Experimental Induction and Three-Dimensional Two-Photon Imaging of Conjunctiva-Associated Lymphoid Tissue

Philipp Steven, Jan Rupp, Gereon Hüttmann, Norbert Koop, Carmen Lensing, Horst Laqua, and Andreas Gebert

Purpose. Conjunctiva-associated lymphoid tissue (CALT) is assumed to be a key location for the generation of adaptive immune mechanisms of the ocular surface, but functional studies of CALT are still lacking. The purpose of this study was to establish an animal model that enables functional analysis of immune mechanisms going on within CALT. In addition, the use of two-photon microscopy, a new optical method, was evaluated for examining complex immunological interactions of CALT by volume (three-dimensional [3-D]) and time-dependence (four-dimensional [4-D]) in vivo.

Methods. The conjunctiva of female BALB/c mice was repeatedly challenged with topical Ochlamydia trachomatis serovar C or a solution of ovalbumin and cholera toxin B. Two-photon microscopy was conducted on explanted, unfixed, and unstained eyes with adjacent nictitating membranes.

Results. After three to five stimulations, CALT was detected exclusively in the nictitating membrane of 73% (C. trachomatis) or 70% (ovalbumin/cholera toxin) of the animals. CALT mainly consisted of CD45R/B220 B cells and CD4+ and CD8+ T cells. Electron microscopy showed intraepithelial lymphocytes and follicles consisting of lymphocytes, dendritic cells, and macrophages. Two-photon microscopy based on tissue autofluorescence allowed all components of CALT to be detected three dimensionally. High-resolution images were generated in tissue depths of 65 μm below the mucosal surface.

Conclusions. This study introduces a novel mouse model for functional investigations of CALT. Topical stimulation with C. trachomatis or ovalbumin/cholera toxin B reliably leads to CALT generation at the nictitating membrane. The use of two-photon microscopy enables groundbreaking 3-D and, in the future, intravital 4-D investigations of immunologic processes initiated in CALT.

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**METHODS**

Fifty female BALB/c mice, 12 weeks of age, were obtained from Charles River Laboratories (Sulzfeld, Germany). Care and treatment of the animals were undertaken in accordance with the regulations of the University of Lübeck and the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. All treatments were performed with the mice under deep anesthesia with ketamine (Ketanest S; Pfizer, Freiburg, Germany) and xylazine (Rompun vet; Bayer Health Care, Berlin, Germany).

**Topical Conjunctival Stimulation with *C. trachomatis* or OVA/CT-B**

Stocks of *Chlamydia trachomatis* (serovar C) were grown on HeLa cells, according to a standard protocol. Elementary bodies were harvested, centrifuged, and diluted with sucrose-phosphate-glutamic acid buffer (SPG). Five microliters containing 5000 IFU *C. trachomatis* were applied topically to each eye of 24 mice with a sterile pipette on day 0, 2, 4, 6, and 8. Twelve female mice received a nontraumatic inoculation of 5 μL of a mixed solution of 0.25 μg/mL ovalbumin (OVA) and 0.1 μg/mL cholera toxin B (CT-B) on days 0, 2, and 4. As a control, six animals received 5 μL SPG topically on days 0, 2, 4, 6, and 8. On days 5, 7, 9, 11, 13, and 15, seven animals (four animals/eight eyes, SPG control) were euthanatized, the eyes, together with adjacent conjunctiva were dissected (Table 1).

**Immunohistochemistry and Transmission Electron Microscopy**

The dissected ocular tissue was either frozen in liquid nitrogen for immunohistochemistry (IHC) or fixed in a solution containing 2% glutaraldehyde and 0.6% paraformaldehyde in 0.1 M Na-cacodylate buffer for transmission electron microscopy (TEM). The specimens were embedded in Araldite (Merck, Darmstadt, Germany) and ultrathin sections were prepared and examined by light and electron microscopy (Axioskop 2; Carl Zeiss Meditec, Oberkochen, Germany; and EM 400 T; Philips, Eindhoven, The Netherlands). Frozen specimens were serial sectioned with a cryostat and stained with toluidine blue. Immunohistochemical analysis was conducted with anti-mouse antibodies directed against CD45R/B220, CD4, follicular dendritic cells (FDCs; BD Pharmingen, San Diego, CA), and CD8 (Santa Cruz Biotechnologies, Santa Cruz, CA). Alexa Fluor 488 or 555 goat-anti-rabbit antibodies (Invitrogen, Carlsbad, CA) or a biotinylated rabbit-anti-mouse antibody (Dako, Glostrup, Denmark) were used as secondary antibodies to reveal labeled cells. Nuclear counterstaining was conducted with Hoechst 33258 (Sigma-Aldrich, St. Louis, MO).

**Two-Photon Microscopy**

The two-photon microscope (Dermainspect; Jenlab, Jena, Germany) was equipped with a tunable infrared femtosecond-laser (720–920 nm tuning range; Wide Band MaiTai; Spectra Physics, Mountain View, CA). After the animals were euthanatized, the eyes, together with adjacent conjunctiva were removed and immediately glued into a six-well plate using Vetbond (3M, St. Paul, MN). Without further fixation, the specimens were covered with DMEM/F12 (Invitrogen) and examined (IR Achroplan LD 40/0.80 W objective; Carl Zeiss Meditec). Autofluorescence image stacks of CALT regions were made at 750 nm excitation wavelength and comprised up to 80 images and volumes up to 200 × 200 × 70 μm.

**RESULTS**

**Histologic Analysis of Induced CALT**

Topical application of *C. trachomatis* or OVA/CT-B reliably induced lymphocyte aggregates in the conjunctiva of the nictitating membrane (Fig. 1, Table 1). Overall expression rates of 73% CALT in *C. trachomatis*-stimulated and 70% in OVA/CT-B-stimulated animals were obtained. The highest expression rates were from day 8 to 11 after first stimulation. Control specimens revealed no organized lymphoid tissue, except one animal on day 9 that showed one small lymphoid aggregate.

Conventional histology and TEM showed lymphoid aggregates of varying shape and size located beneath the outer surface of the nictitating membrane (Fig. 2). The covering stratified squamous epithelium was thinned, presenting only two to four cell layers and containing numerous lymphocytes within intraepithelial pockets (Fig. 3). Blood and lymphatic vessels were located in close relation to or within the aggregates. Lymphatic capillaries possessed a thin endothelium with overlapping cellular processes and displayed a large lumen relative to the thickness of the wall in comparison to blood capillaries. The blood capillaries were fed by two prominent vessels that emerged from the adjacent tarsal conjunctiva and extended along either margin to the apex of the nictitating membrane. Certain blood vessels showed a thickened endothelium with cytoplasmic extensions that protruded toward the lumen. Intravascular lymphocytes were preferentially located at the aggregate-facing side of the blood vessels, migrating across the vessel wall (Fig. 2c). IHC showed that the lymphoid aggregates contained lymphocyte subsets, typical for primary lymphoid follicles. The center of these follicles was predominantly composed of CD45R/B220- B cells (Fig. 2). A few CD8+ and CD4+ T cells (Fig. 2) were located within the center but mainly surrounding the B cells.

**Table 1. Stimulation Protocol and CALT Expression**

<table>
<thead>
<tr>
<th>Day</th>
<th><em>C. trachomatis</em> (n = 48 eyes)</th>
<th>OVA/CT-B (n = 24 eyes)</th>
<th>Control (n = 12 eyes)</th>
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<tr>
<td></td>
<td>Stimulation</td>
<td>CALT/Eyes</td>
<td>Stimulation</td>
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<tr>
<td>0</td>
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<td>15</td>
<td>6/8</td>
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whereas a few CD8+ T cells were detected within the epithelium. Follicular dendritic cells were not detected by IHC.

No morphologic differences or differences in the composition of lymphocyte subsets were noted between C. trachomatis and OVA/CT-B-induced aggregates. C. trachomatis-stimulated animals did not show any signs of destruction of the conjunctiva. Apart from the follicles generated, the mcosa of the stimulated animals did not differ from that of the control animals and showed no accumulation of inflammatory cells.

Two-Photon Microscopy of CALT

Two-photon microscopy of fresh, unfixed conjunctival tissue produced high-contrast autofluorescence images of the lymphoid follicles. The nonlinear effect of two-photon excitation resulted in a strong signal and low noise by producing autofluorescence in a small focal volume as a precondition for the generation of high-resolution images in scattering tissues (Fig. 4). Pictures that featured a resolution better than 1.0 μm were made at depths of 65 μm or more. At a 730-nm excitation wavelength, sections parallel or perpendicular to the surface revealed epithelial cells with bright cytoplasm and dark nuclei. Superficial, intermediate, and basal cell layers of the epithelium could be distinguished by the reduction of cell size and shape (Figs. 4b, 4c). The transition between basal epithelial cell layers and subepithelial lymphocytes was clearly demarcated by the nonfluorescing basal lamina (Fig. 4d). In the subepithelial tissue, fibroblasts, macrophages, and lymphocytes could be readily distinguished by their morphology and fluorescence intensity. According to light and electron microscopic observations, the lymphocytes showed a thin rim of cytoplasm surrounding their dark round nucleus, whereas fibroblasts were larger and possessed spindle-shaped processes of the cytoplasm. Macrophages displayed the highest intensity of autofluorescence with clearly detectable cytoplasmic inclusions (Fig. 4i). All cells were located within a network of bright fluorescing fibers of the connective tissue. Fifteen to 20 μm beneath the basal cell layer of the epithelium, lymphoid follicles extended downward, and individual lymphocytes could be identified at depths of 65 μm below the conjunctival surface (Figs. 4e, 4f). In close relation to the follicles, displaying clearly visible vessel walls, blood capillaries filled with erythrocytes (not shown) and lymphatic vessels containing typical endothelial folds (Figs. 4e, 4f) were located.

**DISCUSSION**

CALT has not yet been included in functional immunologic studies. Instead, based on merely morphologic studies, the
Earlier investigations did not report that CALT is present in the conjunctiva of unstimulated laboratory BALB/c mice, and the authors did not report whether the nictitating membrane was included in the morphologic analysis. Our findings showed CALT exclusively on the outer surface of the murine nictitating membrane. We interpret the specific location of CALT as a consequence of a tear-film–dissolved pathogen accumulation before drainage through the efferent tear duct, whose openings are located opposite the nictitating membrane. Therefore, the duration of mucosa pathogen contact is likely to be the longest in this area, leading to the induction of CALT. In analogy, BALT is mostly located at bifurcations of the bronchi where airflow turbulences likewise might accumulate airborne pathogens.

Topical conjunctival challenge with C. trachomatis (Sero- var C) or ovalbumin with cholera toxin B reliably generated CALT in 70% (OVA/CT-B) or 73% (C. trachomatis) of the eyes investigated. Even though an invasion of chlamydiae could be demonstrated by immunohistochemical detection of intracellular inclusions (data not shown), the mice did not develop ocular surface destruction or further clinical signs of inflammation such as lid thickening or erythema. The frequency and extension of OVA/CT-B-induced CALT was similar to C. trachomatis-induced CALT and the cellular and structural components of CALT were comparable in both experiments. In the control group, CALT was present in only 1 (8%) of 12 eyes. The formation of lymphoid follicles within two weeks after initial stimulation is concordant with previous findings of Sakimoto et al.17 The early conjunctival response stands in contrast to the 14-day latent period of systemic immune responses, but may be characteristic of the investigated nictitating membrane of the mouse.

Within CALT, CD45/B220+ B cells and CD4+ and CD8+ T cells were found predominantly. Further studies should be focused on the functional relevance of better defined T- and B-cell subsets in the two types of stimulation. Whereas topical stimulation of the conjunctiva with ovalbumin is known to induce T-cell energy,29 chlamydia infection of the conjunctiva is associated with T- and B-cell activation.30,31 Ocular surface diseases in humans exhibit a broad spectrum from most common conditions such as dry-eye syndrome and conjunctival allergy to less frequent but severe conditions, such as corneal transplant rejections, that often comprise difficulties in treatment. Our novel animal model allows various ocular surface diseases to be directly investigated in the near future with regard not only to CALT. Accepting the demonstrated requirement of previous pathogen stimulation, the continuing investigation of antigen-uptake and immunologic response related to CALT opens up fascinating perspectives.

We showed in the present study that two-photon microscopy has the potential to be a key technology in the study of antigen uptake, antigen processing, and immune reactions in the conjunctival compartment of living animals over hours. Without the use of intravitral dyes, tissue preparations or transgenic animals (e.g., expressing green fluorescent protein variants) CALT and its overlying epithelium is directly imaged at high resolution. All components of CALT lymphoepithelium, lymphoid follicle, and adjacent blood and lymphoid vessels can be visualized in three dimensions (3-D), and time lapse imaging of a certain tissue volume will achieve a 4-D analysis. In contrast to confocal microscopy, the investigation of highly scattering tissues such as lymphoid tissue is feasible in depths up to 90 μm with an unrivaled low risk of phototoxic damage. Assuming an ideal technical setup, a theoretical lateral resolution of 0.575 μm could be achieved that allows differentiating single mitochondria or lysosomes, for example. Besides fluorescence intensity, measurements of fluorescence-lifetime (FLIM) and spectral analysis of the excited fluorescence give

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**FIGURE 3.** Electron microscopy of CALT. (a) A lymphoid follicle containing densely packed lymphocytes and dendritiform cells (arrows) is located below the epithelium (E) within the subepithelial space. (b) Lymphoepithelium: Lymphocytes (arrow) are located within epithelial pockets. (c) Intravascular lymphocytes penetrate the vessel wall (arrows) at the follicle-facing side. Magnification: (a) ×2100; (b) ×2900; (c) ×3200.

function of CALT has been derived from the well-established theory of MALT function.24 In this context, it is concluded that B cells proliferate within CALT, recirculate and consecutively produce IgA in the conjunctiva and the lacrimal gland. It has even been hypothesized that CALT is involved in an immune privilege of the ocular surface.25 The present study demonstrates that, under defined experimental conditions, mice generate organized lymphoid tissue in the nictitating membrane, with high reproducibility. This tissue consists of a lymphoepithelium, lymphoid follicles with B- and T-cell zones, adjacent blood vessels that have thickened endothelia as well as lymphoid vessels. As these components are characteristic for MALT, the term murine CALT is justified. In this context, we encounter the nictitating membrane as part of the conjunctiva (Fig. 1), for several reasons: (1) Both regions, nictitating membrane and the rest of the conjunctiva (bulbar and tarsal) are covered by the characteristic multilayered epithelium. (2) This epithelium seamlessly extends from the nictitating membrane toward the rest of the conjunctiva. (3) Nictitating membrane and conjunctiva share one network of blood vessels. These findings are in agreement with those in earlier reports,26 and therefore a functional differentiation or compartmentalization seems not to be adequate.

FDCs were not found within the generated follicles, indicating that cells having ultrastructural features of dendritic cells could represent interdigitating dendritic cells (IDCs). The paucity of FDCs fits the findings in BALT in mice.27 BALT has been investigated in detail28 and contains lymphoid follicles bearing a B-cell zone and a T-cell zone that structurally resemble CALT. Although BALT is not present in the normal lung of adults, the functional relevance of BALT for the mucosal immune protection is generally accepted, and the term bronchus-associated lymphoid tissue is well established.

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further functional information—for example, intracellular NAD(P)H metabolism—and could lead to characteristic “fingerprints” of the different tissue components. Fluorescent microspheres or semiconductor nanoparticles (quantum dots) may be promising tools, because their highly defined emission spectra can easily be detected by means of spectral two-photon microscopy. As described for lymph nodes, lymphocyte tracking will enable analyzing the immediate reaction of CALT regarding chlamydia infections of mice.

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References


FIGURE 4. Two-photon microscopy of CALT. (a) Diagram of CALT with adjacent lymphatic vessel (L). Large arrow: orientation of the two-photon microscope objective. The section planes correspond to the images (b–e). (b) View parallel to the surface. Superficial epithelial cells had dark nuclei (arrows) and fluorescing cytoplasm. (c) Epithelial cell size decreased with scanning depth. (d) Subepithelial space with lymphocytes (arrows). Dotted line: transition between basal cell layer and subepithelial tissue. The lymphocytes, featuring a thin cytoplasmic rim, formed a lymphoid follicle. (e) A lymphatic vessel (L) with typical endothelial folds (arrow) was located in spatial relation to the follicle. (f) At a 65-μm depth, individual lymphocytes within the follicle were still distinguishable. Scale bar, 50 μm. (g) Three-dimensional reconstruction of the image stack. Two orthogonal section planes demonstrate epithelium, follicle and lymphatic vessel. Individual lymphocytes are distinguishable in the reconstruction (arrows). (h) Individual orientation of section planes allows extensive analysis of the 3-D structure of CALT. (i) High-resolution autofluorescence images of macrophages (top left), fibroblasts and connective tissue fibers (top right), lymphocytes (arrows, bottom right), and cartilage of the nictitating membrane (bottom left). Scale bar, 10 μm.
24. Knop E, Knop N. Eye-associated lymphoid tissue (EALT) is continuously spread throughout the ocular surface from the lacrimal gland to the lacrimal drainage system (in German). Ophthalmologe. 2005;100:929–942.