Induction of Ocular Inflammation by T-Helper Lymphocytes Type 2

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PURPOSE. Sight-damaging ocular inflammation is often mediated by T-helper (Th) lymphocytes. The population of Th cells is divided into two major subsets, designated Th1 and Th2, that differ by their cytokine production and biological activities. In the present study, the capacity of Th1 and Th2 cells to induce ocular inflammation was examined.

METHODS. Ocular inflammation was induced in transgenic (Tg) mice that express hen egg lysozyme (HEL) in their lens, by adoptively transferring Th cells that transgenically express HEL-specific receptor. Th1 and Th2 populations were polarized in vitro, and their selective cytokine production was determined by conventional methods. Levels of ocular inflammation were monitored by conventional histologic methods. Infiltrating cells were collected from sections of inflamed eyes by microdissection, and their cytokine production was examined by RT-PCR.

RESULTS. Th1 cells were highly immunopathogenic, producing disease in naive recipients at numbers as low as 0.12 × 10^6, whereas Th2 cells were inactive in these recipients, even at 30 × 10^6. Th2 cells, however, produced inflammation when transferred into sublethally irradiated recipients. Distinctive histopathologic changes characterized ocular inflammation induced by the two types of Th cells. Cytokine analysis of infiltrating cells in recipient mouse eyes, as well as of splenocytes of these mice demonstrated that the transferred cells retained their type specificity. Coinjecting Th2 and Th1 cells did not alleviate the ocular disease in naïve recipients and even exacerbated the immunopathogenic process in irradiated recipients.

CONCLUSIONS. Th2 cells are capable of inducing ocular inflammation, but only in immunodeficient mice, and are profoundly inferior to Th1 cells in their immunopathogenic capacity. (Invest Ophthalmol Vis Sci. 2002;43:758–765)

Inflammatory reactions in the eye often produce irreversible damage to ocular tissues and loss of vision. A major portion of these inflammatory processes are mediated by T-helper (Th) cells. This population of lymphocytes consists mainly of two cell subsets, designated Th1 and Th2, that differ in their cytokine production and their immunologic function. Th1 cells produce mainly interferon-γ (IFN-γ) and interleukin-2 (IL-2) and generate effective cellular immunity, whereas Th2 cells produce IL-4, -5, -10, and -13 and are associated with humoral and allergic immune responses. The participation of these two lymphocyte subsets in immunopathogenic processes has been examined in animal studies. Th1 cells are highly immunopathogenic and capable of inducing inflammatory diseases, such as experimental autoimmune encephalomyelitis (EAE) and diabetes. Moreover, in two animal disease models, EAE and experimental diabetes, Th2 cells were found to mediate disease in immunocompromised recipients.

To examine the pathogenicity of lymphocytes in immune-mediated ocular inflammation, we have developed an experimental system whereby lymphocytes sensitized against hen egg lysozyme (HEL) induce severe intraocular inflammation when adoptively transferred into recipients that transgenically express HEL in their eyes (denoted HEL-Tg mice). HEL, a neo self-antigen in the recipient mouse, is expressed under control of the α-crystallin promoter, and this animal disease has therefore been suggested as a model for the condition designated lens-associated uveitis. Using HEL-specific T-cell receptor (TCR) transgenic (Tg) mice made it possible to obtain polarized Th1 and Th2 subpopulations and dissect their pathogenicity in this experimental system. Th1 lymphocytes induced intense ocular inflammation, whereas Th2 cells were found to be inactive in untreated naïve recipients. Th2 lymphocytes induced ocular inflammation, however, when adoptively transferred into recipients treated with sublethal irradiation.

MATERIALS AND METHODS

Mice

HEL-Tg mice, on the FVB/N background, were generated as detailed elsewhere. HEL-specific TCR Tg mice, on the B10.BR background, designated 3A9, were a generous gift from Mark Davis (Stanford University, Stanford, CA). Tg mice from each of the two lines were mated to produce (FVB/N x B10.BR)F1 hybrids, expressing either one of the two transgenes. Only such F1 hybrid mice were used in all experiments of the present study. The mice were housed in a pathogen-free facility, and all manipulations were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation of HEL-Specific Th1 and Th2 Cells

Th1 and Th2 cells expressing HEL-specific TCR were prepared as follows: Spleen and lymph node cells of 3A9 mice were pooled, and the T-cell fraction was partially purified on enrichment columns (R&D Systems, Minneapolis, MN), followed by purification of CD4 cells by a magnetic sorting system (SuperMACS; Milteny Biotec, Sunnyvale, CA), using beads directly coupled to anti-mouse CD4. Purified (>97%) CD4 cells were then cultured at 2.5 × 10^5/mL in RPMI-1640 medium supplemented with 50 μM 2-mercaptoethanol (2-ME), antibiotics, and 10% fetal bovine serum (complete medium) with 10^5 irradiated syngeneic wild-type splenocytes, as antigen-presenting cells (APCs), in the
presence of 2 μg/mL HEL (Sigma, St Louis, MO), 10 ng/mL IL-12 (Sigma), and 10 μg/mL anti-IL-4 antibody (PharMingen, San Diego, CA) for Th1 or 0.2 μg/mL HEL, 10 ng/mL IL-4, 10 μg/mL anti-IFN-γ antibody, and 10 μg/mL anti-IL-12 antibody (all from PharMingen) for Th2.

After 3 days, cultured cells were expanded with 40 IU/mL IL-2 (Chiron of cellular inflammation). The severity of ocular inflammation was scored by two investigators in a masked fashion, evaluating separately the level of infiltration, and levels of proteinaceous exudate.

**Assessment of Histologic Changes**

The severity of ocular inflammation was scored according to tissue structural changes, intensity and surface marker expression were analyzed. Using a cytoperm kit with monensin (GolgiStop; Pharmingen), according to instructions and an established protocol, 21 polarized cells on the day of adoptive transfer were recultured for 6 to 8 days, cells were adoptively transferred as described earlier.

**Adoptive Transfer of Th1 and Th2 Cells**

Upon injection, the cell preparations were more than 90% alive and blastic, and more than 97% CD4 positive. Recipient HEL-Tg mice were either naive or irradiated with 450 rads, 4 to 5 hours before cell injection. Seven days after injection, recipient mice were killed, their spleens were harvested for cytokine release cultures, and their eyes were fixed in 10% formalin. Ocular tissue sections were prepared by conventional methods.

**Flow Cytometric Analysis**

To quantify the degree of polarization, intracellular cytokine production and surface marker expression were analyzed. Using a cytometric cytokoper kit with monensin (GolgiStop, Pharmingen), according to the manufacturer’s instructions and an established protocol, polarized cells on the day of adoptive transfer were recultured for 6 to 8 hours in 40 ng/mL phorbol myristate acetate, 1 μM ionomycin, and monensin; fixed; permeabilized; stained with FITC-conjugated anti IFN-γ antibody and phycoerythrin (PE)-conjugated anti IL-4 antibody and analyzed by flow cytometry. Polarized cells were also analyzed for surface expression of IL-18 receptor (IL-18R) and T1/ST2, selective markers for Th1 and Th2 cells, respectively. Staining for IL-18R was performed with goat anti-mouse purified IL-18R antibody and a PE-conjugated donkey anti-goat secondary antibody (R&D Systems), and T1/ST2 was detected with an FITC-conjugated rat anti-mouse T1/ST2 antibody (Morwell Diagnostics GmbH, Zurich, Switzerland).

**Th2 Cell Sorting**

Th2 cells, after an initial round of stimulation and a 3-day expansion with IL-2, were harvested and stained with antibody against both IL-18R and T1/ST2. Cells expressing IL-18R were eliminated, and only the strongest 10% to 20% of cells stained with T1/ST2 were collected. Sorted cells were resuspended in complete medium and cultured at 2.5 × 10^5/mL with 10% irradiated syngeneic APCs, in the presence of 0.2 μg/mL HEL, 40 IU/mL IL-2, 10 ng/mL IL-4, 10 μg/mL anti-IFN-γ antibody, and 10 μg/mL anti-IL-12 antibody. After incubation for 3 days, cells were adoptively transferred as described earlier.

**Cytokine Measurements**

For measurement of cytokine release by the polarized cell cultures, supernatants were collected before cell injection. Cytokine production by lymphocytes of recipient mice was determined in cultures of splenocytes of these animals, collected after their deaths, 7 days after cell injection. Splenocytes were cultured for 48 hours, at 5 × 10^6/mL, with 10 μg/mL HEL in RPMI-1640 supplemented with 50 μM 2-ME, antibiotics, and a serum replacement (HL-1; BioWhittaker, Walkersville, MD). Supernatants were stored at −70°C until they were assayed, using kits obtained from Endogen (Woburn, MA).

**Microdissection**

Cytokines produced by inflammatory cells infiltrating mouse eyes were identified by the microdissection method, as described in detail by Zhuang et al. In brief, frozen sections of inflamed eyes were stained with hematoxylin and eosin (H&E) and rinsed in 10% glycerol in TRIS-EDTA buffer (Quality Biological, Gaithersburg, MD). Samples of inflammatory cells were collected under light microscopic visualization, using a laser capture microdissection unit (Pixcell II; Arcturus, Mountain View, CA) and immediately transferred into 200 μL of ex-
traction reagent (Trizol; Life Technologies, Grand Island, NY). RNA was isolated by chloroform extraction and isopropanol precipitation according to the manufacturer’s instruction. After digestion with DNase, total RNA was used for RT-PCR amplification. cDNA was synthesized using a reverse transcriptase system (Superscript II RNase H Reverse Transcriptase System; Life Technologies) with random primers (Promega, Madison, WI). PCR was performed with 2 μL cDNA, 3.0 pmol of each 32P-labeled primers, 4.0 nmol of each dNTP, 1 × buffer (GeneAmp; Perkin Elmer, Hayward, CA), 1.0 U polymerase (AmpliTaq Gold; Perkin Elmer) and a final concentration of 1.5 mM MgCl2. The running conditions for PCR were hot start at 94°C for 9 minutes, 40 cycles of denaturing at 94°C for 45 seconds, annealing for 40 seconds at 58°C for IFN-γ and at 57°C for IL-4, and extension at 72°C for 120 seconds. The sequences of primers were: for IFN-γ, sense, 5’-CTTCTGAGTGGTCGTTAC-3’ and antisense, 5’-CAGTCTCAGCCGAGATTC-3’ and for IL-4, sense, 5’-CCAGCTTGGTATGGAGGAGC-3’ and antisense, 5’-GATGCTTCAGTCTGGAGGAGC-3’ and antisense, 5’-GATGCTTGGTATGGAGGAGC-3’ and antisense, 5’-GATGCTTGGTATGGAGGAGC-3’ and antisense, 5’-GATGCTTGGTATGGAGGAGC-3’. The expected size of PCR products were 236 bp for IFN-γ and 357 bp for IL-4. The positive control for IL-4 was purchased from Clontech Inc. (Palo Alto, CA), whereas those for IFN-γ and β-actin were

**Figure 2.** Histopathologic changes in eyes of irradiated recipient mice injected with Th1 or Th2 cells. Eyes were examined 7 days after injection of 0.5 × 10^6 Th1 or 10 × 10^6 Th2 cells. Inflammatory changes were scored at 8 and 4 in the recipients of Th1 and Th2 cells, respectively. (A) Anterior segment of an untreated HEL-Tg control mouse eye. (B) Anterior segment of the Th1 cell recipient eye showing cellular infiltration in the limbus, ciliary body and iris, as well as in the vitreous included in this frame. Proteinaceous exudate is seen in the anterior chamber and vitreous (asterisks). (C) Higher magnification of frame B. The cellular infiltration consists mostly of MNLs. (D) Anterior segment of the Th2-cell-recipient eye showing cellular infiltration in the limbus, adjacent cornea, and anterior chamber, as well as in the retina and vitreous included in this frame. (E, F) Higher magnification of frame D. Most infiltrating cells were PMNs, many of which had the morphology of eosinophils (arrows). (G) Posterior segment of an untreated HEL-Tg control eye. (H) Posterior segment of the Th1-cell-recipient mouse eye. The retina was edematous, folded, and partially detached. Proteinaceous and cellular exudates are seen in the vitreous and subretinal space (arrowhead). (I) Higher magnification of frame H, at the infiltrated blood vessel. Most infiltrating cells are MNLs. (J) Posterior segment of the Th2-cell-recipient mouse eye. Cellular infiltration in the retinal vessels (enlarged in K) and vitreous (enlarged in L). The infiltrating cells consisted mainly of PMNs, with many showing the morphology of eosinophils (K, L; arrows). Co, cornea; CB, ciliary body; Ir, iris; Le, lens; Vi, vitreous; Re, retina.
prepared from a murine Th1 cell line and from a normal mouse eye, respectively.

RESULTS

Th Lymphocyte–Induced Disease in Naive Recipients

Varying numbers of HEL-activated and polarized Th1 or Th2 cells from 3A9 mice were adoptively transferred into naive HEL-Tg recipients. Seven days later, eyes of recipient mice were examined histologically for inflammatory changes. Th1 cells were highly immunopathogenic, producing disease in a dose-dependent manner, with inflammation being observed in eyes of recipients injected with as few as 0.12 × 10^6 Th1 lymphocytes (Fig. 1). In contrast, Th2 cells failed to produce disease in naive recipients, even at numbers as high as 30 × 10^6 per recipient (Fig. 1).

Th2 Lymphocyte–Induced Inflammation in Eyes of Irradiated Recipient Mice

Irradiation promotes adoptive transfer of cellular immunity, 25,26 and Th2 lymphocytes were reported to exert pathogenicity in other experimental systems when adoptively transferred into immunodeficient recipients. 9,10 We examined, therefore, the pathogenicity of polarized Th2 cells in sublethally irradiated recipients. Contrary to their inactivity in naive recipients, Th2 cells induced ocular inflammation in irradiated recipients. The pathogenicity of Th2 was found, however, to be remarkably inferior to that of Th1 cells, with the minimum number of cells capable of inducing inflammation being 2.5 × 10^5 for Th2, but 0.06 × 10^5 for Th1 cells (Fig. 1).

Histopathologic Changes in Recipient Eyes: Differences between Th1- and Th2-Induced Inflammation

Figure 2 demonstrates typical changes in eyes of recipient mice. A common feature in all affected eyes was cellular infiltration in cavities and tissues throughout the eye. Dense accumulation of infiltrating cells was particularly seen in dilated blood vessels of the limbus, optic nerve head, and retinal inner cell layers, suggesting that these are the sites of entry for the infiltrating cells. The lens structure is badly disrupted in HEL-Tg-recipient mice because of the transgene expression, and lenticular material is released into the eye cavities. 17,19

Of particular interest are differences between pathologic changes distinctive for recipients of Th1 and Th2 cells. Remarkably, whereas mononuclear leukocytes (MNLs) comprised the most of the cells in eyes of Th1 cell recipients (Figs. 2C, 2I), mostly polymorphonuclear leukocytes (PMNs), with many eosinophils, were seen in eyes of Th2 recipients (Figs. 2E, 2F, 2K, 2L). Eosinophils were identified by their typical morphology of two lobed nuclei and acidophilic cytoplasm (Figs. 2F, 2K, 2L). In addition, eosinophils were identified in eyes of Th2 cell–recipient mice by a stain that highlights the cyanide-resistant peroxidase of these cells. 27 Other distinctive features are the presence of proteinaceous exudate in the aqueous, vitreous, and subretinal space (Figs. 2B, 2H), as well as marked inflammatory changes in the retina (Fig. 2H) in Th1 but not in Th2-cell recipients (Figs. 2D, 2I). A characteristic feature in recipients of Th2 cells was PMN–eosinophil infiltration of the cornea adjacent to the limbus (Figs. 2E, 2F).

Pathogenicity of Th2 Cell Preparations Is Not Due to Contaminating Th1 Lymphocytes

The distinctive histopathologic changes in recipients of Th2 cells mentioned earlier suggest that contaminating Th1 cells do not play any major role in the pathogenic process. To further rule out this possibility, the purity of the two polarized cell preparations was examined by comparing their cytokine production profiles and specific cell surface markers. Cytokine production was determined in cell culture supernatants, just before cell injection, as well as intracellularly, by flow cytometric analysis. As seen in Figure 3A, Th1 cells released high levels of IFN-γ, but no IL-5, whereas Th2 cells released IL-5, but...
A. Intracellular Cytokines

Th1

IFN-γ

IL-4

98.8%

5.1%

M1

M1

Th2

2.4%

50.4%

M1

M1

B. Surface Markers

IL-18R

T1/ST2

70.7%

2.7%

M1

M1

7.6%

72.4%

M1

M1

Relative Fluorescence Intensity

Cell Number

FIGURE 4. Expression of type-specific intracellular cytokines and surface cell markers by polarized Th1 and Th2 cells. All cells were tested after two cycles of activation and before being adoptively transferred. (A) Intracellular expression of IFN-γ and IL-4 was assessed by staining the cells with antibodies against the two cytokines. (B) The surface markers IL-18R and T1/ST2 were detected on polarized Th cells by specific monoclonal antibodies.

no IFN-γ. Th1 cells also showed strong production of intracellular IFN-γ and no IL-4, whereas a considerable proportion of Th2 cells expressed IL-4 but essentially no IFN-γ (Fig. 4A). The high level of enrichment of the two subpopulations was also demonstrated by the presence of subset-specific markers. As seen in Figure 4B, the great majority of Th1 and Th2 cells stained with their subset-specific markers (IL-18R and T1/ST2, respectively) but not with the marker of the other subset.

To further eliminate contaminating Th1 cells, Th2-cell preparations were purified by cell sorting that included elimination of lymphocytes that expressed the IL-18R and collection of the 10% to 20% of cells that strongly expressed the T1/ST2 marker (see the Materials and Methods section). The sorted Th2 cells induced inflammation in irradiated recipients, similar to the routinely purified Th2 preparations (not shown).

To examine whether Th2 cells could have converted in vivo into Th1 cells, we tested the cytokine production of spleen cells from recipient mice injected with polarized Th2 lymphocytes. As shown in Figure 3B, these spleen cells released high levels of IL-5, but almost no IFN-γ after stimulation in culture with HEL.

Identification of Cytokines Produced by Inflammatory Cells Infiltrating Eyes of Recipient Mice

To identify cytokines produced by the cells that infiltrate the recipients’ eyes, samples of approximately 50 to 100 inflam-
Inflammatory cells were collected by microdissection from selected areas of affected eyes. The cells were collected from two areas where high concentrations of infiltrating cells were seen: the limbus and the vicinity of the optic nerve head. RNA extracted from the pooled cell samples was used to identify by RT-PCR the mRNA of two type-specific cytokines: IL-4 and IFN-γ. As shown in Figure 5, cells collected from recipients of Th1 cells expressed IFN-γ, but no IL-4, whereas the opposite pattern was depicted by cells collected from the Th2-cell recipients.

**Cotransfer of Th1- and Th2-Cell Preparations**

To examine the potentially inhibitory effect of Th2 cells on Th1 cells, mixtures of the two cell types were adoptively transferred to recipient mice that were either naive or irradiated. Adding 10^6 Th2 cells to 1 x 10^6 or 0.5 x 10^6 Th1 cells did not inhibit the disease induced by the Th1 cells alone in naive mice and even enhanced the severity of disease in irradiated recipients. In addition, the ocular infiltration in the latter recipients contained a substantial proportion of PMNs (not shown).

**DISCUSSION**

The novel experimental system described herein made it possible to compare polarized populations of antigen-specific Th cells for their capacity to induce inflammation in eyes in which the antigen is expressed. Th1 cells were found to be highly pathogenic, whereas Th2 lymphocytes failed to induce disease in naive recipients. It is important to note, however, that Th2 cells produced inflammation in eyes of irradiated recipients. The possibility that contaminating Th1 cells were involved in the pathogenic effect of Th2 preparations was ruled out by cytokine analysis of the infiltrating cells (Fig. 5), as well as of splenocytes of recipient mice (Fig. 3B).

The poor pathogenicity of Th2 cells could be attributable to their inability to enter the eye and reach their target antigen and/or to their poor capacity in mediating the inflammatory process. Eye tissues are sequestered by well-defined blood-ocular barriers that restrict cellular passage, and it is possible that these barriers restricted Th2-cell entrance to the eye. It is noteworthy, however, that LaFaille et al.9 did not find any difference between Th1 and Th2 cells in migration into the central nervous system. In addition, both blood–brain and blood-ocular barriers allow passage to all activated lymphocytes.29–31 The other notion, that Th2 cells are poor mediators of inflammatory processes is in accord with our observation that even when producing ocular inflammation, Th2 cells are inferior to Th1 lymphocytes (Fig. 1). Similarly, Th2 cells were found less efficient than Th1 cells in inducing EAE and diabetes.9,16 The difference between the two lymphocyte subsets could be attributed to different cytokines and chemokines they use to mediate inflammation. Thus, similar to observations in other experimental diseases,9,16 most of the infiltrating cells in recipients of Th2 cells consisted of PMNs, whereas mainly MNLs were found in eyes of recipients of Th1 cells (Fig. 2). In addition, proteinaceous exudate was found in eyes of recipients of Th1 cells but not in those of Th2 cells (Fig. 2), suggesting that only cytokines released by Th1 cells affect the blood-
ocular barrier. More investigation is under way to identify the cytokines and chemokines involved in mediating the ocular inflammatory process in the two groups of recipients.

The irradiation effect that allowed pathogenicity by Th2 cells could be attributable mainly to elimination of a large proportion of the recipients’ lymphocyte population. This notion is in line with the observation that both EAE and experimental diabetes can be induced by Th2 cells only in immunodeficient recipients. The conditions that facilitate lymphocyte pathogenicity in such animals are not clear. It is conceivable, however, that in addition to offering “space” in their lymphoid organs, immunocompromised mice are deficient in population(s) of regulatory cells, such as CD25+ lymphocytes, that suppress other lymphocyte activities. 32

Of particular interest are our results in recipient mice injected with mixtures of Th1 and Th2 cells. Th2 cells did not inhibit the inflammation induced in naive recipient mice by Th1 cells, but even enhanced the inflammatory process in irradiated recipients. An inhibitory effect of Th2 cells on the pathogenicity of Th1 cells was demonstrated in several studies, but our data are in line with other publications in which no such effect was observed. 6,9,15 The difference between these two groups of studies may be due to variability in multiple features, including the major cytokines released by the Th2 cells, their local concentration, and the polarization stage of the Th1 cells.

The need to use irradiated recipients in the experimental system in this study limits its usefulness to some degree. Nonetheless, this system provides a useful new tool to differentiate between the activities of Th1 and Th2 cells in the process that brings about ocular inflammation. Indeed, preliminary data we have collected in an extension of the present study indicate that different groups of cytokines, chemokines, and chemokine receptors are upregulated in eyes of Th1 or Th2 cell recipients. These differences in mediators are in accord with the finding recorded in this study of different populations of inflammatory cells in eyes of the two groups of cell recipients. It is also of note that the profiles of infiltrating cells in recipients of Th1 and Th2 cells resembled those seen in eyes with experimental autoimmune uveoretinitis in wild-type mice or in IFN-γ-deficient mice, respectively.

To summarize, in the present study we examined for the first time the capacity of polarized Th1 and Th2 cells to induce immune-mediated ocular inflammation targeted at a neo self-antigen. Th1 cells were highly immunopathogenic, whereas Th2 cells were inactive in naive recipients, but induced disease in irradiated recipients.

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References


