QUANTUM DOT-BASED IN VIVO IMAGING OF INFLAMMATION

By

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To my grandfathers, Mr. K. Appurao and Mr. M. Mahalingam

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CHAPTER I

INTRODUCTION

Role of Leukocytes and Endothelial Cells in Disease

Inflammation is a natural response of the body to invasion by pathogens as well as tissue injury. However, undesirable provocation of this response is a detrimental feature in a variety of diseases, such as atherosclerosis, asthma, rheumatoid arthritis, and multiple sclerosis[1-3]. This immune defense mechanism is a complex process involving a number of cell types and molecular mediators which control their interactions. The multi-step cascade is initiated by the recruitment of leukocytes, bone marrow-derived cells, from the circulation to the inflamed tissue site. This process of transmigration from the blood is characterized by initial rolling and eventual attachment of the leukocytes to the endothelial cells which line the blood vessel walls. A number of cell adhesion molecules (CAMs) on both leukocytes and endothelial cells facilitate these first steps as well as the extravasation of leukocytes into tissue, which is thought to occur by mechanical retraction of lateral endothelial junctions, among other proposed models[4-6]. Once this process has occurred, the essential functions carried out by leukocytes in the decomposition of damaged tissue and the destruction of bacteria can now be part of a deleterious cascade which damages healthy tissue and surrounding structures through phagocytic and cytotoxic activities[1,7,8]. Conventional therapies administered as anti-inflammatory measures such as non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids have mixed results in the clinic, and are often characterized by significant side-effects and far-reaching complications resulting from long-term usage. Specifically-

targeted therapeutic measures in current development are intended to interrupt one or more steps involved in the inflammatory sequence by the inhibition of key molecules, with the objective of preventing transendothelial migration. The success of clinical diagnostic procedures and therapeutic strategies which employ this molecular level approach is contingent upon a detailed understanding of the myriad of mechanisms and mediators involved in specific inflammatory processes.

Decades of research in the pathogenesis of inflammatory diseases has revealed a number of therapeutically-significant CAMs, now classified into subsets based on their locations and functions. The selectin family consists of three CAMs, all of which serve to facilitate leukocyte rolling along the endothelial lining. P-selectin, expressed on platelets and endothelial cells, can be rapidly trafficked to the cell surface upon exposure to inflammatory stimuli, such as the cytokine TNF-α and various interleukins. The therapeutic inhibition of P-selectin surface expression is under study[9-11]. The interaction between leukocyte-expressed P-selectin glycoprotein ligand 1 (PSGL-1) and P-selectin is known to be highly involved in rolling processes[12]. L-selectin is expressed on leukocyte subsets, and also binds with PSGL-1 on the same cell surface in an interaction that is thought to promote enhanced cell tethering. It is also involved in lymphocyte homing and trafficking in Peyer's patches through its interaction with mucosal addressin cell adhesion molecule 1(MAdCAM-1) in various lymphoid organs[13]. E-selectin, a CAM exclusively expressed on endothelial cells, is thought to be a mediator for rolling of leukocytes along stimulated endothelium. Primary ligands which interact with E-selectin are being studied extensively in the development of inhibitory therapies[14-16].

Integrins and immunoglobulin CAM subfamilies also have a critical role in inflammation. The inhibitory targeting of these subtypes is a very active clinical research area. Certain integrins are expressed exclusively on leukocytes. Over 20 types have been classified, which are active upon the association of α and β subunits to form dimers. Their general role can be summarized by the mediation of interactions between leukocytes and extracellular matrix proteins and various counter-receptors on neighboring leukocytes and endothelial cells[17,18]. The capacity of integrins to promote such activity is enhanced upon inflammatory activation of the host cell. Of these many roles, the primary involvement of integrins in the inflammatory cascade is now known to be their binding to the Ig family of CAMs, which are expressed on multiple cell types in the vasculature[17,18]. Primary integrins involved in these associations include lymphocyte function antigen (LFA-1) and membrane-activated complex (also called Mac-1), which aid in leukocyte recruitment by promoting tethering on the endothelial surface; LFA-1 has another significant function in enhancing the response of lymphocytes to antigens[19-23]. Two other integrins, $\alpha_4\beta_7$ and very-late antigen 4 (VLA-4, or $\alpha_4\beta_1$), are expressed on lymphocytes and monocytes and govern rolling and adhesion processes of those cells along inflamed endothelium[24,25]. LFA-1 and Mac-1 are counter-receptors for the IgCAMs intracellular adhesion molecules 1 and 2 (ICAM-1 and ICAM-2), respectively.

The various roles of ICAM-1(CD54) on the endothelial surface are of major importance in inflammation. For example, inhibition of ICAM-1 dependent signaling through the inhibition of the messenger protein kinase C was shown to inhibit lymphocyte transmigration[26]. Other functions include regulation of the endothelial cell cytoskeleton through actin rearrangement[27], as well as the formation of focal adhesion

complexes (FACs), which connect the cell's internal cytoskeleton to the surrounding extracellular matrix[28]. Several other roles for ICAM-1 have been proposed but require further investigation. The other integrins mentioned, VLA-4 and $\alpha_4\beta_1$, bind to vascular cell adhesion molecule 1(VCAM-1) and MAdCAM-1, respectively. The role of MAdCAM-1 in lymphocyte homing was previously discussed, and relates closely to the role of VCAM-1 in many respects. VCAM-1 (CD106) is a crucial component of inflammatory processes, as it is linked directly to processes which mediate endothelial cell shape, viability, and leukocyte transmigration. These activities are conducted through the activation of NADPH oxidase, which has the additional VCAM-1 induced effect of reactive oxygen species (ROS) production in activated endothelium[29-31]. The generation of low-level ROS by VCAM-1 induction has been hypothesized to be a transient, local signal, the scavenging of which could likely reverse many of the deleterious effects of VCAM-1 activation[32]. However, further studies are necessary to probe this specific function more closely. A third important immunoglobulin CAM is platelet-endothelial cell adhesion molecule 1 (PECAM-1), a protein involved in endothelial cell junction formation as well as homophilic leukocyte interactions. It is through the latter that PECAM-1 (CD31) is thought to mediate the adhesion and transmigration of monocytes, neutrophils, lymphocytes, and eosinophils, making it an equally important targeting factor in the treatment of inflammation. Indirect therapies may play an important role in the inhibition of PECAM-1 related processes, as various cytokines have been shown to enhance its affinity to leukocyte subsets[7]. PECAM-1 also is connected through signal transduction to various cytoskeletal proteins such as α and γ-catenins, important in endothelial junction formation[33,34].

A number of other clinically-important mediators of inflammation can also be found on the endothelium, particularly in junctions. Three types of junctions have been classified: adherens, complexus adherents, and gap junctions[1]. A variety of junction proteins await extensive characterization. The functions of these proteins range from the recruitment of matrix metalloproteinases (MMPs) by the claudin junction protein[35,36], to the lateral modulation of endothelial junctions by junction adhesion molecules (JAMs)[1,6] and cadherins (namely N- and VE-cadherins), which are thought to enhance leukocyte transmigration in their absence[37]. It is not clear whether JAM-mediated interactions are primarily heterophilic or homophilic, nor is it clear if lateral translocation of all or some of the junctional proteins are a requirement for effective leukocyte extravasation[7].

Leukocyte rolling, adhesion, and transmigration across endothelium is an essential defense mechanism, but is also a critical factor involved in the pathogenesis of various diseases. Clinical interventions involving the inhibition of one or more CAMs and/or their corresponding ligands are believed to be effective treatment strategies for inflammatory diseases. However, the results in clinical trials have been mixed at best, and warrant extensive further studies[1]. There are a few explanations for the limited success of such therapies. One is that early results observed in animal studies may not necessarily be applicable to human clinical trials. In addition, certain inhibitory therapies may be effective for reasons different than those which were initially hypothesized, which would affect the use of one strategy in the management of another disease. Perhaps most importantly, one CAM, having been chosen for a certain function, may also participate in another unknown function which could be an impediment to successful inhibition. However, there are a number of reasons which also suggest that this method

of treatment will be an effective one in the future. The knowledge of the structure and functions of CAMs and other inflammatory mediators increases daily, and in many cases the function of a certain protein is a consistent feature in many disorders, making treatment planning easier. The future of these therapies will rely on the development of techniques which probe the functions of a multitude of proteins *in vivo*, in order to develop small-molecule inhibitors and combinatorial treatment approaches which provide the highest probability of successful management of the disease.

In Vivo Imaging of Inflammation at the Cellular and Molecular Levels

In the effort to elucidate the biological processes and key molecular players in inflammation, *in vivo* imaging techniques, beginning with intravital microscopy (IVM) have been used for over a century in an attempt to understand the processes of recruitment and extravasation. While these methods provide the benefit of confirming *in vitro* observations obtained by reductionist and retrograde extrapolation approaches, they also have the potential to rapidly identify the biological activity of multiple molecular species at a time. This may be especially useful in determining physiological and pharmacological effects of a given therapy, for example, when the effects of a small-molecule inhibitor are known for one receptor but not various others. IVM has been used with success in the past to visualize leukocyte-endothelial interactions[38], with the transition to epifluorescence IVM to study such adhesions in the microvasculature in modern research[39-42]. It was determined using IVM-based methods how lymphocytes migrated to certain lymphatic organs[43]. Further work using IVM resulted in the now

widely-accepted concept that adhesion cascades with multiple complex steps dictate when and where leukocytes can access a given tissue[44,45].

Techniques based on IVM used to probe leukocyte-endothelial cell interactions are not without drawbacks. The inability of the illumination source to penetrate deep into tissue limited many studies to "*in vivo*" studies of prepared cremaster muscles[46,47], bat wings[48], ear chambers[49], and other tissues with adequate optical translucency for imaging. An obvious caveat with such studies involved the fact that such dissections in themselves were pro-inflammatory stimuli that could compromise the validity of such observations as they apply to the *in vivo* immune response. The developing needs of researchers in this regard resulted in the introduction of non-invasive techniques with enhanced tissue penetration for visualization of leukocyte activity. These techniques included bioluminescence imaging and magnetic resonance imaging (MRI), and (single photon) computed tomography. However, a major limitation was still inherent in the use of these methods in inflammation research. The spatial and temporal resolution was not adequate to accurately probe cell-cell interactions in the circulation. Furthermore these modalities did not permit the measurement of dynamic physiological parameters that might be used to assess the progression of an inflammatory disease, such as leukocyte velocity or the number of adhesions formed at a particular junction.

The advent of widefield and confocal fluorescence microscopy adapted for *in vivo* imaging provided the resolution required in time and space to characterize inflammatory processes in substantial detail. These techniques, in widespread use today for *in vivo* imaging in real-time, allow for non-invasive imaging of tissues. However, these excitation techniques, which rely on high energy wavelengths to excite exogenously-

administered fluorochromes, have a limited depth of field of about 100 μm in tissue. Furthermore, the high-energy excitation light is associated with phototoxicity risks as well as photobleaching of the injected species, which limits long-term imaging of *in vivo* inflammatory activity[50-53]. Nevertheless, numerous significant studies unraveling the mechanisms of inflammation have been reported with these techniques, with considerations in experimental design to the above-mentioned factors. An excellent example involves various studies of inflammation using a scanning laser ophthalmoscope (SLO), a confocal imaging modality adapted for *in vivo* imaging of the fundus. This technique, introduced by Richard Webb of the Schepens Eye Institute[53], is characterized by a lower intensity of light for excitation as opposed to conventional brightfield microscopy. SLO employs a laser for illumination and a PMT for photon collection. The laser illuminates the fundus point by point in a rapid rastering pattern that permits high-resolution imaging of structures on the micron scale. This intensity of light was demonstrated to be approximately 1% of the light needed by a brightfield ophthalmoscope to form a similar-quality image[54-56]. Nishiwaki and colleagues introduced a technique for utilizing the SLO as a leukocyte tracking device to image their trafficking in various disease states[57-59]. Acridine orange, a DNA intercalating dye with spectral properties similar to fluorescein, was employed to visualize the (nucleated) leukocytes in an endotoxin-induced uveitis (EIU) mouse model, a study tool used to simulate human inflammatory conditions. Due to the very high resolution of the SLO, individual leukocytes were visible, and could be further distinguished as either *free-flowing* leukocytes, or *rolling* leukocytes which adhere to and roll along the endothelium of the blood vessels. Honda and colleagues would later take advantage of SLO imaging

technology to characterize leukocyte trafficking patterns in diabetes, and to study the contribution of leukocytes to retinal flow disturbances in the presence or absence of various endothelial surface proteins and other stimuli (such as VEGF)[57-67]. With these studies, the retina was demonstrated to be an excellent tissue for studying inflammation non-invasively *in vivo*. However, SLO and other confocal imaging strategies, while reducing the amount of excitation light, were still limited by spatial resolution as well as the eventual bleaching of flurophores which impede long-term studies.

The limitations of single-photon imaging strategies was addressed with the introduction of multiphoton intravital microscopy (MP-IVM)[68]. This modality is based on a principle of quantum physics, in which two photons of inadequate energy to excite a fluorophore arrive at the same time at a given point in space, to combine to excite a fluorophore as if it were a single, higher-energy photon. For example, if two incoming photons of wavelength 1200 nm (relatively low-energy photons) arrive coincidentally in time and space, the fluorophore interacts with a "single photon" with twice the energy of the original two photons (i.e., 600 nm). When this strategy is optimized with regards to the absorption and emission spectra of the fluorophore, infrared light (commonly from an infrared Ti:sapphire laser) can be harnessed for penetration of tissue to excite a conventional organic dye. A penetration depth of over five-fold over classical confocal techniques was demonstrated with MP-IVM, for imaging of lymphocyte migration *in vivo*[51,69,70]. The outlook for MP-IVM in elucidating various mechanisms of inflammation is promising.

Leukocyte and Cell Adhesion Molecule Detection Techniques

Current techniques for imaging inflammation have employed conventional organic fluorophores to track cells and CAM expression *in vivo*. Dyes used to label leukocytes include acridine orange [16], sodium fluorescein, fluorescein derivatives such as calcein-AM, and indocyanine green. Of these techniques, AO is the preferred dye, as it can be administered intravenously (as opposed to the labeling of donor leukocytes), and has spectral characteristics similar to fluorescein which makes it suitable for ophthalmoscopic devices already configured for fluorescein angiography. In addition, AO can penetrate cell membranes and label nucleic acids. This feature is used to label leukocytes, which have nuclear material and thus are preferentially labeled in fluorescence imaging compared to red blood cells and platelets. However, there are several notable disadvantages with this technique. First, since all nuclear material in the blood is stained, leukocytes cannot be distinguished from one another, since they all have nuclei, and in addition, endothelial cells also fluoresce, which can introduce difficulties in distinguishing adhesion formation from free-flowing leukocytes without high-resolution capabilities obtained with SLO. The inability to distinguish multiple cell types is an impediment to characterizing individual components of an immune response in various pathologies. However, since a majority (over 60%) of circulating leukocytes are neutrophils, AO fluorography can still provide an accurate assessment of neutrophil function *in vivo*. Another challenge in using AO for *in vivo* studies is the low quantum efficiency (~20%) available, and the tendency of the dye to photobleach rapidly. As a result, many murine studies involving AO were performed with constant venous infusion of AO[60,62,71,72]. It is also important to note that AO is a carcinogen[73] and thus cannot be

used in human studies. Also, AO is phototoxic to lysosomes[74], which adds to the many problems in performing long-term studies of inflammation. Thus, an intravenously-injected fluorescent probe capable of long-term *in vivo* targeting and imaging of leukocyte subsets with no immediate adverse effects on cell function is highly desirable in the development of techniques to study inflammation.

Many CAMs and other molecular mediators of inflammation, such as cytokines, are just now being characterized. As a result, imaging techniques to quantitatively study molecular expression and/or interactions are just beginning to be developed. Methods used to study CAMs are primarily based on refinements of existing technology. *Ex vivo* MRI of ICAM-1 in autoimmune encephalitis in mice was accomplished using a liposome-conjugated contrast agent. Fluorescence microscopy was used to localize the areas of interest, to compensate for the spatial resolution constraints which impeded cellular-level studies[75]. The technique employed in this study did not permit real-time monitoring of endothelial expression *in vivo* at high spatial resolutions, although it did highlight the ability of contrast agents to amplify signals due to inflammation. Studies concerning the expression of VCAM-1 and ICAM-1 in oxazolone contact hypersensitivity were carried out using radiolabeled monoclonal antibodies[76]. While a quantitative evaluation of antibody uptake could be used to evaluate molecular expression, acquisition of real-time quantitative data were not possible, and thus only a limited knowledge of the temporal component of molecular expression could be achieved. A similar study involved the above technique, but was combined with IVM to visualize adhesions in further detail. Both radiolabeled antibody techniques were also limited by low signal to noise ratios[76,77]. Weissleder and colleagues developed

magnetooptical probes based on antibody-conjugated paramagnetic nanoparticles and Cy-5.5, a near infrared dye, for imaging of VCAM-1 *in vivo*[78]. The same laboratory also developed a peptide-conjugated Cy-5.5 probe which was accumulated in cells expressing VCAM-1[79]. While both novel strategies improved on the spatial resolution limitations of previous techniques, neither gathered real-time data on VCAM-1 expression, but instead took snapshots at timepoints separated by hours. Furthermore, in some experiments TNF-α, a potent inflammatory stimulant, was used to induce VCAM-1 expression, which leaves a question as to whether such a technique could adequately detect CAMs in inflammatory diseases in very early stages, an ideal time for treatment.

Quantum Dot Nanocrystals for in vivo Imaging

Studies of inflammation have been performed with conventional fluorophores for IVM, paramagnetic conjugates for MRI, and radiolabeled conjugates for X-ray detection of molecular expression. Limitations of these approaches include the inability to image multiple cell types or molecules at one time and low signal to noise ratios. Probes which are capable of surface functionalization for targeting multiple species deep in tissue, and imaging them in real-time with high signal to noise ratios would facilitate the detailed study of leukocyte-endothelial cell interactions in inflammation. The advent of semiconducting nanocrystals, or quantum dots (QD), has the potential to provide these features. In the synthesis of QD, a nanometer-sized crystal (usually CdSe) is capped with a larger bandgap, secondary layer of ZnS for enhanced optical behavior. The absorption of a photon of light by the semiconducting material and subsequent emission of a lower energy photon results in fluorescence. For biological labeling, antibodies or peptide

sequences can be conjugated by traditional chemistry. This feature has made QD a powerful option for use as a site-specific biomarker. The optical properties of QD provide for size-tunable emission wavelengths; smaller QD are towards the blue emission spectrum, while larger QD are towards the red spectrum. This property allows for the synthesis of a variety of QD having different emission wavelengths which make multispecies imaging *in vivo* a possibility. Emission wavelengths of QD include deep infrared wavelengths, which allow for multiphoton excitation modalities for enhanced tissue penetration. In addition, QD have quantum efficiencies beyond 60% in many cases and do not fade under continuous excitation, which make them highly desirable candidates in scenarios in which long-term imaging with high signal to noise ratios are needed. Bioimaging applications of QD to date have included detailed imaging of tumors[80-82], mapping of sentinel lymph nodes[83], and *in vitro* and *in vivo* imaging of ligand-conjugated QD uptake in cells[84,85].

The unique properties of quantum dots make them suitable agents for *in vivo* imaging of leukocyte trafficking in the circulation and tissue, as well as real-time, long-term imaging of CAM molecular expression. QD can be imaged using conventional IVM or MP-IVM, making it a flexible technique, and the ability to image multiple species in inflammation will certainly yield high-impact discoveries involving molecular mechanisms of inflammatory disease and avenues for treatment.

Objectives

Inflammation is a complex process involving a number of cell types and molecular mediators. Imaging techniques that are currently used to probe inflammatory activity for diagnostic and therapeutic purposes are limited by low signal to noise ratios

and an inability to image multiple species simulatenously. Furthermore, continuous long-term quantitative assessment of inflammatory mediators has not yet been performed with these methods. Quantum dot nanocrystals offer a number of desirable optical properties which make them suitable candidates for monitoring live cell trafficking *in vivo*, as well as molecular expression in real-time. We seek to develop quantum dot-antibody conjugates to continuously track specific leukocyte subtypes in the retinal microcirculation, an area in which the *in vivo* circulation can be non-invasively accessed. In addition, we will use quantum dot conjugates for real-time imaging of expression of inflammatory mediators at the molecular level. Retinal imaging using fluorescence microscopy will be performed with these conjugates to assess leukocyte trafficking and the upregulation of the cell adhesion molecules PECAM-1 and VCAM-1 in health and in inflammatory diseases by experimental induction of uveitis and diabetes in mouse and rat models, respectively. The overall objective of our work is to establish an imaging probe capable of application to various fluorescence imaging modalities such as brightfield microscopy, scanning laser ophthalmoscopy, and multiphoton intravital microscopy, for the purpose of examining multiple components of the inflammatory response at the cellular and molecular level. It is hoped that such a technique will facilitate the identification of therapeutic targets and early diagnostic markers in disease.

CHAPTER II

QUANTUM DOT-BASED *IN VIVO* IMAGING OF INFLAMMATION

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Introduction

Inflammation is a complex process involving many mediators at the molecular and cellular level. General indicators of inflammatory activity in the body involve leukocyte rolling and tethering along vessel walls, followed by emigration into tissue[6,7,45]. While these steps are essential in natural immune defenses, undesirable inflammatory responses are thought to play a role in disease such as, for example, multiple sclerosis and diabetes[1]. Various strategies have been developed to probe cellular and molecular involvement in inflammation *in vivo* to observe processes as they actually occur in the body, reducing the need to develop accurate *in vitro* models which simulate the physiologic, geometric, temporal, and biological components of the disease. *In vivo* imaging of inflammatory mechanisms involving cell adhesion molecules or leukocytes for the purpose of staging the course of the disease or developing treatment strategies is a challenging procedure. Disadvantages of current techniques include limited optical accessibility to tissue, invasiveness[46,47], low or unstable signal intensity due to the use of organic fluorophores[59,63,67], or low spatial and temporal resolution achieved by the use of radiolabeled antibodies[76] or magnetic resonance probes[78]. Detailed *in vivo* studies of inflammatory activities at the molecular and cellular level would be enhanced by probes which permit specific, long-term, and continuous imaging. Additionally, imaging strategies which can minimize the impact of tissue autofluorescence for a higher signal to background ratio are needed. Imaging of quantum dots (QD) in the retinal circulation offer the promise of achieving these goals, due to the unique optical accessibility of the retina, as well as quantum dot optical properties, such as size-tunable emission

wavelengths, amenability to surface functionalization, and high-intensity fluorescence with a resistance to photobleaching[84,85].

Results

.In this study, we present a quantum dot -antibody conjugate-based approach for *in vivo* imaging of cell adhesion molecules and leukocyte subsets using fluorescence microscopy. Continuous imaging was performed in rat retinal tissue, which provided a direct, minimally-invasive view of the circulation with sufficient optical quality to visualize vascular targets. Compared to untreated controls, specific labeling of VCAM-1 was evident in streptozotocin (STZ)-treated diabetic rat models. Furthermore, detection of *ex vivo* neutrophils as well as endogenous, circulating quantum dot-labeled neutrophils was achieved, demonstrating the utility of quantum dot-based imaging for long-term imaging of leukocyte recruitment, proliferation, and interactions with the endothelium.

***In vivo* detection of VCAM-1 in the retinal circulation**

In order to determine the utility of quantum dot bioconjugates in detecting inflammatory mediators in diabetes *in vivo*, we systemically administered quantum dot-antibody conjugates directed at rat VCAM-1, a cell adhesion molecule expressed on the luminal surface of the endothelial lining[76,77,86]. Quantum dots were conjugated to mouse anti-rat VCAM-1 antibodies, and blocked with Fc-specific mouse anti-rat $F(ab)_2$ to reduce nonspecific binding[87]. Long-Evans streptozotocin-induced diabetic rats and untreated rats were administered equal doses of VCAM-1-targeted and control bioconjugates through tail vein injection shortly before imaging by fluorescence

microscopy of the fundus[88]. Video sequences of the retinal circulation in diabetic rats indicated enhanced VCAM-1 levels relative to control (Figure 1). Targeting of VCAM-1 was evident within minutes after injection. Injection of QD conjugates through the tail vein is observed as a fluorescent plug in the retinal circulation approximately 6 seconds later, which rapidly moves through the circulation. As unbound conjugate is washed out of the circulation, conjugates bound to VCAM-1 are observed as QD-specific fluorescence emission due to "tethers" along the walls of the endothelium. With increasing circulation time, VCAM-1 labeling accumulated on vessel linings in major veins as well as in the microcirculation. No such accumulation was detected in untreated controls, and unbound conjugate was rapidly cleared. The distribution of VCAM-1 was significantly weighted towards the microcirculation and veins. As opposed to *in vivo* studies of VCAM-1 in TNF-α induced inflammatory models[78], which reported diffuse, dense staining along the vessel walls, we observed a more punctate distribution of VCAM-1 along the endothelium.

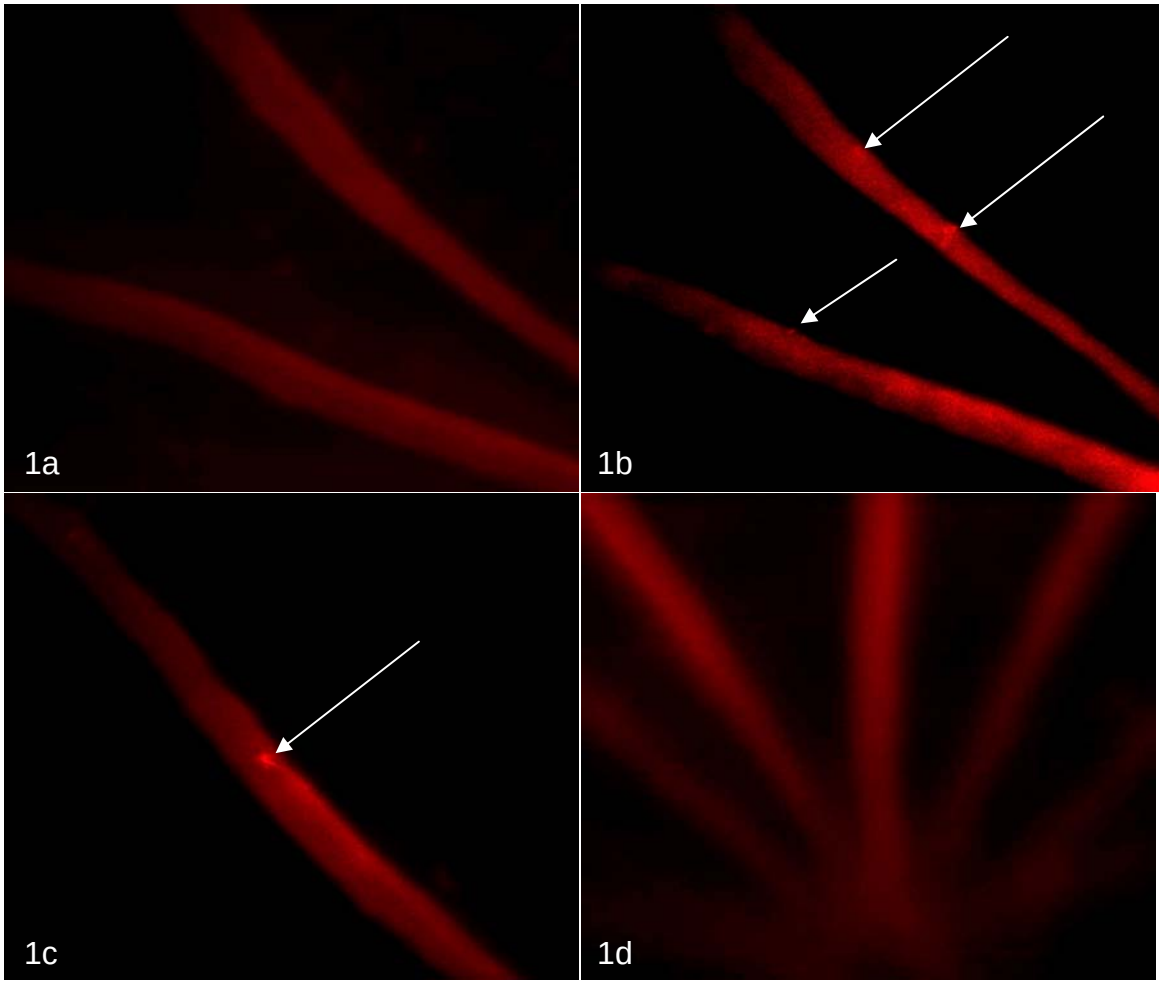

Figure 1: *In vivo QD*-labeling of VCAM-1. STZ-treated and untreated Long-Evans rats were administered 500 nM systemic doses of QD 605/anti-VCAM-1 conjugate and observed via *in vivo* fluorescence microscopy. 1a Background in QD605 channel prior to injection in diabetic rat 1b or 1c: Tethered VCAM-1 adhesions are detected by QD after unbound conjugate is cleared from the circulation. Unbound conjugate is still visible in the background. However, tethered QD are brightly visible and remain fixed along the vessel walls in STZ-treated rats. 1d After washout of QD/VCAM-1 conjugates, healthy rats had no visible tethers in the retinal circulation.

Validation of *in vivo* bioconjugate specificity to VCAM-1

We sought to validate our *in vivo* observations of VCAM-1 levels with high-resolution *ex vivo* analysis, which involved high-magnification fluorescence microscopy and dual-laser scanning. Retinal tissue scanning using a dual-laser, dual-PMT acquisition system provides for a macroscopic view of major arteries and veins, as well as the microcirculation, at a 5μm spatial resolution. Comparative laser scanning analysis of

control and diabetic retinal tissues in the specific emission channel of the QD conjugate is indicative of enhanced VCAM-1 levels in STZ-treated tissue (Figure 2). Densities of QD fluorescence throughout the retina indicated widespread VCAM-1 staining.

In order to determine the specificity of the QD-VCAM-1 conjugates, we co-administered maleimide-activated QD585 and QD565/isotype control IgG with the QD/VCAM-1 conjugate in both experimental groups, at equal concentrations of QD and/or antibody employed in the conjugation chemistry and dosage steps. Analysis of retinal tissue by fluorescence microscopy confirms that the labeling of VCAM-1 by our QD conjugates is specific (Figure 2a-e). Furthermore, increased expression of VCAM-1 in diabetic retina is again confirmed using fluorescence microscopy. These observations are consistent with *in vitro* studies suggesting that VCAM-1 expression on the endothelial cell surface is increased in high glucose medium[89].

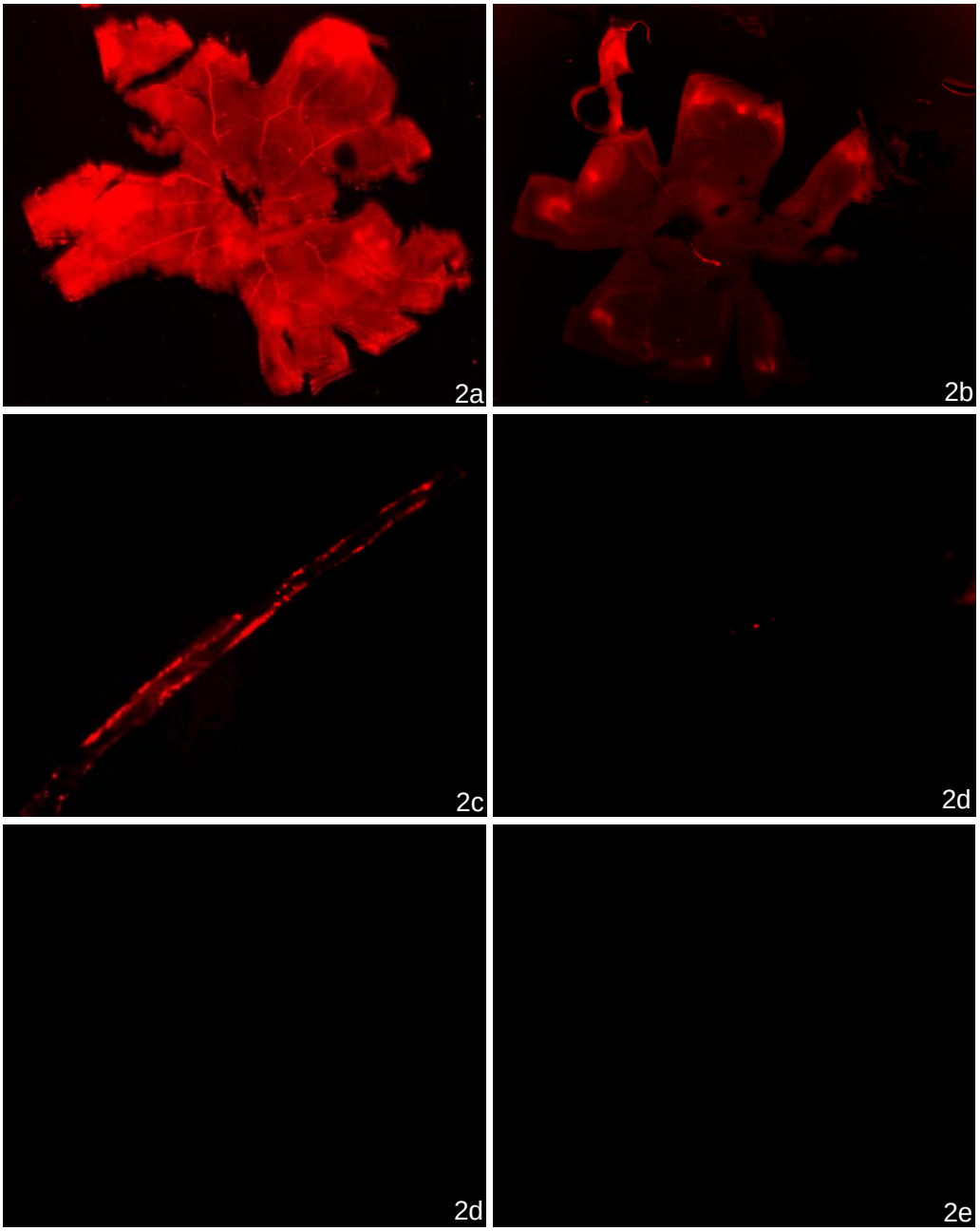

Figure 2 Tissue labeling of VCAM-1 by QD conjugate. Retinal flat mounts were prepared from control and STZ-treated rats after sacrifice for detailed analysis of VCAM-1 staining. 2a,b Retinal flat mounts from a control rat and diabetic rat administered QD/VCAM-1 via tail vein catheterization. Tissue was scanned using a 532nm laser with PMT bandpass set to 670+/- 20nm (details in supplementary methods). 2c Fluorescence microscopy at 1000X magnification of retinal vasculature in diabetic rat 1 hour after administration of QD655/VCAM-1. Positive endothelial staining of VCAM-1 is visible. 2d Healthy rat retinal vasculature is indicative of weak levels of VCAM-1 expression. 2e QD565/Isotype control fluorescence channel at 100X. Fluorescence analysis indicates no significant Fc-region mediated antibody binding as indicated by lack of QD565-KLA IgG fluorescence. 2f Nonspecific QD585 uptake in retinal tissue of diabetic rats was negligible.

QD conjugate specificity to neutrophils and signal intensity

In addition to detecting tethered adhesion molecules on endothelial surfaces, our technique was also used to image cellular mediators of inflammation. A key challenge in the imaging of circulating leukocyte subsets *in vivo* is the development of *specific* targeting methods. Neutrophils, known to be crucial "first responders" in a number of inflammatory diseases, have well-known phagocytic functions in immune defense, and as a result *in vivo* tracking techniques have utilized radiolabeling or intracellular organic dyes as opposed to antibody-based techniques, in which nonspecific uptake of the conjugate could occur by certain leukocyte subsets[2,90]. To investigate if these mechanisms would impose limitations on QD conjugate usage during *in vivo* observations of leukocyte trafficking, flow cytometric analysis of lysed whole rat blood incubated with QD585-RP-1 Mab conjugates was performed. RP-1 is an antigen reported for specificity to neutrophils in rat peripheral blood, but not other leukocyte subsets[91]. Leukocytes were readily distinguished by their scatter properties, and analysis of monocyte, lymphocyte, and granulocyte subpopulations indicate specific staining of polymorphonuclear cells *exclusively*, the vast majority of which consist of neutrophils in peripheral blood (Figure 3a). Analysis of Percoll-isolated neutrophils incubated with QD-isotype control conjugates and nonspecifically-targeted QD indicated mean fluorescence levels similar to unlabeled neutrophils (Figure 3b). QD-RP-1 conjugates provided enhanced signal detection relative to the positive control, phycoerythrin-RP-1 (PE-RP-1). PE has many applications in flow cytometry due to the fluorophore's ideal spectral fit to flow cytometers equipped with a 585/42 bandpass filter, and its relatively high resistance to bleaching. Our observations indicate that leukocytes labeled with QD

conjugates exhibit a 1-decade enhancement in fluorescence intensity compared to PE-labeled leukocytes. Furthermore, quantitative analysis of the ability of QD-RP-1 labeled neutrophils to adhere to tissue culture plastic upon Concanavalin A stimulation indicated adherence by 27% of total cells, within the established range suggested for the preservation of neutrophil function (Supplementary Methods).

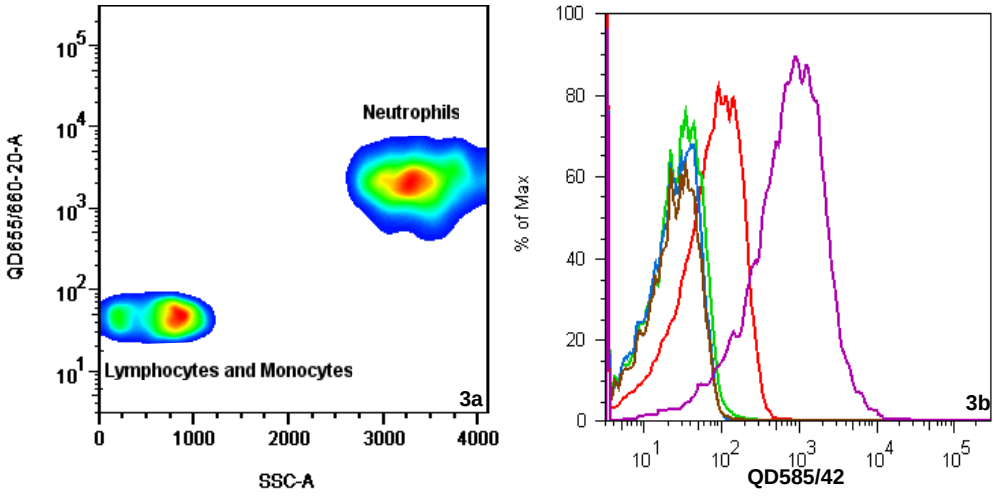

Figure 3 Flow cytometric analysis of QD/RP-1 specificity to neutrophils in lysed whole blood. 3a: Mean fluorescence of neutrophils is almost 2 log decades higher than monocytes and lymphocytes when lysed whole blood is incubated with the QD/RP-1 conjugate. 3b: Neutrophil-gated population of lysed whole blood incubated with PBS (--), QD/isotype control antibody conjugate (--), QD/unconjugated only (--), PE-RP-1 (--), or QD/RP-1 (--). QD/RP-1 fluorescence is log decades higher than controls, and is significantly higher than the PE conjugate.

In vivo imaging of leukocytes in the retinal circulation

In vivo analysis of endogenously-labeled leukocytes indicates that QD are high-intensity probes suitable for studies of leukocyte trafficking. QD605-RP-1 was administered by tail vein catheterization to diabetic and control rats shortly following the conclusion of QD targeting of VCAM-1 in those animals. A period of unbound conjugate washout reduced the background fluorescence at a sufficient level to commence analysis. Labeled spheres appearing to have the shape and speed of

circulating neutrophils were observed in major vessels as well as the microcirculation, although at very low levels compared to detection performed using acridine orange fluorography (unpublished data) in diabetic and healthy rat models. High velocity leukocyte movements through vessels were characterized by streaks (Figure 4a, 4b), whereas movements through the microcirculation were often sufficiently slow enough to be captured as spots by our technique. No leukocyte rolling phenomena could be observed, although in the diabetic rat model, stationary or adherent neutrophils could be visualized in the periphery (Figure 4c, 4d). Such an observation highlights the utility of QD for continuous imaging of leukocyte-endothelial cell adhesions, an application in which dye-based strategies are limited due to their fading characteristics. Neutrophil-mediated plugging of capillaries has long been linked to inflammatory complications in ocular pathologies such as retinal ischemia[92], and may be a significant factor in diabetes-induced complications. The ability to continuously image leukostasis suggests a useful role of QD-based imaging in staging the course of disease or assessing response to therapies. Furthermore, the intensity of the specific QD-neutrophil signal raises a question as to whether the RP-1 antigen may be upregulated in diabetic-induced inflammation. RP-1 was shown in previous studies to be upregulated in response to neutrophil activation[91], which suggests that the relative intensity of labeled neutrophils may be an indicator of activation; this in turn may be a determinant of adhesion potential.

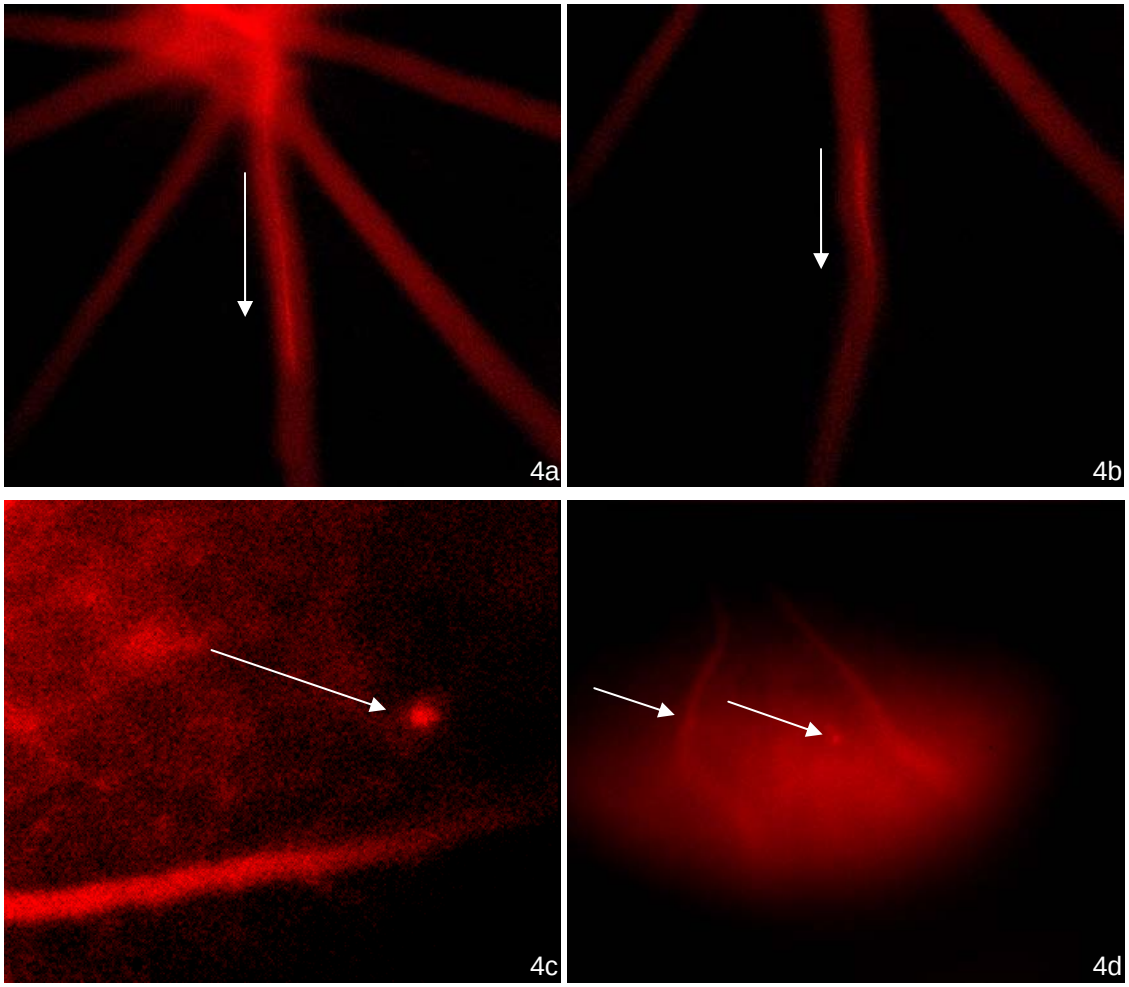

Figure 4 *In vivo* imaging of QD-labeled leukocytes in retinal vasculature. QD605-RP-1 was injected by tail vein administration to an STZ-induced diabetic rat model, and imaged using metal halide and xenon arc flash excitation sources. 4a,b A moving labeled neutrophil moves rapidly through a major vessel (in the direction of the arrows), leaving a streak. 100 msec exposure per frame with a 110 msec interval between frames. Total magnification 40X. 4c,d Neutrophils appearing to be stagnant were observed for several consecutive frames in the diabetic model. No stagnant cells were visible in untreated control retinas. Total magnification 40X, 100 msec per frame.

Utility of QD conjugates for *ex vivo* labeling and systemic reinfusion

Various *in vivo* studies in immunology involve the systemic administration of donor cells which have been genetically altered or stimulated to present a specific antigen, for the purpose of studying an evoked response under controlled conditions. We investigated whether quantum dots were suitable for these purposes by incubating donor rat neutrophils isolated using density gradient medium with QD565-peptide conjugates from Quantum Dot Corporation, which are nonspecifically endocytosed by

cells. Analysis of the labeled cells by flow cytometry and fluorescence microscopy indicated significant uptake of the QD-peptide conjugate, and intense labeling. *In vivo* imaging of reinfused neutrophils revealed very few neutrophils initially; however, these cells were visible in the microcirculation as well as major vessels. We hypothesize that many reinfused cells may have been trapped in the pulmonary circulation for a period of 20 minutes or more, as reported by other donor cell reinfusion studies[93]. While this impeded the rapidity of our *in vivo* imaging technique, it is important to note that donor cells labeled nonspecifically by QD are, as expected, visible in the retinal circulation. Further studies comparing QD-peptide and acridine orange-labeled isolated neutrophil intensity and stability under long-term illumination *in vitro* indicated initial weak fluorescence and rapid fading of the acridine orange within 45 seconds, with QD-internalized cells having several-fold higher initial intensities followed by no change in signal intensity even after an hour of observation (Supplementary Figure I). These data suggest that donor cells could be labeled by QD as an alternative to organic dyes for long-term imaging in which a high signal to noise ratio is required.

In a related study, in order to investigate whether QD probes could be useful as *nonspecific* probes for the identification of all leukocyte subsets, a procedure usually performed with the DNA intercalating dye acridine orange, lysed whole blood was incubated with QD conjugated to mouse anti-rat CD45 (leukocyte common antigen), which is known to react equally well with all three leukocyte subsets. Flow cytometric analysis indicated that QD probes are more intense than acridine orange, and labeled all three leukocyte subsets *in vitro* (Supplementary Figure II). This data suggests that QD probes could be substituted for acridine orange in various protocols, which may be

desirable as the dye is characterized by a low quantum efficiency (<20%) as well as a tendency to rapidly photobleach[94].

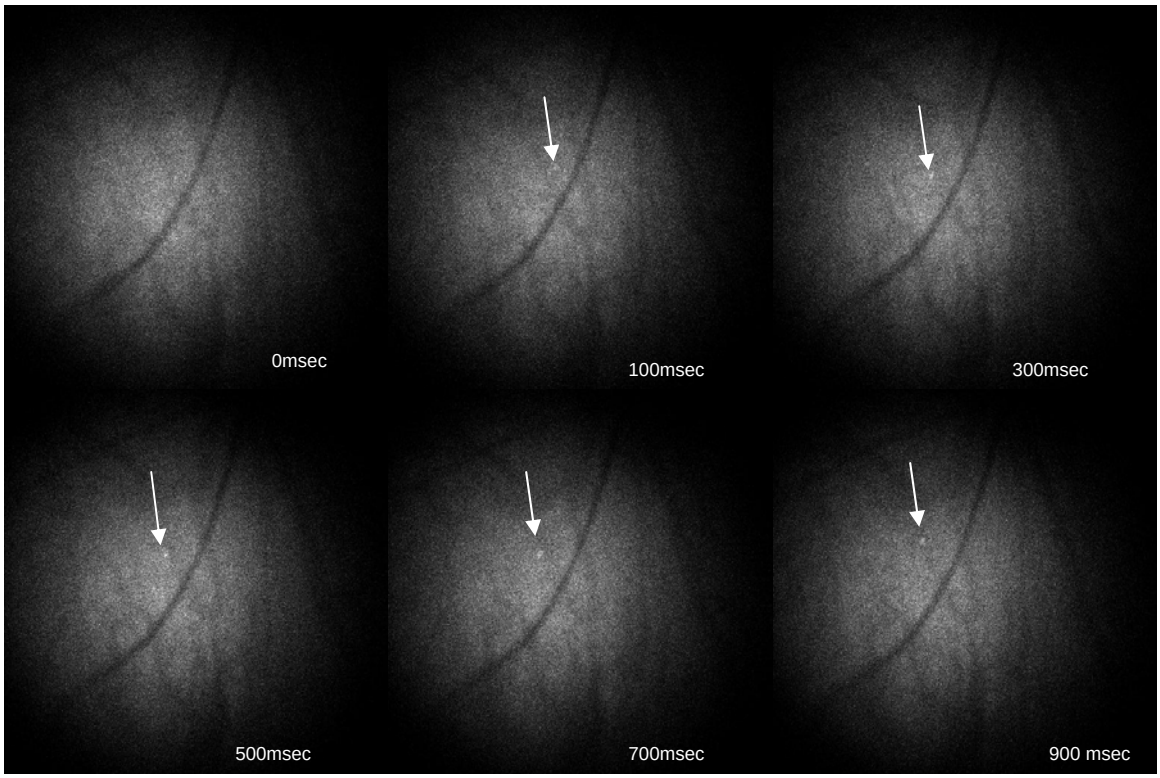

Figure 5 *In vivo* imaging of QD-labeled donor neutrophils. Qtracker 565 was used to stain Percoll-isolated rat neutrophils; the stained and washed neutrophils were then reinfused via tail vein. In vivo sequences of Qtracker-labeled neutrophils are indicative of high signal to noise ratios, due to the uptake of multiple QD-peptide complexes by the cells.

Discussion

The ability to label and image cell adhesion molecules and circulating cells *in vivo* is a major advancement in imaging techniques which seek to probe inflammatory events at the cellular and molecular level. Our technique utilized quantum dots to demonstrate for the first time *in vivo* detection of VCAM-1 expression in diabetes. As VCAM-1 levels were found to be enhanced compared to untreated controls, our studies support *in vitro* work which suggests a link between VCAM-1 levels and diabetic

complications. Thus, our observations warrant further investigation into the spatial and temporal regulation of VCAM-1, and its potential as an early diagnostic marker of diabetes-induced inflammation. Furthermore, our approach enabled the detection of circulating as well as stagnant neutrophils in major vessels as well as the microcirculation. We have shown that quantum dots are specifically-targeted probes with high signal to background ratios, which make them suitable candidates to replace organic dyes in some *in vivo* imaging modalities. In addition, a feature of semiconducting nanocrystals is size-tunable emission spectra, and the need for only one excitation source of any wavelength below the emission peak. Thus, quantum dots could be used in conjunction with *in vivo* fluorescence microscopy to characterize complex leukocyte-endothelial cell interactions using a multispectral imaging approach. The method described in this study uses the optically transparent, readily accessible retinal imaging window to probe inflammatory events at the cellular level, avoiding relatively invasive approaches. Our technique is widely applicable and accessible to a number of immunological laboratories which seek to probe leukocyte-endothelial interactions at the cellular and molecular scales. The QD bioconjugation techniques employed in this study used well-known and simple biochemical techniques readily-accessible to any molecular biology laboratory. Furthermore, QD-based targeting can be utilized in a number of other imaging modalities, such as multiphoton intravital microscopy and scanning laser ophthalmoscopy, a high-resolution device which also takes advantage of the non-invasive retinal window for imaging the circulation. The usage of QD in a research setting using animal models of disease will certainly provide a wealth of information concerning multiple molecular expression profiles in a variety of diseases. Such multi-pronged

approaches which examine both the cells and molecules involved in disease on an *in vivo* and *in vitro* level are likely to have a major impact on the development of detailed, early diagnostic techniques, as well as the identification of high-impact therapeutic routes.

Methods

Conjugation of Antibodies to Quantum Dots

Maleimide-activated CdSe/ZnS quantum dots emitting at 605nm and 655 nm were purchased from Quantum Dot Corporation (Hayward, CA). Antibodies to rat VCAM-1, CD45 (leukocyte common antigen), keyhole limpet antigen (KLA) as an isotype control IgG2a kappa, and RP-1 were purchased from BD Pharmingen (San Jose, CA). Nanocrystals were conjugated to reduced antibodies using a modified protocol from the manufacturer. Briefly, 125 uL of a 1 uM stock solution of nanocrystals in sodium borate buffer (pH 8.5) was reacted with 15 uL of 10 mM SMCC in DMSO for 1 hour at room temperature. 500 uL of 1 mg/mL antibody in PBS with 0.1% sodium azide (pH = 7.4) was incubated with 25 uL of 14 mM 2-iminothiolane (Traut's reagent) in sodium borate buffer at pH=8.0 (Pierce, Rockford, IL) for 1 hour at room temperature. Reacted quantum dot and reduced antibody solutions were desalted over PBS-equilibrated NAP-5 columns (Amersham Biosciences, Chicago, IL) to remove excess SMCC or Traut's reagent. Purified quantum dot and antibody fractions were then co-incubated for 1 hour. The reaction was quenched by reaction of solution with 13.9 uL of 10 mM betamercaptoethanol, followed by a 30 minute incubation at room temperature. The conjugate was then stored at 4ºC overnight. Reaction mixture was purified from unreacted antibody by size-exclusion chromatography using Superdex 200 gel slurry

(Pierce). Conjugate concentrations were determined using a Nanodrop ND-1000 Spectrophotometer using known extinction coefficients at a given measurement wavelength from Quantum Dot Corporation. Final conjugate concentrations ranged from 1.5 – 2 uM.

Animal Preparation for In Vivo Imaging

All animals were prepared, imaged, and euthanized according to standard Vanderbilt University Institutional of Animal Care and Use Committee-approved protocols. Male Long-Evans rats were purchased from Harlan (Indianapolis, IN), and were divided into control and diabetic groups (n=3 per group). Streptozotocin (STZ) was purchased from Sigma-Aldrich (St. Louis, MO). A solution of 65mg/mL STZ was prepared immediately before injection in 0.1mM sodium citrate buffer (pH=4.5). Diabetes was induced by intraperitoneal injection of 1uL of this solution per gram of body weight. Blood glucose measurements were taken from a drop of tail vein blood dawn from pin prick to verify hyperglycemia in treated groups. Prior to *in vivo* imaging, rats were anesthetized by intraperitoneal injection of 15/85% ketazine/xylazine. Tail vein catheterization was peformed for injection of quantum dot conjugates and controls. Both pupils were dilated with 1 drop each of 2.5% phenylephrine hydrochloride and 1% tropicamide ophthalmic solutions. For weight and blood glucose measurements of the rats used, please refer to the Appendix.

In vivo imaging of VCAM-1 and neutrophils

A Nikon TE2000U inverted fluorescence microscope (Nikon, Japan) was used for imaging of the retinal vasculature. Our technique was based on a previously-published experimental design for retinal imaging of tracers[88]. Anesthetized and dilated rats were placed on a modified Ludl microscope stage. The eye was placed on a +6 diopter plano concave lens (Edmund Optics) covered with a drop of Goniosol and embedded in the stage directly above a 4x or 10x Plan Apo objective. For VCAM-1 imaging, an Exfo X-Cite 120 metal halide excitation lamp source (Exfo Life Sciences) was routed through a FITC HQ excitation filter, a CRX50V dichroic mirror (Nikon), and a 655/40 emission filter (Omega Optical, Rockingham, VT). For neutrophil imaging, a Perkin Elmer FX-5400 xenon arc flash lamp or the X-Cite 120 lamp were used. The flash lamp source was set to a 20 Hz pulse at 24 VDC using a function generator and DC power source. Excitation light was passed through the same filter configuration as used for VCAM-1 imaging, except that a 565/20 or 605/20 emission filter (both from Omega Optical) was used. Imaging was performed using either a Andor iXon 885 EMCCD 12-bit camera (Andor Bioimaging, Belfast, Ireland), a Hamammatsu C7780 36-bit color CCD camera, or a Roper Photometrics 512B. Exposure settings were normally 100 msec for VCAM-1 or leukocyte tracking, with adjustments in gain and exposure time made as necessary using Image Pro Plus 5.1 software (Media Cybernetics) for the C7780 and 512B, or Andor iQ or iXon software for the 885 EMCCD. Continuous sequences of 70-100 frames were acquired in quick succession, with less than 110 msec between exposures for leukocyte tracking.

Ex vivo analysis of rat retina

Animals were sacrificed with 150mg/kg sodium pentobarbital (Sleepaway) administered via tail vein catheter. Eyes were extracted and either embedded and sectioned, or enucleated in 4% paraformaldehyde. Retinal tissue was removed from enucleated eyes and prepared for fluorescence microscopy using a drop of Aqua Poly-Mount solution. A Nikon TE2000U inverted fluorescence microscope was used to analyze retinal tissue flat mounts and sections. The C7780 used for *in vivo* imaging was also used for tissue analysis at various magnifications ranging from 100x – 1000x total magnification. For whole retinal tissue analysis, tissue mounts were scanned by a Genepix 4000b microarray scanner (Axon Instruments) with 532 and 635 nm lasers, with dual PMTs configured with 530-595 and 670/10nm bandpass filter sets, respectively.

Flow cytometry

Whole blood was collected by cardiac puncture from male Long-Evans rats into BD Vacutainer tubes lined with K3EDTA. Erythrocyte lysis was then peformed by incubation with BD PharMLyse at a 20:1 ratio of lysis buffer to whole blood for 15 minutes in the dark at room temperature to obtain 500 uL of a diffuse red suspension of white blood cells. Another fraction was set aside for isolation of pure neutrophils. The solution was then centrifuged at 400g in a Allegra X-22R unit with swinging bucket rotor (Beckman) at room temperature. The pellet was rinsed in 500 uL BD Pharmingen staining buffer (pH = 7.2) with 0.5% BSA, and 0.1% sodium azide as a metabolic inhibitor. Each pellet was then resuspended and rinsed twice more with staining buffer. Each 500 uL suspension of leukocytes were incubated with the following: 50 nM each of

QD585/RP-1 conjugate, QD525/CD45 conjugate, and QD585/isotype control IgG conjugate, 1 ug PE/RP-1 conjugate, 5 ug/mL acridine orange in 0.9% isoosmotic saline, and 10 nM Qtracker 525nm conjugate. A separate unlabeled fraction was also retained for analysis. A purified fraction of neutrophils was obtained from rat whole blood by density gradient centrifugation on Percoll. 2 mL of whole blood was layered on a discontinuous 2-step Percoll gradient consisting of 4 mL 1.0815 g/mL layered on 4 mL 1.1005 g/mL sterile medium in a 15 mL centrifuge tube. The gradient was centrifuged at 400g using a fixed-angle rotor (Allegra X-22R centrifuge, Beckman). Mononuclear cells and platelets formed a band at the top of the gradient, with granulocytes in the middle, and erythrocytes at the bottom. The pure granulocyte suspension was obtained using a Pasteur pipet and was estimated to be approximately 94% pure following lysis of contaminating erythrocytes. Isolated neutrophils were washed twice in PBS (pH=7.4), and incubated with 50 nM QD585/RP-1 conjugate, 1 ug PE/RP-1, 50 nM QD585/isotype control IgG conjugate, and 50 nM QD585 which were activated but not conjugated to antibodies. In addition, an unlabeled sample was retained. All samples were analyzed using a BD LSRII multicolor flow cytometer equipped with UV, blue, or green wavelength lasers. Bandpass filters were set at 585/42nm, 530/35nm, or 565/20nm. Analysis was prepared using Treestar Flowjo and BD FACSDiva software.

Conclusions and Future Directions

This work has outlined a quantum dot-based *in vivo* imaging technique for imaging inflammatory mediators at the cellular and molecular level. Advantages of this approach include a high signal to background ratio, continuous digital acquisition, narrow

emission spectra, and a uniform excitation source for all size-tunable nanocrystals. Our technique permitted imaging real-time molecular expression of VCAM-1, which plays a critical role in various diseases as well as endothelial cell homing in angiogenesis. Previous methods of imaging inflammatory mediators involved the use of radiolabeled antibodies and conventional organic fluorophores, which were limited by low signal to noise ratios, lack of specificity, and a challenge in facilitating long-term imaging applications. The ability to detect molecular expression in inflammation using high intensity nanoscale materials is a significant achievement, as it permits the development of early detection strategies which may expedite therapeutic interventions before tissue damage. The ability to also visualize *ex vivo* and *in vivo* labeled leukocyte subtypes will facilitate studies which seek to characterize individual components of an immune response in inflammatory disease. Furthermore, it will aid in studying detailed leukocyte-endothelial interactions in a host of diseases.

Future work is directed at the development of higher-intensity quantum dot probes for improved imaging of inflammatory mediators in diabetes using our technique. Improvements will include the use of antibody fragments as opposed to whole antibodies which include the Fc portion, to maximize circulation time and reduce nonspecific binding without a blocking step. Also, we will use our method to characterize molecular expression patterns for a variety of key adhesion molecules in diabetes. This will be achieved by the use of a multispectral imaging apparatus which can be used in conjunction with our fluorescence microscopy setup in order to spectrally-delegate regions of a CCD chip devoted to different emission bands, customized to narrow quantum dot wavelengths. With this data we will attempt to develop a more detailed

understanding of the function, and spatial regulation of multiple endothelial cell surface markers in this disease, to develop more accurate early diagnostic tools and therapeutics. The technique presented here demonstrates the utility of quantum dot nanocrystals for high resolution imaging of inflammatory processes.

APPENDIX A: SUPPLEMENTARY TABLES AND FIGURES

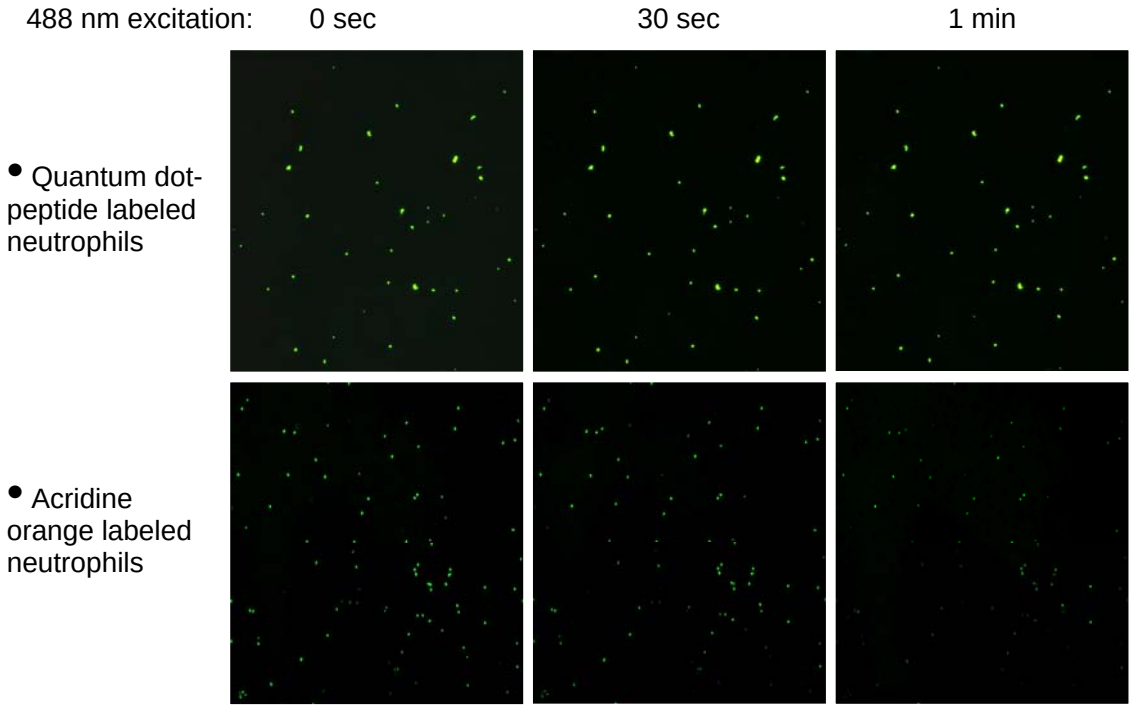

Figure I: Fluorescence microscopy time-lapse images of rat neutrophils labeled with either 10 nM QD565nm-peptide conjugates or the DNA intercalating dye acridine orange at 10 ug/mL of cell suspension. The fluorescence peak of the filter configuration was 490 nm, the optimal spectra for acridine orange, but suboptimal for QD, which are more intensely excited at UV wavelengths. Nevertheless, cells labeled with QD-peptide conjugates are more intense in all images, with acridine orange-labeled cells having low intensity and noticeable photobleaching after only 30 seconds. QD-peptide labeled neutrophil intensity was preserved for the entire hour of observation. This observation has important implications for *in vivo* imaging of leukocytes, especially in situations where long-term observations of stagnant leukocytes are used in diagnosis and therapy efficacy assessments.

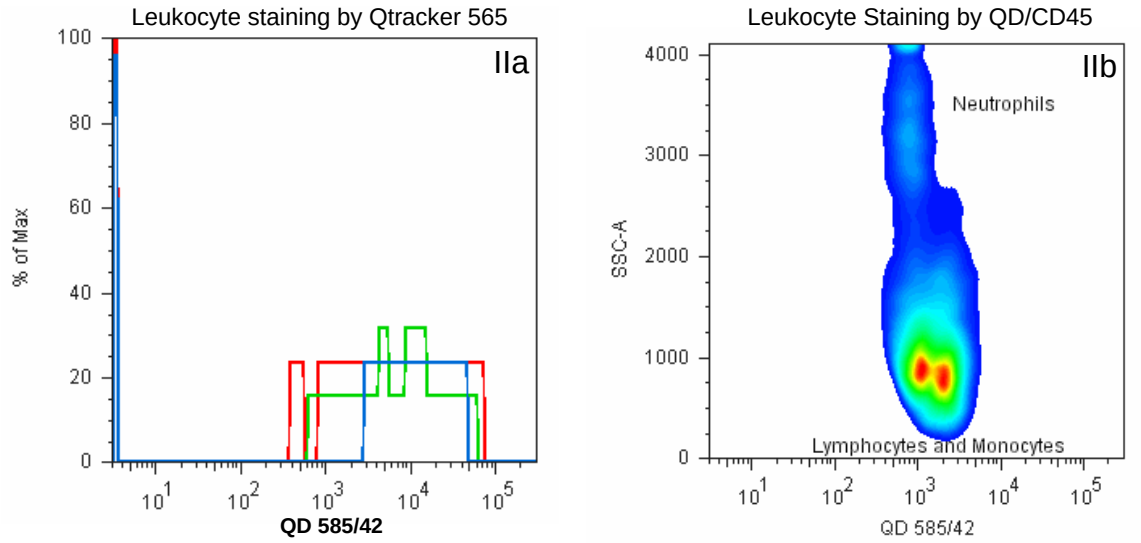

Figure II: Flow cytometric analysis of lysed whole blood from Sprague-Dawley rats labeled with Qtracker 565 peptide conjugate and QD-CD45 (leukocyte common antigen) conjugate. All three leukocyte subsets, monocytes (--), lymphocytes (--) and neutrophils (--) were labeled by the conjugates. Qtracker, a peptide conjugate, required longer incubation times, but also had lower targeting efficiency than antibody conjugates. Fluorescence intensity of labeled cells by both Qtracker (IIa) and QD/CD45 (IIb) was several log decades higher than unstained (not shown for clarity). Either method may be a suitable alternative for applications in which *ex vivo* labeled cells are reinfused *in vivo* for imaging purposes.

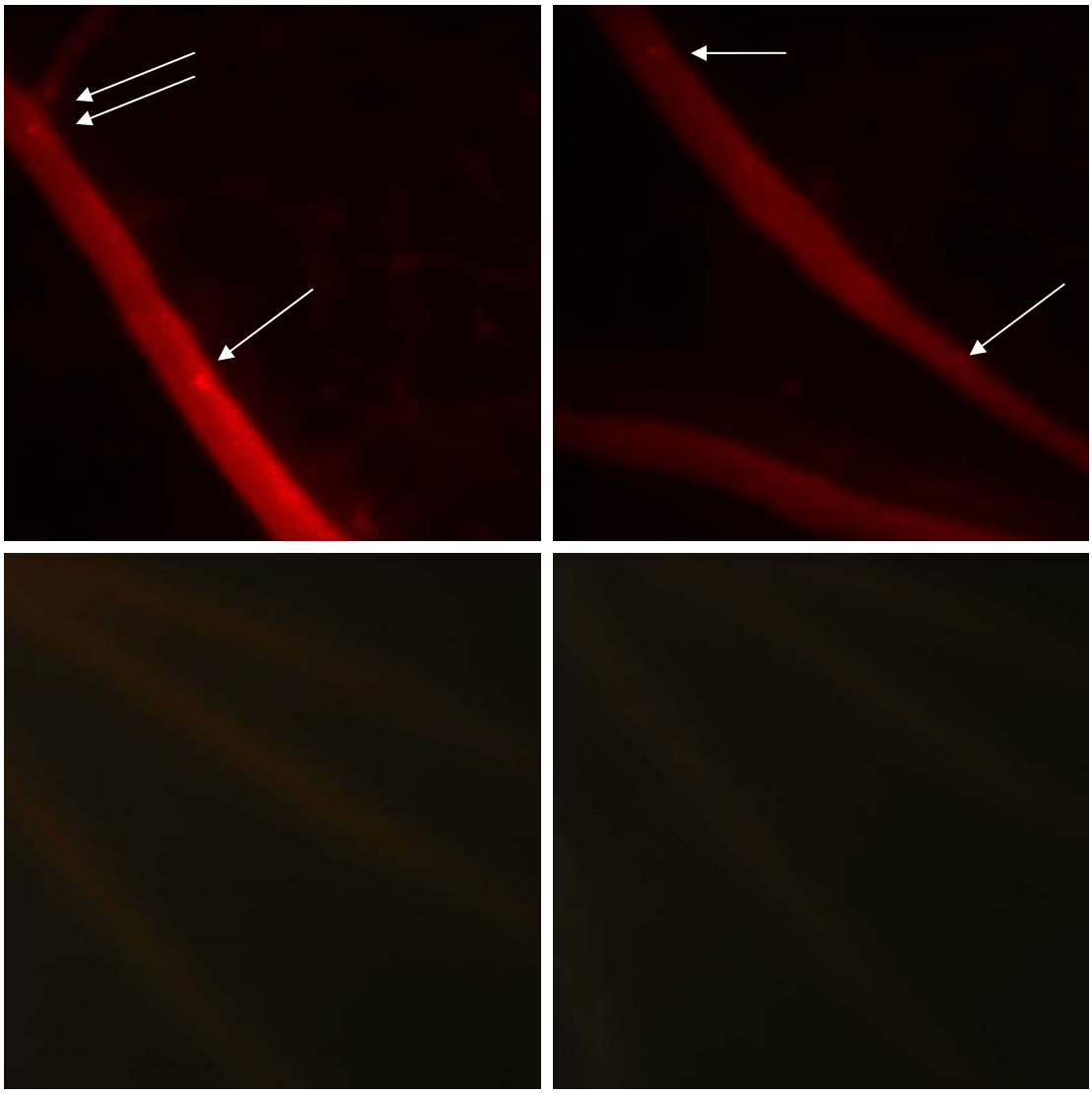

Figure III: Additional *in vivo* fluorescence microscopy CCD sequence extractions of STZ-induced diabetic and untreated Long-Evans rats. IIIa,b Bright, punctate spots indicate immediate VCAM-1 staining by QD conjugates shortly after injection. The QD conjugates, harnessed for their superior intensity properties compared to organic dyes, appear bright over background. The ability to detect small molecules even at low expression levels has important implications for early-detection of inflammatory disease markers which signal upcoming diabetic complications. IIIc Untreated control rat. Extraction of a CCD sequence showing enhanced brightness in vessels due to injection of conjugate. IIId As unbound conjugate clears, no VCAM-1 tethers can be observed in the untreated control rat. These observations reinforce the hypothesis that VCAM-1 expression may be used to stage inflammatory disease in the retina in the progression of diabetes.

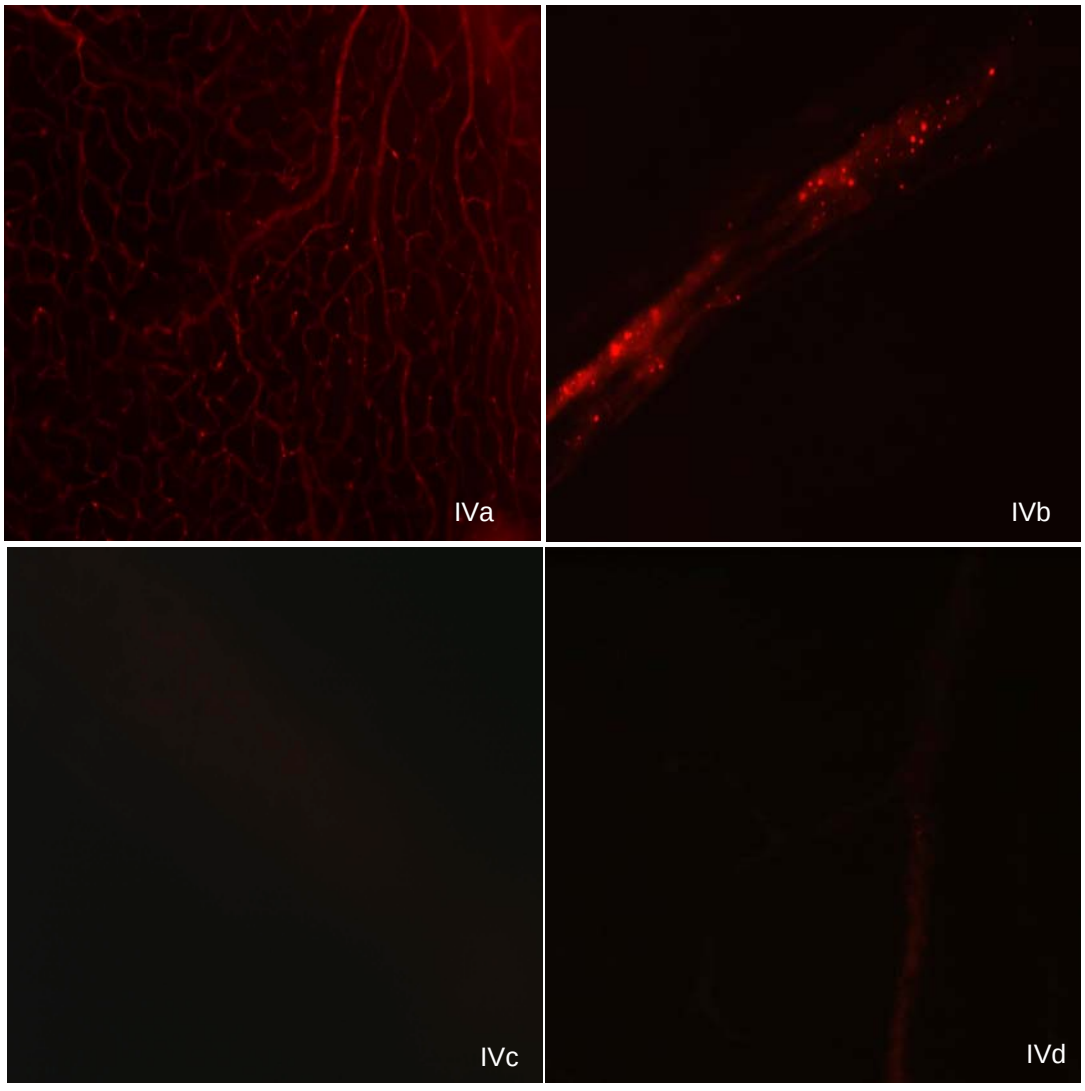

Figure IV: Observation of retinal tissue sections in control and STZ-treated diabetic Long-Evans rats following administration of QD/VCAM-1 conjugate. IVa 400x oil immersion image of STZ-treated retina. Dense staining was visible in the microcirculation *in vivo*, and the observation could be correlated to tissue studies. IVb 1000X magnification of a capillary bundle, densely stained with QD/VCAM-1. IVc,d Even with CCD gain adjustments and compensation, only very low levels of VCAM-1 were detected in non-treated retina. These sections are representative of the brightest signal observed.

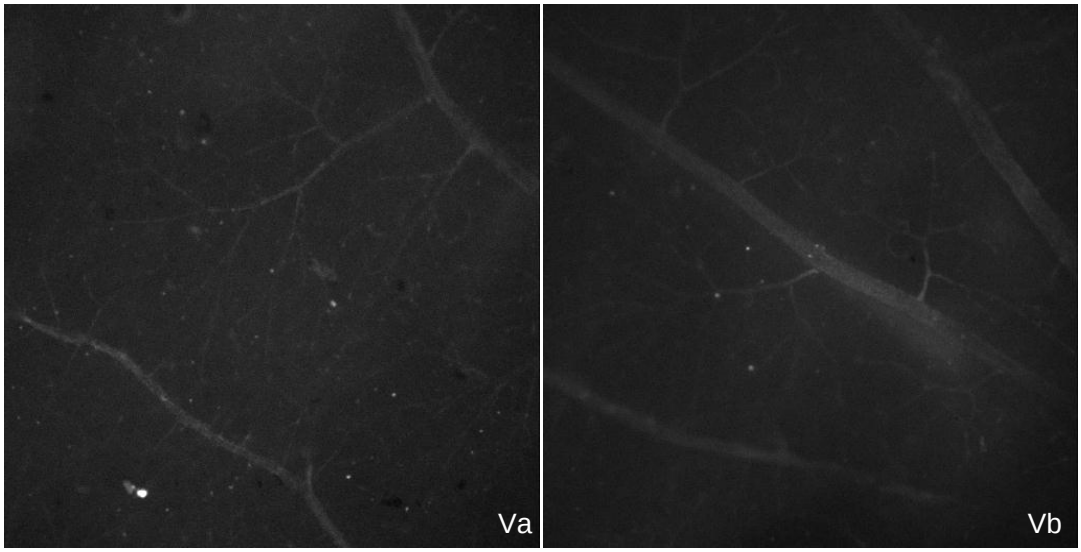

Figure V: Observation of retinal tissue sections after administration of *ex vivo* – labeled QD-peptide neutrophils in STZ-treated rat. Labeled neutrophils are visible long after *in vivo* imaging, and can be used to track extravasation and endothelial interactions in tissue, for example, using 3D reconstructions based on z-stacks. This image was taken using a Roper Scientific 512B 12-bit CCD with the Nikon TE2000U inverted fluorescence scope under 488 excitation with a narrow emission passband (565WB20, Omega Optical).

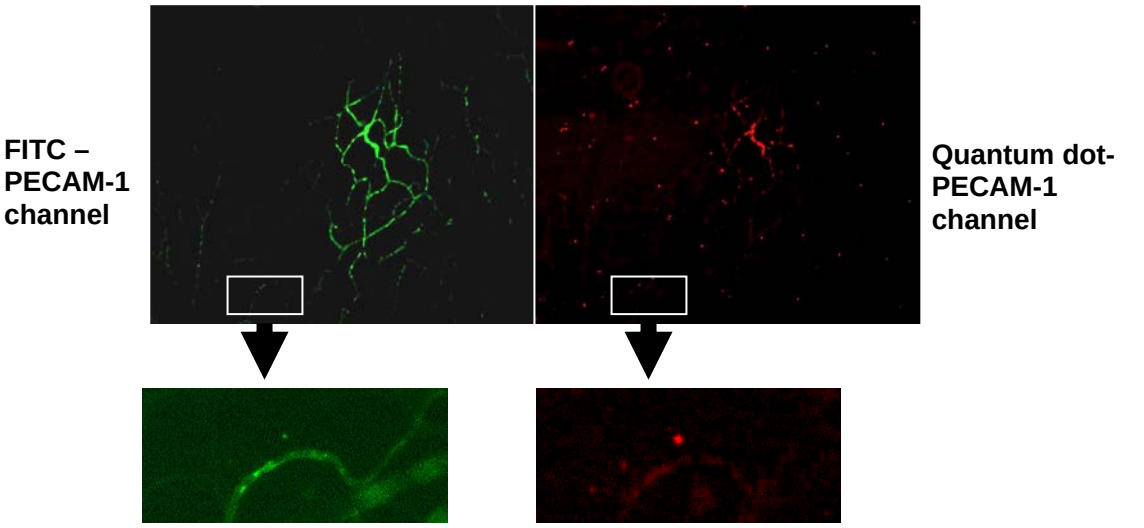

Figure VI: Detection of PECAM-1 on leukocytes and endothelial cells in tissue sections following *in vivo* administration of specifically-targeted QD conjugates. QD-PECAM-1 (M-20) conjugates were prepared according to the aforementioned protocols and were injected *in vivo* in a mouse model of endotoxin-induced uveitis (EIU) in order to assess the distribution of the cell adhesion molecule on leukocytes and endothelial cells. As an indicator of *in vivo* specificity, FITC-PECAM-1 (M-20) conjugates at an equal molar concentration were also injected. Tissue analysis by fluorescence microscopy above indicates a punctate staining pattern for PECAM-1 in the red (QD) channel that is not readily visible in the green (FITC) channel. Image processing and contrast enhancement reveal that FITC conjugates had indeed labeled the same punctate regions which the QD had labeled; this is shown in the inset maps. However, FITC were not as intense so as to readily enable visualization in tissue. This reflects on the fact that PECAM-1 is only weakly expressed on leukocytes. Thus, detection of weakly-expressed cellular surface antigens can be enhanced with QD conjugates.

Table I: Descriptions of weight and blood glucose measurements for untreated and STZ- treated Long-Evans rats used in this study.

Specimen	Weight	Blood Glucose (resting)
Male Long-Evans treated 6/21/05	**487g**	**266 mg/dL**
Male Long-Evans treated 6/21/05 #2	**490g**	**252 mg/dL**
Male Long-Evans treated 4/27/05	**309g**	**369 mg/dL**
Male Long-Evans untreated 4/18/05	**555g**	**68 mg/dL**
Male Long-Evans untreated 4/14/05	**562g**	**66 mg/dL**
Male Long-Evans untreated 4/5/05	**480g**	**66 mg/dL**

APPENDIX B: Supplementary Methods

Density Gradient Centrifugation of Sprague-Dawley Neutrophils for in vivo imaging and functional assays

In order to separate rat neutrophils from whole blood, in a 15 mL polypropylene centrifuge tube, 2 mL of male adult Sprague-Dawley rat blood was layered upon a 2-step isoosmotic Percoll gradient which consisted of a 4 mL 1.0815 g/mL Percoll carefully pipetted on top of a 4mL 1.1005 g/mL Percoll layer. Care was taken not to cause mixing or splashing at the interface of the gradient layers, as only a sharp change in density will promote isopycnic banding. Two counter-balanced 15 mL centrifuge tubes containing the same Percoll layers were layered at the top with Percoll density marker beads, which have color-coded silica beads designed to separate by their respective densities following centrifugation, as a quality control agent and as an aid in finding the cell band of interest.

The Percoll gradients were then placed in a 22º fixed-angle rotor and centrifuged at 400xg for 30 minutes at room temperature (Allegra X-22R, Beckman Coulter). Mononuclear cells and platelets formed a band at the top of the gradient, with granulocytes in between the two gradient layers. Erythrocytes were mainly situated at the bottom of the tube in a plug. Using a small Pasteur pipet, the PMN band was carefully

aspirated by running the pipet along the side of the tube, taking care not to disrupt the gradient or encourage excessive mixing. The granulocytes were then resuspended in 100 mM PBS. To ensure that no erythrocytes had contaminated the PMN layer, the suspension underwent erythrocyte lysis with 20 parts PharMLyse 1X lysis buffer to 1 part whole blood, for 15 minutes as room temperature in the dark, until a clear, red suspension was obtained. Cells were rinsed three times with PBS to ensure complete removal of lysis buffer. A sample of the suspension was analyzed by flow cytometry to confirm the concentration of neutrophils and the preservation of scatter properties. The suspension obtained from this method was found to purify a 95% pure sample of neutrophils.

Neutrophil functional assay: Adherence to tissue culture plastic

Neutrophils adhere to endothelial cells in the initial steps leading to diapedesis. Should neutrophils be labeled on their cell surfaces, it is important to ensure that the cells maintain a key component in their function, which is reversible adherence to a substratum in response to chemotactic factors. We sought to evaluate the general functional stability of QD-labeled neutrophils.

870,000 Percoll-isolated neutrophils were incubated with 50 nM QD/RP-1 conjugate. Cells were lysed with 20 parts BD PharMLyse ammonium chloride lysing solution to 1 part whole blood, and incubated in the dark for 15 minutes until a diffuse red solution was visible. 1 mL of a 2.3×10^6 cells/mL suspension was added to each of three 16-mm diameter wells of a 24 well plate. Wells were incubated for 30 minutes in a 37ºC incubator. Wells were then rinsed 3 times with 100 mM PBS (pH=7.4), and blotted dry. To each well 1 mL of 0.1% Triton X-100 was added, and the well plate was placed

on a shaker for 30 minutes. The suspensions were then withdrawn, and cells were counted using a flow cytometer configured to detect neutrophil scatter properties. The fraction of adherent cells to suspended cells was deemed acceptable if it was between 20-27% of total. Gallin and colleagues have found that using this technique, approximately 20-30% of control neutrophils will adhere to polystyrene[95].

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