

Unique Gene Expression Profiles of Donor-Matched Human Retinal and Choroidal Vascular Endothelial Cells

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PURPOSE. Consistent with clinical observations that posterior uveitis frequently involves the retinal vasculature and recent recognition of vascular heterogeneity, the hypothesis for this study was that retinal vascular endothelium was a cell population of unique molecular phenotype.

METHODS. Donor-matched cultures of primary retinal and choroidal endothelial cells from six human cadavers were incubated with either *Toxoplasma gondii* tachyzoites (10:1, parasites per cell) or *Escherichia coli* lipopolysaccharide (100 ng/mL); control cultures were simultaneously incubated with medium. Gene expression profiling of endothelial cells was performed using oligonucleotide arrays containing probes designed to detect 8746 human transcripts. After normalization, differential gene expression was assessed by the significance analysis of microarrays, with the false-discovery rate set at 5%. For selected genes, differences in the level of expression between retinal and choroidal cells were evaluated by real-time RT-PCR.

RESULTS. Graphic descriptive analysis demonstrated a strong correlation between gene expression of unstimulated retinal and choroidal endothelial cells, but also highlighted distinctly different patterns of expression that were greater than differences noted between donors or between unstimulated and stimulated cells. Overall, 779 (8.9%) of 8746 transcripts were differentially represented. Of note, the 330 transcripts that were present at higher levels in retinal cells included a larger percentage of transcripts encoding molecules involved in the immune response. Differential gene expression was confirmed for 12 transcripts by RT-PCR.

CONCLUSIONS. Retinal and choroidal vascular endothelial cells display distinctive gene expression profiles. The findings suggest the possibility of treating posterior uveitis by targeting specific interactions between the retinal endothelial cell and an infiltrating leukocyte. (*Invest Ophthalmol Vis Sci.* 2007;48:2676-2684) DOI:10.1167/iovs.06-0598

Posterior uveitis is a heterogeneous group of inflammatory disorders that primarily involve the retina and/or choroid. Because the retina contains the photoreceptors, as well as other nonregenerating neural cells that are critical to visual processing, posterior uveitis is the most sinister form of inflammatory eye disease. One widely quoted report estimates that uveitis accounts for 10% of visual disability in the United States.¹ In a second study, conducted in Western Europe, approximately half of individuals with inflammation involving the posterior segment of the eye had visual impairment.² Posterior uveitis may be an inflammatory response to an infection (e.g., with *Toxoplasma gondii* or herpes family viruses), or it may be primarily immunologically mediated (i.e., related to systemic diseases, such as sarcoidosis or multiple sclerosis, as well as to eye-selective inflammations).³⁻⁵ Inflammation of the retinal vasculature is present in most forms of the disease, either as an isolated entity or in association with inflammatory lesions of the retinal and/or choroidal stroma.⁶ Elegant experiments applying intravital microscopy to a rodent model of posterior uveitis, experimental autoimmune uveoretinitis (EAU), demonstrate that leukocytes migrate via the retinal microvasculature,⁷ and this migration is localized to retinal postcapillary venules.⁸ In both the experimental model and the human disease, adhesion molecules and chemokines expressed by the vascular endothelium are responsible for the migration of leukocytes into the posterior segment of the eye.^{9,10}

Vascular endothelial cell diversity is a concept that recently has attracted scientific attention, largely due to global gene expression profiling made possible by microarray technology. Although the endothelium is a continuous organ, different segments of the vascular tree experience different sets of conditions and perform different functions. Microarray profiling confirms that these differences are reflected at a molecular level; vascular endothelial cells within different organs express specific sets of molecular signals, and differences most likely provide part of the explanation for both physiological and pathologic organ-specific processes. "Definitive support"¹¹ for endothelial diversity was provided in 2003 by Chi et al.,¹² who used gene microarrays to study 53 endothelial cell cultures taken from 14 different sites, including five arteries, two veins, four microvascular circulations (i.e., skin, lung, intestine, and myometrium), and three other sources (i.e., nasal polyps, bladder, and myocardium). Their work revealed that transcriptomes of large vessel and microvessel endothelia differed, transcriptomes of arteries and veins were nonidentical, and gene expression varied with organ location. Smaller studies have suggested that diverse patterns of gene expression also distin-

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guish different endothelial populations within an organ.^{13,14} Yet, although much is now known of the phenotype and consequent functions of several extraocular vascular beds, to date there has been minimal research directed at defining the unique properties of human ocular microvasculature, either at the level of mRNA or the level of protein.

The posterior eye contains two distinct microvascular beds.¹⁵ The retinal microvasculature derives from the central retinal artery, which is the first branch of the ophthalmic artery, and drains to the central retinal vein. Retinal arteries and veins lie within the nerve fiber layer, but branches may extend as deeply as the inner nuclear layer. The choriocapillaris is a rich vascular network plexus that forms the bulk of the choroid and is fed by short posterior ciliary branches of the ophthalmic artery. Consistent with clinical and experimental observations that posterior uveitis involves the retinal vasculature and recent recognition of vascular endothelial cell diversity, we hypothesized that retinal vascular endothelium was a cell population of unique molecular phenotype. As the retinal vascular endothelium is the leukocyte "gate" of the eye in posterior uveitis, molecules present in relatively high abundance on this endothelium hold promise as potential therapeutic targets. To test our hypothesis, we compared the expression of more than 8500 genes by retinal endothelial cells with expression by choroidal endothelial cells, using donor-matched primary cell cultures. Forming the middle coat of the posterior eye, the choroid lies immediately adjacent to the retina, and its vasculature derives from the same arterial source, yet the choroidal vessels are not primarily involved in posterior uveitis. Both cells in unstimulated cultures and cells cultured in the presence of inflammatory stimuli, specifically, *T. gondii* tachyzoites and lipopolysaccharide (LPS), were studied.

MATERIALS METHODS

Overview of Experimental Design of Microarray Study

Retinal and choroidal vascular endothelial cells were isolated from both eyes of six human cadaveric donors. Endothelial cells of each subtype from each donor were separately pooled and cultured. In a first series of experiments (experiment 1), donor-matched cultures of retinal and choroidal endothelial cells derived from three donors were incubated with either *T. gondii* tachyzoites or medium alone. In a second set of experiments (experiment 2), cultured retinal and choroidal endothelial cells derived from the other three donors were stimulated with LPS or medium alone. Whenever possible, two replicate dishes of endothelial cells were subjected to each set of conditions. Subsequent to incubations, mRNA was extracted from a cell lysate prepared from each dish of cells, and cRNA derived from each mRNA preparation was separately hybridized to one oligonucleotide expression array.

Isolation and Culture of Retinal and Choroidal Vascular Endothelial Cells

Human eyes were obtained from six human cadaveric donors via the Oregon Lions Eye Bank. The use of human cadaveric tissue for the purpose of these experiments was approved by the Institutional Review Board of Oregon Health and Science University. Age at death and gender for the six donors were as follows: a 4-year-old male (donor 1), a 37-year-old female (donor 2), a 44-year-old male (donor 3), a 36-year-old male (donor 4), a 46-year-old male (donor 5), and a 49-year-old female (donor 6). No donor had a history of ocular disease. Death to isolation time varied from 8 to 19 hours. The cornea, if present, was excised, and the iris was separated from the eye wall. Vitreous was removed from the posterior eyecup, and retina was separated from the underlying choroid. Subsequently, the choroid was separated from the

eye wall and the retinal pigment epithelium was gently removed with a cotton bud.

The choroid and retina were separately treated with graded solutions (0.25–3 mg/mL) of type II collagenase (Sigma-Aldrich, St. Louis, MO) in HEPES-buffered MCDB-131 medium (catalog no. 8537; Sigma-Aldrich), supplemented with 2% fetal bovine serum (FBS; Hyclone, Logan, UT) and 1 μ g/mL amphotericin B (Invitrogen-Gibco, Grand Island, NY), at 37°C and 5% CO₂, until the tissue was visibly digested. In the case of retinal tissue, digestion with type II collagenase was preceded by treatment with dispase (Invitrogen-Gibco). An overnight incubation with 0.3 mg/mL dispase achieved optimal digestion. Subsequently, endothelial cells were purified from stromal cells by using magnetic beads (Dynabeads; Dynal-Invitrogen, Brown Deer, WI) coated with mouse monoclonal anti-human CD31 antibody (BD-PharMingen, San Diego, CA). Cells were cultured in MCDB-131 medium supplemented with 10% to 20% FBS, 1 μ g/mL amphotericin B and endothelial growth factors (Clonetics, Cambrex Bioscience, Walkersville, MD; EGM-2 SingleQuot catalog number cc-4176, but without addition of gentamicin, hydrocortisone, and FBS), at 37°C and 5% CO₂, and passed with the use of 0.05% trypsin (Invitrogen-Gibco). Cultured cells used in the experiments described herein were at passage 5 or less. Immediately before stimulation, retinal and choroidal vascular endothelial cells were grown to confluence simultaneously in separate 10-cm polystyrene tissue culture dishes. They demonstrated cobblestone morphology and 99% purity.

Culture of *T. gondii* Tachyzoites

The *T. gondii* tachyzoites (RH strain) were maintained by serial passage in confluent monolayers of human neonatal dermal fibroblasts (Cascade Biologics, Portland, OR) grown in DMEM (catalog no. 12100; Invitrogen-Gibco), supplemented with 44 mM sodium bicarbonate, 1% heat-inactivated FBS, 50 U/mL penicillin, and 50 mg/mL streptomycin (Gibco-Invitrogen), at 37°C and 5% CO₂. When approximately 50% to 90% of the fibroblast monolayer had lysed, the culture supernatant, containing numerous tachyzoites, was either taken to infect vascular endothelial cells, or between 0.05 and 1.0 mL was used to infect a fresh monolayer of fibroblasts.

Treatment of Vascular Endothelial Cells with *T. gondii* Tachyzoites or LPS

Once retinal and choroidal vascular endothelial cells had achieved confluence in the tissue culture dishes, the medium was changed to MCDB-131 supplemented with 5% heat-inactivated FBS and endothelial growth factors, a medium that we have found to be conducive to viability of both endothelial cells and tachyzoites. After overnight incubation, the medium was again changed as follows. In experiment 1, separate dishes of choroidal and retinal endothelial cells were treated with 10 mL of either 3.0 \times 10⁶/mL tachyzoites in modified MCDB-131 medium or medium alone. In experiment 2, separate dishes of choroidal and retinal endothelial cells were treated with 10 mL of either 100 ng/mL *Escherichia coli* 055:B55 LPS (Sigma-Aldrich) in modified MCDB-131 or medium alone. Plates were then incubated at 37°C and 5% CO₂ for 4 hours. After the incubation, cell supernatants were removed. A 0.6-mL aliquot of RLT lysis buffer (Qiagen, Valencia, CA) was evenly spread across the cells of each plate, and the plates were immediately stored at –80°C.

Isolation of Total RNA from Vascular Endothelial Cells

On thawing, cell lysates were collected using a cell scraper and immediately homogenized using spin columns (Qiashredder; Qiagen). Total RNA was isolated with a kit (RNeasy Mini Kit; Qiagen), according to manufacturer's instructions, including the optional on-column DNase treatment. RNA was eluted from the column using nuclease-free water (Ambion, Austin, TX), with two rounds of 50 μ L yielding a total elute of 100 μ L. To concentrate the RNA, it was mixed in a solution containing 10 μ L of 2 M sodium acetate at pH 4.0 (Stratagene, Cedar Creek, TX) and

TABLE 1. Differences in Expression of Selected Genes for Retinal Versus Choroidal Endothelial Cells from Donor 6, According to Microarray and Quantitative Real-Time RT-PCR

| Gene Transcript | Primer Pair ^{Reference (if applicable)} (Product Size) | Difference (x-Fold) | |
|-------------------|---|---------------------|---------|
| | | Microarray | qRT-PCR |
| E-selectin | for: 5'-GAGCCTTCAGTGTACCTCATC-3' rev: 5'-GACAATTCATGTAGCCTCGCTC-3' (296 bp) | 5.5 | 5.8 |
| ICAM-1 | for: 5'-TAAGCCAAGAGGAAGGAGCA-3' rev: 5'-CATATCATCAAGGGTTGGGG-3' ²³ (282 bp) | 15.3 | 4.4 |
| VCAM-1 | for: 5'-CGTCTCATTGACTTGCAGCACC-3' rev: 5'-GTGATCGGCTTCCCAGCCTG-3' (276 bp) | 24.4 | 10.2 |
| CCL2 | for: 5'-CCTTCTGTGCCTGCTCATAGC-3' rev: 5'-GGTCCATGGAATCGTGAACCCAC-3' (248 bp) | 13.9 | 109.2 |
| CXCL6 | for: 5'-GCTGACAGAGCTGCGTTGCAC-3' rev: 5'-CCAGACAACTTGCCTCCCGTTC-3' (149 bp) | 112.9 | 94.1 |
| CXCL8 | for: 5'-ACAAGAGCCAGGAAGAAAC-3' rev: 5'-CTACAACAGCCACACAATA C-3' ²⁴ (452 bp) | 11.8 | 24.9 |
| CX3CL1 | for: 5'-ACCTGTAGCTTTGCTCATCCA-3' CT rev: 5'-AACAGAACCAGGCATCAT-3' ²⁵ (78 bp) | 27.0 | 9.4 |
| IFN- γ R1* | | 2.2 | 2.1 |
| TLR-1 | for: 5'-CTGCCAAATGGAACAGACAA-3' rev: 5'-GCCACAAAAACAGAAGAGCTG-3' ²⁷ (107 bp) | 3.7 | 3.8 |
| MMP-10* | | 7.2 | 2.9 |
| IL-32 | for: 5'-ATCCCCTGTCCCGGATGTTGAGGAT-3' rev: 5'-GTTGAGAGCACTTCTGGGGTGTGAG-3' ²⁸ (283 bp) | 104.9 | 263.8 |
| Ephrin B2 | for: 5'-GATCCAACAAGACGTCAGAAAC-3' rev: 5'-CTCCGGTACTTCAGCAAGAGG-3' (230 bp) | 3.7 | 2.6 |
| PECAM-1 | for: 5'-CCCAGTTTGGAGGTCATAAAAGGA-3' rev: 5'-AGGCAAAGTTCCACTGATCGA-3' ²⁹ (68 bp) | 1.4 | 1.4 |
| vWF | for: 5'-GGAAGACCCAGTGTGATGAT-3' rev: 5'-GTCTTCTGCACTCCAGCTT-3' ³⁰ (715 bp) | 1.0 | 1.9 |

* Primer sequences for IFN- γ R1 and MMP-10 were obtained from BD-Clontech.²⁶

110 μ L of 100% isopropanol and precipitated overnight at -20°C . RNA pellets were washed with graded solutions of ethanol, dried in air, and subsequently raised in 10 μ L of nuclease-free water. The quantity and purity of the RNA were determined by spectrophotometry.

Gene Expression Microarray

Microarray assays were performed in the Affymetrix Microarray Core, a unit of the Oregon Health and Science University Gene Microarray Shared Resource. RNA was amplified and labeled using the one cycle target labeling method and hybridized (GeneChip Human Genome Focus Array; Affymetrix) according to the instructions to the manufacturer.¹⁶ The Human Genome Focus Array contains probe sets designed to detect 8746 human gene transcripts, verified from the National Center for Biological Information's RefSeq database (www.ncbi.nlm.nih.gov/locuslink/refseq/). Array processing was performed on the Fluidics Station (Affymetrix) using GeneChip Operating System (GCOS) software (ver. 1.2; Affymetrix), yielding cell fluorescence intensity (CEL) files. The distribution of fluorescent material on the array was determined with the confocal laser scanner (GeneChip Scanner 3000; Affymetrix).

Statistical Assessment and Analysis of Data from Microarrays

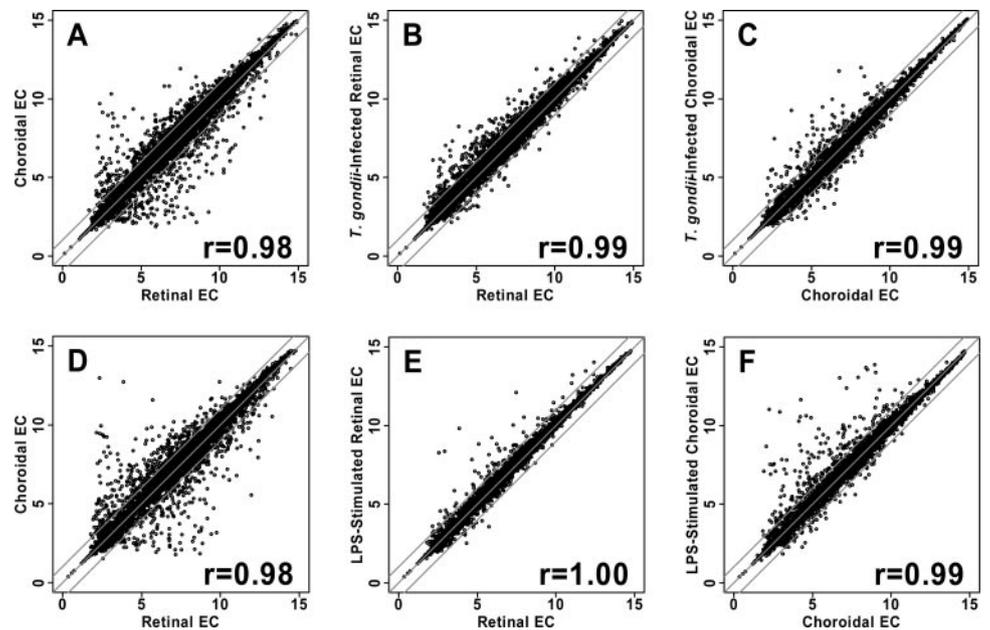
Data preprocessing and normalization were performed with the "affy" and "gcrma" packages of the Bioconductor project (<http://www.bioconductor.org>) that run above the R statistical language environment. The CEL files were imported into the R environment. Perfect match (PM) probe data were corrected for background noise using the GeneChip Robust Multiarray Analysis (GCRMA), which has been described by Wu et al.¹⁷ Corrected PM probe data were normalized with the algorithm based on rank invariant probes, reported by Li and Wong.¹⁸ Gene expression values were determined by using a linear model estimated by the median polish algorithm, according to the description of Irizarry et al.¹⁹

After the data preprocessing, differences in gene expression between endothelial cell subtypes and responses to tachyzoite infection or LPS stimulation were explored using scatterplots and Pearson correlation, and multidimensional scaling plots. We used the significance analysis of microarrays (SAM).²⁰ This method has been developed to deal with a small number of samples, and uses the concepts of the false-discovery rate (FDR)²¹ and the q value.²² The FDR differs from more conventional corrections for multiple comparisons. Instead of controlling for false positives, it controls for the expected ratio of false positives among significantly expressed genes. The q value is a posterior Bayesian probability, and it refers to the minimum FDR at which a test is deemed to show a statistically significant difference. For pairwise comparisons in this analysis, the FDR was set at 5%, with a significant difference in gene expression defined as one yielding $q \leq 0.05$. An additional requirement for statistical significance was that changes in gene expression were unidirectional for all three donors in each experiment. Gene ontology (GO) annotation was performed on March 30, 2006, by using the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 2.1, which is available on the Web site of the National Institute of Allergy and Infectious Disease (<http://david.abcc.ncifcrf.gov/>). We analyzed Molecular Function, Biological Process, and Cellular Component for genes differentially expressed by unstimulated retinal and unstimulated choroidal vascular endothelial cells.

Quantification of Gene Expression by Real-Time RT-PCR

cDNA was synthesized from total RNA using oligo(dT)-primed M-MLV reverse transcriptase (Promega, Madison, WI) for 2 hours at 37°C . Relative expression of gene products, normalized to *GAPDH*, was determined in triplicate on a thermocycler (Chromo4; Bio-Rad Laboratories, Hercules, CA) with SYBR Green nucleic acid dye (iQ SYBR Green Supermix; Bio-Rad Laboratories). A standard amplification consisted of a pre-cycling hold at 95°C for 10 minutes, followed by 40

FIGURE 1. Scatterplots with correlation coefficients in \log_2 scale showing gene expression by (A, D) unstimulated retinal versus choroidal endothelial cells, (B, E) unstimulated versus stimulated retinal endothelial cells, and (C, F) unstimulated versus stimulated choroidal endothelial cells of (A–C) one representative donor in experiment 1 (donor 2), in which endothelial cells were stimulated by infection with *T. gondii* tachyzoites, and (D–F) one representative donor in experiment 2 (donor 6), in which endothelial cells were stimulated by incubation with LPS. Lines in scatterplots show, from top to bottom, twofold upregulation for the y -axis variable, no change, and twofold upregulation for the x -axis variable.



cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 65°C, and extension for 45 seconds at 72°C. Fluorescence was determined during a 1-second hold at the end of each cycle. Melting curves were obtained after product formation. Annealing temperatures and read temperatures were previously established with control product. Subsequent to the PCR, samples were run on a gel to confirm product size. Data were analyzed with the thermocycler system software (Chromo4 Opticon Monitor 3; Bio-Rad Laboratories). Known concentrations of purified control product, simultaneously amplified, were used to generate a standard curve from which the relative expression of experimental samples could be read. Primer pairs were synthesized by Integrated DNA Technologies (Coralville, IA). Sequences appear in Table 1, with the exception of the *GAPDH* primer pair sequences, which were 5'-AGCTGAACGGGAAGCTCACTGG-3' and 5'-GGAGTGGGTGCTGCTGTGAAGTC-3'.³¹

RESULTS

Global gene expression patterns by cultured human donor-matched retinal and choroidal vascular endothelial cells were examined by scatterplots, Pearson correlation, and multidimensional scaling. The scatterplots demonstrated a very strong correlation between the gene expression measured for arrays of unstimulated cells of retinal versus choroidal origin, with Pearson correlation coefficients ranging from 0.95 to 0.99. High coefficients were also measured for comparisons of the data obtained from unstimulated endothelial cells of either origin with matched cells infected with *T. gondii* tachyzoites ($r = 0.98$ – 1.00) or incubated with LPS ($r = 0.96$ – 1.00). Representative scatterplots with Pearson correlation coefficients appear in Figure 1. Multidimensional scaling plots simplified the data sets such that differences between samples could be viewed as two-dimensional distances. Points representing samples with similar gene expression were clustered, and those representing divergent profiles were relatively far apart. As shown in Figure 2, retinal and choroidal endothelial cells had distinctly different patterns of gene expression, despite a high degree of correlation. Although these plots also identified differences in gene expression profiles of individual human donors and differences between cells of the same subtype from the same donor that had been stimulated by tachyzoites or LPS, the greatest differences in profiles were observed when retinal

endothelial cells were compared with choroidal endothelial cells.

To determine the number of genes differentially expressed in unstimulated retinal and choroidal endothelial cells, we used SAM and set the FDR at 5%, with the additional requirement that any differences in expression be unidirectional across all donors. In experiment 1 (donors 1–3), 455 genes showed significant differences in expression, with expression of 208 genes being higher and expression of 247 genes being lower in retinal cells in comparison with choroidal cells. This group included 318 genes (162 genes higher and 156 genes lower) showing a twofold average difference in relative expression. In experiment 2 (donors 4–6), 586 genes showed significant changes in expression, with expression of 252 genes being higher and expression of 334 genes being lower in retinal endothelium in comparison to choroidal endothelium; this group included 326 genes (155 genes higher and 171 genes lower) showing a twofold average difference in relative expression. As expected, there was an overlap in the gene lists identified in the two experiments. However, for no gene was expression seen to be higher in retinal or choroidal cells in one

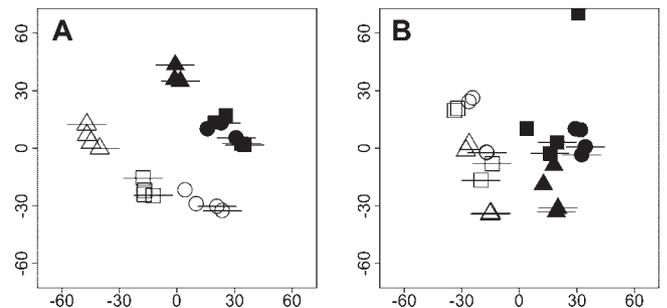
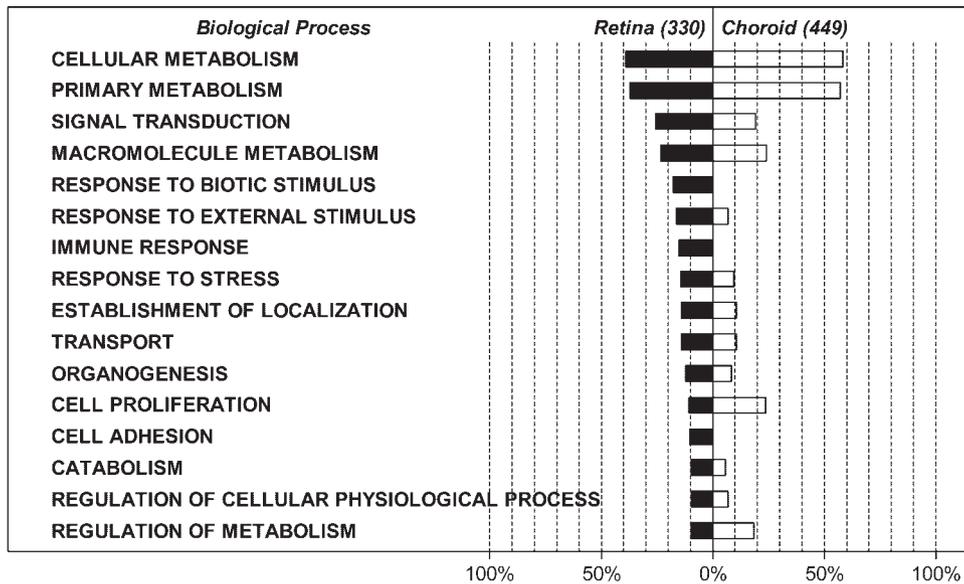


FIGURE 2. Multidimensional scaling plots demonstrating global gene expression for all donors in (A) experiment 1, in which endothelial cells were stimulated by infection with *T. gondii* tachyzoites, and (B) experiment 2, in which endothelial cells were stimulated by incubation with LPS. Filled symbols: retinal samples; open symbols: choroidal samples. Symbols without crossbar represent unstimulated control samples and symbols with crossbar represent stimulated samples. (A) Triangles: donor 1; squares: donor 2; and circles: donor 3. (B) Triangles: donor 4; squares: donor 5; circles: donor 6.

A



B

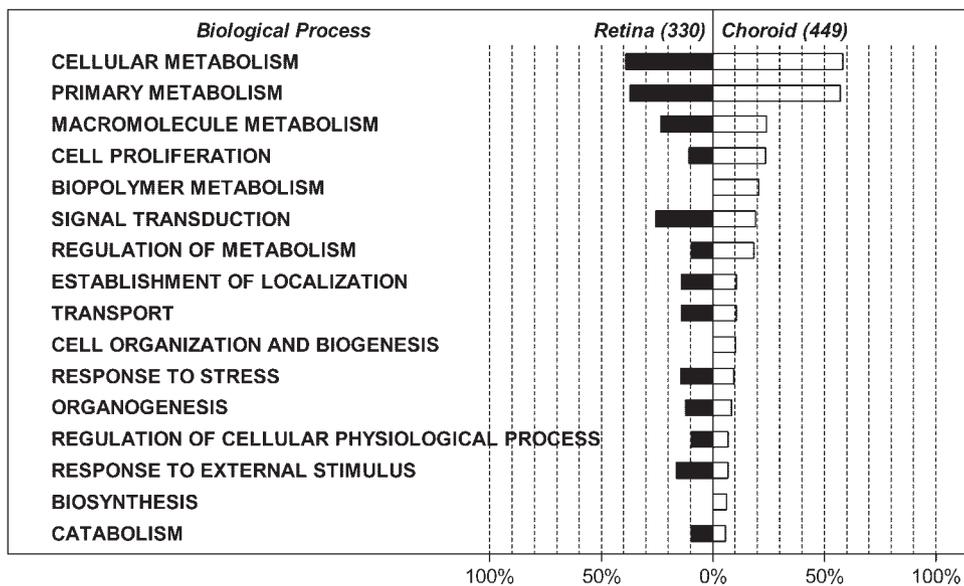


FIGURE 3. GO annotation (according to DAVID ver. 2.1, level 3) by Biological Process for the 330 transcripts that were present at significantly higher levels in unstimulated retinal endothelial cells (*left*) and the 449 transcripts that were present at significantly higher levels in unstimulated choroidal endothelial cells (*right*). Each *horizontal bar* indicates the percentage of transcripts falling within the stated functional category with respect to the total number of significantly upregulated genes for that tissue. (A) Results for the 15 categories that contained the highest percentages of the 330 transcripts that were present at higher levels in retinal endothelial cells. (B) Results for the 15 categories that contained the highest percentages of the 449 transcripts that were present at higher levels in choroidal endothelial cells.

experiment, but lower in the same cell subtype in the other experiment. Overall, of the 8746 gene transcripts on the microarray chip, 330 (3.8%) showed higher relative expression in retinal endothelial cells and 449 (5.1%) showed higher relative expression in choroidal endothelial cells in one or both experiments. Lists of differentially expressed genes, with respective α -fold changes and q values, appear in Supplementary Tables S1 and S2, available online at <http://www.iovs.org/cgi/content/full/48/6/2676/DC1>.

We compared the patterns of gene expression for retinal and choroidal endothelial cells using GO annotation (DAVID ver. 2.1, level 3). This method allowed us to classify separately the 330 transcripts that were present at significantly higher levels in retinal cells and the 449 genes that were present at significantly higher levels in choroidal cells, according to the function of the respective protein products (under the organizing principles of Biological Process, shown in Fig. 3, and Molecular Function, shown in Supplementary Fig. S1, [http://](http://www.iovs.org/cgi/content/full/48/6/2676/DC1)

www.iovs.org/cgi/content/full/48/6/2676/DC1), and by cellular location of these proteins (under the organizing principle of Cellular Component, shown in Fig. 4). Of note, the group of 330 transcripts that were present at higher levels in retinal cells included a larger percentage of transcripts encoding molecules assigned to "immune response," as well as related processes (i.e., "response to biotic stimulus," "response to external stimulus," "response to stress," and "cell adhesion"; Fig. 3A). Indeed, none of the 449 transcripts present at higher levels in choroidal cells were classified under the heading, "immune response." Within these functional groups, several transcripts encoding well-characterized proteins involved in leukocyte trafficking and inflammation appeared to distinguish retinal and choroidal endothelial cells, being expressed at a significantly higher level by retinal cells; these transcripts included the cell adhesion molecules E-selectin, intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1 and the chemokines, CCL2, CXCL6, CXCL8, and

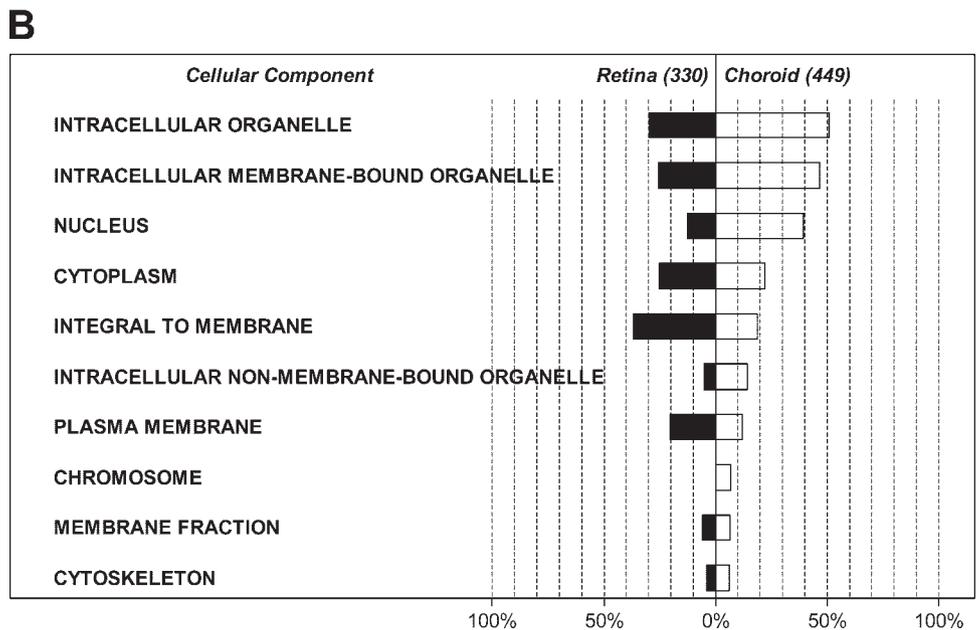
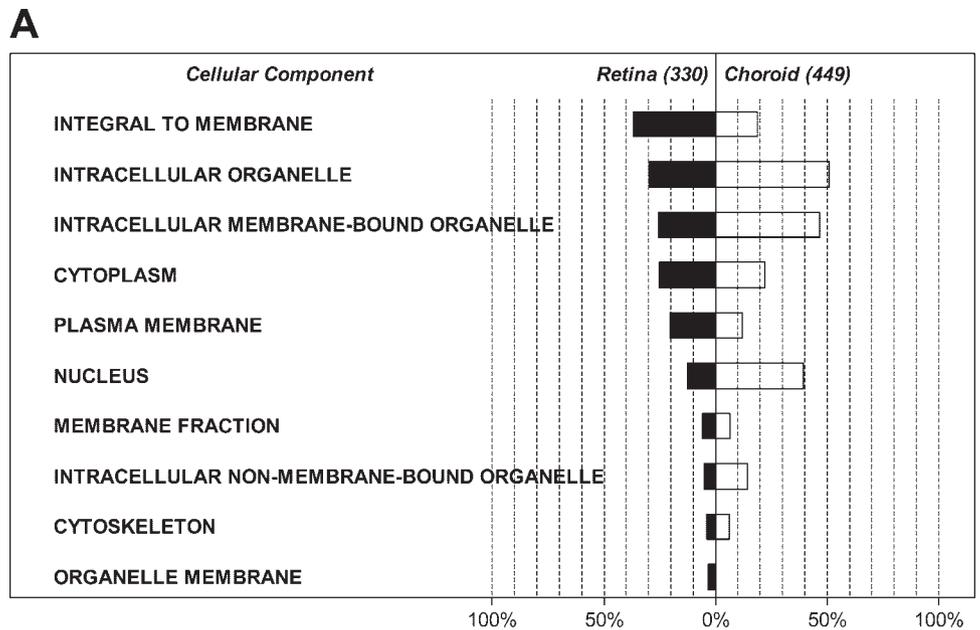


FIGURE 4. GO annotation (according to DAVID ver. 2.1, level 3) by Cellular Component for the 330 transcripts that were present at significantly higher levels in unstimulated retinal endothelial cells (*left*) and the 449 transcripts that were present at significantly higher levels in unstimulated choroidal endothelial cells (*right*). Each *horizontal bar* indicates the percentage of transcripts falling within the stated location category with respect to the total number of significantly upregulated genes for that tissue. (A) Results for the 10 categories that contained the highest percentages of the 330 transcripts that were present at higher levels in retinal endothelial cells. (B) Results for the 10 categories that contained the highest percentages of the 449 transcripts that were present at higher levels in choroidal endothelial cells.

CX3CL1. Also of interest, since proteins that participate in inflammation are often expressed on the cell surface, was the observation that a larger percentage of the 330 “retinal endothelial transcripts” encoded proteins that were integral

to membrane and, specifically, were associated with the plasma membrane, in comparison to the corresponding percentage for the 449 “choroidal endothelial transcripts” (Fig. 4A).

TABLE 2. Number of Gene Transcripts Significantly Up- or Downregulated in Retinal and/or Choroidal Endothelial Cells in Response to Infection with *T. gondii* Tachyzoites or Incubation with *E. coli* LPS with the FDR Set at 5%

| Endothelial Cell Type | <i>T. gondii</i> Tachyzoite | | <i>E. coli</i> LPS | |
|-----------------------|-----------------------------------|-------------------------------------|-----------------------------------|-------------------------------------|
| | Number of Upregulated Transcripts | Number of Downregulated Transcripts | Number of Upregulated Transcripts | Number of Downregulated Transcripts |
| Retinal | 62 | 5 | 116 | 4 |
| Choroidal | 64 | 63 | 489 | 90 |
| Retinal and Choroidal | 34 | 3 | 106 | 4 |

Total number of gene transcripts studied is 8746.

Table 2 reports the number of gene transcripts that were found to be significantly increased or decreased in retinal and/or choroidal vascular endothelial cells in response to stimulation with *T. gondii* tachyzoites or LPS. As expected, there was an overlap between the transcripts significantly upregulated by each endothelial cell type in response to the two inflammatory mediators. For example, suppressor of cytokine signaling 1 [SOCS-1], VCAM-1, and monocyte chemoattractant protein (MCP)-1 were all significantly increased in both retinal and choroidal cells after infection with tachyzoites or incubation with LPS. However, differences in expression profiles were observed, both in relation to cell type and inflammatory stimulus, as illustrated by the following scenarios. ICAM-1 was upregulated only in retinal endothelial cells after exposure to tachyzoites, but in both cell types after exposure to LPS. E-selectin was upregulated in choroidal endothelial cells alone after stimulation with tachyzoites, but was upregulated on retinal and choroidal cells after stimulation with LPS. Early growth response (ERG)-1 was expressed at a significantly higher level by retinal and choroidal cells infected with tachyzoites and by neither cell type incubated with LPS. In contrast to the effect on choroidal cells, very few transcripts were significantly downregulated in response to either stimulus in the retinal cells. Lists of up- or downregulated transcripts including changes (x -fold) and q values, for the two cell subtypes and for the two inflammatory mediators, appear in Supplementary Tables S3 and S4, <http://www.iovs.org/cgi/content/full/48/6/2676/DC1>.

Gene expression differences in retinal and choroidal endothelial RNA samples from one donor (donor 6) were evaluated by real-time PCR, for comparison with the results of the microarray study. We selected 12 transcripts that were relevant to the development of inflammation and that analysis of the microarray data identified as having significantly higher gene expression by unstimulated retinal versus unstimulated choroidal endothelial cells (i.e., E-selectin, ICAM-1, VCAM-1, CCL2, CXCL6, CXCL8, CX3CL1, interferon gamma receptor 1 [IFN- γ R1], toll-like receptor 1 [TLR-1], matrix metalloproteinase 10 [MMP-10], interleukin [IL]-32, and ephrin B2). We focused on these molecules because the underlying goal of our work was to extend understanding of the pathogenesis of posterior uveitis and to identify potential new therapeutic targets for this disease at the level of the retinal vascular endothelium, and because we observed that the group of transcripts that was present at significantly higher levels in retinal endothelial cells included a larger percentage of those encoding molecules involved in inflammation. In addition, we evaluated two transcripts encoding well-established endothelial cell markers that showed no significant differences in expression between cell subtypes on analysis of the microarray data (i.e., platelet endothelial cell adhesion molecule [PECAM]-1 and von Willebrand factor [vWF]). The changes in expression obtained with the two assays were not identical, which was expected given the different methodologies. However, for the 12 genes that were significantly more highly expressed in retinal versus choroidal endothelial cells by array, we consistently observed increased retinal expression of more than twofold for transcripts by real-time RT-PCR. For the two control transcripts, expression levels were less than twofold different between the retina and choroid. Overall, the real-time RT-PCR results, which are shown in Table 1, are consistent with the results from the microarray study, in that they also show retinal and choroidal endothelial cells to have distinct patterns of gene expression.

DISCUSSION

We have studied the molecular profile of human retinal microvascular endothelium, by comparing the gene expression of donor-matched primary cultures of retinal and choroidal endo-

thelial cells using oligonucleotide microarrays. Not unexpectedly, given that both cell subtypes are microvascular endothelial in type, we noted a very close correlation between gene expression of unstimulated retinal and unstimulated choroidal cells. However, setting the FDR at 5%, we demonstrated significant differences in expression of approximately 9% of 8746 genes in at least one of two separate experiments, each involving three different human donors. From a global perspective, differences in gene expression between the retinal and choroidal endothelial subtypes were more marked than were the interdonor differences or changes observed after stimulation with *T. gondii* tachyzoites or LPS. Real-time RT-PCR examination supported the microarray results, by confirming differential expression of selected transcripts that were identified by the array as being expressed at significantly higher levels in retinal endothelial cells in comparison to choroidal endothelial cells. Previous work from our laboratory using relatively small spotted arrays that detected less than 1000 genes and less sophisticated statistical analysis revealed some difference between the microvasculatures of iris and retina.³² The present data provide strong support for our hypothesis that retinal vascular endothelium is indeed composed of a cell population of unique molecular phenotype.

Currently, there is widespread interest in the heterogeneity of vascular endothelium, and the implications of this diversity for cell and microbial trafficking, angiogenesis, and tissue responses to circulating exogenous and endogenous stimuli such as hormones, pathogens, and medications.^{11,33-35} This diversity has been elegantly exemplified in a study in which Arap et al.³⁶ administered a phage-display random peptide library by intravenous injection into a patient who had been declared brain-dead, before removal of cardiorespiratory support. Subsequent screening of tissue biopsy samples for phage inserts that homed to selected tissue sites revealed that different groups of peptides homed to fat, skin, bone marrow, muscle, prostate, and liver. In a similarly impressive project, of particular relevance to our study, Chi et al.¹² defined the expression profile of 53 human endothelial cell cultures taken from 14 different sites, by using the cDNA microarray. In addition to differences between arteries and veins and between large and small vessel endothelial cells, significant differences in vascular endothelial gene expression profiles were related to the anatomic site. The latter paper calls for investigators "... to define the full range of diversity of endothelial cells. ..." ¹² In the present study of human retinal and choroidal vascular endothelial cells, we have confirmed substantial diversity between the transcriptomes for endothelial cells isolated from two adjacent microvascular networks within a single organ. In a separate pilot study (data not shown), we used two-dimensional difference gel electrophoresis to compare protein preparations isolated from similarly procured retinal and choroidal endothelial cells and confirmed that retinal-choroidal endothelial diversity persists at the protein level (Zamora DO et al. *IOVS* 2006;47:ARVO E-Abstract 5160).

Our data highlight a major difference between retinal and choroidal endothelial cells—that the former express higher levels of transcripts encoding proteins related to immunologic reactions, including cell adhesion molecules and chemokines that direct leukocyte trafficking, as well as molecular mediators involved in the generation of an immune response. Although the eye is an immune-privileged site where survival of corneal allografts is often excellent and intraocular inflammation is uncommon, it is clear that privilege can be overcome, resulting in transplant rejection or uveitis.^{37,38} In the majority of forms of inflammatory or infectious posterior uveitis, retinal vessels show evidence of inflammation,⁶ and in EAU, infiltrating inflammatory cells migrate via the retinal vasculature.⁷ These observations indicate that although the retinal endothelium

may contribute to the blood-retinal barrier and ocular immune privilege, it is also the site where leukocytes move into the eye in the event of inflammation. Biological Process GO annotation indicated that the group of transcripts present at higher levels in retinal cells included a relatively large percentage of transcripts encoding proteins related to the immune response, and, consistent with this, Cellular Component GO annotation showed that this same group included a relatively larger percentage of transcripts encoding proteins integral to the plasma membrane. Consequently, when ocular immune privilege is breached and posterior uveitis is initiated, the retinal vascular endothelium is appropriately constituted to facilitate leukocyte migration.

Primary endothelial cells from different tissue sites are known to react differently to different inflammatory stimuli, such as cytokines and LPS, both by overall gene expression³⁹ and in terms of molecules relating specifically to the immune response, including cell adhesion molecules and chemokines.^{32,39-41} In our experiments, endothelial cells were stimulated with either *T. gondii* tachyzoites or LPS. Tabular comparisons indicated that there was up- and downregulation of both common and different genes by retinal and choroidal endothelial cells in these two situations. An interesting observation, which is not explicable at the present time, was that retinal endothelial cells showed very little gene downregulation in relation to either inflammatory stimulus.

Relative upregulation of ICAM-1 gene expression on retinal microvascular endothelium, which overall was approximately 11-fold more highly expressed on retinal versus choroidal endothelium, according to our microarray data, is of particular interest because studies in EAU have implicated this cell adhesion molecule in T-cell migration into the eye during posterior uveitis. Treatment with a blocking antibody directed against ICAM-1, or its ligand, lymphocyte function-associated antigen (LFA)-1, are reported to suppress inflammation in this model,^{42,43} and upregulation of ICAM-1 is noted during EAU.⁸ We observed 2.4- and 4.2-fold upregulation of ICAM-1 gene expression by retinal endothelial cells after exposure to *T. gondii* tachyzoites and LPS, respectively. Although immune-mediated uveitis is in general CD4-positive T-cell mediated, one rare cause of posterior uveitis which often coexists with intracranial pathology is primary central nervous system (CNS) lymphoma,⁴⁴ a diffuse non-Hodgkin B cell lymphoma. The source of the B cells that cause this tumor remains unknown, as there are no lymphoid collections within the CNS. A common theory is that malignant B cells home to this location.⁴⁵ Significantly, ICAM-1 has been shown to be an important mediator of B-cell migration into the brain; B cells migrate more efficiently across unstimulated cultured human brain endothelial cells than T cells, and this movement is significantly reduced by blocking ICAM-1.⁴⁶ Also defying explanation is why infections with *T. gondii* parasites preferentially evolve within the retina and brain in humans. ICAM-1 may be a receptor for *T. gondii* tachyzoites or leukocytes infected with the parasite. Recent studies have implicated ICAM-1 in the passage of tachyzoites across the gut epithelium⁴⁷ and in the binding of infected monocytes to placental trophoblast cells.⁴⁸ Conceivably, the relatively high expression of ICAM-1 on retinal endothelium might provide receptors for the parasite and/or inflammatory cells that concurrently enter the eye. ICAM-1 has also been implicated in diabetic retinopathy,^{49,50} another disease that selectively involves retinal vasculature. Results of other studies suggest that ICAM-1 is expressed at dissimilar levels on different extraocular human endothelial cells: for example, Kanda et al.⁴¹ reported that ICAM-1 was expressed at a relatively higher level on cultured human aortic endothelium in comparison to vena caval and dermal microvascular endothelia. Differences in methodology prevent us from drawing con-

clusions about ocular versus extraocular endothelial expression of ICAM-1.

Experiments in which cell cultures are used attract criticism because of the potential for cell phenotype to change in vitro. Gene expression is influenced by the local microenvironment, which for endothelium includes not only basement membrane and various neighboring cell populations, but also blood-borne molecules, cells, and organisms. However, the method allows for generation of sufficient amounts of RNA for the microarray and for confirmatory real-time PCR analysis, as well as controlled conditions of any stimulation procedure. Our technique for isolation of ocular vascular endothelial cells is previously published,³¹ with verification of endothelial cell phenotype by a variety of methods. Cells form capillary-like networks when grown on a provisional extracellular matrix, take up acetylated low density lipoprotein, and express vWF and CD31. We use purified primary cell cultures at an early passage to minimize phenotypic drift. In general, culturing different cell populations under the same sets of conditions results in a change toward a common phenotype. Despite this, we found a significant percentage of genes to be differentially expressed by cultured retinal and choroidal endothelial cells. Nonetheless, methods using intact tissue are essential in assessing the significance of our results. Another concern when studying cultured cells is the possibility of contamination by other cell populations that are not effectively removed during the isolation process. The striking correlation in gene expression between retina and choroid supports the concept that we achieved highly purified cultures. In addition, selected markers of potential contaminants (glial fibrillary acidic protein and vimentin [Müller cells and astrocytes] and neuron-specific enolase [neurons])⁵¹ were not differentially expressed in unstimulated cultures of retinal versus choroidal endothelial cells (Supplementary Tables S1 and S2 <http://www.iovs.org/cgi/content/full/48/6/2676/DC1>).

We have demonstrated significant differences between microvascular endothelial cells of retinal versus choroidal origin. On the surface, this observation may seem unlikely, given that these cells derive from the same ophthalmic arterial circulation and that the retina and choroid lie directly adjacent to one another. However, our findings are very consistent with the concept of vascular heterogeneity, as well the different anatomic structure and physiological functions of these two tissues, and the fact that these tissues are often separately involved in different diseases. The findings may be particularly relevant to the understanding of basic mechanisms responsible for homing of leukocytes or microbes to the retina, as occurs in posterior uveitis. They also may be relevant to other diseases that have a similar predilection for the retinal microvasculature, such as diabetic retinopathy. Because an interaction between the retinal vascular endothelial cell and a migrating leukocyte is essential for posterior uveitis to proceed, this interaction represents a rate-limiting factor in disease pathogenesis. Consequently, our findings suggest the possibility of targeted therapies that are directed toward interactions with the retinal vascular endothelium that occur only at that disease site.

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