Nasolacrimal Duct Closure Modulates Ocular Mucosal and Systemic CD4+ T-Cell Responses Induced following Topical Ocular or Intranasal Immunization

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Both topical ocular and topical intranasal immunizations have been reported to stimulate the ocular mucosal immune system (OMIS) and the systemic immune system. Nasolacrimal ducts (NLDs) are the connecting bridges between the OMIS and nasal cavity-associated lymphoid tissue (NALT). These ducts drain topical ocularly administrated solutions into the inferior meatus of the nose to reach the NALT. Inversely, NLDs also drain intranasally administrated solutions to the mucosal surface of the eye and thus the OMIS. This unique anatomical connection between the OMIS and NALT systems provoked us to test whether the OMIS and NALT are immunologically interdependent. In this report, we show that both topical ocular administration and topical intranasal administration of a mixture of immunodominant CD4+ T-cell epitope peptides from herpes simplex virus type 1 (HSV-1) glycoprotein D (gD) emulsified with the CpG2007 mucosal adjuvant are capable of inducing local (in conjunctiva) as well as systemic (in spleen) HSV-peptide-specific CD4+ T-cell responses. Interestingly, surgical closure of NLDs did not significantly alter local ocular mucosal CD4+ T-cell responses induced following topical ocular immunization but did significantly enhance systemic CD4+ T-cell responses (as measured by both T-cell proliferation and gamma interferon (IFN-γ) production; P < 0.005). In contrast, NLD closure significantly decreased ocular mucosal, but not systemic, CD4+ T-cell responses following intranasal administration of the same vaccine solution (P < 0.001). The study suggests that NALT and the OMIS are immunologically interconnected.

Mucosal administration of vaccines, such as by the oral, sublingual, intranasal, or intravaginal route, has emerged as the most suitable and practical method of vaccine delivery to reduce the risk of spreading infectious diseases by parenteral injection with contaminated syringes and needles (1, 3, 4, 8, 10, 28, 35, 49). We recently demonstrated that topical ocular mucosal immunization (as in eye drops) of synthetic peptides, selected from herpes simplex virus type 1 (HSV-1) glycoprotein D (gD), together with CpG2007 mucosal adjuvant successfully induced strong local (in conjunctiva) and systemic (in spleen) HSV-specific B- and T-cell responses (10, 31, 32, 34, 35). These results have since highlighted the topical ocular mucosal route as a novel needle/syringe-free mucosal vaccine delivery approach to stimulate the ocular mucosal immune system (OMIS), also known as eye-associated lymphoid tissue (EALT), and the systemic immune system (10, 31, 32, 34, 35). However, the in-depth cellular and molecular mechanisms of ocular mucosal and systemic immune responses to topical ocular immunization remain to be fully elucidated (11). Here we studied the OMIS in further detail in connection with the anatomically bridged nasal cavity-associated lymphoid tissue (NALT).

The OMIS is a uniquely structured integrated system composed of conjunctiva, cornea, lacrimal glands, and the lacrimal drainage system (19, 22). The OMIS can be engaged to induce effective ocular mucosal and systemic B- and T-cell responses (5, 10, 34–37, 39). The vascular nature of the OMIS (both circulatory and lymphatic systems) is involved in the process of aqueous outflow and lacrimal gland/duct drainage. The anatomical connection between the OMIS and NALT suggests an immunological connection and interdependency (6, 10, 35). The integrated nature of the OMIS and NALT systems may be important for ocular immunoprophylactic and immunotherapeutic vaccine development (5, 10, 34–37, 39). It is hoped that NALT will provide or at least contribute to ocular mucosal immune protection (10, 24, 35, 40). The anatomical details of rabbit nasolacrimal ducts (NLDs), the connecting overpass between the OMIS and NALT, are illustrated below (see Fig. 4) and described in references 35 and 24. Vaccine solutions topically administrated to the ocular surface are conducted by NLDs into the inferior meatus of the nose, where they reach the NALT system (10, 24, 35). Inversely, intranasally administrated vaccine solutions are also drained by the NLDs to the mucosal surface of conjunctiva, where they reach the OMIS (10, 24, 35, 40, 47, 49). The aim of this study was to investigate
whether the anatomical connection of the OMIS and NALT through NLDs results in immunological interconnectivity and interdependency. We selected rabbits as a working model and surgically sealed their NLDs to anatomically separate the OMIS and NALT. We then monitored the magnitude of ocular mucosal and systemic T-cell responses (measured by both T-cell proliferation and gamma interferon (IFN-\(\gamma\)) production) following either topical ocular or topical intranasal immunization with HSV-1 gD peptides along with the mucosal adjuvant CpG2007. The levels of local ocular mucosal (conjunctiva) and systemic (in spleen) T-cell responses were then compared between rabbits with sealed NLDs and open NLDs. Our results showed the following: (i) both topical ocular and topical intranasal administration of a mixture of synthetic peptides bearing immunodominant CD4\(^+\) T-cell epitopes of HSV-1 gD emulsified with the mucosal CpG2007 adjuvant induced strong local ocular mucosal and systemic CD4\(^+\) T-cell responses; (ii) the surgical closure of NLDs did not affect the local ocular mucosal T-cell responses upon topical ocular immunization but significantly enhanced the systemic CD4\(^+\) T-cell responses; (iii) in contrast, the NLD closure significantly abrogated the ocular mucosal, but not systemic, CD4\(^+\) T-cell responses upon topical intranasal immunization with the same vaccine formulation. Altogether, these results suggest that the ocular and nasal mucosal immune systems are immunologically interconnected and interdependent.

MATERIALS AND METHODS

Topical ocular and topical intranasal administrations of HSV-1 gD peptides. Six to eight-week-old New Zealand White rabbits (Western Oregon) were used in all experiments except when otherwise specified. Twelve peptides were selected within the HSV-1 glycoprotein gD sequences, each 28 to 35 amino acids long and bearing at least one CD4\(^+\) T-cell epitope (2) (see Fig. 2). The purity of peptides was between 75 and 96%, as determined by reverse-phase high-performance liquid chromatography (RP-HPLC) (Vydac C18) and mass spectroscopy (Voyager matrix-assisted laser desorption ionization-time-of-flight [MALDI-TOF] system) (2). Rabbits were immunized in accordance with ARVO (Association for Research in Vision and Ophthalmology), AALAC (American Association for Laboratory Animal Care), and NIH (National Institutes of Health) guidelines. The studies were conducted with the peptide immunogens emulsified in CpG2007 oligodeoxynucleotide (ODN) adjuvant (Coley Pharmaceutical Group, Wellesley, MA). First, each peptide was dissolved in phosphate-buffered saline (PBS), and 1 mg/ml stock was stored at 4°C. For emulsion preparation, 25 \(\mu\)g (25 \(\mu\)l) of each peptide was pooled together in a total volume of 100 \(\mu\)l. Twenty-five \(\mu\)g of CpG was added to the pooled peptide solution and mixed thoroughly by vortexing. In terms of individual peptide, the delivery dose was 12.5 \(\mu\)g per eye or nasal cavity. After four peptides were pooled together, the delivery dose was 50 \(\mu\)g per eye or nasal cavity. Briefly, 100 \(\mu\)g of peptide and 25 \(\mu\)g of CpG2007 were emulsified and immediately applied topically to the eyes or intranasally in 50 \(\mu\)l solution. Rabbits (10 per group) were immunized on day 0, 14, and 21 with a mixture of three groups of peptides: G1 (gD12-29 \ gD22-52 \ gD72-104 \ and \ gD121-152); G2 (gD12-29 \ gD146-179 \ gD200-234 \ and \ gD157-180); and G3 (gD96-125 \ gD200-209 \ gD228-257 \ and \ gD332-358) or with CpG2007 alone (nonimmunized controls).

Surgical closure of nasolacrimal ducts. Rabbits were anesthetized, and both left and right nasolacrimal ducts (NLDs) were closed (as illustrated in Fig. 1). Briefly, the NLDs of the rabbit located in the nasal third of the lower cul-de-sac were identified, and the edges were lightly coagulated under a Zeiss operating microscope. The NLDs were then sutured closed with interrupted 7-0 Vicryl ophthalmic sutures by stitching with nylon surgical thread. The canal closure was verified by placing 2 drops of 0.25% fluorescein ophthalmic solution in the conjunctival sac of the eye every 10 days until the end of the experiment. Nonsealed NLDs allows the solution to pass through the nasal pharynx to the tip of the nose, resulting in readily detected fluorescence within 30 s under cobalt blue light. Rabbits were immunized 14 days after NLD closure.

Organ and tissue isolation. Ten days after the final immunization, rabbits were euthanized. The bulbar and palpebral conjunctiva tissues were excised and collocated in Hanks balanced salt solution (1×). Conjunctiva tissues were spun down at 1,600 rpm for 5 min at 4°C and digested with collagenase type I (Gibco, Carlsbad, CA) at 3 mg/ml for 3 h at 37°C with occasional vortexing every 15 min. The digested tissue suspension was passed through a 70-μm nylon cell strainer and spun down at 1,600 rpm for 5 min at 4°C. This process was repeated twice, and conjunctival cell suspension was obtained. Rabbit peripheral blood mononuclear cells (PBMC) and spleen cells were isolated by centrifugation on a ficoll density gradient (Amersham Pharmacia) and washed twice. The pellet was resuspended in Hanks balanced salt solution (1×), passed through a 40-μm cell strainer, and spun down at 1,600 rpm for 5 min at 4°C. This process was repeated twice. The final pellet was resuspended in complete RPMI 1640 medium and kept on ice. The live cells were counted using trypan blue.

T-cell proliferation assay. Single-cell preparations from conjunctiva, spleen, and PBMC were suspended in RPMI 1640 medium containing 10% fetal bovine serum and plated at 3 \times 10^6 cells/well in round-bottomed 96-well plates in a total volume of 200 \(\mu\)l. Cells were stimulated with or without immunizing peptides at 37°C and 5% CO\(_2\). After 3 days, cultures were pulsed with 1 \(\mu\)Ci of \(^3\)H per well for 20 h before harvesting and counting in a MicroBeta plate liquid scintillation counter (Wallace, Trilux). Results are expressed as \(\Delta\) cpm (\(\Delta\) cpm = cpm in the presence of antigen [Ag] – cpm without antigen) and as stimulation indices (SI) (SI = cpm in the presence of antigen/cpm without antigen). A positive proliferative response was indicated by a SI value of \(>1\). Fifty percent of the proliferating cells were measured using carboxyfluorescein diacetate succinimidyl ester (CFSE) staining. Briefly, CFSE-labeled conjunctiva cell suspensions were stimulated in the presence of different gD peptides, and the CD4\(^+\) T cells were allowed to proliferate for 4 to 5 days. Total cells were harvested and then stained with monoclonal antibodies (MAbs) specific to rabbit T-cell markers.
CD4 and CD3 for fluorescence-activated cell sorter (FACS) acquisition, and the analysis was done using gated CD4+ T cells. There is no standard formula available in the literature for quantifying the number of dividing lymphocytes in CFSE assays. Several quantitative methods, using different graphical and computer-based procedures and different formulas, have been proposed (revised in the work of Hawkins et al. [17a]). This is mainly because every system is different and because in a CFSE-labeled lymphocyte population, many factors affect the final number of dividing CFSELow lymphocytes. In addition to variable division rates, stimulated lymphocytes are also subject to variable death rates because they are subject to continuous apoptosis and necrosis due to toxicity of CFSE and sometimes of the immunogen itself. The following formula was used in the present study. The number of dividing cells = the number of gated CD4+ CFSELow cells × the number of total CD4+ lymphocytes (i.e., CD4+ CFSELow plus CD4+ CFSEhigh lymphocytes)/the number of total gated lymphocytes. The ratio of [CD4+ CFSElow + CD4+ CFSEhigh] cells/total lymphocytes serves as a normalization factor between one sample and another and one experiment and another. Because we acquired 100,000 cells, we report the y axis as the absolute number of cells per 100,000 cells.

**RNA cytokine expression levels.** For real-time PCR, tissue samples from conjunctiva and spleen from all sets of rabbits were collected and stored at −80°C. The RNeasy minikit from Qiagen was used to isolate RNA from all tissues according to the manufacturer’s instructions. To determine the quality (optical density at 260/280 nm [OD260/280]) and quantity (concentration) of RNA, 1-μl RNA samples were analyzed by using a Nanodrop spectrophotometer (ND-1000). To prepare cDNA, the RNA was reverse transcribed by reverse transcriptase, following the instructions provided with the Qiagen Sensiscript synthesizer kit. The cDNA was stored at −20°C until use. For real-time PCR assays, cDNA was diluted in water equivalent to 5 to 10 ng input RNA. Purified RNA was treated with RNase-free water and subjected to PCR using specific primers. Primers were designed using the Primer 3 Internet software program (the Whitehead Institute, Cambridge, MA), and their specificities were confirmed by a BLAST Internet software-assisted search of the nonredundant nucleotide sequence database (National Library of Medicine, Bethesda, MD). The following primers were used for IFN-γ detection: sense, 5′-CATCAGATGTGGCAAATGGT-3′; forward, 5′-ATGGTCTCGAGCCAGTTCAG-3′. The primer cycle was performed by swabbing eyes of each rabbit once daily (1 to 10 days) with a Dacron swab and transferring each swab to a 75-mm sterile tube containing 0.5 ml of culture medium (34, 35). Aliquots (100 μl) of 10-fold serial dilutions were placed on confluent monolayers of rabbit skin cells in six-well plates, incubated at 37°C for 1 h, and overlaid with medium containing 1% methylcellulose. The plates were incubated at 37°C for 3 days and stained with 1% crystal violet, and the viral plaques were counted.

**Immunohistochemistry.** Conjunctiva and spleen tissue from euthanized rabbits were collected, immediately embedded in Tissue-Tek (optimal cutting temperature compound), snap-frozen, and stored at −80°C. Sections of 10 μm thick were cut, fixed in acetone (10 min, RT), air dried, and finally stained at −80°C. For immunostaining, tissue sections were rehydrated in PBS (10 min, RT) and double stained with FITC-labeled anti-CD11b (clone M1/70; Serotec) and PE-labeled anti-TLR9 (clone 26C932; Imgenex) or with their respective isotype controls for 60 min at RT. After three successive washings in PBS (5 min each), sections were stained with 14.3 μM DAPI (4′,6′-diamidino-2-phenylindole) ( Molecular Probes, California) for 2 min at RT. The tissue sections were washed three times with PBS (5 min each), mounted in 50% glycerol-PBS, and analyzed under a fluorescence microscope.

**Statistical analysis.** Figures represent data from two independent experiments. The data were expressed as the mean ± standard error of the mean and were compared by Student’s t test using the STATVIEW II statistical software program (Abacus Concepts, Berkeley, CA) and by two-way analysis of variance (ANOVA) using Graphpad Prism 4 software. Differences were considered significant when P values were <0.05. All P values were two tailed unless stated otherwise.

**RESULTS**

**Topical ocular administration of HSV-1 gD peptides induced local and systemic T-cell responses and protected against HSV-1 replication in eyes of rabbits.** The immunogenicity and protective efficacy of the 12 selected gD peptides in rabbits (Fig. 2) were assessed following topical ocular immunizations. The 12 peptides were divided into 3 groups by randomly pooling 4 peptides together: group 1 (G1) (gD1–29, gD22–52, gD77–104, and gD121–152); group 2 (G2) (gD49–82, gD146–179, gD200–234, and gD257–317); and group 3 (G3) (gD96–123, gD176–206, gD228–257, and gD332–355). Three groups of rabbits (10 rabbits per group) were immunized topically on the eyes with a mixture of 4 peptides emulsified in Cpg2006 mucosal adjuvant. For immunization purposes, we used a cocktail of four peptides rather than a single peptide. Previously it was shown that in mice, immunization with a mixture of 4 gD peptides induced better T-cell-mediated protective immunity than any individual peptide (5, 6). This information could be advantageous for testing of vaccine efficacy with outbred populations, such as rabbits and humans. In order to avoid biasing the T-cell immunogenicity of each gD peptide, four gD peptides were randomly chosen in each mixture, and all the 12 peptides of gD were covered in the 3 mixtures.
Two weeks after the third immunization, single-cell suspensions were isolated from conjunctiva tissue digestion and spleen of each rabbit and restimulated in vitro with the immunizing gD peptides. All 3 groups induced a level of T-cell stimulation in conjunctiva of immunized rabbits that was higher than that for the CpG control (Fig. 3A). The G2 peptides induced the highest response (Fig. 3A) \((P < 0.005)\).

Compared to the CpG adjuvant control, G2 peptides also induced a significant systemic T-cell response in the spleen (Fig. 3B) \((P < 0.005)\). Phytohemagglutinin (PHA) induced similar levels of T-cell responses in both peptide- and mock-immunized rabbits. These results indicate that following topical administration, the G2 peptide group was more immunogenic than G1 or G3.

To test whether any of the vaccine groups of peptides were efficient at reducing HSV-1 replication in the eye, 10 rabbits were immunized three times with G1, G2, and G3 peptides as described above and then ocularly challenged with \(2 \times 10^5\) PFU of HSV-1 per eye. HSV-1 replication in both eyes was monitored daily from day 1 to day 12 postchallenge, as described in Materials and Methods. The amount of infectious virus in the tear samples was determined by standard plaque assays. During the period of peak replication (days 4 and 5 postinfection), all three vaccinated groups had less virus in their tears than nonimmunized rabbits \((P < 0.005)\), with G2 producing the biggest decrease (Fig. 3C). Because the G2 peptide group appeared to be the most immunogenic and protective, from this point onward, the G2 peptide group was used as the test immunogen.

Topical ocular versus topical intranasal immunization with immunodominant HSV-1 gD peptides (G2 peptides). We next compared the magnitude of the CD4+ T-cell responses induced following topical ocular versus intranasal administration of G2 peptides. Ten rabbits were immunized, either topically on the eyes or intranasally, with G2 peptides plus CpG2007. Two weeks after the third immunization, T cells were isolated from conjunctiva and spleen of each rabbit, labeled with CFSE, and restimulated in vitro with the individual gD peptides comprising G2. To selectively detect the CD4+ T-cell responses, the stimulated T cells were stained with anti-rabbit CD4-PE and analyzed by FACS. Gated CD4+ T cells from peptide-immunized rabbits were examined for CFSE incorporation in proliferating T cells. Both ocular and intranasal immunizations with G2 peptides induced strong CD4+ T-cell responses specific to the gD 49–82, gD146–179, gD287–317 peptides in conjunctiva (Fig. 4A). The absolute numbers of gD 49–82- and gD 287–317-specific CD4+ T cells were higher in the conjunctiva following topical ocular administration than following intranasal administration \((P < 0.005)\). In contrast, the route of immunization did not significantly affect CD4+ T-cell proliferation in the conjunctiva when stimulation was done with two other peptides, gD146–178 and gD200–234 from G2. In spleen tissue, the magnitude of CD4+ T-cell responses was lower than that in conjunctiva for 3 of the 4 peptides, and no differences were detected between nasal and ocular immunization (Fig. 4B). Altogether the results show that both topical ocular and topical intranasal administration of HSV-gD peptides plus
CpG\textsuperscript{2007} can induce local ocular mucosal and systemic T-cell responses.

Surgical closure of NLDs did not affect local ocular mucosal CD4\textsuperscript{+} T-cell responses, induced following topical ocular immunization, but significantly enhanced systemic CD4\textsuperscript{+} T-cell responses. In order to assess whether NALT contributed to the immune responses induced following topical ocular immunization, we surgically closed the NLDs, the connecting bridge between the OMIS and NALT, as illustrated in Fig. 1, and then immunized rabbits topically on the eyes with G2 peptides plus CpG. The T-cell responses induced in the conjunctiva and spleen were measured by CFSE assay following individual gD peptide stimulations. Overall, except for gD\textsubscript{146-179}, the absolute number of CD4\textsuperscript{+} T cells specific to gD peptides detected in the conjunctiva was not affected by NLD closure (Fig. 5A).

To confirm this effect, we also measured the IFN-\(\gamma\) mRNA level in the conjunctiva tissues collected from immunized rabbits with and without sealed NLDs, using real-time PCR. The level of IFN-\(\gamma\) mRNA in the conjunctiva was not affected by NLD closure (Fig. 5B), consistent with the results of the CFSE assay. In contrast, the absolute number of proliferating gD-peptide-specific CD4\textsuperscript{+} T cells in the spleen, as measured by CFSE assay, increased considerably from around 5 CD4\textsuperscript{+} T cells/10\textsuperscript{5} total spleen cells before NLD closure to up to 270 CD4\textsuperscript{+} T-cells/10\textsuperscript{5} total spleen cells after NLD closure (\(P < 0.0001\)) (Fig. 5C). Similarly, the levels of IFN-\(\gamma\) mRNA in the spleen significantly increased following NLD closure (\(P < 0.0005\)) (Fig. 5D). The data (i) show that while closure of NLDs did not alter local ocular mucosal CD4\textsuperscript{+} T-cell responses (in conjunctiva) following topical ocular immunization, it dramat-
FIG. 4. HSV-1 gD peptides induced local and systemic T-cell responses in both topical ocular and nasal immunization setups. Rabbits (n = 5) were immunized either ocularly (gray bars) or nasally (black bars) with a mixture of 4 immunodominant CD4+ T-cell epitopes from HSV-1 glycoprotein gD (25 μg of each gD peptide, gD49–82, gD146–179, gD228–257, and gD332–358) and 25 μg of CpG (adjuvant) three times every 14 days in a total volume of 50 μl and delivered as a drop. Ten days after the last immunization, individual conjunctiva and spleen lymphocytes were isolated. The cells were stained with CFSE (2.5 mM) and stimulated (3 × 105 cell/well) in vitro in the presence or absence of 10 μg/ml of the individual HSV-gD peptides gD49–82; gD146–179; gD228–257; and gD332–358, respectively. The CFSE-labeled cells were harvested, washed, and stained with PE-anti-rabbit CD4 antibody. Dividing cells in conjunctiva (A) or spleen (B) were analyzed by flow cytometry, and the following formula was used to calculate the absolute number of dividing cells in the present study. No. of dividing cells = no. of gated CD4+ CFSElow cells × no. of total CD4+ lymphocytes (i.e., CD4+ CFSElow + CD4+ CFSEhigh lymphocytes)/no. of total gated lymphocytes.

**DISCUSSION**

In the present study, we confirm that topical ocular immunization may be a useful route for development of needle-free mucosal vaccines that induce both local ocular immunity and systemic immunity (36, 37, 39). The CD4+ T-cell responses induced following topical ocular versus topical intranasal immunizations of rabbits using HSV-1 gD peptide CD4+ T-cell epitopes mixed with the mucosal adjuvant CpG2007 were compared. We showed that detectable HSV-1-specific CD4+ T-cell responses were induced at the local (con-
junctiva) and systemic (spleen) levels. This provides new data in the quest to develop an effective ocular mucosal vaccine against HSV-1, a serious pathogen that infects the eye and leads to blindness worldwide. NALT seems to influence the ocular mucosal and systemic T-cell responses induced by topical ocular and intranasal immunization through a yet-to-be-determined mechanism.

Human vaccines are traditionally administered by intramuscular injection (4). The effectiveness of mucosal immunization in humans has been demonstrated by the success of the oral polio vaccine (Sabin) as well as attenuated strains of Salmonella enterica serovar Typhi and Vibrio cholerae (1, 12). Other than these early-developed vaccines, few mucosal vaccines have been available clinically, despite recent progress in understanding the cellular and molecular working mechanisms of the mucosal immune system. Currently, only a nasal influenza vaccine and an oral rotavirus vaccine are available to the public, but numerous mucosal vaccines, including a topical ocular

FIG. 5. Nasolacrimal duct blockage enhanced the systemic immune response induced by topical ocular immunization with HSV-1 gD peptides. Rabbits (n = 5) with closed (black bars) or open (gray bars) nasolacrimal canals were ocularly immunized with 4 immunodominant CD4+ T-cell epitopes from HSV-1 glycoprotein gD (25 μg of each gD peptide; gD49-82, gD146-179, gD228-257, and gD332-358) and 25 μg of CpG mucosal adjuvant) three times every 14 days as a 50-μl drop per eye. Ten days after the last immunization, individual conjunctiva and spleens were harvested and lymphoid cells were isolated. The cells were stained with CFSE (2.5 μM) and stimulated in vitro (3 × 10⁵ cell/well) in the presence or absence of 10 μg/ml of the individual HSV-gD peptides gD49-82, gD146-179, gD228-257, and gD332-358 for 5 days at 37°C and 5% CO₂. The CFSE-labeled cells were harvested, washed, and stained with PE-anti-rabbit CD4 antibody. Cycling cells were analyzed by flow cytometry, and the following formula was used to calculate the absolute number in the present study: no. of dividing cells = no. of gated CD4+ CFSElow cells × no. of total CD4+ lymphocytes (i.e., CD4+ CFSElow + CD4+ CFSEhigh lymphocytes)/no. of total gated lymphocytes. Panels A and C represent the absolute numbers of gD-peptide-specific CD4+ T cells in conjunctiva and spleen, respectively. The amount of IFN-γ transcript produced in the conjunctiva (B) or spleen (D) following topical ocular immunization with gD peptides plus CpG (G2 peptides) or PBS (Mock) was measured by real-time PCR and is represented as an average of results from 2 independent experiments.
mucosal herpes vaccine, are in development (reviewed in references 4, 10, 35, and 49). We have recently demonstrated that ocular mucosal immunization, as eye drops, of T-cell epitope peptides selected from HSV-1 glycoprotein D (gD) mixed with CpG2007 mucosal adjuvant induced strong local (in conjunctiva) and systemic (in spleen) HSV-specific T-cell responses (35). In order to further advance our efforts toward the development of an ocular herpes mucosal vaccine, it is necessary to appreciate and understand the anatomical and functional uniqueness of the OMIS compared with the better-known systemic immune system. Here we studied the OMIS in further detail in connection with the anatomically bridged NALT, and provided evidence of immunological connection and interdependency between OMIS and NALT.

Until recently, studies have demonstrated that many mucosal subunit vaccine formulations administered topically to the eyes are poorly immunogenic and in the absence of a strong adjuvant may induce immunologic tolerance (2, 13). CpG has recently been used as an efficient and safe mucosal adjuvant (16, 38, 41). Coadministration of peptides along with CpG can induce robust mucosal and systemic B- and T-cell responses (29, 48). In a rat model, intranasal administration of antigens mixed with CpG induced ocular immunity as measured by secretion of tear IgA (7, 29, 48). The immune stimulation by
the CpG adjuvant requires engagement of Toll-like receptor 9 (TLR-9) on APCs, which induces intracellular signaling and subsequently triggers a predominantly Th1-type immune response (1, 25, 33). Interestingly, in this study, we demonstrated that TLR-9 is highly expressed in the rabbit conjunctiva resident CD11b+ cells. Studies published thus far support the view that Th1-type responses dominate after CpG coadministration with an immunogen (16, 38, 41). By employing the CpG 2007 adjuvant, we showed that induction of the CD4+ Th1 immune response could be achieved with topical ocular immunizations. In addition, topical ocular delivery of a peptide-CpG 2007 formulation also induced local and systemic IgA and IgG1 responses (not shown). Results in Fig. 8 shows CD11b+ TLR9+ cells migrated to the spleen upon immunization. We never checked the level of CD11b+ TLR9+ cell expression in the spleen before immunization. At this moment, all we can say is that the increase in CD11b+ TLR9+ cells in the spleen could be (i) an immunization effect or (ii) a migration effect. Further studies are needed to investigate this interesting observation.

The mechanism by which ocular immunization influenced the systemic immune response is not yet understood. It has been shown that topical ocularly applied antigens reach the conjunctiva, cervical draining lymph nodes (DLN), spleen, and liver and probably other compartments (10, 11, 35). Similarly, nasal immunization induced a distal immune response in the genital tract (11, 35, 50). It is possible that conjunctiva APCs, such as dendritic cells, capture antigens from the surface of the eye and migrate to the local draining lymph nodes and spleen. Despite emphasis on antibody and CD8+ T-cell responses, there is growing evidence to support a pivotal role for CD4+ T cells in antiherpesvirus immunity (15, 16, 26, 30, 42, 44). In several animal models, CD4+ T cells are required for protection from HSV challenge (15, 17, 18, 27, 30, 42). In humans, CD4+ T cells are stimulated in vivo following an HSV infection, and the integrated CD4+ memory response to HSV-1 appears to occur in up to 0.2% of circulating CD4+ T cells (15–18, 26, 30, 42). Severe herpetic infections are often seen in immunocompromised individuals with impaired T-cell immunity, such as AIDS and organ transplant patients, where the immune defect is predominantly displayed in CD4+ T cells. Our findings, along with the important role of CD4+ T cells in support of both B-cell and CD8+ T-cell functions, suggest that a successful strategy against HSV-1 infection should consider topical ocular delivery of a peptide-CpG 2007 vaccine formulation. Our data show that induction of CD4+ T-cell responses can control viral replication in the eyes (either directly or
indirectly via IFN-γ). However, CD4+ T cells are not the only immune effector involved in viral replication and subsequent protection in eyes. We do not exclude that other cellular factors, such as effector CD8+ T-cell functions (e.g., direct cytotoxicity and/or via IFN-γ), also contribute to controlling viral replication. With this in mind, Fig. 3 shows that G2 peptides with the highest CD4+ T-cell responses in conjunctiva (Fig. 3A) and spleen (Fig. 3B) are also the best for controlling viral replication in eyes (Fig. 3C).

In this study, we selected rabbits as a working model to investigate whether the anatomical connection of the OMIS and NALT through NLDs results in immunological interconnectivity and interdependence. From a practical standpoint, rabbit NLDs are significantly larger than those of mice and thus relatively easier to surgically manipulate for efficient closure (Fig. 1), and the rabbit eye offers plentiful amounts of tissues for in vitro characterization of T-cell responses. In addition, compared to mice, the surfaces of rabbit and human eyes are relatively immunologically isolated from systemic immune responses (19, 21–23, 43). In addition, the rabbit OMIS, also known as eye-associated lymphoid tissue (EALT), closely resembles human EALT (21, 23), while the mouse differs (23).

FIG. 8. CD11b+ cells expressing TLR9 are mobilized following topical ocular immunization. Detection of CD11b+ TLR9+ cells in the conjunctiva and spleen by immunofluorescence staining is shown. Conjunctiva and spleen tissues from rabbits immunized ocularly or intranasally with gD peptides, with or without NLD closure, were snap-frozen in liquid nitrogen and processed for immunostaining as described in Materials and Methods. The sections were doubly stained with FITC-labeled anti-rabbit CD11b and PE-labeled anti-human TLR9. The sections were counterstained with DAPI for nuclear localization (blue). The arrows show the doubly positive CD11b+ TLR9+ cells as orange to yellow dots.
Microanatomy and immunohistological studies indicate that the rabbit conjunctival mucosa is comparable to that of humans and has a typical follicular ultrastructure with an abundance of “conjunctival lymphoid follicles” (CLF), whereas no lymphoid tissue was identified in mice (23, 34, 36, 38).

Mucosa-associated lymphoid tissue (MALT) specifically protects mucosal surfaces. The OMIS comprises the conjunctiva-associated lymphoid tissue and the lacrimal drainage lymphoid tissue (19). The integrated nature of the OMIS and NALT systems is important for ocular immunoprophylactic and immunotherapeutic vaccine considerations. It is hoped that the nasal mucosa and probably also that of the lacrimal drainage system contribute to the integrity and protection of the ocular surface by the reflex stimulation of aqueous tears and through the mechanism of lymphocyte recirculation (20). In rats, topical ocular delivery of a particulate antigen (Ag uptake) is greatest at ocular sites, particularly the conjunctiva, but there is also Ag uptake in NALT (14, 19, 21, 38). In some cases, the induction site for Ag-specific immune responses was traced to NALT rather than the ocular surface (24). Therefore, it was suggested that NALT functions as a primary inductive site for ocular immune responses, at least in rodent models (24). However, this remains controversial and unresolved for humans because of complex interactions between the OMIS and NALT under both normal and pathogenic situations, which need to be fully elucidated. In this study, by using a simple surgical procedure in rabbits, we disconnected the OMIS from NALT and assessed the immunogenicity of a peptide-plus-CpG vaccine formulation administered either ocularly or intranasally. Using this approach, we showed that in rabbits, NALT interacts with the OMIS through the nasolacrimal ducts. Topical ocular immunization-induced T-cell responses in the conjunctiva did not appear to be modulated by NALT. However, NALT appeared to down-modulate systemic immune responses. Conversely, nasal immunization efficiently induced OMIS T-cell responses. The mechanisms by which NALT down-modulated systemic immune responses induced following topical ocular immunization remain to be identified. It is possible that the nature of the immune response induced by NALT during topical ocular immunization could generate suppressive cells or factors that down-modulate the systemic Th1 immune response induced by topical ocular immunization. One can speculate that in topical ocular immunization, the peptide antigens are getting separated from the CpG adjuvant when they reach the NALT, which could induce peripheral immunological tolerance. This has been described for many models of tolerance mediated via intranasal exposure to protein or peptide antigen (45, 46). Further investigations using the approach of anatomically separating the OMIS and NALT in rabbits are ongoing in our Laboratory. The present study shows for the first time that stimulation of the eye-associated lymphoid tissues not only induces local mucosal immune responses (conjunctiva) but also successfully induces systemic immune responses (spleen). The systemic response confirms that the OMIS is part of the mucosal immune system. The OMIS comprises the conjunctiva-associated lymphoid tissue and the lacrimal drainage lymphoid tissue. Although this study showed that topical ocular delivery of vaccine under the NLD-closed condition affected the T-cell immunogenicity in both the conjunctiva and spleen, it is also possible that such manipula-

tion could affect T-cell immunogenicity in the DLN as well. Moreover, the optimal protection against ocular HSV-1 likely requires Th1 immunity at both conjunctiva and DLN sites. Local Th1 responses at both conjunctiva and DLN sites are believed to play a critical role during both initial and recurrent ocular infections.

In conclusion, there are three principal findings in the present report: (i) topical ocular and intranasal administrations of T-cell epitope gD peptides emulsified with CpG9007 induced local ocular mucosal (in conjunctiva) and systemic (spleen) HSV-1 epitope-specific T-cell responses; (ii) surgical closure of NLDs, the connecting bridge between the OMIS and NALT, enhanced systemic but not local ocular mucosal T-cell responses following topical ocular immunization; and (iii) closure of NLDs decreased ocular mucosal but not systemic T-cell responses induced following intranasal immunization. These results confirmed that NALT and the OMIS are immunologically interdependent.

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