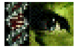
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Endotoxin upregulates CCR7 and its ligands in the lymphatic-free mouse iris

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Purpose: Using time lapse intravital microscopy and histology, we previously reported that we could not detect migration of antigen-presenting cells from the iris to the regional lymph node. Dendritic cells (DC) in other peripheral tissues migrate to lymph nodes in response to chemokines, CCL19 (ELC) and CCL21b (SLC), that activate the CCR7 receptor. We hypothesized that DCs in an inflamed iris might show a different chemokine receptor and ligand profile, thus explaining the DC's inability to migrate.
Methods: Eyes of 35 BALB/c mice were injected intravitreally with 2 μl of 250 ng *E. coli* lipopolysaccharide (LPS) or phosphate buffered saline (PBS). Five mice served as naïve controls. After 3 and 6 h, the iris-ciliary bodies were dissected and pooled in groups of five. Total RNA was isolated, and reverse-transcriptase polymerase chain reaction (RT-PCR) for chemokine receptor and ligand mRNA was performed. In addition, one eye from each of the three animals was taken 6 h after LPS injection for immunohistology (IHC).
Results: The naïve iris, the iris after PBS injection, and the iris after LPS injection contained CCR5 mRNA at approximately equal levels and did not have detectable CCR6 mRNA. No CCR7 mRNA expression was found in the naïve iris, but it was weakly expressed in PBS-injected eyes and was approximately 3.4 fold upregulated after LPS injection. This was confirmed by IHC with no staining for CCR7 in the control iris but positive staining in the inflamed eyes. Transcripts for the CCR7 ligands, CCL19 and CCL21b, were found after LPS or PBS injection but not in naïve iris-ciliary bodies.
Conclusions: The clear upregulation of CCR7 and its ligands in the inflamed iris suggests that another mechanism prevents iris DCs from migrating. Other possibilities include the absence of co-factors, inhibitory substances, the lack of lymphatics inside the eye, or inadequate biological activity of these chemotactic factors and ligands.

It is generally agreed that dendritic cells (DCs) in peripheral tissues respond to an inflammatory stimulus with maturation and migration to draining lymph nodes where they present antigens to passing lymphocytes [1]. One major chemokine receptor (CCR) involved in this process is CCR7, and its ligands are CCL19 (ELC) and CCL21b (SLC) [2,3]. Two other CCRs that are believed to be present in resting, non-migrating DCs are CCR5 and CCR6 [4-6].

Although antigen-specific T-cell expansion has been shown to occur in the draining lymph node after intraocular antigen stimulation [7], we have not been able to detect migration of antigen-presenting cells from the iris to the regional lymph node [8]. It is reasonable to think that differences exist between the eye and other non-lymphoid tissues as the eye is an immunoprivileged site in many respects including the absence of lymphatic vessels [9]. We hypothesized that iris DCs might show a different CCR/ligand profile such as lacking the ability to upregulate CCR7 upon stimulation, thus explaining the non-migratory behavior.

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Previous studies on chemokine receptors and ligand expression in the rat iris [10,11] and whole mouse eyes [12] have aimed at the T-cell response and gene expression changes three days after induction of ocular inflammation with a systemic stimulus. To investigate factors most likely relevant to DC trafficking in the iris/ciliary body (CB), we used the mouse model of endotoxin-induced uveitis (EIU) that we employed in prior studies [8,13] and investigated the expression of CCR5, CCR6, CCR7, CCL19, and CCL21 in the inflamed iris.

METHODS

Use of the animals has been reviewed and approved by the OHSU IACUC. Procedures followed ARVO guidelines.

To induce EIU, 250 ng *Escherichia coli* 055:B5 lipopolysaccharide (LPS, Sigma Chemical Co., St. Louis, MO) in 2 μl phosphate buffered saline (PBS) with 0.25% human serum albumin (α-therapeutics, Los Angeles, CA) was injected into the vitreous body of anesthetized BALB/c mice (35 eyes; female, 6- to 10-week-old). Control mice were injected with 2 μl PBS alone (35 eyes) or not injected at all (10 eyes).

The presence of intraocular inflammation was confirmed after 3 and 6 h with intravital microscopy [13]. The animals were sacrificed and the iris-ciliary bodies (CB) were carefully dissected. Also spleen tissues were harvested from naïve BALB/c mice. Samples were immediately transferred into RLT-Buffer (Qiagen, Valencia, CA) and then stored at -80 °C

until use. Tissues from groups of five eyes were pooled and total RNA was isolated using a Qiagen RNAeasy minikit and treated with DNase I (Invitrogen Life Technologies, Carlsbad, CA) to eliminate genomic DNA contamination.

For each sample, cDNA was synthesized from 150 ng of total RNA with reverse transcriptase MMLV-RT (Invitrogen) and oligo (dT) primers. Intron-spanning primer pairs were used for all transcripts except CCR7. Primers for β-actin were used as a positive control of reverse transcriptase reaction and an additional DNA contamination control. The cDNA was multiplied using 40 cycles of conventional hot-start and touch-down polymerase chain reaction (PCR) and RedTaq DNA polymerase (Sigma Chemical Co.). Table 1 gives the primers used in the different experiments. 2% agarose gels including 0.04% ethidium bromide were used to analyze PCR products. Representative bands were extracted and sequenced to confirm specificity of amplification. For CCR7 cDNA, quantitative PCR was also performed using a MJ Research Chromo4 PT cycler and Sybr Green (Bio-Rad Laboraties, Hercules, CA) as a fluorochrome. The reaction conditions were optimized for the primer pairs in terms of annealing temperature and magnesium and primer concentrations. After 40 cycles, melting curves were obtained to confirm reaction specificity. Additionally 2% agarose gels containing 0.04% ethidium bromide were used to analyze the real time PCR products. Statistical analyses were performed using the Mann-Whitney U test for a nonparametric sample distribution ($p<0.05$ was considered significant). Each experiment was repeated at least three times.

In addition, one eye from each of the three animals was taken 6 h after LPS injection for immunohistology (IHC). The spleen was used as a positive control. Tissues were snap frozen in OCT compound (Miles, Elkhart, IN) and cut into 10 μm cryo-sections. Monoclonal antibodies to CCR7 (Santa Cruz Biotechnologies, Santa Cruz, CA), major histocompatibility class II (MHC) I-A (BD Biosciences PharMingen, San Diego, CA), and respective isotype controls were used in a standard avidin-biotin alkaline phosphatase immunohistochemical staining protocol. Biotinylated rabbit anti-goat (Vector Laboratories, Burlingame, CA) was used as the secondary antibody

Table 1. Reverse-transcriptase polymerase chain reaction primer sequences

Primer	Sequence 5´- 3´	Gene, Exon	Expected product size cDNA (genomic)
CCR7 F	TTC AAG AGG CTC AAG ACC ATG	Exon3	516 bp
CCR7 R	CTC AAA GTT GCG TGC CTG GAG		
CCR6 F	TTCCCTTTCTACACCAGATCTG	Exon 5	182 (302) bp
CCR6 R	CCCAGGAGGCCAAAGACAC	Exon 6	
CCR5 F	TTTGTACAGCTCTCCTAGCC	Exon 1	215 (1150) bp
CCR5 R	CACAAAACCAAAGATGAATACCAG	Exon 2	
CCL19 F	CGCACACAGTCTCTCAGGCTC	Exon 1	110 (1090) bp
CCL19 R	GCAGCAGTCTTCCGCATCAT	Exon 2	
CCL21 F	ACTTGCGGCTGTCCATCTC	Exon 1	200 (300) bp
CCL21 R	GGATGGGACAGCCTAAACTTGG	Exon 2	
Primer qPCR			
CCR7 F	TTC AAG AGG CTC AAG ACC ATG	Exon 3	160 bp
CCR7 R	CTGAAGAAGCTTAACTTATAG		

The table shows the primer sequences used in the experiments showing their origin on the respective gene and the expected product size. Intron-spanning primers were chosen for all transcripts except for CCR7 where the intron-size prohibited this. In the table, F refers to forward/sense strand and R refers to reverse/antisense strand.

Table 2. mRNA expression profiles of the different chemokine receptors and ligands of CCR7

	Naïve iris/CB	Iris/CB after PBS	Iris/CB after LPS	Naïve Spleen
CCR5	++	++	++	++
CCR6	–	–	–	++
CCR7	–	+	++	++
CCL19	–	+	++	++
CCL21	–	+	+	++

The table lists the expression of mRNA found in naive iris/CB tissue and after sham injection compared to inflamed iris/CB showing upregulation of CCR7 and its ligands by LPS, whereas no change in CCR5 or CCR6 expression could be seen. Spleen tissue was used as a positive control. CB: ciliary body; -: no expression of mRNA, +: weak expression, ++: strong expression.

for all stains. All stains were visualized using Fast Red (BioGenex, San Ramon, CA). After staining, six randomly chosen slides with 18 consecutive sections of each eye were evaluated for positive staining for CCR7. The frequency of CCR7 positive cells was compared to MHC class II (I-A) positive cells.

RESULTS

The mRNA expression profiles of the different CCRs and ligands are shown in Table 2. The three chemokine receptors investigated had unique expression profiles in iris/CB. CCR5 was constitutively expressed in all tissues examined whereas CCR6 mRNA was never detected except in the spleen control. In contrast, CCR7 mRNA levels varied with the presence of inflammation. No CCR7 mRNA was found in the naïve iris, but it was weakly expressed in PBS-injected eyes and was three- to four-fold upregulated after LPS injection. Quantitative real-time reverse-transcriptase (RT)-PCR was used to measure the difference in relative expression precisely. At the 6 h time point, the LPS-treated iris/CB had significantly more CCR7 mRNA ($p<0.03$; Figure 1). CCR7 mRNA expression at the 3 h time point showed a similar pattern, but the increase did not reach statistical significance (data not shown).

Immunostaining for CCR7 was consistent with the mRNA results. CCR7 staining was detected in about one cell per section in 15 out of 52 evaluated consecutive sections of inflamed eyes but was negative in naïve eyes (Figure 2). About two cells in the iris of each section stained positively for the MHC class II marker I-A. Staining with the isotype-matched control IgG did not reveal any positive staining.

The mRNA expression profiles of the two chemokines studied were similar but not identical (Table 2). Neither CCL19 nor CCL21 mRNA was detected in naïve iris/CB, and both were present at low levels after PBS injection. Only CCL19 was strongly upregulated after LPS injection.

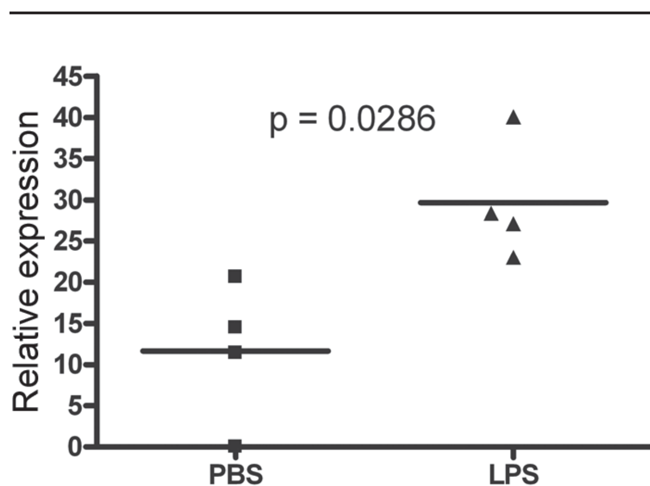

Figure 1. CCR7 transcript levels in sham-injected and inflamed mouse iris. Quantitative RT-PCR was performed with mRNA obtained 6 h after saline or LPS injection. LPS significantly increased CCR7 transcript levels in the iris.

DISCUSSION

CCR5 has been reported to be downregulated by maturing DCs [14]. We detected CCR5 mRNA in approximately equal levels in both naïve and stimulated iris tissue. Downregulation may be occurring at a later time point or may not apply to resident cells in the iris.

CCR6 has been reported to be expressed by immature, CD34+ DCs but not by monocyte-derived DCs [5]. In accordance with this, we could not detect CCR6 mRNA in iris tissue.

Our data showed that CCR7 mRNA levels are increased in inflamed iris-ciliary body. This expression was subsequently confirmed on the protein level with positive staining for CCR7 in dendriform cells by immunostaining. Counting positive cells on consecutive sections that were stained with different antibodies led to the estimate that about 15% of MHC class II positive cells also stained positively for CCR7. We do not think detection of increased CCR7 levels can be attributed to infiltrating T cells or neutrophils because of the positive cells' dendriform morphology and serial staining for MHC-class II. Our data do not allow us to distinguish between the two most likely candidates, dendritic cells and macrophages.

Recent experiments of Dana and coworkers have suggested that corneal antigen presenting cells (APCs) migrate to the lymph node after upregulation of CCR7 although actual cells in motion have not been captured [15]. Blockade of CCL21 reduced the number of CCR7+ cells seen in the draining lymph node by only 40%. Therefore, the authors hypoth-

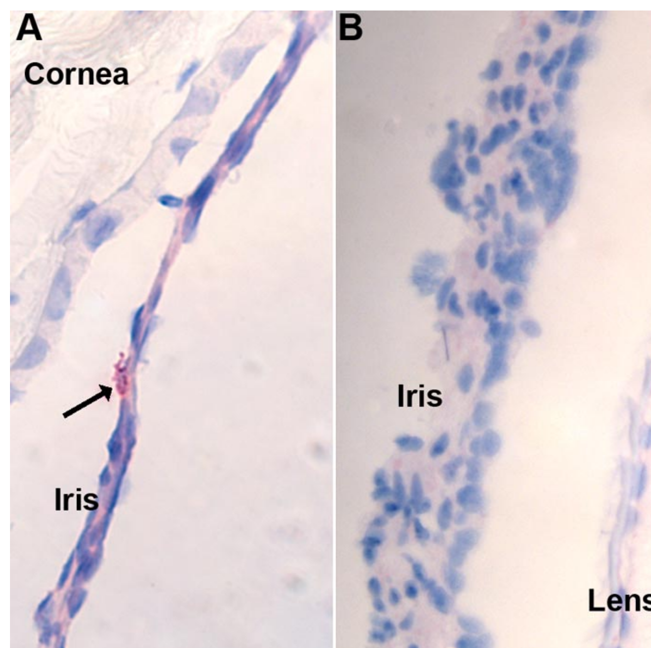

Figure 2. Scattered CCR7+ cells are seen in the iris of eyes with endotoxin-induced uveitis. **A** shows that the dendriform cell (arrow) is stained positively for CCR7 in an iris section of an eye 6 h after LPS injection. **B** shows the isotype-matched IgG control. No staining for CCR7 was observed in the naïve iris (picture not shown). Original magnification 400x

esized a possible role for VEGFR3, also shown to be upregulated on activated DC [16], is to draw DCs toward the lymphatics in addition to CCL21.

The detection of mRNA for two CCR7 ligands, CCL19 and CCL21, in the iris after inflammatory stimulus was of particular interest given the absence of lymphatics inside the eye and the known role of these chemokines in recruiting DC to lymphatic vessels. Lymphatics are known to be a major source of CCL19 and CCL21. However, Sallusto and coworkers [6] showed production of CCL19 by human DCs in culture after LPS stimulation. Scapini and coworkers [17] found that neutrophils have the ability to express and release CCL20 and CCL19 when cultured with either LPS or TNF-α. Foxman and coworkers [12] also detected CCL19 and CCL21 RNA in their comprehensive study on CCR and CCL expression in the mouse eye, but they used whole eyes not just the iris. High endothelial venules in lymph nodes also produce CCL21 [14], which might be present in the inflamed iris, having been found in the retina during the onset of experimental autoimmune uveitis [18]. Kanao and Miyachi [19] saw lymphangiogenesis and VEGFR expression at the dorsal iris in newts nine days after lens pricking or transplantation of dendritic cells that had engulfed the destroyed lens. In an immunohistological study of uninflamed mouse eyes, no typical lymphatic vessels but a large population of LYVE-1(+) and VEGFR3(+) macrophages were found inside the eye [20]. These cells may represent resident precursor cells necessary for the de novo formation of ocular lymphatic vessels in pathologic conditions. We saw expression of the CCR7 ligands after 6 h, certainly too early for lymphangiogenesis to have occurred.

Our data suggesting that a DC chemokine receptor and its ligands are expressed in inflamed iris-ciliary body are particularly intriguing in light of our inability to detect migration of DCs in an iris stimulated with a variety of methods. It remains to be determined if these mRNAs are translated into active proteins at an effective level and if the receptor's signaling pathways are functional or inhibited by immunosuppressive factors in the eye.

The inability of antigen-presenting cells to migrate from the eye has profound implications for understanding the ocular immune response. As CCR7 and its ligands are the best described mechanism to account for migration of APCs to a regional lymph node, it is plausible to hypothesize that ocular APCs would lack either CCR7 or that the ligands would be absent. Our data refute these hypotheses. Alternative explanations such as the absence of a co-factor(s), inadequate biological activity of the ligands or receptor, and/or an active inhibitor must account for the immobility of iris APCs.

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