacent to one of the side windows on the stator prior to starting the homogenization to ensure that the vessels were taken into the homogenizer generator and exposed to sufficient shearing forces to begin the tissue disruption process. The cooled samples were inspected for any remaining large vessel fragments and homogenized for an additional 15–20 sec at 30,000 rpm if necessary before being placed back on ice. To increase the aqueous volume of the lysate prior to phase separation, 100 μ l of RNase-free water was added to each sample and thoroughly mixed. The sample lysates were then transferred to 2.0 ml Phase Lock Gels – Heavy (Eppendorf, Hamburg, Germany). Chloroform (100 μ l) was added to each sample and thoroughly mixed. The samples were then centrifuged for 1 min at 12,000 rcf, and the upper aqueous layer was transferred to a new tube. The RNA was precipitated in isopropanol and washed in 75% ethanol according to the standard TRIzol protocol. The RNA pellet was then resuspended in RNase-free water and subsequently DNase-treated with DNA-free (Ambion, Austin, TX) according to the manufacturer's instructions. RNA quantity and integrity were assessed by examining the relative intensity of 18s and 28s rRNA bands using an Agilent 2100 Bioanalyzer and RNA6000 Pico LabChip Kit (Agilent Technologies, Santa Clara, CA). RNA samples were immediately used in downstream applications without exposure to freeze-thaw cycles.

Microarray hybridization

25–80 ng samples of total RNA from TD, AA, AV, ML, MA, and MV ($n=6$ for each vessel) as well as 1 μ g of Rat Universal Reference RNA (Stratagene, La Jolla, CA) were subjected to two rounds of linear amplification using the Amino Allyl MessageAmp aRNA Kit (Ambion) according to the manufacturers instructions. Briefly, mRNA is reverse transcribed to form cDNA containing a T7 promoter. After conversion of cDNA to double-stranded DNA, T7 RNA polymerase is used to generate numerous antisense copies of each mRNA. For the second round of amplification, 250 ng of amplified RNA (aRNA) was utilized. Following each round of amplification, the quantity and integrity of the aRNA was assessed by examining the distribution of the RNA profile using an Agilent 2100 Bioanalyzer and RNA6000 Nano LabChip Kit (Agilent Technologies). Samples with aRNA distributions centered on an RNA length of less than 500 nt were discarded and reamplified. During the final round of *in vitro* transcription, 5-(3-aminoallyl)-UTP was incorporated into the aRNA, which was then chemically coupled to NHS esters of either Cy5 or Cy3 dye (GE Healthcare, Piscataway, NJ). For each vessel sample, 1 μ g Cy5-coupled vessel aRNA was paired with 1 μ g Cy3-coupled Rat Universal Reference aRNA yielding a total of 36 arrays.

The paired aRNA samples were fragmented by hydrolysis (RNA Fragmentation Reagents, Ambion) and hybridized to

glass arrays spotted with the Array-Ready Oligo Set for the Rat Genome Version 3.0 (Operon Biotechnologies, Huntsville, AL). This array set contains 26,962 oligonucleotide probes representing 22,012 rat genes and 27,044 rat gene transcripts. Array preparation and hybridization was performed by the Department of Medical Physiology Microarray Laboratory, Texas A&M Health Science Center, Temple, TX. Briefly, the oligos were printed on aminosilane-coated glass slides using an OmniGrid Arrayer (Genomics Solutions, Ann Arbor, MI). Prior to hybridization, the arrays were incubated in 5X SSC, 0.1% SDS, and 0.2% bovine serum albumin (BSA) for 45 min at 48°C with 35 rpm orbital agitation, washed twice in nuclease-free water for 5 min each, washed twice in isopropanol for 5 min each, and then maintained at 48°C until hybridization. The fragmented aRNA samples were then denatured for 5 min at 95°C, diluted approximately 4 fold using SlideHyb Glass Array Hybridization Buffer (Ambion), and then added to the array surface. The arrays were then covered with a Hybrislip (Grace Bio-Labs, Bend, OR) and incubated for 18 hrs at 48°C. Following hybridization, the arrays were washed 6 min at 48°C in 1X SSC and 0.1% SDS, 6 min at room temperature in 1X SSC and 0.1% SDS, and then twice in 0.1X SSC at room temperature for 6 min each. The arrays were then spun dry and immediately imaged at 10 μ m resolution using an Axon GenePix 4000A scanner (Molecular Devices, Sunnyvale, CA).

Microarray data analysis

Image analysis and data extraction were performed using GenePix Pro 6.0 software (Molecular Devices). Unprocessed fluorescence intensity data was exported to Microsoft Excel 11.2 software for quality control evaluation. Data points were error-flagged as absent if the Cy3 (reference) channel failed any of the following requirements: 1) at least 40% of feature pixels exhibiting fluorescent intensity greater than local mean background + 2SD, 2) a median feature intensity greater than median local background intensity by at least 10 units, 3) a ratio of median feature intensity to median local background intensity greater than 2.5. The flagged raw feature and GenePix-specified local background intensity values for both dye channels were then imported into GeneSpring GX 7.3 software (Agilent Technologies) and normalized by: 1) subtracting the local background intensity from the feature intensity for each channel, 2) setting all Cy5 measurements less than 0.5 units to 0.5, 3) applying a per-gene and per-array, intensity-dependent (LOWESS) normalization utilizing 20% of data for smoothing and a Cy3 channel cutoff of 0.5, and 4) dividing each normalized Cy5:Cy3 ratio by the median normalized Cy5:Cy3 ratio for that gene across all arrays. Values obtained from replicate oligos on the same array were averaged. Because every sample is compared to the same arbitrary reference sample, the normalized Cy5:Cy3 ratios for each gene were directly comparable across all arrays. To identify and remove those genes with poor reference (Cy3) channel measurements, we excluded flagged measurements according to the following criteria: 1) If any measurements were flagged as absent in the reference channel, or 2) if a gene is flagged as absent on more than 50% of all arrays. The data discussed in this paper have been deposited in NCBI's Gene Expression Omnibus (11) and are accessible through GEO Series accession number GSE17015 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE17015>).

Normalized and filtered log Cy5:Cy3 expression ratios were exported from GeneSpring and imported into the R software environment version 2.8.0 (<http://www.r-project.org/>) for statistical analysis using limma. Limma (linear models for microarray data) fits a linear model to the expression data for each gene in the array and then uses empirical Bayes and other shrinkage methods to borrow information across genes to moderate the variances toward a pooled estimate thereby adding stability to analyses with limited sample sizes.^{46,47} Differential expression was determined using moderated limma F-tests and limma two-sample t-tests (depending upon the comparison) with a false discovery rate (FDR) <1% and with multiple test correction of the *p*-values via Benjamini-Hochberg algorithm (6). A summary of the analytical approaches that were used is shown in Figure 1. Complete lists of all differentially expressed genes can be found in Supplementary Tables 1–12. (Supplementary Tables 1–12 can be found in the online article at www.liebertpub.com/lrb.)

For hierarchical clustering of the vessel samples, the normalized Cy5:Cy3 ratios from those genes found to be differentially expressed in the comparison of all six vessel groups were used to cluster all 36 vessel samples in GeneSpring using standard (cosine) correlation with complete linkage. For hierarchical clustering of genes and the generation of a heat map image, those genes that were found to be differentially expressed within the three thoracic samples or the three mesenteric samples were pooled and clustered according to their expression across all 36 samples using standard (cosine) correlation with complete linkage. To evaluate the discrepancy between the numbers of genes found differentially expressed in the thoracic vessel limma F-test versus the mesenteric vessel limma F-test, the mean squared deviation between each group (MSB) and the mean squared deviation within each group (MSW) were calculated in R using a Welch correction for unequal variances in the three thoracic vessels and three mesenteric vessels for each of the 23,842 genes present in at least 50% of the arrays. The MSB or MSW values for each gene were compared between the mesenteric and thoracic vessels using a one-tailed Wilcoxon signed rank test ($p < 0.01$).

GO (Gene Ontology, <http://www.geneontology.org/>) and KEGG (Kyoto Encyclopedia of Genes and Genomes, <http://www.genome.jp/kegg/>) category enrichment analysis was performed using the WebGestalt Gene Set Analysis Toolkit version 2.0 (<http://bioinfo.vanderbilt.edu/wg2/>) (60). Those genes within our differentially expressed gene lists that mapped to Entrez Gene accessions were compared to the reference dataset of all genes on the array that mapped to Entrez Gene accessions. Significant GO/KEGG enrichment was determined using hypergeometric tests ($p < 0.01$, minimum of 3 differentially expressed genes in an enriched category). If multiple categories contained the same enriched genes, only the most inclusive category is described in most cases. Only the GO biological process domain was considered for these analyses.

Microarray annotations

To provide uniform annotations for the microarray experiment described above, the Operon Rat Genome Version 3.0 Oligo Set sequences were mapped to the NCBI Rat mRNA Reference Sequence (RefSeq) database³⁶ downloaded on September 3, 2008 using the basic local alignment search tool

FIG. 1. Summary of microarray data analysis workflow.

(BLAST)¹ according to the “BLAST Analysis for the MAQC project” guidelines.⁴⁴ BLAST results were restricted to those that matched $\geq 96\%$ of the oligo sequence and then mapped to the Entrez Gene database.²² To select the single most informative annotation accession for each oligo, preference was given to 1) greater sequence homology, 2) RefSeq accessions over predicted RefSeq accessions, 3) RefSeq accessions with an associated Entrez Gene accession, and 4) Entrez Gene accessions with more GO/KEGG annotations. Those oligo sequences that did not map to the RefSeq database were mapped to the NCBI rat mRNA database according to the same guidelines listed above. Selected sequences were mapped to the Ensembl rat transcript database.¹⁷ A total of 22,689 oligos were successfully mapped to an mRNA accession and a total 22,571 oligos were mapped to an Entrez Gene Accession.

Quantitative, real-time polymerase chain reaction (qPCR)

Primers for the rat genes of interest (Table 1) were designed using Beacon Designer 5.10 software (Premier Biosoft International, Palo Alto, CA) and purchased from Eurogentec (San Diego, CA). To determine primer efficiency and efficacy, Rat Universal Reference RNA was reverse transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to the manufacturers instructions. For each primer set, triplicate 25 μ l qPCR reaction mixtures were prepared using iQ SYBR Green Supermix (Bio-Rad), 500 nM of forward and reverse primers, and a seven-point, five-fold serial dilution of cDNA (starting with 100 ng). qPCR was performed on an iCycler iQ Multicolor Real-Time Detection System with software version 3.1 (Bio-Rad) using the following thermal

cycling protocol: 2 min at 95°C followed by 40 cycles of 15 sec at 95°C and 45 sec at 60°C. Primer efficiency was calculated as described previously.³⁴ Primer sets that exhibited nonspecific amplification products in their melt curve analysis or failed to have a minimum efficiency of 1.85 (85%) across a minimum of five cDNA concentrations were discarded and redesigned.

For qPCR analysis of genes of interest, 500 μ g samples of vessel aRNA and 1 μ g of Rat Universal Reference aRNA obtained from the first round of linear RNA amplification for the microarray experiments ($n=6$ for each vessel) were quantified by UV spectrophotometry (A260) and reverse transcribed to cDNA as indicated above. qPCR was performed in triplicate using 5 ng of cDNA per reaction. For 18s rRNA expression analysis, 40–750 μ g of total RNA ($n=6$ for each vessel) and 1 μ g of Rat Universal Reference RNA was quantified using the Agilent 2100 Bioanalyzer, reverse transcribed as indicated above, and analyzed using the 18s rRNA Control Kit (Eurogentec) according to the manufacturer’s instructions. During the course of these experiments, no suitable internal control (“housekeeping”) genes were identified for this large multi-vessel comparison (see Results). As such, qPCR results were normalized to the RNA concentration. Relative mRNA expression was calculated for each gene of interest using an efficiency-corrected version of the $-\Delta\Delta C_t$ method²¹ in which the primer set efficiency is raised to the power of the C_t difference between the sample and the reference cDNA; triplicate C_t measurements were averaged prior to the calculation of relative mRNA expression values for each sample.

To facilitate the use of parametric statistical tests, the relative mRNA expression values were log transformed to convert the ratiometric distributions to normal distributions prior to averaging, error calculation, and statistical analysis.

TABLE 1. qPCR PRIMER SEQUENCES

Accession	Rat Gene	Forward Primer	Reverse Primer
NM_030851	Bradykinin receptor B1 (Bdkrb1)	TCCAGCCCTCTAACCGAAGC	GCCAAAGAAGCAGATAGTGATGAC
NM_017244	Cellular retinoic acid binding protein 2 (Crabp2)	CGCGTCCAGTATTCTAGTTGA	TTCCAGTTGCCAGAAAAGTTAGG
NM_031022	Chondroitin sulfate proteoglycan 4 (Cspg4)	CATGTTACGCGTCATCATCCC	TTGCGTTTGCGGAGGTAGAA
NM_012944	Dopamine receptor D4 (Drd4)	CCTGGTCAGTGTGTACCT	ACTCGCATGGAAGATGGTGTAG
NM_012698	Dystrophin (Dmd)	GCCCTTGTCTGGATCTCTGA	TGTTGTGCTCTTGTCTCCAGAC
NM_012722	Elastin (Eln)	AGGACAAGGAAATCAGACAGCAG	GCTCCTGGGTGGGAAGAGAT
NM_001013078	Fibrous sheath interacting protein 1 (Fsp1)	TGGGAAGCTGAAAGAGATCAATGA	CACCTACACTCGGGCTCAAG
NM_001079064	Forkhead box C2 (Foxc2)	CTTCTGTAACGAGTGGGATTT	CAGTTGGGCAAGATGAAAACCTTAT
AB017801	Glyceraldehyde-3-phosphate dehydrogenase (Gapdh)	GAGAAACCTGCCAAGTATGATGAC	GAAGAATGGGAGTTGCTGTGAAG
XM_223087	Laminin, beta 3 (Lamb3)	GGCTTCTCATCCAGCAGGTC	TCATCTGCGTAGCACTGTAGC
NM_001007604	Large ribosomal protein P1 (Rplp1)	ACAACATGGCTTCTGTCTCTGAG	AGCATTGATCTTATCCTCCGTGAC
XM_001077796	Lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1)	CAAAACAAGGCTTCTAGTCCAATCC	GCAATGGGTCCCTGTAATCAAGTG
NM_031020	Mitogen activated protein kinase 14 (Mapk14)	CACACTGCTGCTTCTCCTCACT	TGTCGGTGATGTCAGATGGC
NM_031622	Mitogen-activated protein kinase 6 (Mapk6)	TCCAAAGTCAGTCAGCCGAGAA	CTTCCTGACCTCACAAACAAAACCTG
NM_053611	Nuclear protein 1 (Nupr1)	AGGCTCTGGAGAGGGAACAC	CCITCTTAGCTCTGCCCATCTAC
NM_031347	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha (Ppargc1a)	TAAATCTGCCGGGATGATGGAGAC	TATATCCATTCTCAAGAGCAGCGCA
NM_019358	Podoplanin (Pdpn)	TCCCAGATGCTCAGAAAAGTTTGT	AGGACTGAGTTTCTGTTCTTGGTIT
XM_223067	Prospero-related homeobox 1 (Prox1)	TCAAATCCCCTAACTGCCTACAAAG	AGACACAGAGAAGTTGGACATCC
NM_145086	Serpine1 mRNA binding protein 1 (Serbp1)	AGTGCTTCTGCTCTCTGATGTAG	AACCAGTGTGTATTATATGGCATCC
NM_019212	Skeletal muscle a-actin (Acta1)	CCTCTCCCTGGAAAAGAGCTA	GCAACGGAAAGCGCTCAIT
NM_031144	β -Actin (Actb)	GCAGATGTGGATCAGCAAGCA	TGTCAAAGAAAGGGGTAAACCGC
ENSRRNOT00000025394	Stabilin-1 (Stab1) [Ensembl Transcript]	CTGGACAACATGACGCTGAGT	TGAGTGGCAGGAAGCAATG
NM_130428	Succinate dehydrogenase complex subunit A (Sdha)	GGAAACACTGGAGGAAGCACAC	TAGGAACGGATAGCAGGAGGTAC
NM_001011977	Tektin 2 (Tekf2)	GCTATCGCTCAGACCAACAATG	CAAACTTCCATCTCCCGAAGC
NM_017200	Tissue factor pathway inhibitor (Tfpi)	TGCCCGAGGAAGACGATGATA	AATAACTCCGTATCATGTGTTGC
NM_053652	Vascular endothelial growth factor receptor 3 (Vegfr3)	CCCTTACAGCCTCCCTATCCA	GGTGACTGCCCCAGTGATATCG

Outlying data points were removed using Dixon's Q test ($p < 0.01$). For the purpose of microarray result validation, a qPCR result was said to validate a microarray result if it reached statistical significance ($p < 0.05$) in a one-tailed, two-sample, Welch t-test analogous to the comparison used in the microarray analysis. For all other comparisons, significance was assessed using one-way ANOVA with a Student's t post hoc test ($p < 0.05$). The qPCR results presented here represent the exponentiated mean log ratio of the relative mRNA expression values. Data are presented as mean $\pm$ SEM. Two-sample t-tests were performed using Excel version 12.1.9 (Microsoft Corporation, Redmond, WA). ANOVA was performed using JMP version 7.0.2 (SAS Institute, Cary, NC).

Immunofluorescence of MHC Class II gene products in isolated rat mesenteric lymphatics

Immunofluorescence of isolated, whole, paraformaldehyde-fixed rat mesenteric collecting lymphatics was performed as previously described.^{7,57} The lymphatic was fixed in 2 % paraformaldehyde in PBS for 60 min and washed in PBS 3 times for 5 minutes each. It was permeabilized in -20°C methanol for 5 minutes and incubated in blocking solution (1% BSA, 5% normal serum in PBS) for 1 hr at room temperature. The vessel was then divided into 2 segments and one was incubated at 4°C overnight with the primary antibodies in blocking solution intralumenally and extralumenally. The second section was given the same treatments except we used the corresponding pre-immune immunoglobulins instead of the primaries as the negative control. Both segments were incubated with the secondary antibodies extra and intralumenally for 1 hr at RT. Primary antibodies used included mouse-anti-mouse β chain of the I-A[k] MHC II, IgG2a (clone 10.3.6, BD-553539, BD Biosciences Pharmagen), mouse anti-rat CD74, IgG1a (clone OX4, sc-53061, Santa Cruz Biotechnology) and mouse anti-rat CD86/B7-2, IgG1 (clone 24F, BD-555016, BD Biosciences Pharmagen). Secondary antibodies used included, goat anti-mouse IgG2a-Oregon Green, goat anti-mouse IgG2a-AF647 and goat anti-mouse IgG1a-AF647 (Invitrogen). The vessel segments were washed in PBS 3 times for 5 minutes each between the two treatments described above. The vessels were then cannulated and tied onto 2 glass pipettes, pressurized at 2 cm H_2O and secured to the stage of a multiphoton/confocal microscope (Leica TCS SP2, Germany) for immediate observation. The vessels were scanned in $0.5\text{ }\mu\text{m}$ z-axis steps with either a Leica HC PL APO 20X (dry, 0.7 na) objective or a Leica U APO 340/cc 40X (water immersion, 1.15 na) throughout the entire vessel thickness at multiple sites within a given isolated lymphatic using a pin-hole size of 1 airy disk. Image reconstruction and orthogonal viewing on the image stacks was performed using the Leica Confocal Software and ImageJ64. The negative controls were produced and analyzed using the same instrumental and image processing procedures with the same parameter settings. Images selected for display are representative of replicate data from animal group sizes of 3–20.

Quantitative comparison of MHC Class II⁺ APCs in rat lymphatics isolated from different sections of the lymphatic network

If the APCs we originally identified in mesenteric prenodal lymphatics are important to antigen processing of lymph

borne agents, then we predicted that there would be significant differences in the expression of these APCs in prenodal versus postnodal lymphatics since the majority of antigens are found in prenodal lymph. Thus we compared the distribution and morphology/phenotype of the resident APCs in prenodal and postnodal lymphatics. To do this we analyzed the characteristics (cell numbers/unit vessel area, cell size, cell shape, the number of dendrites, length of dendrites) of the MHCII⁺ APCs imaged in isolated rat prenodal mesenteric afferent lymphatics ($n=17$) and isolated rat postnodal lymphatics (data from the mesenteric efferent lymphatics and thoracic duct segments were pooled as they did not exhibit significant differences in APC, $n=6$). To do this we analyzed the APCs in average projections reconstructed from the bottom half of each vessels z-stack using Image J.

Results

Microarray analysis

For the present investigation, we examined the relative gene expression in lymphatics, arteries, and veins isolated from both the rat thoracic and mesenteric tissue beds. Following quality control filtering, a total of 23,801 genes were evaluated for differential expression in each of the six vessel groups. Using a limma-based F-test, we identified 1444 genes as being differentially expressed in at least one vessel group. Hierarchical clustering of the 36 vessel samples across these 1444 genes revealed that the vessels clustered first according to their blood versus lymphatic lineage followed by their tissue bed (Fig. 2), and thus, the differences in gene expression between the large thoracic blood vessels and the small mesenteric blood vessels is greater than the differences between the arterial and venous lineages. All vessels clustered tightly with their sample group with the exception of one AV sample that clustered with the MV sample group. The two lymphatic groups appear to cluster the tightest, showing marked distinction both from the blood vessels and between themselves highlighting the unique physiology of these vessels within the cardiovascular system.

To examine the transcriptional differences between the three vessel lineages in the two vascular beds, we identified 127 genes differentially expressed among the three thoracic vessels and 943 genes differentially expressed among the three mesenteric vessels. In an attempt to ascertain why there was a marked difference in the number of genes found differentially expressed in the thoracic versus mesenteric tissue beds, we examined the mean squared deviations within and between (MSW and MSB, respectively) the three vessel groups from the thoracic tissue and compared them to the mesenteric vessels for each of the 23,801 genes analyzed. We found that the within group variances (MSW) were significantly greater in the thoracic vessels while the between group variances (MSB) were significantly greater in the mesenteric vessels (data not shown). While the increased sample variance within the thoracic vessels is evident, those genes that were upregulated in the mesenteric lymphatics versus the mesenteric artery and vein generally displayed a similar pattern of expression in the thoracic vessels (Fig. 3, region a). A similar pattern of conservation is observed among those genes downregulated in the lymph versus the blood vessels; however, there was considerably more variability, particularly in the AV sample group (Fig. 3, region b).

FIG. 2. Hierarchical clustering of the 36 vessel samples across the 1444 genes identified by limma F-test as being differentially expressed in at least one vessel type (FDR <1%). Vessels cluster first according to their lymph versus blood vessel lineage followed by their tissue bed of origin. All vessels cluster within their own vessel group except for AV1, which clusters with the MV group.

To identify the transcriptional differences that most significantly distinguish muscular lymphatics from both arteries and veins, we focused our present investigations on those genes that were differentially expressed in the comparisons of TD to AA (55 upregulated, 121 downregulated) and AV (7 upregulated, 22 downregulated) as well as ML to MA (380 upregulated, 466 downregulated) and MV (135 upregulated, 395 downregulated). Among the four comparisons, there was reasonably good agreement among those genes identified in the lymphatic versus artery and the lymphatic versus vein comparisons within a single vascular bed, with greater than 60% of the genes found in the smaller gene sets also represented in the larger gene sets (Fig. 4). Those genes identified in the lymphatic versus artery or the lymphatic versus vein comparisons also showed good agreement between the two vascular beds.

Functional category enrichment analysis

In order to evaluate the physiological significance of the genes identified in the lymph-blood vessel comparisons, we performed functional category enrichment analysis on the

FIG. 3. Heat map representation of vessel-specific gene expression patterns. The 127 and 943 genes identified by limma F-test as being significantly differentially expressed in at least one vessel type among the three thoracic or mesenteric vessels, respectively, were collectively clustered according to their expression across all 36 vessel samples. Red indicates high expression; green indicates low expression. There is a profound separation of those genes that are up-regulated in the lymphatics relative to blood vessels (region a) versus those genes that are downregulated in the lymphatics versus blood vessels (region b).

eight gene lists indicated in Figure 4. Compared to both MA and MV, the ML were significantly enriched in genes related to “catabolic processes” (GO:0009056; 17 genes versus MA, 9 genes versus MV) as well as “mRNA transport” (GO:0051028; 4 genes versus MA, 3 genes versus MV). Additionally, the ML were enriched relative to the MA with genes related to “translation” (GO:0006412; 19 genes) and “ribosome” (KEGG; 7 genes) cumulatively suggesting the existence of altered gene and protein expression strategies employed by the lymphatic vessels. There were also several immunological genes upregulated in the ML versus the MA and MV including enrichment in the “antigen processing and presentation” (GO:0019882 and KEGG; 5 genes versus MA, 2 genes versus MV) category as well as the high affinity IgE receptor “Fc epsilon RI signaling pathway” (KEGG; 6 genes versus MA, 3 genes versus MV).

Among the genes that were downregulated in the ML versus the MA, there was significant enrichment in genes related to the “cellular localization” of compounds within the cell (GO:0051641; 26 genes) and “regulation of cell proliferation” (GO:0042127; 15 genes). Additionally, there were

FIG. 4. Representation of the number of genes upregulated (A) and downregulated (B) in lymphatics versus arteries or veins. The numbers in the overlapping sections of the circles indicate the number of common genes present between the two indicated comparisons.

several genes related to carbohydrate metabolism in particular "glycolysis" (GO:0006096; 5 genes). Relative to the MV, the ML exhibited downregulation of several intracellular signaling genes including "small GTPase mediated signal transduction" genes (GO:0007264; 20 genes), the "inhibition of adenylate cyclase activity by G-protein signaling" genes (GO:0007193; 3 genes) and genes related to "axonogenesis" (GO:0007409; 7 genes). Both the MA and MV also showed significant enrichment in genes related to "oxygen transport" (GO:0007409; 3 genes) relative to the ML. The small numbers of genes in the thoracic vessel comparisons precluded the identification of significantly enriched functional categories in these gene lists.

Validation of microarray results using qPCR

To confirm the accuracy of our microarray results, we selected 15 genes from the microarray results representing a variety of cellular processes and validated their differential expression using qPCR (Table 2). Seven of the genes (representing 12 pair-wise comparisons) were found to be significantly different via qPCR in accordance with the microarray data; however, the qPCR results for chondroitin sulfate proteoglycan 4 (Cspg4) and stabilin 1 (Stab1) displayed a much greater fold change than observed in the microarray. Fibrous sheath interacting protein 1 (Fsip1) and mitogen-activated protein kinase 6 (Mapk6) showed substantial upregulation and downregulation, respectively, in most lymphatic versus blood vessel comparisons and the qPCR data showed a similar trend but only reached significance in the mesenteric comparisons. Strangely, dystrophin (Dmd), laminin, beta 3 (Lamb3), tissue factor pathway inhibitor (Tfpi), and the mesenteric comparisons for dopamine receptor D4 (Drd4) were all found to be differentially expressed by microarray and qPCR; however, the polarity of the expression differences were found to be exactly opposite between the two detection methods.

Analysis of lymphatic marker gene expression using qPCR

Much of the current lymphatic research relies intensively on the use of lymphatic marker proteins to identify and track

LECs in various experimental preparations. Many of these lymphatic markers were originally characterized by their ability to distinguish LECs from blood vascular endothelial cells in dermal microvascular preparations and, thus, may predominantly reflect the expression profile of LECs from initial lymphatics. To examine the utility of common lymphatic marker genes in identifying collecting lymphatic endothelial cells and collecting lymphatic vessels, we examined the mRNA expression of four putative lymphatic endothelial cell marker genes as well as a fifth gene intensely studied in lymphatic development (Fig. 5). Consistent with its role as the master control gene for the lymphatic endothelial cell phenotype, we see that Prox1 is significantly upregulated in both the TD and ML and undetectable in both arteries examined. While LYVE-1 is considered one of the most reliable markers of endothelium from initial lymphatics, recent reports have demonstrated down regulation of LYVE-1 in mouse collecting lymphatics.^{23,55} Indeed, we see that the rat collecting lymphatic vessels express LYVE-1 mRNA at levels very similar to the AV, although, lymphatic LYVE-1 expression is still significantly greater than that observed in all other blood vessels tested. Podoplanin (Pdpn) expression has been shown to remain present in the collecting lymphatic vessels of the mouse skin,^{10,23} and similarly, we see significantly elevated levels of podoplanin in the ML versus all other vessels tested. Strangely, the TD exhibits levels of podoplanin mRNA expression similar to that found in the larger blood vessels. VEGFR3 was one of the first markers identified for the postnatal lymphatic endothelium,¹⁸ and given its role in responding to the primary lymphatic growth factors VEGF C and D, it is not surprising that both ML and TD exhibit very high levels of VEGFR3 mRNA relative to blood vessels. FOXC2 has been intensively investigated for its role in congenital lymphedema-distichiasis as well as the maturation of collecting lymphatic vessels and their intraluminal valves,³³ and we do see that Foxc2 mRNA is expressed in relatively high quantities in the lymphatic vessels versus the blood vessels. These data do support the use of Prox1 and VEGFR3 as lymphatic vessel markers among collecting lymphatics, arteries, and veins; however, the utility of the commonly used marker, Pdpn, may be limited in large lymphatics such as the TD.

TABLE 2. qPCR VALIDATION OF MICROARRAY DATA

Gene	Accession	Detection	Fold Change in:			
			TD vs. AA	TD vs. AV	ML vs. MA	ML vs. MV
Acta1	NM_019212	qPCR			0.514	
		microarray			0.384	
Bdkrb1	NM_030851	qPCR			2.750	
		microarray			2.039	
Crabp2	NM_017244	qPCR			2.815	3.225
		microarray			2.398	2.503
Cspg4	NM_031022	qPCR	0.057		0.004	
		microarray	0.268		0.198	
Dmd	NM_012698	qPCR			0.127*	0.213*
		microarray			11.944	11.309
Drd4	NM_012944	qPCR	0.120		2.622*	1.759*
		microarray	0.316		0.253	0.163
Fsip1	NM_001013078	qPCR	1.466+	1.651+	3.489	1.526
		microarray	6.572	6.111	6.715	5.320
Lamb3	XM_223087	qPCR			0.145*	
		microarray			1.956	
Mapk6	NM_031622	qPCR	1.205+		0.329	0.301
		microarray	0.153		0.144	0.074
Mapk14	NM_031020	qPCR	1.701		0.669+	
		microarray	1.726		2.308	
Nupr1	NM_053611	qPCR	0.156			
		microarray	0.182			
Ppargc1a	NM_031347	qPCR				1.125+
		microarray				2.051
Serbp1	NM_145086	qPCR	1.623		1.749	1.692
		microarray	2.162		2.536	2.603
Stab1	ENSRNOT00000025394	qPCR	5.885		17.021	
		microarray	2.944		4.473	
Tfpi	NM_017200	qPCR	3.956*		2.473*	1.546*
		microarray	0.143		0.071	0.134

All data above was found to be significant in both microarray analysis (FDR <0.01) and qPCR analysis ($p < 0.05$) unless otherwise indicated. $n = 6$ for all qPCR data.

*denotes significance but fold change is opposite to that observed in the microarray.

+denotes NOT significantly different ($p < 0.05$) by qPCR.

Identification of internal reference genes for lymphatic-vascular comparisons

Nearly all of the commonly used methods for measuring mRNA expression rely upon the normalization of the calculated expression values to some variable representative of the

quantity of tissue that is being analyzed. In many cases, the preferred normalization strategy utilizes an internal reference gene ("housekeeping gene") that is presumably stably expressed among all samples of interest. In order to identify a suitable internal reference gene for the present lymphatic-vascular comparisons, we evaluated the expression of the

FIG. 5. mRNA expression of putative lymphatic endothelial cell marker genes in rat collecting lymph and blood vessels as determined by qPCR. Expression level is presented relative to ML. Statistical significance is displayed using a connecting letters report; for each gene, sample groups not connected by the same letter are significantly different ($p < 0.05$). Error bars represent ± 1 SEM.

FIG. 6. qPCR analysis of “housekeeping gene” expression in thoracic and mesenteric lymph and blood vessels. For each gene, expression level is presented relative to the sample group with the highest expression. Statistical significance is displayed using a connecting letters report; for each gene, sample groups not connected by the same letter are significantly different ($p < 0.05$). There is marked variability in the mRNA levels of common and proposed internal reference genes across the different vascular lineages and tissue beds. Error bars represent ± 1 SEM.

three most commonly used internal reference genes as well as two housekeeping genes that were identified as being stably expressed across all six vessel groups in the microarray comparison (Fig. 6). When normalized to RNA concentration, the commonly used internal reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) displays significant variability in gene expression across the six vessel groups, particularly among the three mesenteric vessels. As demonstrated in the microarray data (see Supplementary Tables 6 and 12), β -actin also exhibits significant differences in gene expression between ML compared to TD and MA; the expression is relatively stable within the remaining comparisons. Surprisingly, 18s rRNA, whose concentration is usually proportional to the total RNA concentration, displays large differences in expression levels particularly with the two lymphatic vessels relative to all the blood vessels. While succinate dehydrogenase complex subunit A (SDHA) was identified in the microarray data as being stably expressed across the six vessel groups, qPCR analysis revealed significant differences in expression across all vessel types for SDHA. Large ribosomal protein P1 (RPLP1) mRNA expression was consistent across most vessels tested; however, the ML in particular displayed pronounced differences in gene expression relative to the remaining vessels. Taken together, β -actin appears to be the most effective internal reference gene among those tested; however, the approximately 1.6 fold range in β -actin mRNA expression would introduce some error in relative mRNA quantitation, and thus, normalization to RNA concentration when possible may be the most consistently accurate method of relative RNA quantitation available for examinations of collecting lymphatic versus blood vessel differential gene expression.

Analysis of “antigen-presentation” gene products in ML

Because of the consistent high enrichment of immunological genes (in particular those related to antigen presentation in ML) and given the presumed role of the lymphatics in immune cell trafficking, we investigated via immunofluorescence histochemistry two MHC class II complex proteins related to the genes upregulated in ML from the microarray (CD74/invariant chain of MHC II and the β chain of the I-A MHC class II). In addition, we determined the lymphatic ex-

pression of CD86, a co-stimulatory protein expressed on APCs that provides the non-antigen specific signals required for T cell activation and an effective immune response. As seen in Figure 7, isolated rat ML contained a large population of cells that strongly express these 3 common APC proteins. These MHCII⁺ APCs in the ML had an average cell body diameter of 15.7 ± 1.0 μ m and each APC covered an average area of 635 ± 94 μ m². They display an extensive dendritic-like morphology with an average of 3.58 ± 0.33 main dendrites per cell and 1.53 ± 0.20 secondary projections from the main dendrites. The average length of the main dendrites was 23.2 ± 1.6 μ m. The APCs were extensively (322 ± 44 cells/mm² of lymphatic wall) and evenly distributed within the wall of the rat mesenteric muscular prenodal lymphatics and represent a significant fraction of the ML cell population. Analysis of a subgroup of the ML APCs that express CD74 provided similar morphometric results (data not shown). Dual staining for the 2 MHC complex proteins indicated that the vast majority of these cells expressed both of the 2 MHC II complex proteins and that the MHCII⁺ cells also typically express the T cell co-stimulatory molecule, CD86, indicating a potential role of the mesenteric lymphatic as a rendezvous for antigen presentation.

Comparison of MHC Class II⁺ APCs in rat lymphatics isolated from different sections of the lymphatic network

While the postnodal lymphatics did have MHCII⁺ APCs within the walls of these lymphatics (data not shown), there were significantly fewer of them per unit area of lymphatic wall (119 ± 32 cells/mm² of lymphatic wall or 36% of the ML cell density) compared to the prenodal lymphatics. In addition, the MHCII⁺ APCs within the walls of the postnodal lymphatics had a much less dendritic-like morphology with fewer dendrites per cell (1.19 ± 0.22 main dendrites per cell or 33% of the ML value).

Discussion

In an effort to characterize the gene expression patterns that may underlie the unique biology of muscularized lymphatics, we designed and conducted mRNA expression microarray

FIG. 7. Immunofluorescence imaging of MHC II/APC proteins in cells within the wall of isolated rat mesenteric prenodal lymphatics. (A–D) Z-axis projections of confocal image stacks of afferent lymphatics. Panel A is a composite of mesenteric collecting lymphatics stained for β chain of the I-A[k] MHC II (green) with high magnifications inset to demonstrate the number, shape and location of MHC II⁺ cells within the lymphatic wall. Panel B is a composite of mesenteric prenodal lymphatics stained for the invariant chain, CD74 of MHC II (green) with high magnifications inset to demonstrate the distribution of MHC II⁺ cells within the lymphatic wall. Panel C is a mesenteric prenodal lymphatic dual-stained for the invariant chain, CD74 of MHC II (top red image) and the β chain of the I-A[k] MHC II (middle green image) to illustrate the colocalization of the majority of MHC II⁺ cells within the lymphatic wall co-expressing both of these MHC II complex proteins (bottom red/green image). Panel D is a mesenteric prenodal lymphatic dual-stained for the β chain of the I-A[k] MHC II (left green image) and CD86/B7-2 (right red image) to illustrate the colocalization of the majority of MHC II⁺ cells co-expressing both of these APC proteins (middle red/green image). In all panels scale bars depict size and the light gray lines represent the edge of the lymphatic outer wall.

experiments to examine the transcriptional profile of rat thoracic ducts and rat mesenteric collecting lymphatics and compared them to regional arterial and venous blood vessels. The clustering of the differentially expressed genes documented that the lymphatic versus blood vessel differences were more profound than between blood vessels, particularly in the Microvessels grouping. From the patterns of genes that were differentially expressed in the microarrays, we tested the expression of some genes by qPCR and others by expression of the gene products by immunofluorescence in some lymphatic tissues. Since the GO analysis indicated an enrichment for “antigen processing and presentation” in the prenodal lymphatics we evaluated the expression via immunohistochemistry of a number of known markers of professional antigen presenting cells and found a unique resident population of cells that display most of the important hallmarks of a professional antigen presenting cell.

From a lymph transport perspective, the TD represents a large, post nodal conduit vessel characterized by relatively

low phasic contractility but strong flow-dependent contractile regulation while the mesenteric lymphatic vessel (ML) represents a small prenodal collecting vessel characterized by very potent phasic contractility but relatively modest flow-dependent contractile regulation.^{12–14} Together, these vessels span the known spectrum of rat collecting lymphatic morphology and contractility. Given the structural similarities and extensive knowledge base of the blood vasculature, the transcriptional profile of TD and ML was compared to that of spatially similar blood vessels (Fig. 1). For TD, the axillary artery (AA) and axillary vein (AV) were selected for comparison due to the similarity of their spatial orientation (a few centimeters distal to the junction of the TD, subclavian vein, and external jugular vein) compared to the section of the TD utilized for these experiments. For the ML, the small mesenteric arteries (MA) and veins (MV) running parallel to the selected ML were utilized for comparison.

While there is a substantial knowledge base for the cellular and molecular biology of the blood vasculature, our

understanding of the molecular events underlying lymphatic physiology remains somewhat limited. The identification of the lymphatic endothelial cell-specific growth factor receptor VEGFR3 in 1995¹⁸ initiated a burst of scientific interest in lymphangiogenesis and lymphatic development; however, until very recently, the outcomes of this research focused almost entirely upon the initial lymphatic network or the isolated lymphatic endothelial cell. Very little attention has been given to the cellular and molecular biology of the muscular collecting lymphatics despite their significant role in the most prolific disease of the lymphatic system, lymphedema.^{26,29} In an effort to begin unraveling the molecular events that underlie the unique physiology of the muscular lymphatics, we constructed a series of microarray experiments to identify the transcriptional profile of these vessels. The rat was selected as the preferred animal model for these experiments to permit correlative comparisons between the molecular profile identified and the extensive lymphatic physiology data available for this animal. Muscular lymphatics were compared to their parallel artery and vein in both the thoracic and mesenteric tissue beds. The thoracic duct and the mesenteric lymphatic span the range of muscular lymphatic phenotypes identified in the rat (large and small, postnodal and prenodal, weak and strong phasic contractility, strong and weak flow-mediated contractile regulation, respectively)¹²; their parallel blood vessels also represent a range of morphological and physiological phenotypes among arteries and veins. Collectively, the comparison of these six vessels will permit the construction of a database of transcriptional heterogeneity within the blood and lymphatic vascular systems.

The hierarchical clustering of the six vessels across all differentially expressed genes (Fig. 2) demonstrated that the lymph versus blood vessel differences were more profound than the transcriptional differences found within the blood vascular network. Indeed, hierarchical clustering of those genes that were differentially expressed among the three thoracic or mesenteric vessels displayed intense transcriptional differences between the lymphatic and blood vessel groups (Fig. 3). Within the reference-based comparison, there was reasonable agreement between those genes identified as differentially expressed in the two different tissue beds; however, 86% fewer genes were identified as differentially expressed within the large thoracic vessels compared to the mesenteric vessels. These differences in the number of differentially expressed genes may reflect further specialization of the three vessel lineages (and thus greater differences between the three lineages) as you approach the microcirculation, since this is where the most critical roles of all of the vascular systems play out. However, variance analysis of the vessel groups revealed that there is significant variability within the sample groups of the three thoracic vessels, which would limit the number of differentially expressed genes identified statistically. The size and structure of the large thoracic vessels does impose additional difficulties when extracting biological materials such as RNA; however, our RNA bioanalyses did not reveal any quantifiable differences in RNA integrity between the thoracic and mesenteric vessel groups.

In the present investigation, we focused our analysis on those genes that were differentially expressed in the comparisons of arteries or veins to the lymphatic vessels. Interestingly in all four lymph-blood vessel comparisons made,

there were two consistent trends in the numbers of differentially expressed genes (Fig. 4). First, there were substantially more genes identified as differentially expressed in the lymphatic-artery comparisons compared to the lymphatic-vein comparisons, which is consistent with the venous rather than arterial developmental origin of lymphatic vessels.⁴⁸ Second, there were substantially more genes (22–214% more) identified as being downregulated compared to being upregulated in lymphatics. This later result suggests that muscular lymphatics express a smaller active gene set than comparable blood vessels, possibly reflecting a more specialized phenotype for the lymphatic vessels. Indeed, the enrichment of numerous genes related to intracellular signaling in the blood vessels suggests that muscular lymphatics may lack or exhibit diminished activity in some of the signaling pathways present in the cells of the blood vessels.

An important result from the functional category enrichment analysis of the lymphatic-specific upregulated genes was the presence of several genes related to antigen presentation and immune function. It should be noted that in these studies, the entire lymphatic vessel as well as any minor remnants of its luminal contents were treated as a single organ. Since we do not purposely occlude the vessel during the isolation procedure, normally the contents of the lymph and blood vessels empty nearly completely during the isolation procedure. Thus, it is possible although unlikely that the presence of any trace luminal elements in the samples may have minor influences that are reflective of differences in components present in the lymph compared to those in blood. However the verification of some of these gene products by immunofluorescence histochemistry in Figure 7 documents that in fact these antigen presenting – MHC II gene products are indeed seen in a significant resident population of cells within the lymphatic wall, since the immunohistochemistry procedure specifically washes the luminal contents out and the location of the cells by 3D microscopy documents they reside in the vessel wall. Thus there is a resident population of potential antigen presenting cells that exhibit most of the gene products necessary for professional antigen presentation and demonstrate a morphology with extensive cell extensions similar to the interdigitating dendritic cells of the lymph node. The question of the exact antigen presenting cell type these APCs represent, i.e., dendritic versus macrophage-derived remains to be determined. With the isolated vessel procedure and other lymph collections methods we have employed in other studies, we rarely seen any of these cells free within the lumen of the lymphatic, further indicating these prenodal lymphatic resident APCs represent a immune cell population that was previously unknown. These data bring up interesting points that are important to our understanding of immunity and lymphatic function. It has long been thought that the prenodal muscular collecting lymphatic vessels are responsible for transporting activated antigen presenting cells and antigen itself from the parenchyma to the lymph node to initiate an adaptive immune response and are thus in a unique position to interact with and modulate the immune response.⁵⁰ Indeed, the clinical significance of the association between lymphatic vessels and immune cells in the treatment of lymphedema was proposed more than two decades ago.³¹ More recent work has documented the impact that lymphatic dysfunction has on immune cell trafficking and the resulting changes in tissue inflammatory cells.^{19,24,37,40,53,56,59}

The identification of immunological genes in the lymphatic samples reflects the presence of immune cells that are resident within the lymphatic vessel wall that may participate in screening for lymph-borne antigens. Given that the lymphatic vascular network represents a preferential path of low-resistance fluid transport from the interstitium, it provides a logical site for cells to capture antigen from the upstream tissue drainage fields. Similarly once antigen presenting cells have captured and processed antigen, the lymphatics provide a direct low-resistance path to the lymph node for complete activation of the adaptive immune response. Indeed, the presence of these genes may reflect a novel mechanism by which lymphatic vessels could increase immune cell transport during an immune response or possibly amplify the immune cell response itself. Verification of this novel potential antigen presentation/processing mechanism in the APCs of the prenodal lymphatics and how they might each control the others systems functions remains to be determined. Additionally, the mechanism by which the adaptive immune response initiated in the lymph node is targeted back to the origin of the antigen also remains unresolved²; however, some form of countercurrent signaling mechanism along the lymphatic vessel from the node back to the tissue could be responsible for this. The enrichment of the catabolic processes gene category is consistent with the antigen processing/presentation pathway, as these mechanisms would be necessary for either foreign or self antigen processing for display. Providing further support for an important role of the lymph, prenodal lymphatics, and their unique resident APCs in immune tolerance and foreign antigen responses. Other functional categories of interest within the lymphatic upregulated genes were "translation," "ribosome," and "mRNA transport" for which the functional significance is not known. It is interesting to consider, however, that the unique phenotype of the muscular lymphatics may be linked to altered protein synthesis strategies, which would be reflected in this present result.

One of the most commonly addressed features of any expression analysis of lymphatic endothelial cells or vessels is the presence of so-called lymphatic marker genes. Because the most common lymphatic markers were either absent from our array oligo set or flagged as absent on our arrays (usually due to a lack of expression in our reference RNA sample), we investigated the expression of these genes using qPCR (Fig. 7). Consistent with previous reports demonstrating uniform expression of Prox1 by endothelial cells throughout the lymphatic system,⁴⁸ we show that Prox1 is expressed in both the TD and ML. It is interesting that a low level of Prox1 mRNA is detected in the venous samples given that lymphatic development begins with the expression of Prox1 in venous endothelial cells that then bud off to form the primitive lymphatic network.²⁸ This low level of venous Prox1 expression may represent the underlying ability of venous endothelial cells to be stimulated to differentiate into lymphatic endothelial cells. Despite the fact that LYVE-1 has been shown to be downregulated in muscular collecting lymphatics,^{23,55} we still see relatively high levels of LYVE-1 mRNA expression in the TD and ML. This may indicate that the downregulation of LYVE-1 by smooth muscle cell contact is mediated at the post-transcriptional level, but this finding warrants further investigation. Podoplanin has been used as a reliable maker of dermal collecting lymphatic vessels,²³ and we do see signifi-

cant upregulation of podoplanin in the ML. However, the levels detected in the TD were considerably lower than those seen in ML and were similar to those observed in the blood vessels. While podoplanin is expressed in cultured endothelial cells derived from the TD,²⁵ there are no reports of its expression in the TD *in vivo*, and the present results may reflect a functional difference in the large muscular lymphatic ducts relative to the smaller muscular collecting vessels. Among the lymphatic marker genes evaluated, VEGFR3 displayed the most consistently high gene expression levels in lymphatics relative to blood vessels underpinning the importance of this receptor in mediating lymphatic vessel growth and development. FOXC2 has been implicated in mediating collecting lymphatic vessel differentiation,³³ and indeed both TD and ML express relatively high levels of FOXC2 mRNA. Interestingly, FOXC2 has also been proposed to play a role in regulating venous valve formation²⁵; however, our present analysis shows that venous FOXC2 mRNA levels are among the lowest of the three vascular lineages. Besides, FOXC2 has been involved in cancer metastasis. Our data provide an evidence for the preferred lymphatic metastasis of the carcinoma.

A consistent technical difficulty that must be overcome when performing mRNA or protein express analysis is the selection of a suitable internal reference gene or protein for use in data normalization particularly when sample sizes are small enough to preclude the reliable use of RNA or protein concentration as a normalization agent. Given the diversity of tissues utilized in these studies, we examined five different candidate internal control genes for use in this analysis of vascular transcriptional heterogeneity. We found that, at the mRNA level, all candidate internal control genes display significant variability in expression across the vessels tested. GAPDH and β -actin mRNA expression have both been shown to be modulated by serum and PO₂ levels,^{43,62} which may explain the trends in differential gene expression observed for these two genes. The most interesting result was the range of 18s rRNA expression among the six vessels. Since 18s rRNA should be reflective of total RNA concentration,^{9,43} the present differences, particularly between blood and lymph vessels, may represent different degrees of rRNA degradation encountered while isolating these tissues. It should be noted, however, that this result together with the upregulation of mRNA transport and RNA splicing genes in lymphatics may in fact represent different mRNA handling strategies employed by lymphatic vessels. Fortunately, the use of amplified RNA in the present study allowed us to accurately normalize our qPCR data to RNA concentration. Within the thoracic vessels, GAPDH shows relatively stable mRNA expression and would thus be a suitable internal reference gene for those three vessels. Studies involving the mesenteric or all six vessels would be much more challenging. β -actin is the most stable internal reference identified for all six vessels; however, its 1.6 fold range in expression would introduce an appreciable amount of error in the qPCR analysis.

In summary, we have demonstrated the application of transcriptional profiling techniques to the analysis of rat lymphatic vessels from different segments of the lymphatic network, and we have described interesting candidate genes that may be responsible for some of the unique biological aspects of these vessels. Importantly we found that the lymphatics had significant enrichment in a number of GO

categories compared to arteries and veins, particularly at the prenodal afferent lymphatic level. An important enrichment that we focused our efforts on was the enrichment of genes associated with immune responses and in particular antigen presentation and processing in the prenodal lymphatics. We further investigated the unique immunological characteristics of the lymphatics and found that the prenodal, muscularized lymphatics of the gut had a large, resident population of cells that display most of the critical characteristics and morphology of professional antigen presenting cells, particularly the dendritic/macrophage classes of cells. In addition, we propose that this early immune response may modulate lymph transport by interacting with other lymphatic cells (lymph pump pacemakers, lymphatic muscle and endothelial cells) in order to optimize lymph flow to accommodate overall immunity. Further studies are needed to document the specific APC cell type and demonstrate their ability to capture, process and display the antigens to other adaptive immune cells at important immune sites. These data serve as strong indicators that the lymphatic and immunological systems are functionally intertwined in a fashion that matches their anatomical perspective and that the lymphatic "immunovascular system" may have important immune-centric roles and regulation in addition to the cardiovascular-centric roles that have been historically defined. A better understanding of the interactions of the lymphatic and immune systems may have significant benefits to our understanding of normal function and numerous disease processes. Last, the transcriptional database generated from these experiments can serve a data mine for future investigations pending further insights into the biology of muscularized lymphatics.

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Author Disclosure Statement

The authors have no conflicts of interest or financial ties to disclose.

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Address correspondence to:

David C. Zawieja
 Department of Medical Physiology
 Texas A&M Health Science Center
 College of Medicine
 702 S.W. H.K. Dodgen Loop
 Temple, TX 76504

E-mail: dcz@tamu.edu