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Regulatory T Cells Control Uveoretinitis Induced by Pathogenic Th1 Cells Reacting to a Specific Retinal Neoantigen

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In many clinical cases, uveitis develops secondary to an infection. This could result from peripheral activation followed by ocular penetration and reactivation of T cells specific for microbial Ags expressed in the retina. To gain insights into the pathophysiology of uveitis, we developed a new mouse model based on stable retinal expression of influenza virus hemagglutinin (HA) neoantigen by adeno-associated virus-mediated gene transfer. One month thereafter, we adoptively transferred HA-specific T cells, which were activated in vitro or in vivo. Intraocular inflammation was clinically and histologically observed in all animals within 15 days. The ocular infiltrate was composed mostly of macrophages and HA-specific T cells with a proinflammatory cytokine profile. Depletion of CD4+CD25+ regulatory T cells exacerbated the disease, whereas HA-specific CD4+CD25+ T cells given i.v. controlled the disease. This novel model should allow to better study the pathophysiology and therapeutic of uveitis. The Journal of Immunology, 2006, 176: 7171–7179.

A n autoimmune diseases result from inappropriately responses of the immune system to self-Ags. Their etiology remains largely unknown, but candidate etiological factors include genetic abnormalities and infections (1–5). Uveitis is a sight-threatening disease affecting the neural retina and uvea. The most common murine model is experimental autoimmune uveitis (EAU) (6, 7), which has been used to describe different aspects of its pathophysiology. Disease development is mediated by Th1 CD4+ cells specific for various retinal autoantigens (8). However, the role of these cells in the pathogenicity of uveitis remains poorly understood, because it is difficult to follow the activation and migration of these rare and undefined T cells. Activated macrophages, attracted by cytokine secretion (6, 9–11), also cause retinal damage due to the release of cytotoxic oxygen and nitrogen metabolites, cytokines, and proteolytic enzymes (12, 13).

In addition to uveitis of autoimmune origin, the contribution of infectious agents in the pathogenesis of idiopathic uveitis has been suspected in different clinical situations. An autoimmune response may be caused by bacterial or viral Ags cross-reacting with self-Ags, although evidence for molecular mimicry is lacking in most cases. We have recently reported the role of the Herpes viruses in the etiology of non-necrotizing retinopathies with typical features of Behçet’s disease, birdshot retinochoroidopathy, and idiopathic retinal vasculitis (14). Klebsiella and other Gram-negative bacteria are also associated with acute anterior uveitis (15). In addition, uveitis is observed in AIDS patients with a past medical history of CMV retinitis (16). Interestingly, uveitis occurs after immune reconstitution following highly active antiretroviral therapy. The pathophysiology may involve a cellular response directed against CMV Ags that are expressed in retinal cells.

Whatever the etiology and the mechanism of uveitis (autoimmune or not, postinfectious or not), it appears that the presence of Th1 cells attracting activated macrophages into the immune privileged ocular site rapidly induces a pathologic uveitis. Thus, even if activated in the periphery, CD4 T cells that react against an Ag expressed in the eye present the risk of inducing uveitis if they are reactivated at this specific site. To test this hypothesis and to improve our understanding of the pathophysiology of the disease, we have developed a new mouse model of uveitis.

Stable expression of a model neoantigen, the influenza virus hemagglutinin (HA), was induced in the retina by gene transfer with recombinant adeno-associated viral (AAV) vector (17, 18). Mice were then adoptively transferred with HA-specific T cells derived from TCR transgenic mice, followed by s.c. immunization with the cognate HA peptide. All animals developed clinical signs and lesions of uveitis within 10–15 days after immunization. Donor pathogenic T cells that exhibited a proinflammatory cytokine profile, identified by a congenic marker, migrated specifically only into the eye expressing HA. We show clearly that the disease could be down-regulated by administration of HA-specific CD4+CD25+ regulatory T cells and up-regulated in the absence of these cells.
Materials and Methods

Animals

Six- to eight-week-old female BALB/c mice were obtained from Charles River Laboratories. The TCR-HA transgenic mice (19) that express a TCR recognizing 1-E-restricted HA epitope 110–120 (SFREPFFHPKE) were backcrossed >10 generations onto a BALB/c genetic background, then bred with congenic Thy-1.1 BALB/c mice to generate Thy-1.1 TCR-HA mice. Mice, bred in our animal facility under specific pathogen-free conditions, were manipulated according to the European Union guidelines.

Viral vectors and methods for intravitreal injection

AAV2/5-HA and AAV2/5-GFP were produced by the Gene Vector Production Network (Genethon). To observe the vitreous and retina, the iris was dilated with 0.5% tropicamide (Novartis). Surgery for subretinal injection was performed under direct retinoscopy using a binocular microscope (Wild M3B; Leica) according to the procedure described previously (20). A 33-gauge needle (Hamilton) was brought into focus between the retina and retinal pigment epithelium, and 2 μl of viral supernatant was injected to produce retinal detachment. For intravitreal injection, 2 μl of viral suspension was injected directly into the vitreous behind the lens.

Preparation of CD25− and CD25+ cells

CD25− and CD25+ cells preparations were obtained from Thy-1.1 TCR-HA mice as previously described (21). Briefly, after mechanical dissociation of spleen and peripheral lymph nodes (inguinal, brachial, axillary, and cervical), cells were stained with biotin-labeled anti-CD25 (7D4; Miltenyi Biotec) and incubated with antibiotin magnetic microbeads and cervical), cells were stained with biotin-labeled anti-CD25 mAb (7D4; Miltenyi Biotec) and incubated with antibiotin magnetic microbeads (Miltenyi Biotec). CD25− and CD25+ cell fractions were then separated using LS columns (Miltenyi Biotec). Less than 0.3% of the CD25− cell preparation contained CD4+CD25+ cells, and the CD25+ cell preparation was 92% pure. CD25− cells from Thy-1.1 TCR-HA were used to induce uveitis as described in the next paragraph. CD25+ cells from Thy-1.1 TCR-HA mice were stimulated by irradiated (20 Gy) BALB/c splenocytes, HA peptide (10 μg/ml), and murine IL-2 (10 ng/ml; R&D Systems) for 4 days. These activated regulatory CD25+ cells were used to control uveitis as described in Fig. 7. When required, cells were labeled with CFSE (Sigma-Aldrich) by incubating with 2.5 μM CFSE in protein-free PBS for 10 min at room temperature just before transfer.

Induction of uveitis

Uveitis was induced in BALB/c mice 1 mo after intraocular injection of the AAV2/5-HA vector using four different experimental procedures (Table I). The first procedure (experimental procedure 1) was performed in the experiments described in Figs. 1–5. BALB/c mice were sublethally irradiated (3 Gy). The next day, mice were injected in the tail vein with 2 × 106 CD25− cells purified from Thy-1.1 TCR-HA mice. The following day, mice were injected s.c. in the footpad with 2 × 106 CD25− cells purified from Thy-1.1 TCR-HA mice. The following day, mice were injected s.c. in the footpad with 2 μg of peptide HA 111–119 (SFE) emulsified in CFA and received an i.p. injection of pertussis toxin (1 μg/ml; Sigma-Aldrich). CD25+ cells were purified from Thy-1.1 TCR-HA mice and incubated with antibiotin magnetic microbeads (Miltenyi Biotec). CD25− and CD25+ cell fractions were then separated using LS columns (Miltenyi Biotec). Less than 0.3% of the CD25− cell preparation contained CD4+CD25+ cells, and the CD25+ cell preparation was 92% pure. CD25− cells from Thy-1.1 TCR-HA were used to induce uveitis as described in the next paragraph. CD25+ cells from Thy-1.1 TCR-HA mice were stimulated by irradiated (20 Gy) BALB/c splenocytes, HA peptide (10 μg/ml), and murine IL-2 (10 ng/ml; R&D Systems) for 4 days. These activated regulatory CD25+ cells were used to control uveitis as described in Fig. 7. When required, cells were labeled with CFSE (Sigma-Aldrich) by incubating with 2.5 μM CFSE in protein-free PBS for 10 min at room temperature just before transfer.

Uveitis grading

Experimentally induced uveoretinitis (EUR) scores were evaluated 10–15 days after intraocular injection of the AAV2/5-HA vector using four different experimental procedures (Table I). The first procedure (experimental procedure 1) was performed in the experiments described in Figs. 1–5. BALB/c mice were sublethally irradiated (3 Gy). The next day, mice were injected in the tail vein with 2 × 106 CD25− cells purified from Thy-1.1 TCR-HA mice. The following day, mice were injected s.c. in the footpad with 2 μg of peptide HA 111–119 (SFE) emulsified in CFA and received an i.p. injection of pertussis toxin (1 μg/ml; Sigma-Aldrich). The second procedure (experimental procedure 2) (Fig. 6, A and B) was performed as in experimental procedure 1 without sublethal irradiation of the recipient mice. In the third procedure (experimental procedure 3) (Fig. 7), uveitis was induced in nonirradiated mice by i.v. administration of 2 × 106 activated HA-specific T cells obtained as follows: CD25− cells purified from Thy-1.1 TCR-HA mice were stimulated by irradiated (20 Gy) BALB/c splenocytes and HA peptide (10 μg/ml) for 4 days. In the fourth procedure (experimental procedure 4) (Fig. 6, C and D), TCR-HA transgenic mice were depleted or not depleted of CD25+ cells. Ten days later, mice were subretinally injected with AAV2/5-HA.

T cell proliferation assay and cytokine detection

Cells from spleens, lymph nodes, and eyes were stimulated by irradiated syngeneic splenocytes (ratio 1:2) in the presence or absence of the cognate HA peptide for 72 h at 37°C in U-bottom 96-well plates. In proliferation assays, spleen or lymph nodes cells were pulsed with 1 μCi of [3H]thymidine for the final 16 h. For cytokine detection, supernatants were collected after 48 h and assayed for TNF-α, INF-γ, IL-2, IL-4, and IL-5 using a mouse Thy1/Th2 cytokine cytometric bead array from BD Biosciences according to the manufacturer’s instructions.

FIGURE 1. Stable expression of HA in the retina after injection of AAV2/5-HA. A, Flat-mount preparations of the retina were performed 4 wk after subretinal (SR) injection of AAV2/5-HA in BALB/c mice. Expression of HA was detected by immunostaining with a biotin-conjugated anti-HA Ab revealed by FITC-streptavidin, and control was incubated with FITC-streptavidin. B, Original magnification was ×200. B, Confocal microscopy on retinal flat mounts (a–c) or cryosections (d) and standard microscopy (e and f) were performed 4 wk after subretinal (SR) (a, b, and e) or intravitreal (IVT) injections of AAV2/5-HA (c, d, and f). a–d, Expression of HA was detected by immunostaining with a biotin-conjugated anti-HA Ab revealed by FITC-streptavidin. Scale bands represent 20 μm (c), 10 μm (a and d), and 5 μm (b). Confocal analysis showed expression of HA on photoreceptor inner segment membranes (a), photoreceptor cell bodies (b), and ganglion cells (c and d). e and f, H&E staining was performed on the following retinal sections: ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), and retinal pigmentary epithelium (RPE). Original magnification was ×400. Mice injected with AAV2/5-HA had normal retinas without inflammatory cell infiltration.
FIGURE 2. Induction of clinical signs of uveitis after injection of HA-specific T cells in mice expressing HA in the retina. Subretinal (SR) or intravitreal (IVT) injections of AAV2/5-HA (●) or AAV2/5-GFP (○) were performed in both eyes of BALB/c mice. One month later, mice were nonlethally irradiated (3 Gy), injected i.v. with CD25^+ cells from TCR HA-transgenic mice, and immunized with the 111–119 HA peptide emulsified in CFA. A, EUR scores were evaluated 9–12 days after immunization by clinical examination (slit lamp biomicroscopy) as described in Materials and Methods. Each point corresponds to one mouse (average of both eyes). Horizontal bars represent the mean of each group. Data are a compilation of six separate experiments. B, Examination of the anterior chamber after (a and c) or before iris dilation (b and d) with tropicamide (0.5%) in BALB/c mice that had received subretinal injection of AAV2/5-HA into the right eye (a and b) and AAV2/5-GFP into the contralateral eye (c and d). Biomicroscopy was evaluated between 9 and 12 days after immunization. The pupil border is outlined. Only mice expressing HA in the eye developed clinical signs of uveitis.

Results

Retinal expression of HA after intravitreal or subretinal injection of rAAV-HA

Long-term expression of enhanced GFP in the retina has been obtained after injection of an rAAV2/5 vector in the eye (17, 26, 27). To induce stable expression of HA in the retina of BALB/c mice, we performed intravitreal or subretinal injections of a rAAV2/5 vector carrying HA (AAV2/5-HA). Thirty days after injection, anti-HA labeling was performed on the retina. Subretinal injection resulted in a strong fluorescent signal in all preparations. Round patches of transduced fluorescent cells were observed at the injection site, localized by the retinal detachment created by the injection (Fig. 1Aa), but were not in controls (Fig. 1Ab). By confocal microscopy of flat retinal preparations, fluorescence was detected in the external segment of photoreceptors (Fig. 1Ba) and in the photoreceptor cellular bodies (Fig. 1Bb). In contrast, after intravitreal injection of AAV2/5-HA, a weak and diffuse fluorescent signal covered the whole internal surface of the retina. The majority of transduced cells were ganglion cells (Fig. 1Bc, c and d), and no signal was seen in retinal pigmentary epithelium-choroid-sclera flat mounts (data not shown).

There was no histologically detectable toxicity or immune reaction due to HA protein expression 30 days after AAV2/5-HA injection. Indeed, sections of the retina after subretinal (Fig. 1Be) or intravitreal (Fig. 1Bf) injections showed no infiltration of inflammatory cells. All retinal layers were normal and well preserved. Similar findings were obtained when analyzed 3 mo after AAV2/5-HA injection (data not shown). Thus, depending on the model, spontaneous uveitis was induced (28) or not induced (29–31) as a result of transgenic retinal expression following subretinal injection of an AAV vector. For the rest of the study, uveitis induction was performed 1 mo after intraocular gene transfer of AAV2/5-HA in mice that expressed retinal HA and had a reconstituted blood-retinal barrier.

Injection of HA-specific T cells in irradiated mice expressing retinal HA induces clinical uveitis

In some models of autoimmunity, diseases were obtained by the transfer of pathogenic cells in sublethally irradiated recipients (32, 33). Thus, 1 mo after intraocular injection of AAV2/5-HA, mice were sublethally irradiated and injected i.v. with T cells derived from TCR-HA transgenic mice. Donor cells from transgenic mice enriched in HA-specific T cells were depleted of CD25^+ cells that
exhibit suppressive function (34–36). The next day, mice were immunized s.c. with HA peptide emulsified in CFA, which induced strong activation of donor HA-specific T cells in draining lymph nodes. The severity of ocular inflammation was determined by slit lamp examination between days 7–15 postimmunization. Subretinal and intravitreal injections of AAV2/5-HA were equally effective in inducing clinical signs of uveitis (Fig. 2A). To analyze whether HA-specific donor T cells affect only HA-expressing eyes, mice were injected subretinally with AAV2/5-HA in the right eye and with AAV2/5-GFP in the left eye. Ocular inflammatory signs were observed only in eyes expressing HA, with posterior synechiae and total (Fig. 2Ba) or partial (Fig. 2Bb) pupillary occlusion. Control eyes, which expressed GFP, did not show any signs of inflammation (Fig. 2B, c and d). For the rest of the study, we report only results obtained with subretinal injections of AAV2/5-HA in both eyes. This model will subsequently be referred to as HA-induced EUR (HA-EUR).

**Retina of HA-EUR are highly infiltrated with inflammatory cells**

Retinal tissues in HA-EUR were analyzed from mice that have received HA-specific T cells expressing the congenic Thy-1.1 marker that allowed detection of pathogenic donor T cells by immunohistology and flow cytometry. Sections showed severe lesions concentrated in the photoreceptor cell layer, disclosing focal destruction of the photoreceptor cells and some bipolar cells with
a fibroblastic scar (Fig. 3a). Immunohistology performed on a flat-mount retina to analyze the presence of Thy-1.1 T cells showed large numbers of infiltrating Thy-1.1 donor T cells around the injection site in the retina (Fig. 3b). In control eyes injected with AAV2/5-GFP, no lesions were detected in tissue sections. All retinal layers were normal and well preserved (Fig. 3c), and no infiltration of Thy-1.1+ T cells were detected by immunohistology on flat-mount retina (Fig. 3d).

To further characterize the composition of cells infiltrating the retina in HA-EUR, we isolated ocular-infiltrating cells and analyzed them by flow cytometry. Whereas no CD4+ Thy-1.1+ T cells were detected in the eyes of AAV2/5-GFP injected animals (not shown), numerous CD4+ Thy-1.1+ T cells were found in eyes injected with AAV2/5-HA (Fig. 4A). Indeed, Thy-1.1+ donor T cells represented half of the CD4+ T cell population, and >80% of them, identified with an anticonalotyptic Ab, were specific for HA. The CD4+ Thy-1.1- cells were infiltrating recipient T cells. CD8+ T cells, CD8+ Thy-1.1+ T cells, and anti-HA donor CD8+ T cells were present in similar proportions (Fig. 4B). Ag-specific effector T cells represent <0.1% of the total T cell repertoire. T cells were not the only infiltrating cell types. Numerous macrophages (CD11b+ CD11c-) and dendritic cells (CD11c+) were observed in eye infiltrates of mice with HA-EUR. Numbers of B cells (CD19+) were very low and were not increased in inflammatory infiltrates compared with what was detectable in the needle-perforated eyes of control mice without injection.

To evaluate effector Thy-1.1+ T cell proliferation in HA-EUR, donor cells were labeled with CFSE before transfer. Loss of CFSE staining is indicative of cell division. Fourteen days after immunization with HA peptide, a significant fraction of donor Thy-1.1+ T cells had gone through more than five divisions in draining lymph nodes (LNs), 34% of them were specific for HA Ag (6.5+). In the eye, all Thy-1.1+ donor T cells had gone through more than five divisions with no intermediate division states, suggesting either local reactivation of uveitogenic T cells or preferential migration of highly proliferating T cells into this site (Fig. 4A).

**Responsiveness to HA and cytokine profiles in HA-EUR**

Fourteen days after HA immunization in the HA-EUR model, we analyzed T cell proliferation to HA. Significant proliferation to HA

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**FIGURE 5.** Proliferative response to peptide HA of infiltrating ocular, draining lymph node cells, and splenic cells. HA-EUR was obtained as described in Fig. 2A after subretinal injection of the AAV2/5-HA vector. Fifteen days after immunization, animals were sacrificed. A. Draining lymph node cells and splenic cells were harvested, and CD3+ T cells (100,000) were stimulated in vitro with HA-peptide (10 μg/ml) and 200,000 irradiated spleen cells from BALB/c mice for 90 h. Proliferative responses were analyzed by [3H]thymidine incorporation for the final 16 h. Data are given as the stimulation index, i.e., the mean counts in cultures with the HA peptide divided by the mean counts in control culture without peptide. B, CD3+ infiltrating T cells (50,000) of the eye were CFSE-labeled and stimulated with or without HA-peptide as described above for 90 h. Cell divisions and surface phenotypes were analyzed by flow cytometry. The graph shows data compiled from three separate experiments. C and D, CD3+ T cells infiltrating draining LN (C) or eyes (D) of HA-EUR mice were stimulated with irradiated spleen cells from BALB/c mice (1:2 ratio) with or without HA-peptide (10 μg/ml). After 48 h, culture supernatants were assayed for TNF-α, IFN-γ, IL-2, IL-4, and IL-5 cytokines by using a cytokine bead assay as described in Materials and Methods. Error bars represent SEM. Results are representative of two separate experiments with a total of six mice. Dotted lines represent the limit of detection of the assay.
was detected in popliteal LNs draining the injection site and cervical LNs draining the eyes (37) and the spleen, but not in non-draining brachial LNs. Low proliferation was observed in control cultures without the cognate peptide (Fig. 5A). This observation indicates that HA-specific T cells were enriched not only in the draining popliteal LNs but also in the spleen and cervical LNs and that these cells were not anergic at the time of EUR.

Such an assay could not be performed on cells infiltrating the eye because of their small numbers. Therefore, we analyzed T cell proliferation of CFSE-labeled cells infiltrating the eye by flow cytometry (Fig. 5B). The majority of donor T cells detected at the end of the culture in the presence of the HA peptide had gone through more than five divisions. A similar profile was observed in cultures without added HA peptide, probably because T cells were activated by HA expressed by virally transduced retinal cells present in the culture. Thus, even in the eyes a significant fraction of HA-specific T cells was reactive. Furthermore, recipient T cells detected in the culture had divided between one and more than five times, with or without HA peptide. This suggests that recipient T cells present in the eye may have been reactive to self-Ags expressed in the tissue.

Because Th1 T cells have been implicated in the pathophysiology of autoimmune uveitis, we analyzed cytokines produced by T cells reacting to HA in draining LNs after in vitro restimulation (Fig. 5C). To measure cytokines produced by cells of the eye, we restimulated T cells in vitro and used a sensitive technique allowing cytokine detection in 50 μl of culture supernatant (Fig. 5D). With or without HA peptide stimulation, significant amounts of the Th1 type cytokine IFN-γ and the proinflammatory cytokine TNF-α were observed. In the absence of added HA peptide, HA-specific T cells were probably reactivated by retinal cells expressing HA. IL-2, IL-4, and IL-5 cytokines were below the levels of detection of our tests. Thus, cells exhibiting a cellular proinflammatory profile were present in the inflamed eyes of mice with HA-EUR.

Up to this point, HA-EUR was obtained after sublethal irradiation (experimental procedure 1 of Table I). However, this manipulation has potentially multiple effects such as homeostatic expansion of HA-specific transferred T cells, depletion of endogenous regulatory T cells (Tregs), and alteration of the blood-retinal barrier, which may weaken the clinical relevance of our model. We thus explored the possibility of inducing uveitis without irradiation. When mice were not irradiated (experimental procedure 2 of Table I), they developed delayed and weak uveitis (compare Fig. 6A to Fig. 2). We thus tested whether a more robust and accelerated uveitis could be obtained upon depletion of endogenous Tregs or by transfer of in vitro preactivated HA-specific T cells.

### Table I. Experimental procedures

<table>
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<th>Experimental Procedure (no.)</th>
<th>Mice</th>
<th>AAV2/5-HA Injection (day)</th>
<th>Irradiation (day)</th>
<th>Transfer of Effector T cells (HA specific T cells)</th>
<th>Immunization CFA-HA Peptide PTX (day)</th>
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*In experimental procedures 1–3, uveitis was induced in BALB/c mice 1 mo after intraocular injection of the AAV2/5-HA vector. In experimental procedure 1, BALB/c mice were sublethally irradiated on day –2. The day after (day –1), the mice were injected in the tail vein with 2 × 10⁸ CD25<sup>+</sup> cells purified from Thy-1.1 TCR-HA mice. On day 0, the mice were immunized with peptide HA (SFE) emulsified in CFA and received an i.p. injection of pertussis toxin (PTX). Experimental procedure 2 was performed identically to experimental procedure 1, but without sublethal irradiation of the recipient mice. In experimental procedure 3, uveitis was induced in nonirradiated mice by i.v. injection of 2 × 10⁶ activated HA-specific T cells (day 0). In experimental procedure 4, uveitis was induced in TCR-HA transgenic mice after subretinal injection of AAV2/5-HA (day 0).

Endogenous CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells play an inhibitory role in the development of HA-induced EUR model

Aviechezer et al. (38) showed that CD4<sup>+</sup>CD25<sup>+</sup> Tregs play a role in experimental autoimmune uveitis (38, 39). To examine a possible contribution of Tregs in the susceptibility to EUR in our model, we analyzed the effect of endogenous Treg depletion in nonirradiated recipients expressing HA in retina and adoptively transferred with anti-HA CD25<sup>+</sup> cells followed by immunization with the cognate HA peptide (experimental procedure 2 of Table I). Clinical signs developed earlier (7 days compared with 15 days) and were more severe in Treg-depleted mice compared with non-depleted control mice (Fig. 6, A and B). Uveitis was also induced by viral transduction of HA directly in TCR-HA transgenic mice (Fig. 6, C and D) as described in the legend of Table I for experimental procedure 4. In this model, in which activation of HA-specific T cells was likely initiated in the eyes or in their draining LNs, nondepleted control mice developed moderate clinical signs 4 days after gene transfer. In contrast, Treg-depleted mice developed severe EUR 2 days after gene transfer. Taken together, these data confirmed that Tregs play a regulatory role in uveitis.

Control of uveitis by TCR HA-specific regulatory T cells

The injection of Tregs specific for target tissues suppresses several autoimmune diseases (40, 41) Thus, we explored whether HA-specific Tregs could be used to prevent development of uveitis. Tested in the above model of HA-EUR (procedure 1 of Table I), administration of HA-specific Treg regulated the disease, but did so most likely by regulating the activation and expansion of T cells in popliteal draining LNs (data not shown). To test the capacity of Tregs to block disease progression directly in target ocular tissue, we modified our animal model of uveitis. Instead of in vivo priming, HA-specific T cells were activated in vitro. These cells were then adoptively transferred into nonirradiated and nonimmunized mice that stably expressed HA in the eyes (experimental procedure 3 of Table I). Severe uveitis developed between 7 and 10 days after cell transfer. Clinical signs and lesions were similar to those observed in the HA-EUR model obtained after irradiation (not shown). We then tested the capacity of HA-specific Tregs to prevent uveitis. Coinjection of freshly purified Tregs had no effect, probably because they could not cross the blood-retinal barrier and enter the eyes (data not shown). However, coinjection of pathogenic T cells and in vitro activated Treg i.v. (2 × 10⁶ cells) reduced clinical signs of uveitis as compared with control mice receiving only pathogenic T cells. Interestingly, even when administered 4 days after pathogenic T cells, systemic injections of
activated Treg decreased the severity of the disease (Fig. 7A). Levels of ocular infiltration by HA-specific pathogenic cells confirmed clinical data. When HA-specific Tregs were administered, numbers of pathogenic T cells, identified using an antyclonotypic Ab, were significantly reduced (Fig. 7B). Finally, to determine the numbers and divisions of preactivated Tregs, these cells, which express the congenic marker Thy-1.1, were labeled with CFSE just before the injection. Flow cytometry analysis showed a correlation between the inhibition of ocular infiltration by anti-HA effector T cells and the number of HA-specific Tregs. Most of the Tregs had been activated in the eye, because they went through cell divisions (Fig. 7C). This experiment shows that in vitro activated Tregs specific for an Ag expressed in the eye are reactivated in situ, allowing regulation of Th1-dependent uveitis.

Discussion

In this work, we describe a new and highly reproducible model of uveitis. In classical models of EAU, the disease was induced by immunization with a retinal Ag (42, 43). However, our model of uveitis could not be obtained simply by immunization with the HA peptide emulsified in CFA in recipient mice expressing HA in their retina (not shown). Unlike classical models, EUR was obtained only after the transfer of retinal Ag (HA)-specific effector cells. These different requirements for disease induction could be due to the nature of the retinal Ag (constitutive self-Ag vs neoantigen expressed only after vector transfer in adult) or the genetic background. Importantly, our model adds the unique possibility of following the pathogenic T cell behavior and migration at a level not possible in previous EAU models. The HA neoantigen expressed in the eye attracts and activates transferred, HA-specific T cells that initiate retinal lesions and clinical signs of uveitis. After initial activation in lymphoid tissues, T cells specific for microbial Ags would be reactivated in the eyes, inducing postinfectious uveitis.

In HA-EUR, posterior segment involvement with vitritis and retinochoroidal infiltrates occurred after a brief episode of anterior uveitis. Focal retinal destruction occurred at the end of the acute phase, followed by chronic retinal inflammation persisting for more than 1 mo (data not shown). Interestingly, similar pathological features and kinetics of ocular inflammation have been observed in previously described models of autoimmune uveitis (24). Additionally, similar clinical signs of uveitis were observed in our model, whether the target Ag was diffusely expressed in the internal retina (intravitreal injection) or locally expressed in the external retina (subretinal injection). Thus, the eye is a particular site that is highly sensitive to the pathologic effects of activated Th1 cells, which induce severe uveitis with similar clinical signs irrespective of its etiology and the localization of the target Ag expression.
In our model, pathogenic T cells could be traced using either a congenic marker or an anticlonotypic Ab, which provides a unique tool for better understanding the pathophysiology of uveitis such as migration, specificity, activation, and effector functions of pathogenic cells. After activation in peripheral draining LN, HA-specific T cells crossed the blood-ocular barrier of both eyes because of its permeability to activated T cells as previously reported (44). Therefore, HA-specific T cells were detected only in the eye expressing HA and not in the control eye expressing GFP. Their unique location is likely due to the fact that APCs presented HA in situ to specific T cells, allowing their retention and/or their local activation (45, 46). Thus, a gene-transferred Ag in the eye can be processed and presented by local APCs at a level effective for recognition by T cells. This was confirmed in our study, because retinal cells transduced with HA activated HA-specific T cells in the absence of exogenous HA. Importantly, not only were HA-specific T cells present in the affected eye, but also recipient T cells and numerous macrophages that have pathogenic roles in EAU (11, 47, 48). In addition, we were able to detect for the first time IFN-γ and TNF-α cytokines directly in the eyes of mice. IFN-γ was probably secreted by activated Th1 type T cells, and TNF-α could have been derived from both T cells and macrophages. Based on these data, the following scenario for the pathophysiology of HA-EUR can be proposed. T cells specific for a retinal Ag are initially activated in peripheral lymphoid tissues. Then, they migrate into the eye and are further activated by local APCs processing the cognate Ag. These T cells secrete cytokines and chemokines that attract other T cells, macrophages, and dendritic cells, leading to the amplification of inflammation and the secretion of IFN-γ and TNF-α, which are detrimental to the integrity of the retina. Recently, Thura et al. (46) showed migration of activated T cells before the induction of uveitis. Ag-specific T cells expressing GFP and adoptively transferred into mice were tracked into ocular tissues 74 h post injection. Flow cytometry analysis suggested that intraocular presentation of specific Ags was a prerequisite for T cell reactivation and the recruitment of inflammatory cells.

Previous reports suggest that CD4+CD25+ natural suppressor T cells control uveitis. When mice were immunized with low doses of interphotoreceptor retinoid-binding protein, no or only moderate EAU was induced. Depletion of CD25+ Tregs exacerbated the disease, because all of the mice then developed severe EAU (38, 39). In addition, Treg deficiency induced by neonatal thymectomy was associated with the development of autoimmune uveitis (38). Accordingly, in our model the depletion of CD25+ Tregs exacerbated clinical signs of HA-EUR, confirming the role of these cells in regulating uveitis. These findings open new prospects in immunotherapeutic interventions of uveitis. We and others have demonstrated the therapeutic potential of Tregs in various autoimmune diseases (40, 41, 49, 50) and graft-vs-host disease (51, 52). In our model of uveitis, administration of specific Tregs in peripheral blood significantly reduced EUR. The effect was also observed when Tregs were injected 4 days after pathogenic T cells, a time when the latter cells have started infiltrating the eyes. To have an effect locally, injection of cells or reagents into the eyes of patients with uveitis is currently being proposed. If efficient, administration of Tregs in situ could have the great advantage of inducing local immunosuppression in this confined organ without affecting the rest of the immune system.

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Disclosures
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