Ag-Specific Recognition, Activation, and Effector Function of T Cells in the Conjunctiva with Experimental Immune-Mediated Blepharoconjunctivitis

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PURPOSE. To investigate antigen (Ag) specificity, activation, and effector function of the Ag-specific T cells involved in the development of experimental immune–mediated blepharoconjunctivitis (EC), an experimental conjunctivitis.

METHODS. EC was induced in Brown Norway rats by injection of ovalbumin (OVA)-specific T cells followed by OVA challenge with eye drops. Eyes, including the conjunctivas, were harvested at different time points after challenge. The dependence of EC onset on the challenging Ag was assessed by challenge with an irrelevant Ag or stimulatory OVA peptides. To show the infiltration of transferred T cells into the conjunctiva, T cells were labeled with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) before transfer. The activation of T cells in the conjunctiva was assessed by measuring phosphorylation of Lck-associated molecules by Western blot analysis. Conjunctivas were also examined by immunohistochemistry and used for reverse transcription–polymerase chain reaction to determine the phenotype of the infiltrating cells and cytokine, chemokine, and chemokine receptor expression. To investigate infiltration of non Ag-specific T cells into the conjunctiva, ragweed (RW)-primed lymphocytes were transferred into OVA-specific T-cell receptor transgenic (DO11.10) mice. The mice were then challenged with RW and the conjunctivas were harvested for immunohistochemistry to detect T cells derived from DO11.10 mice.

RESULTS. EC was induced only when challenged with OVA protein or stimulatory OVA peptides, and CFSE-labeled transferred cells were found in the conjunctiva. Phosphorylation of Lck and an 85-kDa Lck-associated molecule were observed in the conjunctiva 6 hours after challenge. Many cytokines and chemokines began to be expressed at 6 hours, and individual expression patterns over time correlated well with the infiltration patterns of different inflammatory cells. In DO11.10 mice that received RW-primed lymphocytes, T cells derived from the recipient mice infiltrated the conjunctiva after RW challenge.

CONCLUSIONS. Ag-specific T cells initiate EC by first infiltrating the conjunctiva, where they become activated by the specific Ag in the conjunctiva. (*Invest Ophthalmol Vis Sci.* 2003;44: 4366–4374) DOI:10.1167/iovs.02-1323

Allergic conjunctivitis (AC)[1] is a term applied to a variety of diseases that range from mild types of AC, such as seasonal and perennial AC, to more severe diseases, such as atopic keratoconjunctivitis (AKC) and vernal keratoconjunctivitis (VKC). In the latter disease, the conjunctivas are infiltrated with inflammatory cells, such as eosinophils and lymphocytes,[2] which can lead to tissue damage, not only in the conjunctiva but also in the adjacent corneal tissues.[3] The corneal damage may result in corneal opacity that ultimately leads to blindness. Although the frequency of severe cases is relatively low,[2,3] severe types of AC are highly sight-threatening. Consequently, we were interested in determining the mechanisms involved in the development of severe AC.

Many experimental studies examining the initiation and progression of severe AC have been conducted, and together their results suggest that although eosinophils are the main effector of the tissue damage,[4–6] antigen (Ag)-specific T cells initiate the eosinophilic infiltration into the conjunctiva.[7] This is because AC is an Ag-specific disease, and eosinophils do not have Ag-specific receptors. To clarify the essential roles played by Ag-specific T cells in the development of severe types of AC, we established an experimental AC model in Brown Norway (BN) rats. This model involves the transfer of Ag-specific T cells and challenge with eye drops containing the Ag in question. The model has been denoted experimental immune-mediated blepharoconjunctivitis (EC).[8–10] In a previous series of experiments, we found that Ag-specific T-cell transfer followed by Ag challenge can induce the infiltration of eosinophils as well as mononuclear cells, which confirms the notion that Ag-specific T cells can promote eosinophilic infiltration. However, there are still a number of questions that should be resolved. One is that it is not clear whether the development of EC is dependent on the challenging Ag. In addition, it has not been shown that transferred Ag-specific T cells actually infiltrate the conjunctiva. It is also not clear whether these cells are activated in the conjunctiva. In addition, because our previous studies investigated only one time point[8–10]—namely, 24 hours after Ag challenge—the events that occur early after challenge remain unclear. Furthermore, the infiltrating cells, which are regulated by complicated cytokine and chemokine interactions, have not been extensively phenotyped.

Resolving these issues will greatly improve our understanding of the mechanisms used by Ag-specific T cells to induce EC. In addition, the information will help define EC as a model of an appropriate subset of severe types of human AC. We investigated these questions in the current study and found that Ag-specific T cells indeed infiltrated the challenge site and that they were then activated. Reverse-transcription–polymerase chain reaction (RT-PCR) studies of conjunctiva harvested at various time points after challenge revealed the rapid expression of cytokines and chemokines that could plausibly be induced by the cytokines released by the activated T cells. These cytokine–chemokine expression patterns also correlated well with the infiltration patterns of the various cell types examined by immunohistochemistry. These observations together support the notion that Ag-specific T cells play a central

role in severe types of AC, as they show that this cell type alone can orchestrate an immune cascade that resembles the immune responses observed in chronic AKC.

MATERIALS AND METHODS

Animals

Male 8- to 12-week-old BN rats (Clea Japan, Tokyo, Japan), Balb/c mice (Japan SLC Inc., Hamamatsu, Japan), and ovalbumin (OVA)-specific TCR-transgenic mice (DO11.10 mice; The Jackson Laboratory, Bar Harbor, ME) were maintained in a pathogen-free animal facility at Kochi Medical School. All animal procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Reagents

Ovalbumin (OVA; grade V) was purchased from Seikagaku Co., Tokyo, Japan. Short ragweed pollen (RW) was obtained from ICN Biomedicals Inc. (Aurora, OH) and bovine serum albumin (BSA, fraction V), phorbol 12-myristate 13-acetate (PMA), and ionomycin from Sigma-Aldrich (St. Louis, MO). Thirty-eight 15-mer OVA peptides that overlap 5 amino acids were synthesized according to the multipin method (Peptide Institute, Inc., Osaka, Japan). OVA peptide 323-339 was synthesized by solid-phase chemistry using *t*-butyloxycarbonyl derivatives of the amino acids and purified by HPLC to at least 95% purity (Funakoshi, Tokyo, Japan). 5-(and-6)-Carboxyfluorescein diacetate sucinimidyl ester (CFSE) was purchased from Molecular Probes (Eugene, OR); antibodies against CD3 (G4.18), CD8 (OX-8), CD25 (IL-2 receptor α chain, OX-39), RT1B (rat major histocompatibility complex [MHC] class II equivalent to I-A, OX-6), RT1D (rat MHC class II equivalent to I-E, OX-17), and streptavidin-phycoerythrin from BD-PharMingen (San Diego, CA); anti-CD4 (OX-38) from Caltag (Burlingame, CA); anti-CD45RA (anti-B cell, OX-33) from Cederlane (Hornby, Ontario, Canada); anti-CD68 (anti-monocyte and macrophage, ED-1) from Serotec (Oxford, UK); anti-major basic protein (MBP) from Biodesign International (Saco, ME); anti-Lck (clone 3A5) and anti-phospho-tyrosine (4G10) from Santa Cruz Biotechnology (Santa Cruz, CA); and anti-FITC (F4/1) from Neomarkers (Fremont, CA). KJ1–26, which recognizes transgenic TCR specific to OVA, was used to detect T cells derived from DO11.10 mice. Anti-phosphatidyl inositol-3-kinase (PI3K) p85 was purchased from Upstate Biotechnology (Lake Placid, NY) and protein G Sepharose from Amersham Pharmacia Biotech (Uppsala, Sweden). All antibodies used in immunohistochemistry were biotin-labeled, apart from anti-MBP, anti CD-68, and anti-FITC, which were unlabeled.

Establishment of OVA-Specific T-Cell Lines

The details of this method have been published elsewhere.[11] Briefly, OVA-primed lymph node (LN) cells were stimulated in vitro with OVA repeatedly. Three days after the second stimulation, live cells were harvested by gradient centrifugation (Lymphocyte Separation Medium; Organon Teknika Corp., West Chester, PA) and used for transfer, CFSE-labeling, flow cytometric analysis, Western blot analysis, and RT-PCR analysis.

Cellular Proliferation Assay and Flow Cytometric Analysis

The method used has been described.[10,11] In the proliferation assay, the concentrations of OVA or BSA in vitro were 0.1, 1, 10, and 100 μg/mL, whereas those of OVA peptides were 10 μg/mL. Data were expressed as stimulation indices or Δcpm.

CFSE-Labeling of OVA-Specific T Cells

OVA-specific T cells collected after the second stimulation with OVA were labeled with CFSE according to a method described previously.[12] Briefly, OVA-specific T cells were suspended in phosphate-buffered saline (PBS) at 5×10^7 cells/mL and incubated with 2 μM CFSE solution for 5 minutes at room temperature. The cells were then washed with 1% FCS-PBS three times and injected into rats. CFSE-labeling was confirmed by conventional immunocytology using anti-FITC antibody. More than 99% of cells became FITC positive after labeling.

Induction and Evaluation of EC

CFSE-labeled or unlabeled OVA-specific T cells (5×10^6/rat) were injected intravenously into syngeneic recipient rats. Four days after transfer, rats were challenged with OVA protein, BSA (both 250 μg/10 μL per eye), or OVA peptides (20 μg/10 μL per eye) in eye drops. The rats were killed either before EC induction; at 15 minutes; or at 6, 12, 24, or 48 hours after challenge, after which their eyes, including conjunctivas, were harvested for immunohistochemistry, RT-PCR analysis, and Western blot analysis. For RT-PCR and Western blot analysis, conjunctivas were frozen in liquefied nitrogen within 5 minutes after death. Each group at a particular time point consisted of at least six rats. To further investigate Ag specificity on the development of EC, RW-primed LN cells from BN rats were stimulated in vitro with RW (50 μg/mL) for 3 days in RPMI 1640 medium supplemented with 10% FCS and 2-mercaptoethanol (ME). These in vitro–stimulated cells (40×10^6) were injected intravenously into BN rats, and the rats were challenged with RW (2 mg/10 μL per eye) or OVA (both 250 μg/10 μL per eye). Twenty-four hours after challenge, eyes were harvested for histologic analysis.

Histologic Analysis

Methods used for conventional histologic analysis have been published elsewhere.[8–11] For immunohistochemical analysis, sections were prepared according to the method of Kawamoto and Shimizu.[13] Briefly, the eyes, including the conjunctivas, were immediately frozen in hexane, cooled with dry ice, immersed in 3.5% carboxymethyl cellulose (CMC) gel, and again placed in cooled hexane until the CMC gel was completely frozen. After trimming, the surface of the CMC block was covered with a polyvinylidene chloride film (Asahikasei Kogyo, Tokyo, Japan) precoated with synthetic rubber cement (Cryoglue type 1; Finetec Co., Ltd., Tokyo, Japan), and 4-μm-thick sections were cut with a cryomicrotome. The films with the sections on them were mounted on cooled glass slides with double-sided adhesive tape (Nitoms Inc, Tokyo, Japan). All samples were fixed for 5 minutes in cooled ethanol and then washed with 0.01 M PBS (pH 7.4). Before the specimens were stained with antibodies, the endogenous peroxidase activity was inhibited with 0.1% NaN_3 and 0.3% H_2O_2 in distilled water for 10 minutes at room temperature. The slides were incubated overnight at 4°C with the antibodies described earlier. The slides incubated with anti-CD68 antibody, anti-MBP antibody, or anti-FITC antibody were then stained with biotinylated secondary antibody (rabbit anti-mouse IgG; Dako, Copenhagen, Denmark) for 1 hour at room temperature. All slides were stained with an avidin-biotin complex kit (Vector Laboratories Inc., Burlingame, CA) and then developed with 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). The average number of stained cells in the six parts of an eye (limbus, fornix, and palpebral conjunctiva of the upper and lower conjunctivas) were then counted by a masked observer using an eyepiece grid with a 400× magnified field. Because the eosinophil numbers were relatively lower than the mononuclear cell numbers, they were counted throughout the conjunctiva. Each group at each time point consisted of six samples.

RT-PCR Analysis of Cytokine and Chemokine Expression

Details of the method have been published elsewhere.[9] In brief, extracted RNA from line cells and conjunctivas by a commercially available homogenizer (Mixer Mill MM 300; Qiagen KK, Tokyo, Japan) was transcribed into cDNA. PCR was performed on a DNA thermal cycler (Applied Biosystems, Foster City, CA) using one 10-minute cycle at 94°C followed by 30 to 50 cycles consisting of denaturation at 94°C for 30 seconds, annealing at the optimal temperature of the primer pairs used for 30 seconds, and extension at 72°C for 90 seconds. The

molecules tested were β-actin, 11 cytokines (IL-2, -4, -5, -6, -10, -12p35, -12p40, and -13; IFN-γ' transforming growth factor (TGF)-β' and tumor necrosis factor (TNF)-α), 7 chemokines (CCL2 [macrophage chemoattractant protein (MCP)-1], CCL3 [macrophage inflammatory protein (MIP)-1α], CCL4 [MIP-1β], CCL5 [regulated on activation, normal T-cell expressed and secreted (RANTES)], CCL10 [eotaxin], CXCL4 [platelet factor (PF)-4], and CXCL10 [interferon-induced protein of 10 kDa (IP-10)], and 3 chemokine receptors (CCR3, CCR5, and CXCR3). The primer pairs, published elsewhere,[10,14–16] were synthesized by Sawady Technology (Tokyo, Japan) or purchased from Biosource International Inc. (Camarillo, CA). Differential cycle analysis indicated the optimal cycle number to use with each primer pair. Thirty-five cycles were chosen for the mRNA for OVA-specific T cells, except for IL-2, -4, and -5 (40 cycles). For conjunctival mRNA, 40 cycles were used for most cytokines, chemokines, and chemokine receptors. The exceptions were β-actin (30 cycles), IL-5 (50 cycles), TGF-β (30 cycles), TNF-α (35 cycles), and CCR5 (35 cycles). The molecular size marker used was X174/*Hae*III digest (Wako, Osaka, Japan). PCR products were electrophoresed in a 2% agarose gel and then stained with ethidium bromide.

Western Blot Analysis and Immunoprecipitation

OVA-specific T cells (10^6/mL) were stimulated in vitro with PMA (40 ng/mL) and ionomycin (2 nM) for 5 minutes in six-well plates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan). After stimulation, the harvested cells were centrifuged and transferred into 1.5-mL microcentrifuge tubes in ice-cold PBS. After another centrifugation, cell pellets were lysed with ice-cold lysis buffer (10 mM Tris-HCl [pH 7.8], 1% NP-40, 150 mM NaCl, 50 μg/mL leupeptin, 1 μg/mL pepstatin A, 20 μg/mL aprotinin, 400 μM EDTA, 400 μM NaOV, 10 mM NaF, and 1 mM phenylmethylsulfonyl fluoride [PMSF]). Protein from conjunctivas was extracted on ice by a homogenizer with the same lysis buffer. Tissue debris was removed by centrifugation (13,000 rpm, 10 minutes 4°C), and the supernatants were applied for immunoprecipitation. The lysates (0.5 mg of T-cell lysates and 2 mg of conjunctival lysates) were incubated at 4°C with protein G Sepharose together with anti-Lck antibody (2 μg) for 2 hours. After extensive washing with ice-cold lysis buffer, 20 μL SDS was added to the pellets, followed by boiling for 5 minutes. Samples were then electrophoresed on a 10% SDS polyacrylamide gel, transferred to nitrocellulose filters, and subjected to Western blot analysis. Membrane filter was blotted with anti-phosphotyrosine (pY) antibody and reprobed with anti-Lck or anti-PI3K.

Detection of Non–Ag-Specific T Cells in the Conjunctiva

RW (50 μg) emulsified with complete Freund's adjuvant (CFA, Difco Laboratories, Detroit, MI) was injected subcutaneously into Balb/c mice. Ten days later, draining LNs were harvested, and single-cell suspensions were prepared. These LN cells (2.5 × 10^6/mL) were cultured in a 75-cm² culture flask (Greiner Bio-One GmbH, Frickenhausen, Germany) at a volume of 20 mL RPMI 1640 medium supplemented with 10% FCS and 2-ME in the presence of RW (50 μg/mL). Three days later, stimulated cells were harvested, and 140 × 10^6 cells were injected intraperitoneally into DO11.10 mice. Four days after transfer, mice were challenged with RW (1 mg per eye) and 24 hours after challenge, eyes were harvested for immunohistochemical analysis to detect KJ1–26–positive cells. As a control, naïve DO11.10 mice were challenged with RW (1mg per eye), and eyes were examined similarly.

RESULTS

Characteristics of the OVA-Specific T-Cell Line

Cell surface marker analysis demonstrated that the EC-generating cell line before its transfer into naïve rats was $CD3^+CD4^+CD8^-CD45RA^-$ and that one third of the cells in the line expressed CD25 (Fig. 1A). These cells also proliferated vigorously against OVA protein in a concentration-dependent manner but did not respond to BSA (Fig. 1B). RT-PCR analysis revealed that the line produced IFN-γ, IL-4, and IL-5 (Fig. 1C). Epitope mapping with overlapping OVA peptides demonstrated that these T cells respond to peptides 15 and 23 (Fig. 1D).

Ag Specificity for the Development of EC

To test whether the development of EC in rats primed by transfer of OVA-specific T cells is dependent on the challenging Ag, rats receiving these T cells were challenged with either OVA or BSA. Inflammatory cell infiltration was induced by challenge with OVA (Fig. 2B) but not with BSA (Fig. 2A). In addition, when recipient rats were challenged with the OVA peptides 15 and 23, which were recognized by these T cells, inflammatory cell infiltration was again observed (Figs. 2C, 2D). To further confirm Ag specificity on the development of EC, recipients of in vitro-stimulated RW-primed LN cells were challenged with either RW or OVA. Challenge with RW (Fig. 2F), but not with OVA (Fig. 2E), induced the infiltration of inflammatory cells into the conjunctiva. To assess whether these transferred T cells themselves infiltrate into the conjunctiva, we labeled them with CFSE just before the transfer. That the cells were properly labeled was confirmed by immunocytology (Fig. 3A, 3B). Rats were killed at various time points after challenge, and their conjunctivas were examined for CFSE-labeled cell infiltration by immunohistochemistry. Most of the infiltrating cells were unlabeled, but 6 hours after EC induction, sparsely distributed CFSE-labeled cells were observed (Fig. 3C). The number of labeled cells did not change when the rats were examined 12, 24, and 48 hours after challenge (Fig. 3C). However, CFSE-positive cells were never detected in the conjunctiva of BSA-challenged rats (data not shown).

Activation of Ag-Specific T Cells in the Conjunctiva

Thus far, we have shown that EC initiation is dependent on the Ag recognized by the transferred T cells and that the Ag-specific T cells actually enter the site of challenge—namely, the conjunctiva. However, it is still not clear whether these infiltrating T cells are actually activated in the site of challenge. To resolve this question, the infiltrating cells were examined for Lck phosphorylation by Western blot analysis, because Lck phosphorylation ensues when T cells are activated by the ligation of their receptors. First, we confirmed that the activation of rat T cells indeed results in the phosphorylation of Lck and its associated molecules. Although phosphorylation of Lck in the OVA-specific line was noted before stimulation, we found the augmentation of Lck phosphorylation, as the protein recognized by the anti-Lck antibody underwent a mobility shift (Fig. 4A). Notably, the blot probed with anti-pY antibody also revealed an 85-kDa band coprecipitated with Lck that was markedly increased by stimulation (Fig. 4B). Reprobing with an anti-PI3K antibody revealed that this protein is not PI3K (data not shown). In the conjunctiva, Lck was observed at minute levels in the conjunctiva before EC induction, but higher levels were detected after challenge, especially 12, 24, and 48 hours after challenge (Fig. 4C). Furthermore, Lck phosphorylation increased 6 hours after EC induction and peaked at 24 hours and then declined (Fig. 4C). Another notable change induced by OVA challenge was the phosphorylation at 6 and 12 hours of an 85-kDa protein that coprecipitated with Lck and was identified by blotting with the anti-pY antibody (Fig. 4D). Similar to the cell line, this band did not react with the anti-PI3K antibody when the same blot was reprobed.
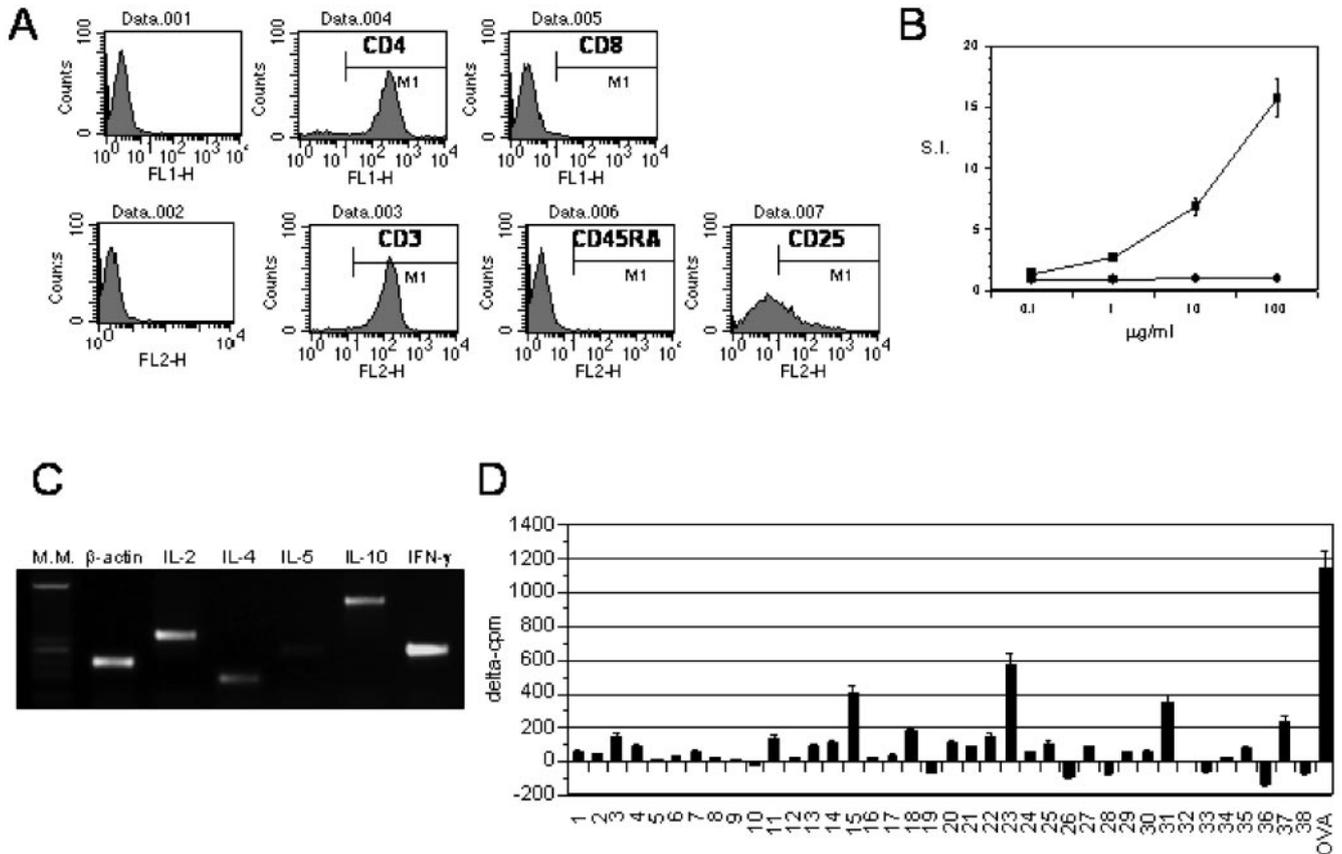
FIGURE 1. Characterization of the OVA-specific T-cell line used to transfer EC. An OVA-specific T cell line was established from OVA-primed LN cells by repeated in vitro stimulation with OVA. (**A**) Phenotypic analysis by flow cytometry. The cell line consisted of CD4$^+$ T cells, a third of which express CD25. (**B**) Ag-specific proliferation. The cell line responded to OVA (■) in a dose-dependent manner but did not respond to BSA (●). Background level was 132 ± 11 cpm. (**C**) Cytokine expression, as measured by RT-PCR. The cell line expressed both IL-4 and IFN-γ in response to OVA, although more IFN-γ production was noted. (**D**) Fine specificity of OVA epitope recognition, as measured by testing the line with overlapping OVA peptides. The concentration of OVA peptides was 10 μg/mL and that of OVA was 100 μg/mL. The line responded to peptides 15 and 23. Background level was 67 ± 9 cpm. One representative result of four reproducible lines is shown.

Phenotypic Analysis of Infiltrating Cells by Immunohistochemistry

To better understand the immunopathologic mechanisms behind EC, we used immunohistochemistry to detail the phenotypes of the cells infiltrating the conjunctiva at different time points. CD3$^+$ T cells became detectable 6 hours after OVA challenge and peaked and plateaued at 12 hours (Fig. 5A). Notably, CD4-positive cells that presumably belong to a macrophage subset existed in the conjunctiva before EC induction (Fig. 5A). Furthermore, CD68-positive cells, which also belong to a macrophage subset, existed before challenge. Their num-

FIGURE 2. The development of EC is dependent on an Ag-specific immune response. Rats receiving OVA-specific T cells were challenged with BSA (**A**), OVA (**B**), OVA peptide 15 (**C**), or OVA peptide 23 (**D**), and eyes were harvested 24 hours after challenge. In addition, rats receiving in vitro–stimulated RW-primed LN cells were challenged either with OVA (**E**) or RW (**F**), and eyes were similarly evaluated. In the transferred OVA-specific T-cell line, EC was induced by challenge with the OVA protein (**B**) and peptides (**C**, **D**) but not with BSA (**A**). In the transferred RW-primed LN cells, EC was induced by challenge with RW (**F**) but not with OVA (**E**). Data are a representative section of 3 to 12 individual rats. Giemsa stain; magnification, ×240.
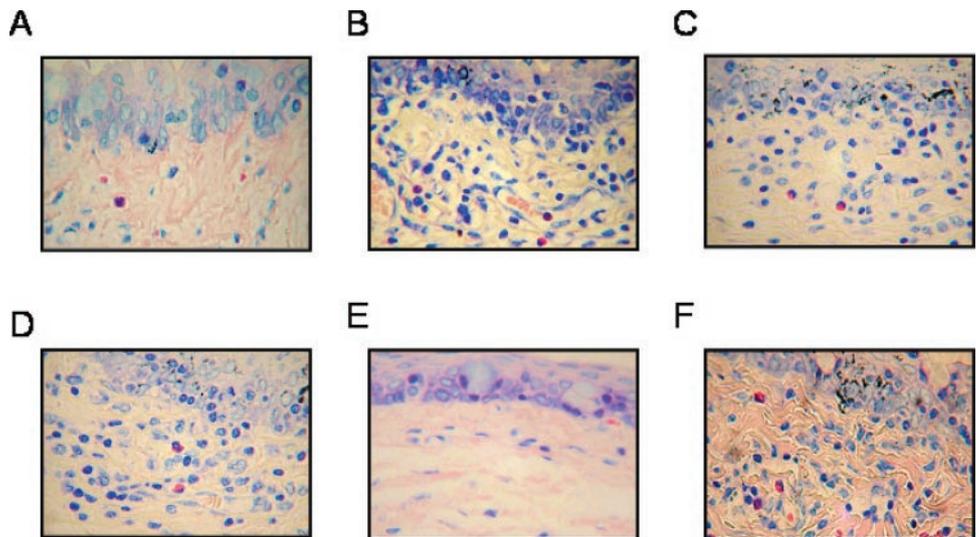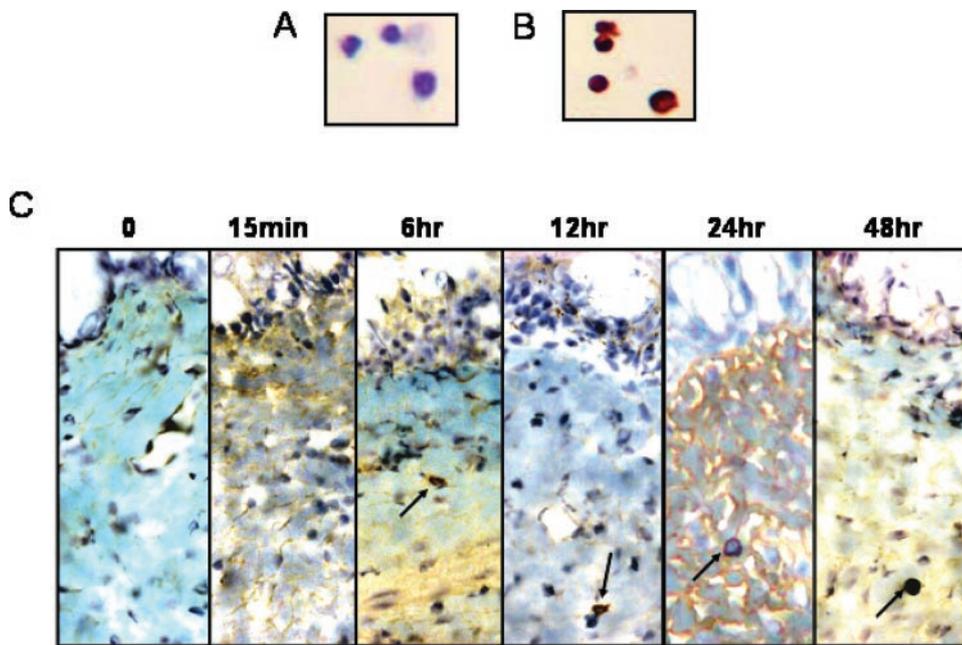
FIGURE 3. Ag-specific T cells infiltrated the conjunctiva. OVA-specific T cells were labeled with CFSE just before transfer. (A, B) The cells were checked for proper labeling by immunocytology (A: unlabeled, B: labeled). (C) The labeled cells were then transferred into rats, and EC was induced by challenge with OVA. Rats were killed at various time points after OVA challenge (three rats per time point). Shown is a representative section of the rats that were killed at indicated time points after EC induction. Although most of the infiltrating cells were CFSE negative, only a few CFSE-positive cells were detected in the conjunctiva. Note that CFSE-positive cells were not detected either before EC induction or 15 minutes after EC induction. Arrows: CFSE-positive cells. Magnification, ×200.

ber increased from 6 hours onward in a time-dependent manner (Fig. 5A). Kinetic changes of RT1B- (Fig. 5A) and RT1D-positive (data not shown) cells paralleled those of the CD68-positive cells. CD45RA-positive cells were not detectable at any time point tested (data not shown). A small number of eosinophils, confirmed by MBP staining, were detectable before EC induction (Fig. 5A). Eosinophil infiltration was first detected at 6 hours, peaked at 12 hours, and then decreased (Fig. 5A). Counts of the cells positive for each marker at the various time points confirmed the differential kinetics of the different subsets of infiltrating cells (Fig. 5B).

Expression of Cytokines, Chemokines, and Chemokine Receptors in the Conjunctiva

To understand the molecular mechanisms behind the infiltration of the inflammatory cells, we examined naïve, un-challenged, and EC-developing conjunctiva at different time points for the mRNA expression of 11 cytokines, 7 chemokines, and 3 chemokine receptors. The observations are presented in Figure 6. IL-12p35 was not detectable at any time point (data not shown). Expression was evaluated by the following criteria: (1) expression is either constitutive (detectable in naïve rats) or inducible; (2) upregulation of expression does or does not occur after challenge; and (3) expression is either transient or persistent (expression continues up to 48 hours after it is initiated).

TGF-β, TNF-α, eotaxin, RANTES, MIP-1α, MIP-1β, IP-10, CCR3, CCR5, and CXCR3 were constitutively expressed, whereas others were inducible (Fig. 6). Upregulation of expression due to OVA challenge was noted for most molecules, except for eotaxin and CCR5 (Fig. 6). Expression of IL-4, -6,
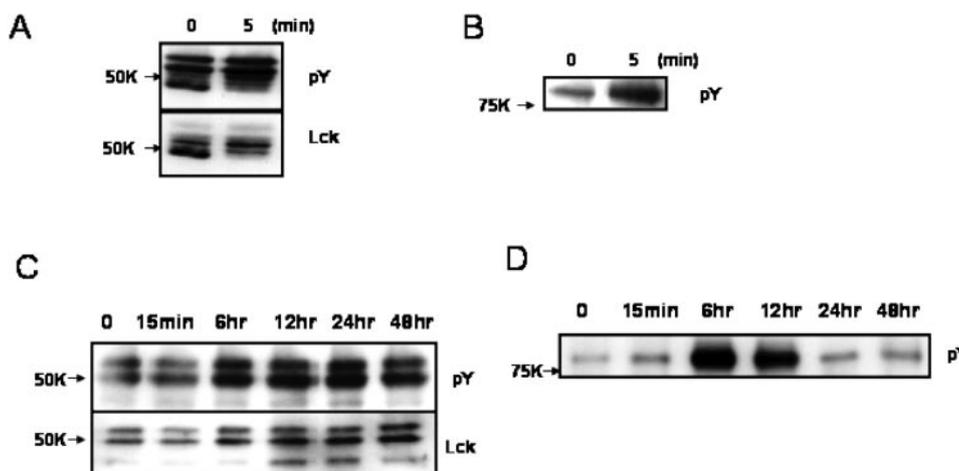

FIGURE 4. Kinetics of T-cell activation in the conjunctiva after OVA challenge. (A, B) The phosphorylation of signaling molecules due to the ligation of the T-cell receptors on the OVA-specific T-cell line was checked by stimulating the cells with PMA and ionomycin for 5 minutes, harvesting them, and subjecting immunoprecipitates of the cell lysates with anti-Lck antibody to Western blot analysis with anti-phosphotyrosine (pY) antibody. The membrane was then reprobed with anti-Lck antibody or anti-PI3K antibody. Stimulation of the line induced phosphorylation of Lck (A) and an 85-kDa protein (B), which was not identified as PI3K (data not shown). These observations were reproduced three times. (C) Phosphorylation of Lck in the conjunctiva. EC was induced by OVA-specific T-cell transfer, and conjunctivas were harvested at different time points after OVA challenge. Cellular extracts were immunoprecipitated with anti-Lck antibody, blotted, and probed first with the anti-pY antibody and then reprobed with anti-Lck antibody and anti-PI3K antibody. Lck was phosphorylated 6, 12, and 24 hours after challenge. (D) Phosphorylation of an 85 kDa Lck-associated molecule in the conjunctiva. Conjunctival extracts immunoprecipitated with anti-Lck antibody were blotted, probed first with anti-pY antibody and then reprobed with anti-PI3K antibody. Marked phosphorylation of an 85-kDa protein 6 and 12 hours after OVA challenge was noted. However, this band was not recognized when the same membrane was reprobed with anti-PI3K antibody. Representative data of three separate experiments are shown (C, D).
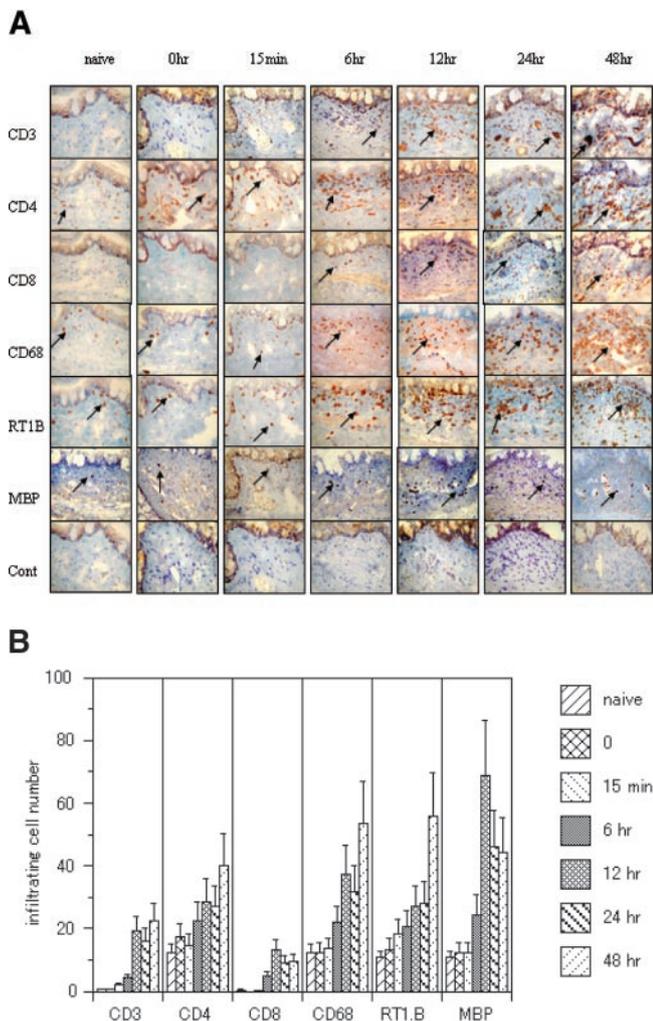
FIGURE 5. Kinetics of infiltrating cell types after OVA challenge. (**A**) Micrographs of cells infiltrating the conjunctiva. EC was induced by OVA-specific T-cell transfer followed by OVA challenge, after which the eyes were harvested at different time points and used for immunohistochemistry. Eyes of naïve rats were also examined. The molecules tested were CD3, CD4, CD8, CD68 (anti-monocyte and macrophage), RT1B (rat MHC class II equivalent to I-A), and MBP (which is produced by eosinophils). As a control, micrographs of sections without antibody treatments are shown (Cont). One representative photograph of six individual samples is shown. *Arrows:* positive cells for each marker. Magnification, ×20. (**B**) The infiltrating cells that were positive for each marker were counted. MBP-positive cells were counted in each section, whereas the cells positive for other markers were counted per field of six different portions. The eosinophil numbers peaked 12 hours after challenge but the macrophage numbers continued to increase over the observation period. An average of six individual rats is shown.

and -13 and MIP-1β was transient, but the expression of the other molecules persisted (Fig. 6).

Infiltration of Non-Ag Specific T Cells in the Conjunctiva

To examine the possibility that non–Ag-specific T cells are recruited to the conjunctiva as well as Ag-specific T cells, we set up experiments to distinguish immunocompetent cells of the recipients from the transferred cells. We transferred in vitro–stimulated RW-primed LN cells into DO11.10 TCR-transgenic mice, in which CD4-positive T cells recognize OVA peptide 323-339. Splenocytes from DO11.10 mice proliferated

against OVA and OVA 323-339, but did not show proliferation in response to RW (data not shown). In naïve DO11.10 mice, challenge of RW injected into the conjunctiva did not induce infiltration of KJ1–26–positive cells in the conjunctiva (Fig. 7A). In contrast, challenge of RW into the conjunctiva of DO11.10 mice that received in vitro–stimulated RW-primed LN cells, induced abundant infiltration of KJ1–26–positive cells, which were derived from recipients and did not recognize RW (Fig. 7B). Thus, it was demonstrated that non-Ag specific CD4-positive T cells were recruited, together with Ag-specific T cells.

DISCUSSION

That Ag-specific T cells play essential roles in the development of experimental AC has been determined by several groups,[17–19] including our own,[8,10] but the precise mechanisms by which these cells promote the pathogenic immune responses involved are still unclear. In this article, we investigated the mechanisms by which Ag-specific T cells drive the development of severe types of AC, by examining when T cells are activated in the conjunctiva, when T cells and other infiltrating immune cells enter the conjunctiva, and which cytokine– chemokine signals are associated with these influxes. We conducted these studies in rats with EC, which is a model for severe types of human AC.

Data in Figures 2A and 2B confirm the notion that the development of EC is dependent on Ag-specific T cells. Furthermore, Figures 2E and 2F indicate that the Ag response is not unique to OVA. In addition, experiments with CFSE-labeled OVA-specific T cells revealed that these cells enter the conjunctiva 6 hours after EC induction, albeit in quite low numbers. That only very few OVA-specific T cells were detected in the conjunctiva of our OVA-challenged rats agrees well with the observation of Prendergast et al.,[20] who found that only a very low percentage of transferred experimental autoimmune uveitis (EAU)–inducing cells can later be detected in the retina.[20] In contrast, challenge with BSA failed to recruit any CFSE-labeled cells into the conjunctiva. Thus, EC is induced by Ag-specific T cells that recognize their specific Ag in the conjunctiva.

Ag recognition leads to T-cell activation[21] which in turn generates various T-cell effector functions. It has never before been investigated whether experimental AC-inducing T cells are actually activated in the conjunctiva. To investigate, we measured T-cell activation in the conjunctiva by examining the phosphorylation of signaling molecules that occurs when the T-cell receptor is ligated. Phosphorylation of Lck was transiently upregulated 6, 12, and 24 hours after EC induction. In addition, an 85-kDa Lck-associated protein in conjunctiva lysates became markedly phosphorylated 6 and 12 hours after EC induction. This protein is clearly associated with Lck, because it was immunoprecipitated by the anti-Lck antibody. It is important to note that in T cells, an Lck-associated 85-kDa protein was phosphorylated by stimulation (Fig. 4B). Thus, it can be concluded that the Lck-associated 85-kDa protein in the conjunctiva is, at least partially, derived from infiltrating T cells. A possible candidate for this molecule is PI3K, because association between Lck and PI3K has been reported.[22] Although the size of PI3K is in concordance with the heavily phosphorylated protein associated with Lck in the conjunctiva (data not shown), the anti-PI3K antibody failed to recognize the 85-kDa band at 6 and 12 hours on the same membrane of immunoprecipitates, using conjunctival lysates. The identity of the phosphorylated 85-kDa protein is thus at present not clear. Nevertheless, given that CFSE-positive cells were detected at the 6-hour time point after challenge and that T-cell activation in
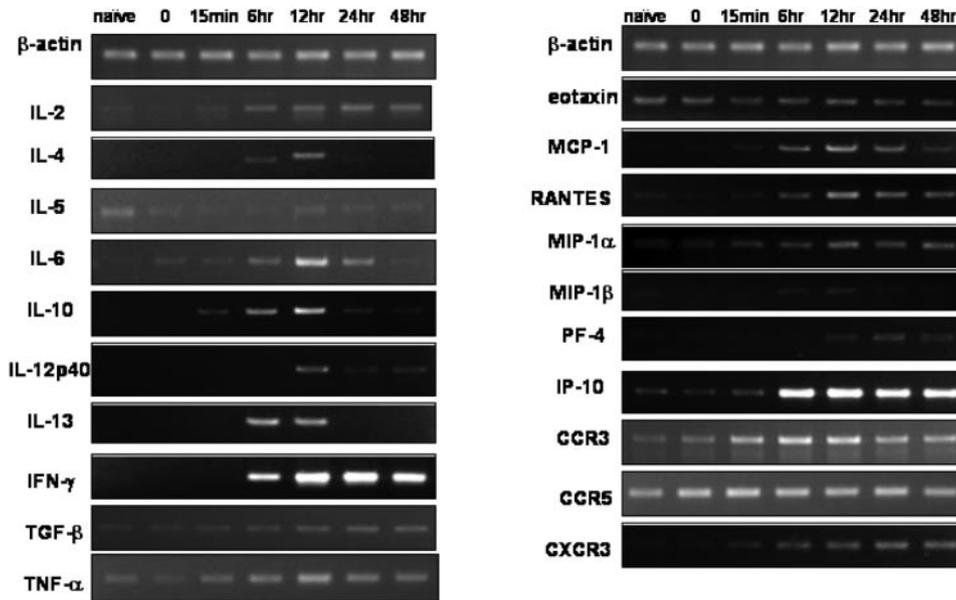
FIGURE 6. Expression of cytokines, chemokines, and chemokine receptors in the conjunctiva after OVA challenge. Conjunctivas were harvested at different time points after EC induction and subjected to RT-PCR for β-actin, 11 cytokines, 7 chemokines, and 3 chemokine receptors. PCR products are shown, except for IL-12p35 (not shown), which was not detectable at any time point. Representative data of six individual samples are shown.

the conjunctiva was observed around the same period, it is likely that this T-cell activation is due to OVA-specific T cells that are encountering OVA in the conjunctiva. Supporting this notion is that the challenging Ag in our studies was the whole OVA protein, which must first be processed into antigenic peptides, bind to MHC class II molecules, and be presented on the conjunctival antigen-presenting cell surface. In vitro experimental data show that 6 to 8 hours are needed for Ag processing that yields stimulatory peptide–MHC II complexes on the cell surface,[23] which fits with the kinetics of CFSE-labeled T-cell infiltration and T-cell activation in the conjunctiva.

It is likely that in EC, the Ag-specific T cell is the prime orchestrator driving the infiltration of other inflammatory cells into the conjunctiva, because the initiation of EC does not involve an early-phase reaction induced by Ag-specific IgE (data not shown). The effector functions of such Ag-specific T cells can be evaluated by assessing which inflammatory cells enter the conjunctiva early after T-cell activation and which T-cell–synthesized cytokines and chemokines are produced at the site

of inflammation.[8–10] Our previous studies examined the presence of only a few of these molecules and cell types at one time point (24 hours after challenge).[8–10] To obtain a more detailed picture of the inflammatory cascade ensuing after pathogenic T-cell activation, we subjected conjunctiva harvested at various time points after OVA challenge to broad-ranging immunohistochemical analyses. We also examined a wide range of cytokines, chemokines, and chemokine receptors involved in inflammatory cell infiltration that are not necessarily produced by T cells, because they can help determine the phenotypes of the infiltrating cells.[24,25]

CXCR3, which is expressed in Th1 cells and macrophages,[26] were expressed from 6 hours onward, which is the same kinetic pattern as that of infiltrating macrophages. Many chemokines, such as MCP-1, MIP-1α, MIP-1β, and RANTES, attract monocytes.[27] All these showed relatively sustained upregulation when EC was induced. This suggests that these chemokines may be responsible for the continuing influx of macrophages into the conjunctiva. CCR3, which is expressed
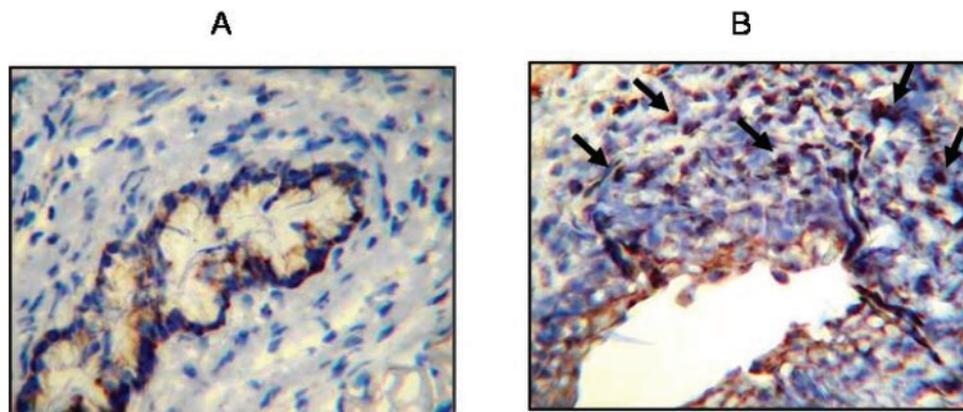

FIGURE 7. Identification of non–Ag-specific T cells in the conjunctiva. (**A**) KJ1–26–positive cells were not detected in naïve DO11.10 mice challenged with RW. Naïve DO11.10 mice were challenged with RW and eyes were harvested for immunohistochemistry to detect KJ1–26–positive cells. (**B**) KJ1–26–positive cells were abundantly identified in the conjunctiva of DO11.10 mice that received RW-primed LN cells. DO11.10 mice were injected intraperitoneally with in vitro–stimulated RW-primed LN cells. Four days later, the mice were challenged with RW and 24 hours after challenge, eyes were harvested for immunohistochemistry to detect KJ1–26–positive cells. One representative of four samples is shown. *Arrows:* KJ1–26–positive cells.

in eosinophils and Th2 cells,[28] showed upregulated expression at 6 and 12 hours after challenge, after which it decreased. This concurs with the observation that eosinophil infiltration peaks 12 hours after challenge and then declines. Eosinophils are attracted by eotaxin, MIP-1α, and RANTES[29] but we could not detect eotaxin upregulation, which indicates that MIP-1α and RANTES may be the major chemokines attracting eosinophils into the conjunctiva in EC. The lack of eotaxin upregulation may be due to low IL-4 expression and abundant IFN-γ expression by the transferred T cells, because IL-4 induces eotaxin expression and IFN-γ inhibits it.[30] IP-10, which binds with CXCR3, attracts activated T cells,[31] and we found that IP-10 expression increased markedly 6 hours after challenge. This sharp increase may induce a T-cell influx at 12 hours. Given that IP-10 is strongly induced by IFN-γ,[32] it is possible that the transferred T cells are the major initial source of IFN-γ. This suggests the following immune cascade: The transferred T cells enter the conjunctiva, become activated by the specific Ag, release IFN-γ, which then causes conjunctival residential cells to release IP-10, which in turn attracts a second influx of T cells.

Another interesting observation is that the kinetics of the Th1 and Th2 cytokines in the conjunctiva differed markedly. The Th2 cytokines IL-4, -10 and -13 were detected between 6 and 12 hours after challenge, and then all declined. In contrast, expression of the Th1 cytokine IFN-γ was sustained up to 48 hours after challenge. These patterns concur with the decline of eosinophil infiltration at 24 hours and the continued increase of macrophage infiltration. These observations are in line with the now generally accepted notion that Th1 and Th2 cells induce the infiltration of monocytes and eosinophils, respectively.[33] It is possible that the patterns we observed may reflect the relatively stronger Th1 immune responses detected in the transferred cell line (IFN-γ > IL-4, Fig. 1C), which may somehow cause the eventual decline of Th2 responses in the conjunctiva.

Because the number of CFSE-positive cells in the conjunctiva was extremely small (Fig. 3), the measurable changes such as phosphorylation of Lck (Fig. 4) and expressions of cytokine, chemokine, and chemokine receptors (Fig. 6) could not be totally attributed to the CFSE-positive cells. Therefore, it is conceivable that the CFSE-negative CD4-positive cells recruited by the inflammatory process became activated and contributed to the detected changes. To demonstrate the infiltration of non-Ag–specific T cells into the conjunctiva, we transferred RW-primed LN cells into DO11.10 TCR transgenic mice, in which immunocompetent cells do not recognize RW (data not shown). Data clearly demonstrate the infiltration of non–RW-specific T cells derived from recipients (Fig. 7B). Thus, non–Ag-specific T cells infiltrated together with Ag-specific T cells into the conjunctiva. Although it remains unclear whether these infiltrating non–Ag-specific T cells in the conjunctiva are activated or not, data support the notion that non–Ag-specific T cells also contribute to the activation of T cells in the conjunctiva and influx of inflammatory cells into the conjunctiva.

The major pathogenic mechanisms of AC include involvement of IgE and degranulation of mast cells. These two mechanisms are not apparently involved in EC induced by transfer of Ag-specific T cells. Thus, EC induced by transfer of Ag-specific T cells, such as seasonal and perennial AC, cannot be regarded as a model of AC. In contrast, EC induced by active immunization followed by Ag challenge has involvement of IgE and thus, could be considered a model of AC. Immunohistochemical analysis and Western blot of the conjunctiva from BN rats with EC induced by active immunization demonstrated the similar pathologic changes and Lck phosphorylation noted in the conjunctiva with EC induced by T-cell transfer (data not

shown). Thus, activation and effector function of T cells may have occurred similarly in the conjunctiva of EC induced by active immunization and EC by T-cell transfer. Therefore, it could be considered that data reported herein may not be restricted to understanding the development of EC induced by T-cell transfer but may be extended to understanding the developing mechanism mediated by T cells in AC.

The immunohistochemical and RT-PCR analyses demonstrate that EC generated by the transfer of pathogenic T cells is a disease that is both Th1 and Th2 in nature, though Th1 is dominant. In certain human AC diseases, such as chronic AKC, both Th1 and Th2 cytokines occur at equivalent levels or Th1 cytokines predominate.[34] Thus, the cytokine pattern in the conjunctiva of EC by transfer of T cells resembles chronic AKC, and therefore EC by T-cell transfer would be a suitable model for chronic AKC. The information in this study helps us understand the immune cascade that may be initiated by Ag-specific T cells and that can lead to the development of chronic AKC.

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