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T cell – antigen-presenting cell interactions visualized in vivo in a model of antigen-specific inflammation

James T. Rosenbaum^{a,b,c}, Mischa B. Ronick^a, Xubo Song^d, Dongseok Choi^e, and Stephen R. Planck^{a,b,c}

^aDepartment of Ophthalmology, Casey Eye Institute, Oregon Health & Science University, Portland, OR 97239

^bDepartment of Medicine, Casey Eye Institute, Oregon Health & Science University, Portland, OR 97239

^cDepartment of Cell & Developmental Biology, Casey Eye Institute, Oregon Health & Science University, Portland, OR 97239

^dDepartment of Computer Science & Electrical Engineering, Casey Eye Institute, Oregon Health & Science University, Portland, OR 97239

^eDepartment of Public Health & Preventive Medicine, Casey Eye Institute, Oregon Health & Science University, Portland, OR 97239

Abstract

Videomicroscopy is being used increasingly to characterize the interaction of T cells and antigen-presenting cells (APCs) within lymphatic tissues but has not been reported, to our knowledge, at sites of inflammation. We employed intravital videomicroscopy to study an anterior uveitis model using DO11.10 T cells and ovalbumin (OVA). T cell movement in iris was consistent with a random walk independent of the presence of recognized antigen and had a lateral speed slower than T cells in lymph node. Lingering of T cells adjacent to APCs suggested that they were physically interacting. This apparent contact demonstrated antigen specificity when comparing results from DO11.10 cells with OVA versus bovine serum albumin (BSA) loaded APCs but not when comparing results from OVA-loaded APCs with DO11.10 versus HA clonotype 6.5 T cells. Further studies with this model system should clarify the contribution of T cell-APC communication at a site of inflammation, infection, or immunization.

Keywords

Uveitis; Intravital microscopy; T cells; Antigen-presenting cells; cell migration; mice

Introduction

T cell-APC interactions have been studied dynamically *in vitro* and components of the molecular structures that comprise the synapse have been elegantly characterized [1,2,3]. More recently *in vivo* documentation of T cell and APC trafficking and interactions have been documented, especially through the use of two photon confocal microscopy [4,5,6,7,8,9]. The

Corresponding author: Stephen R. Planck, Ph.D., Casey Eye Institute, L467AD, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239, Phone: 503-494-5110; Fax: 503-494-6875, E-mail: plancks@ohsu.edu.

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majority of these studies involve the lymph node or other lymphoid organ such as the bone marrow or thymus [10,11]. In contrast, the characteristics of T cell interaction with APCs at a site of inflammation are not well understood. Presumably the functional consequence of this interaction differs from the communication that results within the lymph node and varies in response to the presence of inflammatory mediators. Dynamic visualization of this process at a single cell level is difficult to achieve.

The eye affords some unique opportunities to image the immune response. The normal cornea is transparent, which not only permits light to enter the eye, but also facilitates the observation of structures posterior to it. The iris is readily seen behind the cornea. The iris is the potential target of T cell mediated inflammation which is known as iritis or anterior uveitis. Although uveitis or intraocular inflammation is a relatively rare disease, it ranks as one of the leading causes of blindness [12,13]. Anterior uveitis can occur in association with systemic inflammatory diseases such as ankylosing spondylitis, juvenile idiopathic arthritis, sarcoidosis, and Behçet's disease [14,15].

We have recently described techniques to label APCs within the iris with fluorescent antigen [16] and we have developed a model of T cell-mediated, antigen-specific inflammation within the iris [17]. Combining these two capabilities has allowed us to characterize the interaction between T cells and APCs in this model of anterior uveitis.

Materials and methods

Mice

Female, 6–13 week old BALB/c mice were used (Jackson Laboratories, Bar Harbor, ME). Transgenic DO11.10 and HA clonotype 6.5 mice, whose T cells recognize ovalbumin peptide (OVA_{323–339}) and influenza hemagglutinin peptide (HA_{111–119}) respectively in the context of I-A^d and I-E^d, had been extensively backcrossed to BALB/c [18,19,20]. They were obtained from Andrew Weinberg (Earle A. Chiles Research Institute, OR) and Hyam I. Levitsky, (Johns Hopkins University School of Medicine, Baltimore, MD), and bred in OHSU animal care facilities. Transgenic mice were 1–10 months old when used in experiments. The animal experimental protocols were in accord with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by our Institutional Animal Care and Use Committee.

T cell preparation and mouse model

Splenocytes were obtained by crushing the spleen of DO11.10 or HA mice and inducing hemolysis with red blood cell lysing buffer (Sigma; St. Louis, MO). Effector cells were generated from splenocytes upon incubation *in vitro* with the appropriate peptide, OVA_{323–339} or HA_{111–119} (2 µg/ml) (Synpep Co.), for four days. T cells were isolated using Lympholyte-M Cell Separation Media (Cedarlane Labs, Ontario, Canada), stained with orange CellTracker CMTMR (5.5 µg/10⁷ cells, Molecular Probes), and injected intravenously into naive BALB/c mice (2×10⁷ cells/animal). After 1–3 days these mice were challenged with a 4-µl intravitreal injection using a 30-gauge needle of 0.5 µg *E. coli* strain 055:B5 lipopolysaccharide (LPS; Sigma) plus 100 µg of either Alexa Fluor 350 (blue)- or 488 (green)-conjugated chicken ovalbumin (OVA; Sigma, Grade V) or bovine serum albumin (BSA; Sigma) in PBS.

Intravital microscopy

Labeled T cells in the iris stroma were observed by intravital epifluorescence videomicroscopy of 17 anesthetized animals with a modified DM-LFS Leica microscope and an Optronics DEI750 camera (Goleta, California). The animals were anesthetized with inhalation anesthesia

consisting of 0.2% isoflurane (Novaplus) in oxygen. This technique has been previously reported in detail [16,21]. CMTMR-labeled cells and pinocytic cells that had taken up green-fluorescent OVA or BSA were imaged simultaneously with a dichroic filter set (fluorescein/rhodamine). Iris cells that take up OVA have dendriform morphology and have previously been characterized for MHC class II, F4/80, and CD11c expression [16]. Because T cell movement in the iris tissue is sufficiently slow, areas of T cell-infiltration in the iris were imaged by time-lapse microscopy for 1–4 hours (1 frame/5 minutes) beginning 24 hours following antigenic challenge.

In vivo data processing and analysis

Iris movements due to animal breathing, eye rolling, and pupil contraction were observed in the time-lapse videos collected. To compensate for this movement all videos were stabilized using MediaCybernetics Image Pro Plus 5.0 software (Silver Spring, MD). All labeled T cells that stayed in the field of view for most of the recording were individually tracked in two dimensions using NIH ImageJ 1.36b (Bethesda, MD). Mean lateral T cell speeds were calculated and a mixed effects model was used to test the differences in speeds between the OVA and BSA-injected groups while mice were treated as a random factor. Mean displacement plots were also prepared in order to characterize T cell motion as directed, random, or constrained and to obtain mobility coefficients. A masked quantifier recorded the duration of probable T cell-APC interactions as the time that a T cell and a protein-loaded antigen presenting cell were within one T cell diameter of one another. All resolvable labeled T cells in the videos were analyzed for a total of 52 T cells in 7 OVA-injected mice and 28 T cells in 10 BSA-injected mice. Select videos were also stabilized by a novel image registration process that we are developing [22].

Ex vivo wholemount preparation and imaging

In some mice, iris wholemounts were prepared 24 hours after antigenic challenge. In addition to fluorescent OVA or BSA-injected mice that received OVA-specific DO11.10 T cells (Groups A and B, respectively), a group of mice were included that were injected with fluorescent OVA after receiving HA-specific T cells (Group C). Immediately prior to euthanasia, mice in each group were injected with either 750 µg FITC-dextran (MW 2000kDa; Sigma) or 50–150 µg Texas Red *Lycopersicon esculentum* (Tomato) lectin (Vector Labs, Burlingame, CA) to label blood vessels. Eyes were enucleated, dissected to remove the posterior of the eye and lens, and fixed in 4% paraformaldehyde at 4°C for 3–18 hours. The anterior segment of the eye, a hemisphere made up of the iris, ciliary body, and cornea, was then cut into the shape of a clover leaf such that it could be flat-mounted on a glass microscope slide in SlowFade Antifade solution (Molecular Probes) and imaged. Z stacks of iris regions where infiltrated T cells were present were acquired by deconvolution fluorescence microscopy with an Applied Precision Deltavision™ image restoration system. This includes the API chassis with precision motorized XYZ stage, a Nikon TE200 inverted fluorescent microscope with standard filter sets, halogen illumination with API light homogenizer, a CH350L Camera, and DeltaVision software. Deconvolution using an iterative constrained algorithm and additional image processing was performed using Imaris Bitplane software. Three-dimensional rotations were used for quantification. All resolvable labeled T cells in the images were analyzed for a total of 218 T cells in 4 Group A mice, 57 T cells in 3 Group B mice, and 75 T cells in 9 Group C mice.

Ex vivo data processing and analysis

A masked individual quantified possible T cell-APC interactions by determining the number of infiltrated CMTMR-labeled T cells that were physically contacting cells labeled by uptake of blue or green OVA. T cells were categorized as one of the following: 1) Associated T cells

were those in apparent tactile contact with an OVA-loaded APC; 2) Proximal T cells were those less than one T cell diameter from the nearest OVA-loaded APC; and 3) Distant T cells were those more than one T cell diameter from the nearest OVA-loaded APC. Intravascular T cells were excluded from these designations. Group 2 and 3 T cell populations were compared with the Group 1 population using chi square analyses.

Results

A network of fluorescent cells, many of which have a dendriform morphology, can be seen in the iris after injection of fluorescently tagged OVA directly into the eye (Figure 1A). We have previously reported that these cells include both dendritic cells and macrophages [23], though Camelo et al found uptake of soluble dextran to occur predominantly by macrophages in rat iris [24]. An antigen-specific accumulation of CD4⁺ T cells occurs after OVA or OVA+LPS injection into the eyes of mice that have previously received an intravenous injection of DO11.10 CD4⁺ T cells (Dullforce et al. [17] and Figure 1B). Significantly more labeled DO11.10 cells are found in the iris of eyes 24 hours after injection with OVA+LPS than with HSA+LPS (Figure 1B) or BSA+LPS (data not shown). Furthermore, if we inject similarly prepared T cells from the HA clonotype 6.5 mouse whose T cells are transgenic for the recognition of an antigen derived from hemagglutinin, many fewer labeled T cells are present within the iris despite the injection of ovalbumin (data not shown).

We used time-lapse intravital videomicroscopy capturing an image every 5 minutes for up to 4 hours to record infiltrating DO11.10 T cells and APCs in mouse irises (Figure 2 and supplemental video 1 and supplemental video 2). As we previously reported, the APCs labeled by ingestion of fluorescent OVA or BSA are relatively stationary [25]. In marked contrast, many of the T cells are seen migrating within the iris stroma.

We analyzed the videos to investigate if the migration of CD4⁺ T cells is altered by the presence of their relevant antigen in the iris. Since the uveitis model employed for this study involves the introduction of a foreign antigen and the use of a TLR 4 agonist, LPS, the APCs should be stimulated and are presumably elaborating chemokines that attract T cells. Mean displacement plots (MDPs) are commonly employed as a method to determine if cell movement is random, directed, or confined as by a structure such as a vessel or a collagen bundle [26]. The MDPs in Figure 3 include data from 42 DO11.10 T cells in the irises of seven mice that had been injected with ovalbumin and in 24 DO11.10 T cells in the irises of ten control mice injected with BSA. The linearity of the two plots indicates that the OVA-specific T cells' pattern of movement was predominantly random in both OVA- and BSA- injected eyes. Likewise the similar slopes indicate that there was no substantial difference in overall motility of these T cells. In support of this conclusion, we found that DO11.10 T cells moved at an average 2-dimensional speed of $1.6 \pm 0.3 \mu\text{m}/\text{min}$ in OVA-injected eyes, and this did not differ significantly if BSA rather than OVA was injected since in this setting the average speed was $1.3 \pm 0.1 \mu\text{m}/\text{min}$ ($p=0.4$). LPS was used to amplify the inflammation. It is possible that the LPS altered the number of cells moving in a directed fashion, but the number of cells entering the iris in the absence of LPS in this model is such that it is difficult to study an adequate number of cells without its concomitant injection. Although the predominant movement of T cells within the iris appears random, visual inspection of videos revealed T cell – APC encounters characterized by what appears to be brief, purposeful migration of the T cell directly toward the APC.

When viewing the videos, one can see individual DO11.10 cells that move toward, remain close to, and migrate away from APCs. To further address the question if the presence of relevant antigen alters the migration of T cells through direct interaction with APCs, we quantified the potential contacts between these cells. Because many iris macrophages and

dendritic cells have long processes that are difficult to see *in vivo*, we defined potential contact as when a labeled T cell was within one T cell diameter of an antigen-labeled cell. A majority of the DO11.10 T cells was in potential contact with at least one APC during the observation periods (mean 99 minutes) and of those cells, a majority was in potential contact with a single APC (Figure 4A). Some cells interacted with two and occasionally three APCs. Cells were often in contact at the beginning or end of the videos, so we determined the percent of time that a T cell was in contact with an APC during the recording period. DO11.10 cells tended to be in contact with OVA-containing APCs longer than with BSA-containing eyes but this did not reach statistical significance, $p=0.10$ (Figure 4B).

In order to improve our quantitation of cell contact, we elected to perform measurements on 3-dimensional images constructed from z-stacks of images obtained by deconvolution microscopy of iris wholemounts. Even with the higher resolution, the 3-dimensional nature of the interaction with extended cell processes was such that it was difficult to determine definitively in all instances if cell contact occurred. Consequently we elected to judge cell contact as (1) definitely associated, (2) proximal (i.e., possibly associated and no more than one cell diameter separating the T cell and the APC), or (3) distant (unassociated). Representative cells meeting these definitions are shown in Figure 5A and the results are in Figure 5B. A significantly higher percentage of DO11.10 cells was in contact with APCs in eyes injected with OVA than in eyes with a control antigen BSA ($p<0.0001$). By this criterion, antigen specificity clearly influences the positioning of T cells. However, this conclusion was not confirmed by comparison of the results obtained with DO11.10 and HA 6.5 T cells in OVA-injected eyes B (Figure 5B). A more exhaustive investigation is needed to determine if these results reflect fundamental differences in the transgenic T cell lines, in responses to the two foreign proteins, or in the efficiencies of APC labeling by the different proteins.

Discussion

We believe that this study is the first to report the interaction between T cells and APCs within an inflamed tissue. *In vivo* imaging of T cell dependent inflammation has been reported in a limited number of tissues which include central nervous system [27,28,29], skin, and synovium [30]. These studies have not included an analysis of T cell-APC interaction to our knowledge. Clearly the APC exerts a profound influence on the function of the T cell. The extent of that influence at a site of inflammation in comparison to effects within a secondary lymphoid organ is unknown. We are undertaking studies to assess if this contact between a T cell and APC within the iris results in activation of the T cell.

It is worthwhile to compare the migration which we quantitate with that which has been described for T cells within a lymph node. Lymphocytes with the T cell zone of a lymph node also follow paths that have been classified as analogous to random walks by mean displacement plots [26]. More recently it has been shown that the vast majority of lymph node T cells actively follow along fibers of the fibroblastic reticular cell network. Presumably the choice of which branch to follow is random unless an inflammatory response alters the presence of or sensitivity to factors that would favor one path over another [31,32]. The absence of this specialized matrix in the iris stroma likely contributes to the slower speed that we observed for T cells migrating in the iris. Estimates of the average lateral speed of T cells in lymph node vary from 4 $\mu\text{m}/\text{min}$ during an activation phase to about 11 $\mu\text{m}/\text{min}$ in the absence of an antigenic challenge [4,6, 33]. These are considerably faster than the 1–2 $\mu\text{m}/\text{min}$ lateral speed reported here and likely reflect tissue-specific differences in the nature of chemotactic signals and/or the extracellular matrix along which the cells must navigate.

The eye is an excellent organ for studies of T cell mediated immunological disease. Ocular inflammation is a common concomitant of inflammation elsewhere in the body in a multitude

of diseases which include rheumatoid arthritis, multiple sclerosis, ankylosing spondylitis, juvenile idiopathic arthritis, sarcoidosis, and Behçet's disease [14]. The corneal clarity allows intraocular inflammation to be imaged without resorting to any surgical trauma. On the other hand, the eye does have some unique immunological characteristics [34] including a lack of lymphatics in the iris, an abundance of transforming growth factor beta and other potential immunosuppressive factors within the aqueous humor, expression of cytokines such as FasL and TRAIL that promote apoptosis (though these are best characterized on the corneal endothelium and not within the iris), and susceptibility to anterior chamber associated immune deviation (ACAID), i.e., the suppression of a T cell mediated immune response when a soluble antigen is injected into the anterior chamber. Accordingly additional studies need to be performed to determine if our observations can be extrapolated to other tissues and diseases.

We were surprised that we could not demonstrate that a greater percentage of T cells were moving in a directed fashion and we could not consistently demonstrate that the presence of a recognized antigen affected the directed nature of T cell migration. An antigen-specific effect is strongly indicated, however, with the use of DO11.10 T cells and an irrelevant antigen, BSA. Furthermore the injection of endotoxin could exert an equal or greater effect relative to antigen vis a vis T cell migration, thus obscuring an antigen specific effect. This is a difficult hypothesis for us to test with the DO11.10 adoptive transfer model because the inflammation which we observe in the absence of endotoxin is mild and results in a very limited number of T cells in the iris.

In summary, our studies show that T cells do seek out antigen presenting cells within an inflamed tissue. This interaction occurs even if the antigen-presenting cell has not ingested an antigen which is recognized by the T cell. The ability to image T cell-APC interactions at a site of inflammation can be used in future studies to analyze the signals which facilitate or inhibit this interaction, the specific T cell and APC subsets involved, the effect of the interaction on T cell function and recruitment of additional leukocytes, and the migratory behavior of the T cell after its encounter with an APC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A. Distribution of OVA-labeled APCs and OVA-specific T cells in an inflamed iris. Image was captured 24 hr after intravitreal challenge with green-OVA+LPS of a mouse that previously received red OVA-specific T cells (supplemental video 1). Note that many of the cells that have ingested the green-OVA have a dendriform morphology and lie adjacent to the dark blood vessels. The red T cells are dispersed in the field of view. Original magnification: 200x. **B.** Antigen-specific accumulation of T cells is most pronounced at 24 hr after intravitreal challenge. Mice that had been injected with DO.1110 T cells received an intravitreal injection of LPS plus either OVA or HSA. The number of infiltrating T cells were counted by intravital

microscopy at the indicated times and the difference observed at 24 hr is statistically significant ($p < 0.002$; $n = 3$ mice)

Figure 2. Selected frames from a time-lapse video (supplemental video 2) in which a red OVA-specific T cell (arrow) approaches and then leaves a green BSA-loaded APC.

Figure 3.

Mean displacement plots for DO11.10 T cells in irises of eyes injected with LPS and either OVA or BSA. The DO11.10 T cell mobility is consistent with a random walk independent of the protein injected.

Figure 4.

A. When BSA was injected, fewer DO11.10 T cells infiltrated the iris stroma at 24 hours but the proportion that interacted with labeled APCs was not significantly different compared to OVA-injected eyes ($p=0.27$). The majority of DO11.10 cells interacted with at least one antigen-labeled APC during the imaging period (71% in OVA-injected eyes, 58% in BSA-injected eyes) and several T cells interacted with multiple APCs. **B.** DO11.10 cells tend ($p=0.10$) to contact OVA-loaded APCs for longer periods of time than BSA-loaded APCs. These data are based on 52 cells in 7 OVA+LPS-injected eyes and on 28 cells in 10 BSA+LPS-injected eyes. Many DO11.10 cells maintained contact with a single APC throughout the entire

imaging period (33% in OVA-injected eyes, 25% in BSA-injected eyes). The mean observation period for each cell was 99 min. The whiskers indicate SEM.

Figure 5.

A. Representative whole-mount image of an inflamed iris prepared at 24 hr post OVA+LPS challenge. Probable APCs are labeled by uptake of Alexa Fluor 350 (blue) ovalbumin, OVA-specific T cells are stained with CMTMR (red), and blood vessels are identifiable by FITC (green)-conjugated dextran. Arrows mark four T cells that illustrate the categories used for quantification. Whereas T cell A is clearly in close physical association with a blue APC (**associated**), T cell B is within one T cell diameter of the nearest APC but, due to the imperfect nature of APC labeling, it is unclear whether cell-cell interaction is taking place (**proximal**). Cell C is clearly not interacting with a nearby OVA-loaded APC (**distant**). Cell D is intravascular and was therefore excluded from the quantification. **B.** Eyes were removed at 24

hr post challenge from mice that received DO11.10 T cells and OVA (218 cells in 4 mice), HA 6.5 T cells and OVA (75 cells in 9 mice), or DO11.10 cells and BSA (57 cells in 3 mice) Images were captured from regions where T cell infiltration was evident. The differences in populations are statistically significant by chi square analysis of the cell counts: 3×3 analysis of all groups, $p=0.0001$; 2×3 analysis of DO11.10 OVA vs DO11.10 BSA, $p=0.0007$; 2×3 analysis of DO11.10 OVA vs HA OVA, $p=0.011$; 2×3 analysis of HA OVA vs DO11.10 BSA, $p=0.0001$.