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J Immunol 2004; 172:6701-6708; doi: 10.4049/jimmunol.172.11.6701
http://www.jimmunol.org/content/172/11/6701

Supplementary Material
http://www.jimmunol.org/content/suppl/2004/05/17/172.11.6701.DC1

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APCs in the Anterior Uveal Tract Do Not Migrate to Draining Lymph Nodes\textsuperscript{1}

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The migration of APCs from sites of infection and their maturation are critical elements in the generation of immune responses. However, the paths by which intraocular Ags migrate to draining lymph nodes are not known because the eye has limited lymphatic vessels. To date, only dendritic cells from the cornea and conjunctiva have been shown to emigrate. We demonstrate that phagocytic APCs in the anterior uveal tissues of the murine eye that ingest fluorescent latex beads do not migrate to regional lymph nodes. The beads are ingested in the uveal tract by cells expressing MHC class II, CD11c, or F4/80. Using intravital time-lapse videomicroscopy to monitor iris APC migration after anterior chamber injection of fluorescent Ag, fluorescently labeled APCs fail to move at multiple observation times, even in the presence of Ag and LPS. Whereas an as yet unidentified ocular nonphagocytic APC subset might migrate from the anterior uveal tissues, it is more probable that immune responses in the draining lymph nodes are engendered by soluble Ag escaping the eye through interstitial spaces. The inability of anterior uveal tissue APCs to migrate to lymph nodes may contribute to deviant immune responses that dominate after Ags are introduced into the anterior chamber. \textit{The Journal of Immunology}, 2004, 172: 6701–6708.

Despite immune privilege, the uvea or inner eye can suffer from a broad spectrum of systemic diseases such as sarcoidosis, ankyllosing spondylitis, or Behcet’s disease (1), as well as local immune-mediated inflammations. Within the mammalian eye are networks of APCs capable of presenting Ag to T cells (2). MHC class II\textsuperscript{+} cells isolated from the anterior segment of the rat eye are effective APCs (3, 4). These APCs have been well characterized in mice, rats, and humans, and comprise two basic types that express macrophage or dendritic cell phenotypic markers (5–7). In the mouse anterior segment, dendritic cells have been defined by their expression of CD11c and MHC class II. Ags introduced into rodent eyes are clearly presented in the submandibular lymph nodes draining the treated eyes (8, 9). Investigators (9, 10) have reproduced in a number of models the work of Egan et al. (8), who used a murine DO11.10 T cell transfer model to demonstrate that T cell responses to ocular Ags occur in the submandibular lymph nodes draining the ipsilateral eye. Yet, how the Ag introduced into the eye induces T cell activation in the draining lymph nodes remains an unresolved question.

Within the ocular tissues, lymphatics are present in the conjunctiva surrounding the globe, but no lymphatics within the globe itself have been discerned through immunohistology (11, 12). The only reported instance of lymph vessels in the globe has been when nascent lymphatics were identified in vascularized corneas (13, 14). The migration of green fluorescent protein (GFP)\textsuperscript{2}–marked CD11c\textsuperscript{+} APCs from one ocular tissue, the cornea, to the draining lymph nodes 24 h after allogeneic corneal transplantation was shown to occur despite the paucity of lymphatics (15). However, the migration of APCs from the protected internal ocular tissues, iris, ciliary body, retina, and choroid has yet to be demonstrated.

In our laboratory using intravital microscopy, we can monitor living cells in the anterior segment of the eye (16, 17). This segment is anterior to the lens and consists of the cornea, iris, ciliary body, limbus, trabecular meshwork, and the aqueous humor-filled anterior chamber (a.c.) (Fig. 1A). We and others (5–7) are able to detect macrophages and dendritic cells in the eyes of living mice after the introduction of fluorescently labeled Abs and protein Ag (7). In addition, we are able, using intravital microscopy and time-lapse videography, to detect the movement of APCs in the dermis of the ear (our manuscript in preparation).

Using a combination of these techniques, we investigated the migration of APCs from the internal tissues of the ocular anterior segment, the anterior uveal tissues, and compared this migration with that of the dermal APCs (18, 19). We marked ocular APCs using either their pinocytic ability to take up fluorescent proteins (20) or their phagocytotic ability to ingest fluorescent latex beads (21), and were able to demonstrate that previously identified phenotypes of ocular APCs take up the beads. Results indicated that, in contrast to dermal APCs, anterior uveal tissue phagocytic APCs do not migrate from their immune-privileged tissue.

Materials and Methods

\textit{Experimental mice}

Female, 6- to 10-wk-old BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) and DO11.10 mice transgenic for a TCR that recognizes an OVA peptide and backcrossed to a BALB/c background (22) (bred in Oregon Health & Science University animal care facilities) were used for these experiments. This work was supported by National Institutes of Health Grants EY13093 and EY13609. J.T.R., S.R.P., and the Casey Eye Institute are recipients of Research to Prevent Blindness awards. 

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Received for publication November 26, 2003. Accepted for publication March 22, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{3} Abbreviations used in this paper: GFP, green fluorescent protein; a.c., anterior chamber; ACAID, a.c.-associated immune deviation; DTH, delayed-type hypersensitivity; i.d., intradermal; TR-OVA, Texas Red-labeled OVA.
studies. In some experiments, BALB/c mice were depleted of neutrophils 24 h before study with an i.v. injection of 150 μg of anti-Ly-6 Ab, RB6-8C5 (BD Pharmingen, San Diego, CA) (23, 24). The animal experimental protocols were in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by our institutional animal use committee.

**Fluorescent reagents**

Fluorescent latex beads were from Molecular Probes (Eugene, OR) (red 605 nm emission or green 500 nm) or from Polysciences (Warrington, PA) (red 570 nm or green 515 nm). Abs were obtained from BD Pharmingen (anti-MHC class II (I-Ad), polyclonal mouse IgG2b and Serotec (Raleigh, NC) (anti-CD11c, anti-F4/80, polyclonal rat IgG2b, and polyclonal hamster IgG). Abs and OVA (Sigma-Aldrich, St. Louis, MO) were labeled with AlexaFluor Green (488 nm), AlexaFluor Red (594 nm), or AlexaFluor Blue (350 nm) (Molecular Probes), as described in Becker et al. (7). Texas Red-labeled OVA (TR-OVA) was obtained from Molecular Probes.

**Microcapillary injections into a.c. and ear dermis**

BALB/c mice were injected in the a.c. of the eye or into the dermis of the ear pinnae (intradermal (i.d.)) with 2 or 3 μl of various combinations of fluorescent 2-μm latex beads (~2 × 10^3 beads), fluorescent OVA (150 μg), or fluorescent Abs (1.5–3 μg). The injections were performed with ultrathin, pulled borosilicate glass needles (outer diameter ~50 μm) and Hamilton syringes under direct visualization through a surgical microscope, and eyes were immediately washed with 0.5 ml of PBS to avoid uptake by the conjunctiva of any marker that leaked during injection.

To generate a strong danger signal, endotoxin-induced uveitis was initiated by injecting into the vitreous cavity 250 ng of Escherichia coli 055:B5 LPS (List Biological Laboratories, Campbell, CA).

**Histology**

High magnification images of iris whole mounts containing fluorescent beads and OVA injected in vivo were obtained by confocal microscopy of tissues harvested at 4 and 24 h postinjection. The iris, ciliary body, and cornea were dissected and cut to lie flat. The tissues were fixed overnight in 10% neutral-buffered formalin and mounted using the Slow-Fade Anti-fade kit (Molecular Probes). Ear pinnae were frozen in OCT compound (Miles, Elkhart, IN), cryosectioned, fixed in 4% paraformaldehyde, and mounted with Slow-Fade. Ear sections and iris whole mounts were imaged by a Bio-Rad (Hercules, CA) 1024ES system attached to an inverted Nikon Eclipse TE300 confocal microscope using Bio-Rad software.

Spleens and lymph nodes (submandibular, superficial cervical, and axillary) were harvested 6, 24, 48, and 96 h after injection of fluorescent labels. All organs were immediately frozen in OCT mounting medium. Sections (10 μm) were mounted using Slow-Fade Anti-fade kit and examined using a fluorescence microscope for the presence of fluorescent beads and OVA. MHC class II, F4/80, CD11c, and CD11b+ cells were also identified in the sections by previously described standard immunohistological methods (7).

**In vivo immunohistology**

This method has been described elsewhere (7) and is given in this work in brief. Mice were injected with green anti-MHC class II (I-Ad), anti-CD11c, or anti-F4/80 Abs; blue OVA, and red fluorescent beads in the a.c. Intravitreal microscopy was performed 6 h later, after excess protein had drained from the aqueous humor and thereby reduced the fluorescent background. Digital videos were recorded in the same area of the iris with three different filters to detect the separate fluorescence emission wavelengths. Comparably labeled mouse IgG2b, rat IgG2b, and hamster IgG Ig served as isotype controls for the anti-MHC class II, anti-F4/80, and anti-CD11c Abs, respectively, and revealed no nonspecific staining.

**Time-lapse video imaging**

Intravitreal epifluorescence videomicroscopy was performed using a modified Leica DM-FLS microscope and either a black and white CF 84/NIR camera (Kappa Scientific, Gleichen, Germany) or a color Optronics DEI-750CE camera (Optronics International, Chelmsford, MA), as previously described (16, 17). The movement of cells marked by uptake of green OVA or red beads was observed using time-lapse intravitreal microscopy recording three frames per minute for at least 1 h at various time points after injection. Adobe After Effects (Adobe Systems, Mountain View, CA) software was used to align frames, which stabilized the time-lapse videos, thereby minimizing the motion artifact due to the animal’s breathing and eye rolling, and aiding in the visualization of cell movements. The movies showed online are ~7 s long (~1 h real time at 30 frames/min) and are best viewed on a continuous loop.

**Results**

**Definition of phagocytic and APCs in the anterior segment**

To study the migration of APCs from the eye, we needed to mark these cells in vivo. When fluorescent OVA was injected into the aqueous humor of the a.c., pinocytic cells of the anterior segment took up the fluorescent protein and could be identified using fluorescence microscopy. Although non-APCs may pinocytose protein, APCs are highly efficient pinocytes (20) and are certainly included among the cells marked by the fluorescent OVA. APCs from the anterior uveal tissues (iris, ciliary body, and anterior choroid), as well as trabecular meshwork cells and some corneal APCs (Fig. 1) were identified in this manner. The fluorescent protein was taken up predominantly by the trabecular meshwork cells in the angle of the chamber (Fig. 1), as expected because the normal outflow of the aqueous humor filters through the trabecular meshwork. Fluorescent cells were also found in the iris, ciliary body, peripheral cornea, and anterior choroid (Fig. 1). Some fluorescent OVA passed through the choroid and sclera in the posterior segment. This flow has been postulated to be a route of outflow called the uveoscleral route (25).

We also sought to mark ocular APCs with larger particulate objects that could not escape the eye in fluid through interstitial spaces. For this purpose, we used 0.04- and 2-μm-diameter fluorescent latex beads, which could be phagocytosed and carried by an APC, but were too large to diffuse through interstitial spaces as free particles. Fig. 1C illustrates the tissues whose cells phagocytosed 0.04-μm beads after they were introduced into the a.c. The same pattern was obtained when either 2- or 0.04-μm-diameter beads were used. The beads were found in the same anterior segment tissues as the fluorescent OVA (Fig. 1C), except the cornea. Most beads found in the cornea were in the endothelium lining the a.c., and only a few were located in the corneal stroma, mainly around the injection site. As the cells of the corneal stroma took up few beads, the beads can be considered to mark only cells in the tissues in direct contact with the aqueous humor: the corneal endothelium, ciliary body, trabecular meshwork, and iris.

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*The on-line version of this article contains supplemental material.*
The dendritic cells and macrophages in mouse iris have been well characterized by in vitro immunohistology (5, 6), and more recently by this laboratory using in vivo immunohistology (7). Most of the iris APCs are dendritiform in morphology, as seen in iris whole mounts taken 24 h after the injection of fluorescent OVA a.c. (Fig. 2A). Many of the same cells that took up fluorescent OVA also phagocytosed beads (Fig. 2A), although some phagocytic cells did not pinocytose the fluorescent OVA, nor did all the fluorescent OVA-labeled cells take up beads (Fig. 2A). The latter observation is probably due to a lack of accessibility to the beads. The flow of aqueous humor sweeps across the iris, causing the beads to accumulate in the angle of the chamber, thus reducing the number of beads accessible to the iris APCs. The iris APCs readily took up the beads with three-dimensional constructs of confocal images revealing ingested beads deep within the iris stroma (see online supplemental data movie 1).

We previously demonstrated that in vivo immunohistology can classify iris APCs into distinct macrophage (CD11b+ CD11c−) and dendritic cell (MHC class II+ CD11c+) phenotypes (7). We used in vivo immunohistology to characterize further the cells that phagocyte the 2-μm latex beads. In this technique, fluorescent Abs to APC markers injected into the a.c. bind to cells expressing their epitopes and are taken up by the cells, resulting in a fluorescent cell. Excess Ab is washed out of the eye with the continuous replenishment of the aqueous humor, and the fluorescent cells are clearly visible during intravital microscopy. Fluorescent Ab is diluted in the blood and is not discernible elsewhere in the body. Control Abs of the same isotype, but irrelevant specificity, produce a scant background fluorescence on intravital microscopic images (7). The mass of injected Abs is 1–2% of the dose used for OVA, so uptake by pinocytosis is below the level of detection. Fig. 2B illustrates the results obtained using a red fluorescent Ab against MHC class II (I-Ad), blue fluorescent OVA, and green fluorescent 2-μm beads. Six hours after injection, green beads have been taken up by cells marked with the blue OVA, of which the majority stained MHC class II+ (red). In vivo immunohistology was performed with fluorescent beads, OVA and anti-MHC class II, F4/80, and CD11c Abs. Of the cells that take up fluorescent OVA, 70% (±27%) were MHC class II+, 49% (±25%) F4/80+, and 81% (±12%) CD11c+. These values are consistent with previous results from our lab (7) and elsewhere (5, 6). Of the cells that took up OVA and also stained for MHC class II, F4/80, or CD11c, 36% (±20%), 40% (±16%), or 47% (±17%), respectively, had ingested the 2-μm-diameter beads.

In summary, fluorescent OVA was clearly taken up by APCs in the eye. Moreover, 2-μm latex beads coinjected were also taken up, albeit at a lower frequency by MHC class II+, F4/80+, and CD11c+ APCs. The beads therefore could be used to discover the migration patterns of these ocular phagocytic APC subsets, with the assumption that the ingested beads do not substantially hinder the ability of the cells to migrate.

We sought to determine whether APCs containing engulfed 2-μm latex beads could migrate to draining lymph nodes. The migration of APCs from the skin to the lymph nodes has been demonstrated by Randolph et al. (21) using 1-μm fluorescent latex beads, and more recently by Bajenoff et al. (26) using 40-nm beads. We verified that similar migration from the skin occurs in our system using 2-μm latex beads. First, we looked at the proliferation of transferred OVA-specific, CFSE-labeled, DO11.10 cells in lymph nodes after intradermal injection of OVA. The results reveal that the superficial cervical, and not the submandibular, lymph nodes drain our intradermal injection site (data not shown). When green fluorescent 2-μm beads were injected into the dermis, they formed a clump at the site of injection. Outside the clump, some beads were ingested by MHC class II+ cells (Fig. 3A). Some fluorescent beads reached the marginal sinus of the draining superficial cervical lymph nodes within 6 h of intradermal injection (see Fig. 4A, top right panel). Twenty-four hours after injection, the beads were observed in the node paracortex, and the cells engulfing them were MHC class II+, F4/80+, and CD11b+ (Fig. 3B). The beads were not found in CD11c− lymph node cells 24 h after injection, but were found at 96 h (Fig. 3B). These results confirm those of Randolph et al., who reported the conversion of immature macrophages into a CD11c+ dendritic cell type during the migration from the dermis and their late arrival in the lymph nodes (day 3).

The intradermal beads do not simply move as free beads through the afferent lymphatics. Randolph et al. (21) previously reported that 1-μm beads injected into close, but distinct areas of the head and ear were not found combined in the same APCs, which would indicate mixing of free beads in the lymph, but in different APCs within the lymph node. We have repeated these experiments with the same results and conclusions (our unpublished data).

From these experiments in the skin, we can conclude that the ingestion of 2-μm beads does not block the migration of dermal APCs to the draining lymph nodes. We also established that beads injected into the subconjunctival tissue are transported to the ipsilateral submandibular lymph node (data not shown). There is no evidence that such latex beads affect the migration of APCs. It is reasonable to presume that the intracellular APCs that phagocytose the beads are not thereby prevented from migrating to lymph nodes.
Given the ability to identify all known anterior segment APC phenotypes in vivo with two markers, their ability to take up fluorescent OVA, and to ingest latex beads, we sought in subsequent experiments to trace the migration of ocular APCs to the lymph nodes that drain the head.

APCs in the anterior uveal tract do not arrive in the lymph nodes draining the head

When OVA is injected into the a.c., the Ag is presented by APCs to T cells in the draining submandibular lymph node. This was first demonstrated by Egan et al. (8). We repeated this experiment observing T cell proliferation in both submandibular and superficial cervical lymph nodes and in no other lymph nodes (data not shown). We checked APC migration to the submandibular lymph node and the nearby superficial cervical node. When fluorescent TR-OVA was injected into the a.c., it was observable in the submandibular lymph nodes within 6 h (Fig. 4A). Cells illuminated by the fluorescent dye accumulated subsequently in the marginal sinus and at the 24- and 96-h time points were observed penetrating into the T cell paracortex (Fig. 4A). Interestingly, no green fluorescent 2-μm beads coinjected a.c. with the TR-OVA were observed in either submandibular (Fig. 4A) or superficial cervical lymph nodes (our unpublished data) at any time.

In contrast, green fluorescent beads arrived in the superficial cervical lymph nodes 6 h after i.d. injection into ear pinnae, and by 24 and 96 h beads could be observed penetrating further into the paracortex (Fig. 4A). TR-OVA coinjected i.d. was observable in the superficial cervical lymph nodes draining the ear by 24 and 96 h after a.c. or i.d. injections, respectively. Green fluorescent beads were only observed in the superficial cervical lymph nodes draining the ear. Green beads in spleen, 24 h after injection into the eye. C. Only TR-OVA+ cells were found in the submandibular lymph nodes draining eyes injected 24 h earlier with TR-OVA, green fluorescent beads, and LPS (original magnification ×100).

Although the introduction of OVA induces a mild inflammation in the eye, it might be speculated that it is not a sufficient danger signal to induce APCs to migrate. However, when LPS was injected into the vitreous of the eye as a danger signal, similar results were obtained with the introduction of beads and TR-OVA into the a.c. The enlarged submandibular lymph nodes contained Texas Red+ cells, but no beads were found 6 and 24 h (Fig. 4C) after a.c. injection. Neither beads nor Texas Red+ cells were observed in the superficial cervical lymph nodes draining LPS-treated eyes.

The beads in the a.c. tend to stay clumped in the angle of the chamber, but some can eventually escape the eye. Occasional beads can be found in the spleen (Fig. 4B) 24 h after injection. No
Iris APCs do not move at any time during OVA- or endotoxin-induced uveitis

Our previous results clearly implied that APCs, which take up latex beads, do not leave the eye. This is in direct contrast to dermal APCs that arrive in the draining lymph nodes within 16–18 h of Ag challenge. Previously, we have used intravital microscopy to monitor the movement of APCs in the ear dermis (our manuscript in preparation). In time-lapse videos covering 1 h, APCs that have ingested beads could clearly be seen moving 12–13 h after injection of the beads. In similar experiments, the movement of cells containing fluorescent beads could be seen 12–13 h after injection of the beads. Three experiments were performed for every time point with consistent results. The intravital microscopy results are best appreciated by viewing the movies online.

The fluorescent OVA injected into the a.c. was taken up by the network of iris APCs previously defined (Fig. 2). At no time point (4, 5, 6, 12, 24, and 48 h) after the injection was any movement of the OVA-labeled cells out of the iris field observed during the 1-h recordings (movie 2). In contrast, a minority of the red beads co-injected with the OVA did move at the early time points (2–6 h). These moving beads could be split into two categories: apparent random wandering, and those that flowed across the surface of the iris toward the angle of the eye. The latter were in the aqueous humor or rolling on the surface of the iris as they were generally out of the focal plane of the iris (best example, movie 6). The majority of beads introduced into the a.c. end up clumped at the trabecular meshwork in the angle of the eye due to the flow of the aqueous humor. The occasional bead-containing cells that wandered, but did not migrate out of the observed area moved in a manner seen previously with time-lapse intravital microscopy of neutrophils infiltrating the iris with endotoxin-induced uveitis (our manuscript in preparation). Neutrophils are the predominant infiltrating cell type at the early stage of OVA-induced inflammation, and are competent phagocytes. It is probable that the moving beads observed in these movies were beads ingested by neutrophils.

The soluble OVA used in these experiments induced only a mild inflammation in the eye. A potential limitation of the experiments is that there is an insufficient danger signal to cause a substantial migration of APCs out of the anterior segment. The injection of LPS into the vitreous of the eye provides a strong danger signal in the ocular tissues, and results in endotoxin-induced uveitis. Despite the ensuing enhanced inflammation, no fluorescent OVA-labeled cells migrated from the observed areas of iris at any time, whether 7, 12, 24, or 49 h after LPS challenge (movies 3–5; our unpublished data). The number of beads moving within the iris was dramatically increased at the 7-h time point (movie 3). This coincides with the previously reported influx of neutrophils attracted by the LPS stimulus (17, 27). At the later time points, 12, 24, and 49 h postinjection, a minority of cells that had ingested beads wandered around in the iris, but no directional migration was observed. There were few free-floating beads left, and the influx of neutrophils had passed (movies 4 and 5). None of the moving, bead-containing cells had taken up fluorescent OVA, and at no time was a bead-containing cell observed to migrate from the iris field of view (movies 3–6).

Neutrophils are the predominant infiltrating cell type early in the inflammation of the iris caused by OVA or LPS (17, 27). The injection of RB6-8C5, a mAb specific to the Ly-6-G Ag expressed on neutrophils, has been consistently shown to deplete completely mice of neutrophils (23, 24). In our hands 24 h after the i.p. injection of 150 μg of RB6-8C5, mice were depleted of neutrophils, as judged by flow cytometry analysis of blood leukocytes. Time-lapse intravital microscopy was performed on RB6-8C5-treated mice 5 h after the injection of OVA and beads, when the peak of neutrophil infiltration would have normally occurred. Despite a flow of beads in the aqueous humor across the iris, no moving beads were observed within the iris (movie 6). The network of cells defined by fluorescent OVA uptake was unchanged in the neutrophil-depleted mice; neither they nor beads in the iris moved appreciably during the hour of observation. This result supports the argument that the beads moving within the iris in the previous time-lapse recordings had been ingested by neutrophils, although other Ly-6-G/C+ cells, such as blood plasmacytoid dendritic cells (28), might also be involved.

It should be emphasized that, despite the distraction caused by some beads’ Brownian-like motion, the majority (~80%) of beads in the focal plane of the iris did not move after a.c. injection. Furthermore, at no time point did APCs defined by the fluorescent beads and OVA migrate from the observed iris, even with LPS-induced inflammation, and, as reported above, no beads were found in the draining lymph nodes.

Discussion

Migration of APCs from the eye

These studies were initiated to discover the route by which Ag from the anterior segment of the eye migrates to draining lymph nodes. It was expected that Ag-bearing ocular dendritic cells migrate to the draining lymph nodes because Ag-specific T cells proliferate in the lymph nodes draining Ag-challenged eyes (8). However, of all the ocular tissues, lymphatics are normally present only in the conjunctiva (11, 12). Nascent lymphatics have been found in inflamed cornea (13, 14), but in no other ocular tissues. While attempting to discover the route of APC migration from the anterior uveal tissues, we believe our experiments actually show that these APCs, whether dendritic cells or macrophages, do not migrate to draining lymph nodes, and therefore, immune responses in the draining lymph nodes are engendered by Ags escaping the eye extracellularly.

Our results clearly show that phagocytic APCs in the anterior uveal tissues do not migrate to draining lymph nodes. In sharp contrast to the skin, no beads are observed in the submandibular and superficial cervical lymph nodes draining the bead if they are injected into the a.c. As phagocytic APCs in the anterior uveal tissues took up the beads (Fig. 2), the lack of beads in the draining lymph nodes at any time up to 96 h after injection revealed that no anterior uveal tissue phagocytic APCs migrated to the nodes.

We argue that actually no anterior uveal APCs migrate to the lymph nodes. The APCs of the anterior ocular segment have been identified by immunohistology (5, 6, 29). Work by McMenamin et al. (5, 6) demonstrated that the iris, ciliary body, and choroid contain a network of macrophages and dendritic cells. Macrophages were identified by the pan macrophage markers, F4/80 and SER4, whereas dendritic cells were MHC class II+, mainly F4/80+ and SER4+. The authors reported only weak expression of CD11c, CD80, and CD86, concluding that most dendritic cells were immature. In a more recent study by our group, we were able to confirm the network of MHC class II+, and F4/80+ APCs described by McMenamin et al., and using the technique of in vivo
immunohistology, were able to define further the expression of CD11c, CD80, and CD86 on iris APCs (7). We were able to identify CD11b^+ iris APCs; however, we have failed to detect other markers found on some dendritic cell subsets (CD8, CD4, and DEC205). Recent work has revealed various types of APCs in the cornea (29). Mature dendritic cells (CD11c^+, MHC class II^+), CD11b^+, CD80^+, CD86^+) are located in the stromal periphery, while immature dendritic cells (CD11c^− MHC class II^−, CD11b^−, CD80^−, CD86^−) are in the central cornea. Macrophages (CD11c^−CD11b^+) were found in the posterior stroma. Importantly for our experiments, in all the studies, anterior segment APCs can be identified either by MHC class II, CD11c, or pan macrophage markers such as F4/80. Our results demonstrate the iris APCs that were able to phagocyte beads were identified by in vivo immunohistology as MHC class II^+, F4/80^+, or CD11c^+ (Fig. 2). Therefore, we believe the fluorescent latex beads may mark all the phenotypes of dendritic and macrophage cells present in the anterior uveal tissues and demonstrate that no APCs migrate from these tissues to the draining lymph nodes.

We cannot absolutely exclude the possibility that a subset of unlabeled cells, which take up Ag, but not beads, could be migrating to the draining lymph nodes. The beads are taken up only by 36−47% of the MHC class II^+, F4/80^+, or CD11c^+ OVA-phagocytic cells. We believe this is the result of poor accessibility as opposed to the presence of nonphagocytic APCs. After ∼200,000 beads are injected into the a.c., the flow of aqueous humor carries most beads to the trabecular meshwork, and relatively few are accessible to the iris APCs lining the a.c. The fluorescent OVA should be taken up by all macrophage and dendritic cell types. Even Langerhans cells, which are considered poor pinocytes, have been marked by their uptake of fluorescent markers in migration studies (30, 31). If cells that took up OVA and not beads did migrate, such movement should have been observed in the intravital time-lapse movies. However, none of the intravitreal movies taken show any movement of OVA-labeled APCs out of the iris at 4, 5, 6, 12, 24, and 48 h post-OVA challenge (movie 2; our unpublished data). Nor when LPS, a strong danger signal, was injected into the vitreous humor did the OVA-labeled cells show any tendency to migrate from the iris in any movie taken after challenge (movies 3−6). The intravitreal movies support the contention that iris cells, which take up Ag, do not migrate to draining lymph nodes.

As yet, we have been unable to induce anterior uveal APCs to migrate despite treatments with a variety of activation stimuli such as LPS (Fig. 4C, and movies 3–5) and anti-CD40 (our unpublished data). We speculate that the immune-suppressive factors in the aqueous humor (32–35), e.g., TGF-β and α-melanocyte-stimulating hormone, that bathe the tissues are repressing any migratory activity of the APCs. In addition, a consequence of the absence of lymphatic vessels is that there are no lymphatic endothelial cells to secrete chemotactic factors to induce APC migration (36).

Routes of Ag out of the eye

Liu et al. (15) have shown that 24 h after the transplantation of corneal allografts expressing GFP, donor CD11c^+ APCs expressing GFP can be found in the ipsilateral lymph nodes. With our a.c. injections, cells in the corneal endothelium take up beads (Fig. 1), but only few phagocytes in the corneal stroma around the injection site, and none in the corneal epithelium, take up the beads. Therefore, we could not assess the migration of the APCs from the corneal stroma or epithelium. The fluorescent OVA observed in the submandibular node 6 h after injection is not caused by corneal APCs taking up the Ag and migrating to the node. Only patchy localization of corneal APCs was observed at 24 h after allograft transplantation, the earliest time point reported by Liu et al. (15), implying that the fluorescent OVA we observed in the node at the 6-h time point (Fig. 4A) exited the eye extracellularly.

The majority of the aqueous humor passes through the filter of the trabecular meshwork and enters directly into venous blood. Extracellular OVA escaping this way would be diluted in the blood. The soluble protein would be taken up by the spleen and would not preferentially localize to the submandibular lymph nodes, as was found in our experiments. There is experimental evidence that a.c.-associated immune deviation (ACAD) is induced by F4/80^+ ocular APCs that exit the eye directly into the bloodstream and migrate to the spleen (37, 38). The discovery of beads in the spleen is consistent with the migration of these ocular APCs into the bloodstream. Based on our intravitreal microscopy results, it is unlikely that iris APCs migrate to the spleen. Although we can speculate that it is APCs of the trabecular meshwork, where most beads accumulate, that pass into the bloodstream and are picked up by the spleen, we have as yet no data identifying which bead-containing cells are involved in this migration. Indeed, we cannot exclude the possibility that free beads may cross the trabecular meshwork epithelium by way of transcellular channels or by transcytosis, and thereby enter Schlemm’s canal and the bloodstream. Although occasional ocular APCs might migrate to the spleen, this does not negate our premise that anterior segment APCs do not migrate to regional lymph nodes, given that the routes through blood and lymph are distinctly separate.

An alternative route through interstitial spaces, the uveoscleral outflow, enables part of the aqueous humor (~16%) (39) to escape the extraorbital conjunctival tissues (40, 41). This route of drainage was first identified in studies from the 1960s of human and monkey eyes (40, 41), but the exact path or paths of the outflow have not been well defined. In the mouse, a potential route of uveoscleral outflow was identified when fluorescent dextran illuminated a trail of tissue through the choroid and eventually into the sclera (25). Indeed, we can show similar results with TR-OVA (Fig. 1B). We hypothesize that this is the route by which soluble Ags escape the anterior segment and arrive in the submandibular lymph nodes.

Immune responses in the submandibular lymph nodes

Soluble Ag injected into the a.c. of the eye causes a deviant immune response, ACAD, which is characterized by abrogated delayed-type hypersensitivity (DTH) responses and Th1 type Ab production (9, 37, 38, 42). The progression of the deviant response has been well studied and is initiated by F4/80^+ APCs in the spleen (37). We postulate that if ocular APCs arrived in the submandibular node, a normal rival immune response would be generated in the lymph node and enable the restoration of DTH responsiveness and Th1-type Ab production. This speculation is supported by the recent work of Itano et al. (43). Soluble Ag injected in the dermis of the ear pinnae arrived in the draining lymph nodes in two waves, the first within 30 min after the injection was composed of extracellular Ag. The second wave arriving 18–24 h later was comprised of Ag-loaded dermal dendritic cells. The first wave of Ag presentation was sufficient to induce full T cell proliferation, but not a DTH response to a subsequent challenge. Only the arrival of dermal APCs in the second wave enabled the creation of DTH responses to subsequent challenge. Given the presence of lymphatics draining most tissues in the body, the arrival of soluble Ags ahead of dendritic cells in lymph nodes would be anticipated upon Ag introduction in most sites. However, the immune responses normally generated have been clearly demonstrated to be dependent upon the arrival of APCs. We postulate that with limited lymphatics draining the eye, anterior uveal tissue dendritic cells do
not migrate to the lymph node and a second wave of Ag presentation is lost. We cannot exclude the possibility that APCs in the conjunctiva picked up some Ag and carried it to lymph nodes. These APCs might not have received the local inflammation signals needed for them to activate immune responses in the lymph node. The proliferation of Ag-specific T cells observed by others and us in the submandibular lymph nodes upon a.c. challenge (unpublished data) (8, 9) is caused solely by the extraocular uptake of soluble Ags. We speculate such T cell activation does not generate DTH responsiveness and allows ACAID to dominate the immune response. Of note, some ocular APCs, CD11c+ dendritic cells from corneal allografts, do arrive in the submandibular lymph nodes (15). Although these cells are poor APCs, they do induce subsequent graft rejection, with a correlation between the number of APCs leaving inflamed corneas and the speed of the cornea’s subsequent rejection (15).

The eye is an immune-privileged organ. Many factors contribute to this role, including the immunosuppressive cytokines and neuropeptides, e.g., TGF-β and α-melanocyte-stimulating hormone (32–35), and the constitutive expression of CD95L (Fas ligand) (44, 45). Physical attributes also help maintain immune privilege, including avascularity of tissue, e.g., the cornea and lens, and the presence of barriers similar to those found in the placenta and brain that inhibit the trafficking of leukocytes through these tissues (34). The apparent lack of APC migration from the anterior uveal tissues is therefore but one of many factors contributing to the immune-privileged status of the eye.

Other immune-privileged organs include the CNS, reproductive organs, and the fetus. For Ags in the brain, immune response in the lymph nodes may be generated in a similar way to the anterior uveal tissues. Soluble Ag introduced into the brain can cause the normal generation of Th2 Ab responses and activation of pre-uveal tissues. Soluble Ag introduced into the brain can cause the organs, and the fetus. For Ags in the brain, immune response in the normal mouse eye. Br. J. Ophthalmol. 83:598.

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