



# Intravital microscopy of leukocyte-endothelial dynamics using the Heidelberg confocal laser microscope in scleritis and allergic conjunctivitis

Lyndell L. Lim,<sup>1</sup> Lani Hoang,<sup>1</sup> Tiffany Wong,<sup>1</sup> Stephen R. Planck,<sup>1,2,3</sup> Mischa B. Ronick,<sup>1</sup> Robert R. Gould,<sup>1</sup> William D. Mathers,<sup>1</sup> James T. Rosenbaum<sup>1,2,3</sup>

<sup>1</sup>Department of Ophthalmology, <sup>2</sup>Department of Medicine, and <sup>3</sup>Department of Cell and Developmental Biology, Casey Eye Institute, Oregon Health & Science University, Portland, OR

**Purpose:** To examine leukocyte-endothelial cell rolling and arrest in human ocular vessels overlying sites of inflammation in various ocular inflammatory diseases in comparison to normal controls using the Heidelberg confocal laser microscope, which provides images with greater clarity and resolution than the tandem scanning microscope that uses white light.

**Methods:** Healthy controls (n=8) and patients with active anterior scleritis (n=7) or allergic eye disease (n=4) were scanned using the Heidelberg confocal laser microscope (HRT II) with the Rostock cornea module attachment for a minimum of 5 min at a depth of 45-120  $\mu\text{m}$  from the conjunctival epithelial surface.

**Results:** There was a marked increase in the number of rolling leukocytes in scleritis patients (534 $\pm$ 119 cells per  $\text{mm}^2$ /min) versus controls (6 $\pm$ 6 cells per  $\text{mm}^2$ /min;  $p=0.0002$ ) or allergic patients (59 $\pm$ 44 cells per  $\text{mm}^2$ /min;  $p=0.009$ ). No statistically significant increase was seen in allergic patients compared to controls ( $p=0.059$ ). A similar pattern was seen in the number of arrested leukocytes in patients with scleritis (56 $\pm$ 23 cells per  $\text{mm}^2$ ) in comparison to either those with allergic eye disease or controls (each=0 cells per  $\text{mm}^2$ ;  $p=0.02$ ).

**Conclusions:** Patients with scleritis have a significantly increased number of rolling and arrested leukocytes in superficial ocular vessels in comparison to patients with mild allergic conjunctivitis and controls. The image quality with this microscope is superior to prior studies with a scanning microscope.

Although leukocyte rolling and arrest are considered fundamental components of inflammation, almost all knowledge of this process in vivo derives from animal studies. The human eye is one of the few organs whose vessels are non-invasively accessible for imaging. To date, this has been limited to a tandem scanning microscope that uses white light [1-5].

Recent advances in confocal microscopes designed for use in patients have provided images of the conjunctival and episcleral vasculature with greater clarity and resolution (Figure 1). In this present study, we sought to examine leukocyte-endothelial cell rolling and sticking in ocular vessels overlying sites of inflammation using the Heidelberg confocal laser microscope in patients with scleritis, allergic conjunctivitis and normal controls.

## METHODS

Healthy controls and patients with active anterior scleritis or allergic eye disease provided informed consent and were prospectively enrolled from March, 2005 through March, 2006. OHSU Institutional Review Board permission was granted for the conduct of this study.

Prior to imaging, the surface of the eye was anesthetized with one drop of 0.5% proparacaine hydrochloride (Ophthetec; Allergan Optical, Irvine, CA) and lubricated with GenTeal Gel (Novartis Ophthalmics, East Hanover, NJ). All subjects were scanned using the Heidelberg Confocal Laser microscope (HRT II, Heidelberg Engineering, Heidelberg, Germany) with the Rostock Cornea Module attachment for a minimum of 5 min at a depth of 45-120  $\mu\text{m}$  from the conjunctival epithelial surface. Images of vessels were captured for at least 20 s per field of view at a rate of 5 frames per s and a magnification of 400X. The ASL-1000 (Advanced Scanning Inc, New Orleans, LA) image shown in Figure 1 was captured as previously described [5].

In those patients with scleritis, vessels overlying areas of active scleritis were imaged, whereas in all other subjects, vessels 2 to 4 mm from the superior limbus were imaged. In one patient with active scleritis, images were taken before and 8 weeks after the institution of systemic immunosuppressive therapy. The average length and diameter of the vessels scanned were 434  $\mu\text{m}$  and 55  $\mu\text{m}$ , respectively.

Twenty clips of the raw movies were stabilized using MediaCybernetics Image Pro Plus Version 5 (Silver Spring, MD) to minimize the effects of eye movement during the recording. The images were then graded according to the number of arrested and rolling leukocytes per  $\text{mm}^2$  of vessel endothelium using a previously published method by Becker et al. [6]. Two graders were used: one who was unmasked as to

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Correspondence to: Dr. Lyndell L. Lim, Centre for Eye Research Australia, University of Melbourne, 32 Gisborne Street, East Melbourne, Victoria 8002, Australia; Phone: +613-9929-8399; FAX: +613-9929-8379; email: limllp@unimelb.edu.au

the diagnosis of the subject (Observer 1) and the other who was masked (Observer 2).

Statistical analysis consisted of an unpaired one-tailed t test based upon the hypothesis that there would be increased rates of leukocyte rolling and arrest in scleritis and allergic conjunctivitis subjects in comparison to controls. Prism 4 software (GraphPad Software, San Diego, CA) was used for all statistical calculations.

**TABLE 1.**

Subjects	Number of subjects	Male/female ratio	Age range in years (Mean)
Normal controls	9	6/3	26-64 (43)
Allergic conjunctivitis	4	1/3	24-35 (30)
Scleritis	7	3/4	43-74 (54)

Table showing specific subject demographics of those taking part in the study.

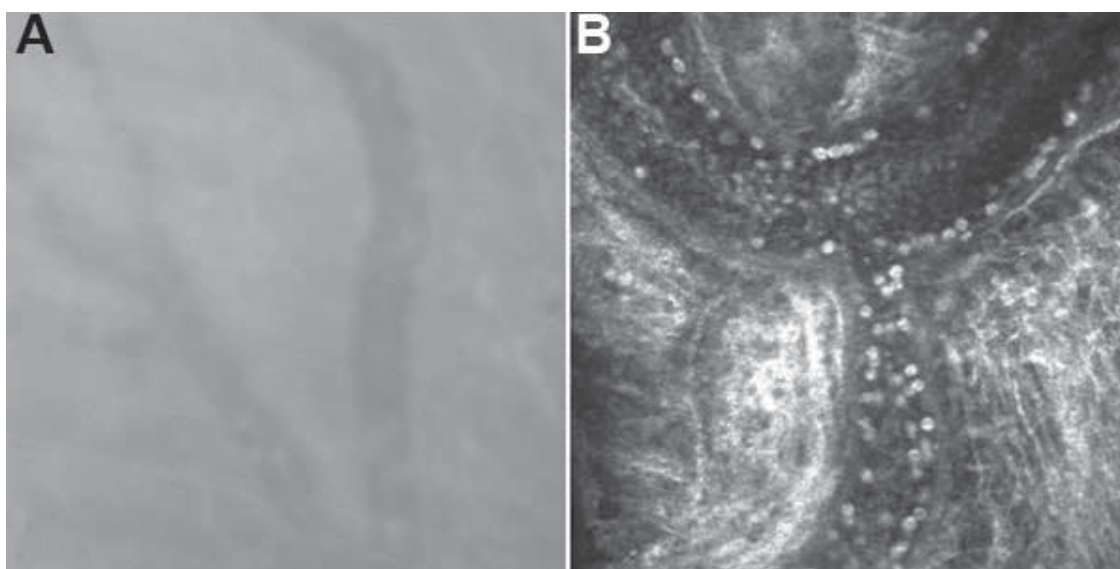


Figure 1. Non-enhanced, representative images of ocular vessels obtained from **A** the ASL-1000 white light scanning confocal microscope (Advanced Scanning Ltd, New Orleans, LA) and **B** the HRTII laser confocal microscope (Heidelberg Engineering, Germany). Magnification of both images was 400X.

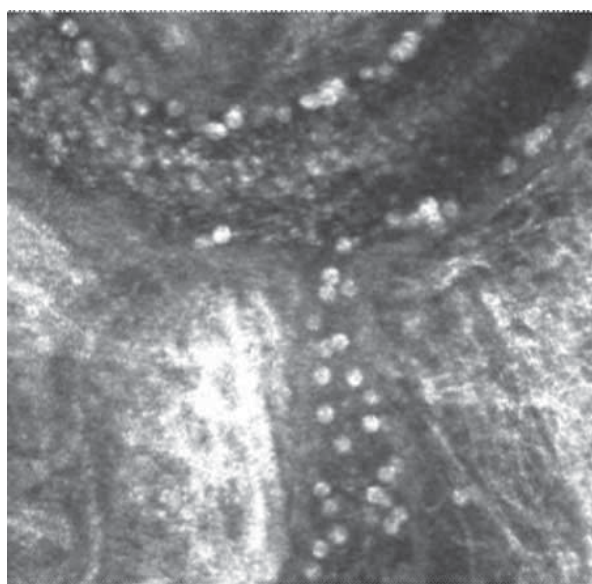


Figure 2. Representative movie of rolling and arrested leukocytes in a patient with scleritis. Large numbers of rolling and arrested leukocytes are evident along the walls of the vessel. There is a quicktime movie of this figure in the online version of this article. A representative frame is included here.

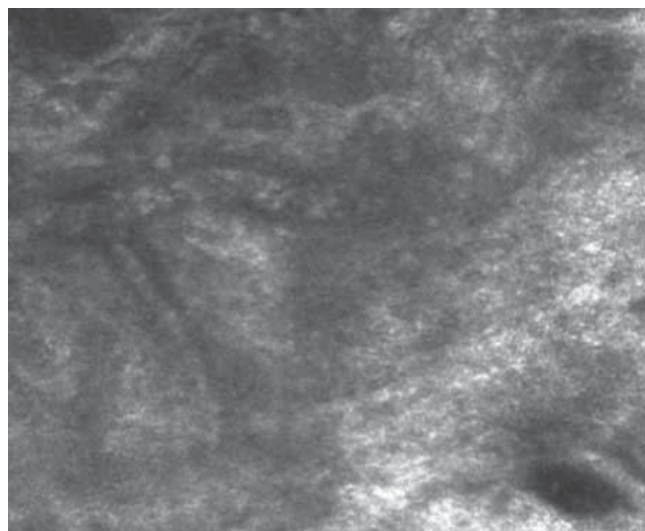


Figure 3. Movie of rolling and arrested leukocytes in a patient with allergic conjunctivitis. Very few rolling leukocytes can be seen, with no arrested cells. There is a quicktime movie of this figure in the online version of this article. A representative frame is included here.

## RESULTS

Nine controls, seven patients with scleritis, and four with mild allergic conjunctivitis were enrolled. Specific demographic data is shown in Table 1 and representative movies from each of these three subject groups are shown in Figure 2, Figure 3, and Figure 4.

As shown in Figure 5 and Figure 6, Observer 1 (unmasked) found a marked increase in the number of rolling leukocytes in scleritis patients ( $534 \pm 119$  cells per  $\text{mm}^2/\text{min}$ ) versus controls ( $6 \pm 6$  cells per  $\text{mm}^2/\text{min}$ ;  $p=0.0002$ ) or allergic patients ( $59 \pm 44$  cells per  $\text{mm}^2/\text{min}$ ;  $p=0.009$ ). No statistically significant increase was seen in allergic patients compared to controls ( $p=0.059$ ). A similar pattern was seen in the number of arrested leukocytes in patients with scleritis ( $56 \pm 23$  cells per  $\text{mm}^2$ ) in comparison to either those with allergic eye disease or controls (each=0 cells per  $\text{mm}^2$ ;  $p=0.02$ ).

Observer 2 (masked) had comparable findings to Observer 1 (Figure 5 and Figure 6), as this observer also found a marked increase in the number of rolling leukocytes in scleritis patients ( $570 \pm 121$  cells per  $\text{mm}^2/\text{min}$ ) versus controls ( $154 \pm 65$  cells per  $\text{mm}^2/\text{min}$ ;  $p=0.004$ ). Again, no statistically significant increase was detected in allergic subjects compared to controls ( $47 \pm 47$  cells per  $\text{mm}^2/\text{min}$ ;  $p=0.15$ ). Additionally, a similar pattern was seen with arrested leukocytes in patients with scleritis ( $62 \pm 17$  cells per  $\text{mm}^2$ ) in comparison to either the controls ( $6 \pm 6$  cells per  $\text{mm}^2$ ;  $p=0.003$ ) and those with allergic eye disease (0 cells per  $\text{mm}^2$ ;  $p=0.02$ ). The only discrepancy seen between the two observers was in the rates of rolling leukocytes recorded in the control group that is most likely due to bias on the part of the unmasked observer.

In the one scleritis patient with pre and post treatment scans, the number of arrested cells prior to treatment (110 cells per  $\text{mm}^2$ ) had decreased to 18 cells per  $\text{mm}^2$  eight weeks after the commencement of treatment with oral prednisone (1 mg/kg) and methotrexate (20 mg/week).

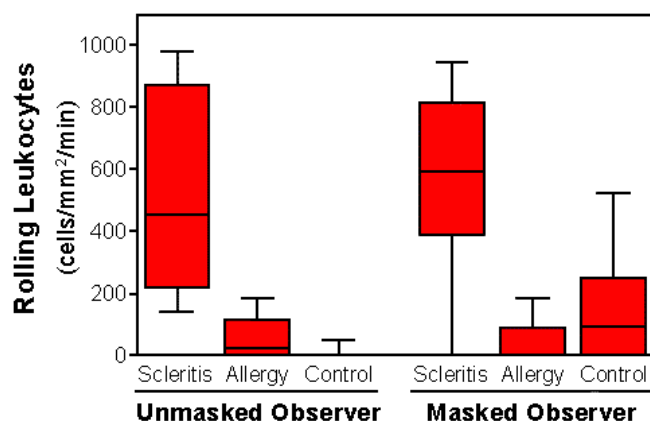


Figure 5. Number of rolling leukocytes detected by Observer 1 (unmasked) and Observer 2 (masked) in patients with scleritis, allergic conjunctivitis, and normal controls. A statistically significant increase in the number of rolling cells was seen by both observers in the scleritis group in comparison to both the allergic conjunctivitis and control groups.

## DISCUSSION

Characteristics of rolling and sticking leukocytes in vessels of patients with various inflammatory diseases have been previously published [1-4], however they are yet to be described in scleritis. In addition, imaging of these leukocyte-endothelial dynamics has not been described in any condition with the Heidelberg confocal laser microscope (HRT II) and Rostock cornea module attachment, which affords far higher image

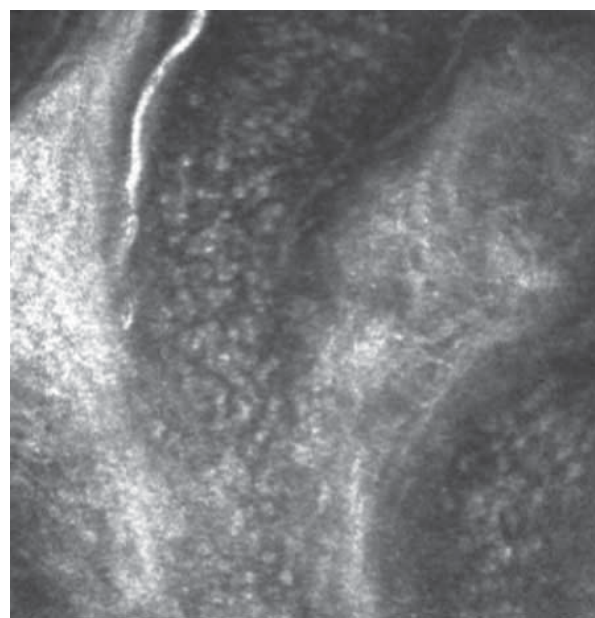


Figure 4. Representative movie of rapidly moving blood cells within a vessel of a normal subject. Note the lack of rolling and arrested leukocytes in comparison to the patient with scleritis (Figure 2). There is a quicktime movie of this figure in the online version of this article. A representative frame is included here.

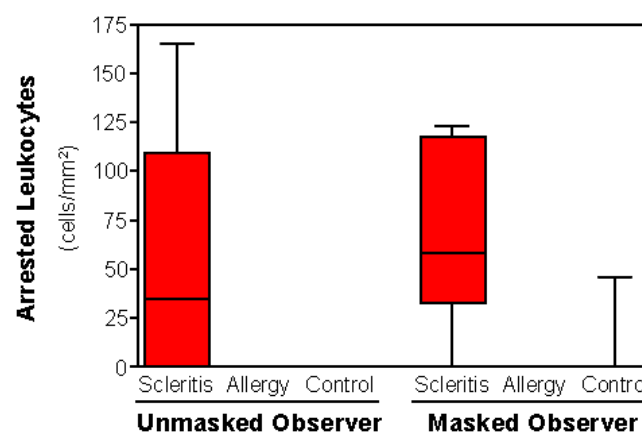


Figure 6. Density of arrested leukocytes detected by Observer 1 (unmasked) and Observer 2 (masked) in subjects with scleritis, allergic conjunctivitis, and normal controls. Again a statistically significant increase in the density of arrested cells was seen by both observers in the scleritis group in comparison to both the control group and allergic conjunctivitis group, where virtually no arrested cells were detected.



quality in comparison to the standard, white light confocal microscopes that are used for human corneal imaging in vivo.

Our results have conclusively shown that leukocyte rolling and arrest are significantly increased in patients with scleritis in comparison to normal controls and those with mild allergic conjunctivitis with good inter-observer agreement. However, our results for allergic conjunctivitis were not as conclusive as previous studies [1]. This discrepancy is most likely due to the patient population, as all of our patients had mild disease, with their diagnosis based upon the presence of mild symptoms of epiphora and pruritus. Examination of these patients revealed only mild conjunctival injection and no other signs of allergic conjunctivitis (such as papillary changes or follicles). In comparison, Helinto et al. [1] exposed the patients directly to an allergen to which the subjects were known to be sensitive, thereby precipitating a much more fulminant allergic inflammatory response. We also cannot exclude the possibility that study of a larger patient population would have demonstrated differences in rolling between allergic conjunctivitis patients and controls that were statistically significant.

Our rates of leukocyte rolling and sticking in scleritis subjects are also far greater than in previously published studies of allergic conjunctivitis and post surgical inflammation. Kirveskari et al. [1-4] described up to  $78.8 \pm 40$  cells/mm<sup>2</sup>/min rolling leukocytes in allergic patients and up to  $53 \pm 34$  cells/mm<sup>2</sup>/min in post surgical subjects. In comparison, our rates in scleritis patients are 10 fold greater. There are many possible explanations for this observation. The first is that the greater numbers of rolling and arrested leukocytes may be a reflection of the far greater degree of inflammation induced by scleritis in comparison to these other inflammatory conditions. This is supported by the reduction in the number of arrested leukocytes seen in the one patient who had pre and post treatment scans. The second possibility is that the improved image quality seen with the laser confocal microscope has resulted in a better ability to visualize and quantify the cells.

Ideally, we would like to correlate the degree of leukocyte rolling and sticking with the severity of the patient's scleritis, and possibly provide some prognostic information regarding the likely course of the patient's disease, based upon the change in leukocyte-endothelial dynamics with treatment. However, at this point, our patient set is too small and lacks sufficient sequential scans to draw any such comparisons or conclusions.

In conclusion, we have shown that laser confocal microscopy is a viable, noninvasive means of visualizing the human vascular bed relevant to several ocular inflammatory diseases and that the image quality with this microscope is superior to that obtained in prior studies with a scanning white light microscope. Patients with scleritis clearly have a significantly increased number of rolling and arrested leukocytes in superficial ocular vessels in comparison to patients with mild allergic conjunctivitis and controls. This demonstration of rolling and arrested leukocytes in scleritis with laser confocal microscopy holds future promise for its application in the assessment of disease severity, recognition of disease subsets, determining efficacy and mechanism of medications, and prediction of disease outcomes.

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