TNFRSF25 Agonistic Antibody and Galectin-9 Combination Therapy Controls Herpes Simplex Virus-Induced Immunoinflammatory Lesions

Pradeep B. J Reddy,^a Taylor H. Schreiber,^b Naveen K. Rajasagi,^a Amol Suryawanshi,^c Sachin Mulik,^a Tamara Veiga-Parga,^a Toshiro Niki,^d Mitsuomi Hirashima,^d Eckhard R. Podack,^b and Barry T. Rouse^a

Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, Knoxville, Tennessee, USA^a; Department of Microbiology and Immunology, University of Miami Miller School of Medicine, Miami, Florida, USA^b; Department of Ophthalmology, Tufts University School of Medicine, Boston, Massachusetts, USA^c; and Department of Immunology and Immunopathology, Faculty of Medicine, Kagawa, Japan^d

Ocular infection with herpes simplex virus 1 (HSV-1) results in a chronic immunoinflammatory reaction in the cornea, which is primarily orchestrated by CD4⁺ T cells. Hence, targeting proinflammatory CD4⁺ T cells or increasing the representation of cells that regulate their function is a relevant therapeutic strategy. In this report, we demonstrate that effective therapeutic control can be achieved using a combination of approaches under circumstances where monotherapy is ineffective. We use a convenient and highly effective monoclonal antibody (MAb) approach with MAbT25 to expand cells that express the tumor necrosis factor receptor superfamily member 25 (TNFRSF25). In naïve animals, these are predominantly cells that are Foxp3-positive regulatory T cells. MAbT25 treatment before or at the time of initial HSV infection was an effective means of reducing the severity of subsequent stromal keratitis lesions. However, MAbT25 treatment was not effective if given 6 days after infection since it expanded proinflammatory effector T cells, which also express TNFRSF25. Therefore, the MAbT25 procedure was combined with galectin-9 (Gal-9), an approach that compromises the activity of T cells involved in tissue damage. The combination therapy provided highly effective lesion control over that achieved by treatment with one of them. The beneficial outcome of the combination therapy was attributed to the expansion of the regulatory T cell population that additionally expressed activation markers such as CD103 needed to access inflammatory sites. Additionally, there was a marked reduction of CD4⁺ gamma interferon-producing effector T cells responsible for orchestrating the tissue damage. The approach that we describe has potential application to control a wide range of inflammatory diseases, in addition to stromal keratitis, an important cause of human blindness.

Chronic tissue damage resulting from infectious or autoimmune diseases often results from a dysregulated host response to persisting antigens (6, 21). The extent of tissue damage in inflammatory lesions can often be limited and even reversed if the balance of cellular or humoral components involved is changed (21). For example, in stromal keratitis (SK), the blinding ocular lesion caused by herpes simplex virus (HSV) infection, lesion severity is minimized if the balance of the host response is changed to emphasize CD4⁺ T cells that have a regulatory function rather than those that are proinflammatory (29). Accordingly, such regulatory T cells (Tregs) can suppress the activity of the proinflammatory CD4⁺ T cells that orchestrate SK lesions (29). Specifically, SK lesions become far more severe in animals with absent or suppressed Treg responses and are reduced in magnitude if animals are given adoptive transfers of Tregs (28, 29) or receive some therapies that expand or activate Tregs (26, 27). Unfortunately, convenient methods to cause Treg expansion and activation, particularly the population that is antigen specific, are limited.

A novel approach recently described by Podack and colleagues may prove to be a convenient method to expand Tregs (24). The approach takes advantage of the fact that Foxp3-positive (Foxp3⁺) regulatory T cells but less so other naïve T cell subsets constitutively express high levels of the tumor necrosis factor (TNF) receptor superfamily member 25 (TNFRSF25) (24). TNFRSF25 has had several other names that include death receptor 3 (DR3), indicating that it plays a role in apoptosis of activated T cells (4). Stimulation of TNFRSF25 on antigen-activated cells may also cause them to produce more effector cytokines. However, stimulation of TNFRSF25 on Tregs with an agonistic antibody in the absence of exogenous antigen rapidly expands Tregs

(24). This Treg population proliferates and expands to 30% or more of total CD4⁺ T cells in healthy animals following a single exposure to anti-TNFRSF25 monoclonal antibody (MAb) MAbT25. Interestingly, the approach was highly effective at reducing lung pathology when used prophylactically in an allergic disease model (24). Whereas the MAbT25 therapy worked well when it was used prior to the induction of lesions, the consequences of such therapy need to be further evaluated in a therapeutic setting. This is because proliferating and activated effector T cells may also express TNFRSF25 (25), and so targeting this receptor in active disease may costimulate both regulatory and effector T cells, with unknown disease consequences.

In the study described in the present report, we have used the SK model to compare the outcomes of MAbT25 treatment at different stages of the disease process. Our results demonstrate that therapy before or at the time of initial infection was an effective means of reducing the severity of subsequent SK lesions. However, if therapy was given 6 days after infection, then lesions became even more severe than those observed in untreated infected animals. Nevertheless, if animals in the therapeutic setting were additionally treated with galectin-9 (Gal-9), an approach that inhibits effector T cell function without impairing Treg activity (27, 33),

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Address correspondence to Barry T. Rouse, btr@utk.edu.

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FIG 1 MAbT25 induces rapid expansion of CD4⁺ Foxp3⁺ cells in HSV-1-infected mice. To demonstrate the effect of MAbT25 on Treg expansion in HSV-1-infected mice, Foxp3-GFP mice were infected with HSV-1 and injected i.p. with a single dose of MAbT25 or isotype on day 1 p.i. (A) FACS plots showing Foxp3⁺ T cells in blood and spleen at different time points in HSV-1-infected mice treated with MAbT25; (B) kinetics of Treg expansion in peripheral blood and spleen of HSV-1-infected mice treated with MAbT25; (C) kinetics of Treg expansion in the peripheral blood and spleen of MAbT25-treated naïve mice. Data represent means $\pm$ SEMs of at least two independent experiments with ≥ 15 mice per group. At least 3 mice were sacrificed at each time point to isolate spleens and blood to measure Treg frequencies. Statistical significance was calculated by Student's *t* test at each time point between the spleen samples isolated from isotype- and MAbT25-treated groups (***, *P* < 0.0001).

the combination therapy recipients showed SK lesions markedly reduced compared to those in monotherapy-treated or untreated animals. The lesions were also significantly diminished compared to those in either control infected untreated animals or infected animals treated only with Gal-9. The beneficial outcome of the combination therapy was attributed to the expansion of the Treg population that additionally expressed activation markers needed to access inflammatory sites, along with a marked reduction in the presence of CD4⁺ gamma interferon-producing (IFN- γ ⁺) effector T cells responsible for orchestrating the tissue damage. Many of the effectors were likely eliminated by apoptosis, like that which occurs *in vitro* when Gal-9 binds to T cell immunoglobulin and mucin domain 3 (Tim-3) receptors expressed by activated effector T cells (1, 33). Our results emphasize that combination therapy, as demonstrated in this study by expanding Tregs and reducing effector T cells, represents a promising approach that may be broadly applicable to control chronic inflammatory diseases in a wide range of diseases.

MATERIALS AND METHODS

Ethics statement. This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Research Council (14a). All animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved animal facilities. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Tennessee (PHS assurance number A3668-01). All experimental procedures were in complete agreement with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. Herpes simplex virus 1 (HSV-1) eye infection was performed under anesthesia (tribromoethanol [Avertin]), and all efforts were made to minimize animal suffering.

Mice and virus. Six- to 8-week-old female C57BL/6 mice and congenic Thy1.1⁺ B6.PL (H-2^b) mice were purchased from Harlan Sprague-Dawley and The Jackson Laboratory, respectively. C57BL/6 green fluorescent protein (GFP)-Foxp3-knock-in mice (Foxp3-GFP) mice were a kind gift from M. Oukka (Brigham and Women's Hospital, Harvard Medical School). HSV-1 strain RE was grown on Vero cells obtained from the

FIG 2 MAbT25 administration before or following HSV infection reduces the severity of SK lesions. C57BL/6 mice infected with HSV-1 were treated with MAbT25 or isotype antibody either 2 days before infection or at 8 h p.i. (A) SK disease progression was scored at different time points starting from day 8 p.i. until day 15 p.i. ($n = 5$ to 6 mice per group). DPI, day postinfection. Two-way ANOVA with Bonferroni's multiple-comparison test was used to calculate significance between the groups (**, $P < 0.01$; ***, $P < 0.001$). (B) Individual SK lesion scores of MAbT25-treated and control mice on day 15 p.i. One-way ANOVA with Tukey's posttest was performed to calculate significance between control and MAbT25-treated groups (**, $P < 0.01$; ***, $P < 0.001$). (C) Eyes from different

American Type Culture Collection (ATCC CCL81). The harvested virus was titrated and stored in aliquots at -80°C until further use.

Anti-TNFRSF25 agonistic antibody and kinetics of Treg expansion. Armenian hamster hybridomas producing antibodies to mouse TNFRSF25 receptor (4C12 or MAbT25, agonistic) were described previously (8). The Armenian hamster IgG isotype control was purchased from eBioscience. MAbT25 was reconstituted in phosphate-buffered saline (200 μl), and a single dose of 20 $\mu\text{g}/\text{mouse}$ was administered by the intraperitoneal (i.p.) route to naïve C57BL/6 Foxp3-GFP mice. Another group of mice was infected with HSV-1, and MAbT25 was administered on day 1 postinfection (p.i.). Blood samples were collected at intervals from MAbT25-treated C57BL/6 Foxp3-GFP mice (HSV infected or uninfected) to record the percentage of CD4^{+} T cells that were Foxp3 positive. Three or more mice were sacrificed at each time point to record the percentage of the Treg population in the spleen.

Corneal HSV infection and clinical scoring. Corneal infections of mice were conducted while the mice were under deep anesthesia induced by i.p. injection of tribromoethanol. Mice were scarified on the corneas with a 27-gauge needle, and a 3- μl drop containing 1×10^4 PFU of virus was applied to the eye. The eyes were examined on different days p.i. for the development and progression of clinical lesions by using a slit-lamp biomicroscope (Kowa Company, Nagoya, Japan). The scoring system was as follows: 0, normal cornea; +1, mild corneal haze; +2, moderate corneal opacity or scarring; +3, severe corneal opacity but iris visible; +4, opaque cornea and corneal ulcer; +5, corneal rupture and necrotizing keratitis (20).

Treatment of mice with MAbT25 and recombinant Gal-9. C57BL/6 mice infected with HSV-1 were divided into multiple groups. In the first experiment, one group of mice received MAbT25 2 days before HSV-1 infection (day -2) and another group received MAbT25 on the same day as the day of infection (day 0, 8 h p.i.). In another experiment, MAbT25 was administered during the clinical phase of disease, i.e., at day 6 p.i. All the control groups were injected with Armenian hamster IgG isotype antibody (eBioscience). In some experiments, C57BL/6 mice were injected with a single dose (20 $\mu\text{g}/\text{mouse}$) of MAbT25 on day 6 p.i., and starting from day 6, these mice also received recombinant Gal-9 (rGal-9; 50 $\mu\text{g}/\text{mouse}$) until day 14 p.i. Another group of mice received rGal-9 (100 $\mu\text{g}/\text{mouse}$) alone starting from day 6 until day 14 p.i. Mice were observed for the development and progression of SK lesions as described in the previous section.

Histopathology. For histopathological analysis, eyeballs from different groups of treated and control C57BL/6 mice were extirpated on day 15 p.i. and stored in 10% formalin. In brief, the samples were put overnight in a Tissue-Tek processor (Sakura), which removes all the moisture content from the samples and embeds it in paraffin. Tissue-Tek was automatically programmed, treating the samples sequentially with 100% alcohol, 100% xylene, and paraffin. Six-micrometer sections were then cut using a microtome and stained with hematoxylin-eosin (H&E).

Confocal microscopy. C57BL/6 Foxp3-GFP mice were infected with HSV as mentioned above. One group of mice was treated with a single dose (20 $\mu\text{g}/\text{mouse}$) of MAbT25 on day 6 p.i., and starting from day 6, these mice also received rGal-9 (50 $\mu\text{g}/\text{mouse}$) until day 14 p.i. Another group of mice received rGal-9 (100 $\mu\text{g}/\text{mouse}$) alone starting from day 6 until day 14 p.i. The mice treated with hamster IgG isotype antibody served as a control group. On day 15 p.i., the mice were sacrificed and the

corneas were isolated and directly examined under a confocal microscope (Leica SP2 LSCM) for GFP-expressing Foxp3⁺ Tregs.

Reagents and antibodies. CD4-allophycocyanin (APC; RM4-5), CD45- peridinin chlorophyll protein (53-6.7), CD11b-APC (M1/70), Ly6G-fluorescein isothiocyanate (FITC; 1A8) IFN- γ -FITC (XMG1.2), CD103-FITC (M290), annexin V-APC, anti-CD3 (145-2C11), anti-CD28 (37.51), and GolgiPlug (brefeldin A) were purchased from BD Biosciences. Foxp3-phycoerythrin (FJK-16S) and IFN- γ enzyme-linked immunosorbent assay kits were purchased from eBioscience. Phorbol myristate acetate (PMA) and ionomycin were purchased from Sigma-Aldrich. Mouse rGal-9 was provided by Gal Pharma, Japan. The antibody-stained cells were acquired with a FACSCalibur apparatus (BD Biosciences), and the data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Flow cytometric analysis. HSV-infected corneas were harvested from different groups of mice at the indicated time points p.i. Six to eight corneas per group were excised, pooled group-wise, and digested with 60 U/ml Liberase (Roche Diagnostics) at 37°C for 45 min. The single-cell suspensions obtained from the corneas were stained for different cell surface molecules for fluorescence-activated cell sorter (FACS) analysis. To measure the number of IFN- γ -producing CD4^{+} T cells, intracellular cytokine staining (ICS) was performed by stimulating the corneal single-cell suspensions with PMA-ionomycin or UV-inactivated HSV-1 as described previously (20). The stained samples were acquired with a FACSCalibur apparatus (BD Biosciences), and the data were analyzed using FlowJo software (Tree Star, Ashland, OR). Cell sorting was performed using a FACSARIA cell sorter (BD Biosciences).

Purification of Thy1.1 CD4^{+} T cells. CD4^{+} T cells were purified from a suspension of single cells from pooled draining lymph nodes (DLNs) from HSV-infected Thy1.1⁺ B6.PL (H-2^b) mice using a mouse CD4^{+} T cell isolation kit according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). The purity was achieved to an extent of 90%. Purified CD4^{+} T cells were used for the proliferation assay.

Quantitative real-time PCR (qPCR). Corneal cells were lysed, and total mRNA was extracted using TRIzol LS reagent (Invitrogen). Total cDNA was made with 500 ng of RNA using oligo(dT) primers. Quantitative PCR was performed using SYBR green PCR master mix (Applied Biosystems, Foster City, CA) with an iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA). The expression levels of different molecules were normalized to that of β -actin using the change in cycle threshold (ΔC_T) calculation. The relative expression between control and experimental groups was calculated using the $2^{-\Delta\Delta C_T}$ formula, as described earlier (31). The PCR primers used were as follows: β -actin, forward (F) primer 5'-CCTTCTTGGGTATGGAATCCTG-3' and reverse (R) primer 5'-GGCATAGAGGTCTTTACGGATG-3'; IFN- γ , F primer 5'-GGATGC ATTCATGAGTATTGC-3' and R primer 5'-GCTTCCTGAGGCTGGAT TC-3'; interleukin-6 (IL-6), F primer 5'-CGTGGAAATGAGAAAAGAT TGTGC-3' and R primer 5'-ATGCTTAGGCATAACGCACACTAGGT-3'; CXCL-1 or KC, F primer 5'-GGGATTCACCTCAAGAACATCC-3' and R primer 5'-TCTGAACCAAGGGAGCTTCA-3'; IL-10, F primer 5'-CC TTTGACAAGCGGACTCTC-3' and R primer 5'-GCCAGCATAAAAC CCTTCA-3'; and transforming growth factor β (TGF- β), F primer 5'-T TGCTTCAGCTCCACAGAGA-3' and R primer 5'-TGGTTGTAGAGGG CAAGGAC-3'.

BrdU proliferation assay. C57BL/6 Foxp3-GFP mice were scarified on the corneas with a 27-gauge needle, and a 3- μl drop containing 1×10^4

groups of treated and control mice were extirpated on day 15 p.i., and images for pathology analysis were taken at different microscope augmentations using a $\times 40$ objective. (D to I) HSV-infected corneas were harvested on day 15 p.i., processed, and stained for different cell surface molecules. (D) FACS plots represent the frequencies of neutrophils in various groups (D, day); (E) bar graphs showing number of neutrophils in the MAbT25-treated group and control groups; (F) FACS plots showing frequencies of CD4^{+} T cells (SSC, side scatter); (G) bar graphs representing CD4^{+} T cell numbers in the cornea; (H) FACS plots showing IFN- γ -producing CD4^{+} T cells (top, IFN- γ^{+} cells on HSV-1 stimulation; bottom, IFN- γ^{+} cells on PMA-ionomycin stimulation); (I) bar graphs depicting the numbers of CD4^{+} IFN- γ^{+} cells. Statistical significance was calculated by one-way ANOVA with Tukey's multiple-comparison test (***, $P < 0.001$). (J) FACS plots showing the CD4^{+} T cell population (DLN) expressing CD44 and CD62L markers. (K) FACS profiles showing the frequencies of CD4^{+} T cells (DLN) producing IFN- γ on PMA-ionomycin stimulation in MAbT25-treated and control groups. Experiments were repeated 3 times with 5 to 6 mice per group. Data represent means $\pm$ SEMs of at least two independent experiments.

FIG 3 MAbT25 treatment increased the frequencies of the Treg population in the cornea and draining lymph nodes. C57BL/6 mice infected with HSV-1 were treated with MAbT25 or isotype antibody on day -2 or on day 0 (8 h p.i.). HSV-infected corneas were processed and stained for Treg population. (A) FACS plots showing CD4⁺ Foxp3⁺ Tregs in the corneas of control and MAbT25-treated groups. (B) Bar graphs representing the percentage of Foxp3⁺ cells. (C) FACS plots showing

PFU of virus was applied to the eye. These mice received MAbT25 2 days before (day -2) HSV-1 infection. Since MAb25-induced expansion of Tregs peaked by day 4 or 5 after treatment, bromodeoxyuridine (BrdU; 1 mg/mouse) was injected (i.p.) on day 4 after MAbT25 injection. Another group of mice received MAbT25 on day 6 p.i. and BrdU on day 9 p.i. Mice were terminated 1 day after BrdU treatment. Spleen and draining lymph node cells were stained first with anti-CD4 and subsequently with anti-BrdU APC from a flow kit from BD Pharmingen according to the manufacturer's instructions. The antibody-stained cells were acquired with a FACSCalibur apparatus (BD Biosciences), and the data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Ex vivo apoptosis assay. Suspensions of single cells from DLNs isolated 12 days p.i. from HSV-infected C57BL/6 Foxp3-GFP mice (isotype and MAbT25 treated on day 6 p.i.) were incubated (1×10^5 cells/well) for 5 h with various concentrations of rGal-9 in the absence or the presence of α -lactose in 96-well flat-bottom plates in a humidified incubator in the presence of 5% CO₂. After the incubation period, cells were transferred to a 96-well U-bottomed plate and stained for the annexin V marker using a kit from BD Biosciences. Additionally, cells were also costained for CD4 and Tim-3 markers (27).

In vitro suppression assay. C57BL/6 Foxp3-GFP mice infected with HSV-1 were divided into multiple groups. Mice in one group were injected with MAbT25 on day 6 p.i., and starting from day 6, these mice also received rGal-9 (50 μ g/mouse) until day 14 p.i. Mice in another group received rGal-9 (100 μ g/mouse) alone starting from day 6 until day 14 p.i. Control groups were injected with hamster IgG isotype antibody (Bioxcell). At day 15 p.i., DLN single-cell suspensions were prepared and CD4⁺ Foxp3⁺ T cells were sorted on a FACS Aria cell sorter to 99% purity. CD4⁺ Foxp3⁺ T cells were then cultured with anti-CD3 (1 μ g/well) and anti-CD28 (0.5 μ g/well) antibodies and carboxyfluorescein succinimidyl ester (CFSE)-labeled CD4⁺ CD25⁻ Thy1.1 responder cells (purified by a Miltenyi biotech kit) in a U-bottom 96-well plate. The suppressive capacity of Tregs toward responder cells in coculture was measured by coculturing Tregs and T conventional cells (Tconv) at different ratios (Treg/Tconv, 1:1 to 1:16). After 3 days of incubation, the extent of CFSE dilution was measured in Thy1.1 CD4⁺ CD25⁻ cells by flow cytometry. Percent suppression by Tregs was calculated by using the formula $100 - [(frequency\ of\ cells\ proliferated\ at\ a\ particular\ Treg/effector\ T\ cell\ ratio)/(frequency\ of\ cells\ proliferated\ in\ the\ absence\ of\ Tregs)]100$ (12).

Statistical analysis. Graphs were prepared and statistical analysis was performed using GraphPad Prism software. Student's *t* test was performed to determine statistical significance, and data are expressed as means $\pm$ standard error of the means (SEMs). Multiple-variable analysis was performed using one-way analysis of variance (ANOVA) and Tukey's multiple-comparison test. A *P* value of less than 0.05 was considered significant.

RESULTS

Administration of TNFRSF25 agonistic antibody expands regulatory T cell population and reduces the severity of SK lesions.

To demonstrate the effect of MAbT25 on Treg expansion in uninfected and HSV-1-infected mice, groups of mice were injected with MAbT25 or isotype MAb following ocular infection with HSV-1 (day 1). Our results demonstrated that exposure of unin-

fected and HSV-infected mice to agonistic MAbT25 resulted in selective expansion of Foxp3⁺ Tregs in the peripheral blood and spleen (Fig. 1A). The percentage of Tregs in peripheral blood and spleen was increased by 3- to 4-fold in the HSV-infected and uninfected mice treated with MAbT25 by day 4 posttreatment, with levels staying elevated for the 14-day observation period (Fig. 1B and C). Treg frequencies in DLNs (data not shown) of HSV-1-infected mice treated with MAbT25 were similar to those in blood and spleen.

To assess the efficacy of MAbT25 administration on the extent of ocular lesions caused by the virus, animals were given a single i.p. injection of MAbT25 or isotype monoclonal antibody either at 2 days before infection (day -2) or at 8 h p.i. (day 0). The disease severity was compared at different times p.i. over a 15-day observation period (Fig. 2A). By day 12 p.i., clear-cut differences in lesion severity were evident between treated and control animals, with the animals treated on day -2 showing reduced lesion severity (Fig. 2A). On the day of termination, 10 out of 14 eyes in the control group showed scores of ≥ 3 , whereas lesions (score, ≥ 3) were present in 3 out of 14 eyes in the group treated on day -2 and 5 out of 14 eyes in the group treated on day 0. The average lesion scores on day 15 p.i. were significantly reduced in the MAbT25-treated groups compared with the control group (day -2) (Fig. 2B).

At the termination of the experiments on day 15 p.i., corneas were collected and processed to identify their cellular composition by FACS analysis. Analysis of corneal infiltrates revealed that the total numbers of inflammatory cells were reduced in MAbT25 recipients compared to the isotype recipients. This pattern of reduced inflammatory reaction in MAbT25 recipients was also evident in histological sections (Fig. 2C). There was a reduction in inflammatory cell numbers (both neutrophils [3- to 6-fold; Fig. 2D and E] and CD4⁺ T cells [2- to 4-fold; Fig. 2F and G]). In addition, functional responses were measured by stimulating the corneal cell population with UV-inactivated HSV-1 or PMA-ionomycin to detect IFN- γ -producing CD4⁺ T cells. Our results showed that the average number of Th1 cells per cornea was reduced by 3- to 5-fold in MAbT25 recipients compared to that in the isotype control-treated mice following stimulation with HSV-1 (Fig. 2H and I). A similar reduction in Th1 cell numbers was observed in MAbT25 recipients following stimulation with PMA-ionomycin (Fig. 2H and I). Furthermore, analysis of activation markers on the DLN cell populations revealed an increased percentage of CD4⁺ T cells that were CD44^{hi} CD62L^{lo} in control mice, whereas higher percentages of cells were CD44^{lo} CD62L^{hi} in MAbT25-treated mice (Fig. 2J). These results indicate that CD4⁺ T cells obtained from MAbT25-treated mice were less activated than those obtained from their isotype-treated counterparts. In addition, the percentage of IFN- γ -producing CD4⁺ T cells in the DLNs of isotype-treated mice was significantly higher

CD4⁺ Foxp3⁺ Tregs in the DLNs of control and MAbT25-treated groups. (D) Bar graphs representing the numbers of CD4⁺ Foxp3⁺ and CD4⁺ IFN- γ ⁺ T cells in DLNs. (E) FACS profiles of CD4⁺ Foxp3⁺ Tregs expressing CD103 in the corneas of control and MAbT25-treated groups. (F) Bar graphs showing the frequencies of CD4⁺ Foxp3⁺ Tregs expressing CD103. (G) FACS profiles of CD4⁺ Foxp3⁺ Tregs expressing CD103 in the DLNs of control and MAbT25-treated groups. (H) Bar graphs showing the frequencies of CD4⁺ Foxp3⁺ Tregs expressing CD103. (I to L) The relative fold change in the expression of mRNA of various cytokines was examined and compared between the control and treated groups on day 15 p.i. by qPCR. mRNA levels for the different cytokines in mock-infected mice were set to 1 and used to determine the relative fold upregulation. Relative mRNA expression of TGF- β (I), IL-10 (J), IFN- γ (K), and IL-6 (L) in MAbT25 and control groups. Each experiment was repeated at least three times with 5 to 6 mice per group per experiment. Data represent means $\pm$ SEMs of triplicate wells with 5 to 6 pooled corneas per group from three independent experiments. Statistical significance was calculated by one-way ANOVA with Tukey's multiple-comparison test (ns, not significant; *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.0001).

FIG 4 MAbT25 administration during early clinical phase of disease was not effective in controlling the severity of SK lesions. (A) C57BL/6 mice infected with 1×10^4 PFU HSV-1 were treated with MAbT25 or isotype antibody on day 6 p.i. (B) SK lesion scores of MAbT25-treated and control mice on day 15 p.i. Experiments were repeated three times with 8 to 10 mice per group. One-way ANOVA with Tukey's multiple-comparison test was used to calculate significance (P value not significant, $P > 0.05$). (C to F) HSV-1-infected corneas were pooled and processed as previously described for FACS analysis. (C) FACS plots showing neutrophils in the corneas of control and MAbT25-treated groups; (D) bar graphs showing neutrophil numbers; (E) FACS plots showing the total CD4⁺ T cells in the corneas; (F) bar graphs showing CD4⁺ T cell numbers. (G) Representative FACS plots showing CD4⁺ IFN-γ⁺ cells. (Top) IFN-γ⁺ cells on HSV-1 stimulation; (bottom) PMA-ionomycin stimulation in MAbT25-treated and control groups. (H) Bar graphs representing CD4⁺ IFN-γ⁺ T cell numbers. (I) FACS plots showing Foxp3⁺ Tregs in the corneas of control and MAbT25-treated groups. (J) Bar graphs representing numbers of Foxp3⁺ T cells. Data represent means $\pm$ SEMs of 6 to 8 mice per group from at least two independent experiments. Statistical levels of significance were calculated by Student's t test (*, $P < 0.05$; ***, $P < 0.0001$).

FIG 5 Comparison of CD4⁺ T cell responses in prophylactic and therapeutic settings following MAbT25 administration. To determine the subset of CD4⁺ T cells proliferating in MAbT25-treated animals following the two treatment modalities (on day -2 or day 6 p.i.), a BrdU proliferation assay was performed. HSV-infected Foxp3-GFP mice were treated with MAbT25 (day -2). BrdU was injected on day 4 after MAbT25 treatment. Another group of mice received MAbT25 on day 6 p.i. and BrdU on day 9 p.i. (A) FACS profiles showing BrdU incorporation in CD4⁺ Foxp3⁺ and CD4⁺ Foxp3⁻ T cells in mice treated with MAbT25 on day -2; (B) bar graphs representing frequencies of CD4⁺ Foxp3⁺ and CD4⁺ Foxp3⁻ cells; (C) FACS profiles showing BrdU incorporation in CD4⁺ Foxp3⁺ and CD4⁺ Foxp3⁻ cells from the mice treated with MAbT25 on day 6 p.i.; (D) bar graphs representing frequencies of CD4⁺ Foxp3⁺ and CD4⁺ Foxp3⁻ T cells. (E and F) DLN samples isolated from MAbT25 and isotype-treated mice on day 15 p.i. were stimulated with UV-inactivated HSV-1; (E) FACS profiles showing frequencies of CD4⁺ T cells producing IFN-γ; (F) bar graphs representing frequencies of IFN-γ-producing CD4⁺ T cells. (G) Bar graphs showing MFI levels of IFN-γ. Data represent means ± SEMs of at least two independent experiments. Statistical significance was calculated by Student's *t* test (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001).

than the percentage in MAbT25-treated mice following stimulation with PMA-ionomycin (Fig. 2K).

To measure the effect of MAbT25 treatment on the composition of T cell subsets in corneal infiltrates at day 15 p.i., groups of

corneas from treated and control animals were collagen digested to recover the corneal cell population. The frequencies of the Treg population in the corneas of MAbT25-treated mice (day -2) were higher (Fig. 3A and B), with an increase in the average ratio of

FIG 6 MAbT25 and rGal-9 combination therapy effectively reduced SK lesion severity in a therapeutic setting. (A) HSV-infected C57BL/6 mice were injected with a single dose of MAbT25 or isotype control antibody on day 6 p.i., and starting from day 6, these mice also received rGal-9 (50 µg/mouse) until day 14 p.i. Another group of mice was treated with rGal-9 (100 µg/mouse) alone starting from day 6 p.i., and disease progression was observed until day 15 p.i. (B) Individual SK lesion scores of MAbT25–Gal-9-, Gal-9-, and isotype-treated groups on day 15 p.i. Experiments were repeated three times with 8 to 10 mice per group. One-way ANOVA with Tukey's multiple-comparison test was used to calculate the level of significance (*, $P < 0.05$; ***, $P < 0.001$). (C to H) HSV-infected corneas were harvested and processed as previously indicated for FACS analysis; (C) FACS plots showing neutrophils infiltrated into the corneas of various

CD4⁺ Foxp3⁺ Tregs to Th1 cells (PMA-ionomycin stimulated) in MAbT25 recipients (1:6 in controls versus 1:1.3 [day -2] and 1:3 [day 0] in MAbT25 recipients). Similar differences in the ratios of the Treg to Th1 cell population were also observed in DLNs obtained from MAbT25-treated mice and those obtained from control mice (Fig. 3C and D). Further phenotypic characterization of Tregs in the corneal pools revealed that a significant proportion of Tregs in MAbT25-treated (day -2) corneas expressed the activation marker CD103 compared to the proportion in isotype recipients (Fig. 3E and F). Upregulation of CD103 expression on the Foxp3⁺ population was also evident in DLNs and spleens between MAbT25-treated mice (for both the group treated on day -2 and the group treated on day 0) and control animals (Fig. 3G and H).

Effect of MAbT25 treatment on inflammatory factors involved in SK lesions. Extracts of corneas collected on day 15 p.i. from control and MAbT25-treated (starting on day -2 and day 0) animals were pooled, and sample pools were analyzed for IFN- γ , IL-6, IL-10, and TGF- β mRNA expression levels using qPCR. The MAbT25 treatment (day -2) resulted in an average 3.5-fold increase in the levels of the anti-inflammatory cytokine TGF- β (Fig. 3I) and a 5-fold increase of IL-10 (Fig. 3J) compared to those in the control group. Additionally, a 5-fold reduction of IFN- γ (Fig. 3K) and an 8-fold decrease of IL-6 (Fig. 3L) were observed in the samples obtained from the MAbT25-treated groups compared to the levels in the control group. Although mice treated with MAbT25 on the same day of infection showed a similar reduction in proinflammatory cytokine levels and upregulation of anti-inflammatory cytokines, the differences compared to the controls were less than those observed in animals treated on day -2.

These studies show that administration of MAbT25 before (day -2) or following HSV infection diminishes HSV-1-induced corneal immunopathology and proinflammatory leukocytic infiltration.

Effect of MAbT25 treatment during clinical phase of SK. In most clinical settings, the challenge is to control inflammatory disease once it is already present. To address this possibility with the SK system, treatment with MAbT25 was begun at day 6 p.i., the early phase of lesions (Fig. 4A). Our results showed that MAbT25 administration at this stage caused enhanced lesion immunopathology in comparison to that in isotype-treated controls, when measured over 15 days. Although the differences in SK lesion scores were not statistically significant when they were compared on day 15 p.i. (Fig. 4B), differences in their cellular composition were readily detectable at day 15 p.i. Pooled corneas from MAbT25-treated animals showed a 1.5- to 2-fold increase in neutrophil infiltration (Fig. 4C and D) and a 1.5-fold increase in CD4⁺ T cells (Fig. 4E and F). Additionally, *in vitro* stimulation of corneal cells with PMA-ionomycin and HSV-1 revealed higher numbers of Th1 cells per cornea in MAbT25 recipients than isotype-treated controls (Fig. 4G and H). However, MAbT25-treated animals also showed a 2-fold increase in Treg numbers compared to isotype-treated controls (Fig. 4I and J). The average ratio of

FIG 7 MAbT25-Gal-9 combination therapy reduced the expression of proinflammatory cytokines and upregulated anti-inflammatory cytokine expression. C57BL/6 mice were injected with MAbT25 or isotype antibody on day 6 p.i., and starting from day 6, these mice also received rGal-9 (50 μ g/mouse) until day 14 p.i. Another group of mice received Gal-9 alone starting from day 6 until day 14 p.i. The relative fold change in expression of mRNA of various cytokines was examined and compared between control and treated groups on day 15 p.i. by qPCR. mRNA levels for the different cytokines in mock-infected mice were set to 1 and used to determine the relative fold upregulation. Relative mRNA expression of TGF- β (A), IL-10 (B), IFN- γ (C), KC (D), and IL-6 (E) in MAbT25-Gal-9-, Gal-9-, and isotype-treated groups. Data represent means $\pm$ SEMs of 5 to 6 corneas per group from at least two independent experiments. Statistical significance was calculated by one-way ANOVA with Tukey's multiple-comparison test (**, $P < 0.01$; ***, $P < 0.001$).

Foxp3⁺ to Foxp3⁻ CD4⁺ T cells remained approximately the same in MAbT25- and isotype-treated mice.

Comparison of T cell responses in prophylactic and therapeutic settings. To determine which CD4⁺ T cell types are proliferating in MAbT25-treated animals following the two treatment modalities, a BrdU proliferation assay was performed. In the group treated on day -2, the majority of cells that underwent proliferation in both spleens and DLNs were Foxp3⁺ regulatory T cells, with a 4-fold increase in the proliferative index (Fig. 5A and B) compared to that for the control animals. However, in the

groups; (D) bar graphs representing numbers of neutrophils in various groups; (E) FACS plots showing CD4⁺ T cells in the corneas of various groups; (F) bar graphs representing total CD4⁺ T cell numbers in the cornea; (G) FACS plots showing IFN- γ -producing CD4⁺ T cells (top, IFN- γ ⁺ cells on HSV-1 stimulation; bottom, IFN- γ ⁺ cells on PMA-ionomycin stimulation); (H) bar graphs depicting CD4⁺ IFN- γ ⁺ T cell numbers. (I) FACS profiles showing frequencies of CD4⁺ T cells producing IFN- γ in DLNs; (J) bar graphs representing their frequencies. Data represent means $\pm$ SEMs of 5 to 6 mice per group from at least two independent experiments. One-way ANOVA with Tukey's multiple-comparison test was used to calculate the level of significance (**, $P < 0.01$; ***, $P < 0.0001$).

FIG 8 MAbT25 and Gal-9 combination therapy expanded the Treg population in the corneas and DLNs. (A) Foxp3-GFP mice infected with HSV-1 were treated with MAbT25 on day 6 p.i., and starting from day 6, these mice also received rGal-9. Another group of mice received Gal-9 alone starting from day 6 until day 14 p.i. (A) On day 15 p.i., the mice were sacrificed and the corneas were isolated and directly examined under a confocal microscope (Leica SP2 LSCM) for GFP-expressing Foxp3⁺ Tregs. (B to E) HSV-infected corneas were harvested and processed as previously indicated for FACS analysis; (B) FACS plots showing Foxp3⁺ Tregs in the corneas of MAbT25-Gal-9-, Gal-9-, and isotype-treated groups; (C) bar graphs showing the percentage of CD4⁺ Foxp3⁺ cells; (D) FACS profiles of Foxp3⁺ Tregs expressing CD103 in the corneas of various groups; (E) bar graphs showing the numbers of Foxp3⁺ Tregs expressing CD103 in the

animals on the day 6 treatment regimen, both Foxp3⁺ and Foxp3⁻ CD4⁺ T cells proliferated (Fig. 5C and D). The proliferative index of CD4⁺ Foxp3⁻ T cells was enhanced by 2-fold, whereas the proliferative index of Foxp3⁺ regulatory T cells was increased by 3- to 4-fold.

The CD4⁺ T cell populations isolated from the DLNs of mice treated with MAbT25 and the isotype control on day 6 p.i. were also compared for levels of cytokine production following HSV-1 stimulation. As shown in Fig. 5E and F, a higher percentage of CD4⁺ T cells isolated from the group that received MAbT25 on day 6 p.i. than from the isotype recipients secreted IFN- γ . Additionally, cells from the group treated on day 6 p.i. showed higher mean fluorescence intensity (MFI) values of IFN- γ (177 ± 7.05) than cells from animals treated with MAbT25 on day -2 (136 ± 8.14), indicating that the amount of IFN- γ secreted per individual cell was also higher (Fig. 5G).

Collectively, these results demonstrated that in the absence of exogenous antigen, TNFRSF25 stimulation selectively expanded the Treg population, whereas after disease was established, the same treatment caused the proliferation of both Tregs and effector T cells. The MAbT25 treatment also stimulated the effector T cells to secrete higher levels of the proinflammatory cytokine IFN- γ , which may contribute to the failure of MAbT25 treatment on day 6 p.i. to reduce the severity of SK.

MAbT25 in combination with rGal-9 is effective in a therapeutic setting. The previous experiments showed that the Treg population expanded by MAbT25 was effective at inhibiting SK lesions when given before clinical disease (prophylactic setting) but failed to reduce established disease (therapeutic setting), likely because MAbT25 stimulated both effector T cells and Tregs. To be effective in a therapeutic setting, Treg expansion by MAbT25 may therefore have to be combined with an agent that limits effector T cell function. Accordingly, experiments were performed using the approach of rGal-9 administration that causes effector cells that express Tim-3 to be eliminated by apoptosis (33). In such experiments, HSV-infected animals were treated with MAbT25 at day 6 p.i., and subsequently, animals either were left untreated or were given daily treatment with rGal-9 (Fig. 6A). Lesion severity was compared with that in untreated animals as well as with that in animals receiving rGal-9 monotherapy (started at day 6 p.i.). The average SK lesion scores were significantly reduced in the MAbT25-Gal-9-treated group than the isotype-treated controls (Fig. 6B). In this experiment, the dose of Gal-9 chosen for use in combination therapy (50 μ g/mouse) was less than that used in Gal-9 monotherapy (100 μ g/mouse).

At the termination of the experiments on day 15 p.i., corneas were collected and multiple pools of three corneas in control and test groups were processed to identify their cellular composition. The extent of neutrophil infiltration into corneas was significantly reduced in the mice that received the combination treatment (Fig. 6C and D) compared to the other groups. The total number of CD4⁺ T cells (Fig. 6E and F) was greatly reduced in the combina-

tion therapy group (6- to 7-fold) compared to that in the control or Gal-9 monotherapy group (1.6- to 1.8-fold). A reduction in the number of Th1 cells was also evident following activation with either UV-inactivated HSV-1 or PMA-ionomycin (Fig. 6G and H) in combination therapy recipients. Furthermore, the percentage of cells producing IFN- γ (PMA-ionomycin stimulated) in the DLNs of combination therapy recipients was reduced compared to that in the Gal-9 monotherapy or control groups (Fig. 6I and J). The expression levels of some cytokines in corneal extracts were also compared between the various treatment groups. Combination therapy resulted in the upregulation of anti-inflammatory cytokines TGF- β (2.8-fold; Fig. 7A) and IL-10 (6.8-fold; Fig. 7B) but a reduction in the levels of proinflammatory cytokines IFN- γ (4.3-fold; Fig. 7C), KC (3.2-fold; Fig. 7D), and IL-6 (6-fold; Fig. 7E) compared to those in the Gal-9-treated or control groups. Although proinflammatory cytokine levels were less in the Gal-9 monotherapy group, combination therapy recipients showed the lowest levels of these proinflammatory mediators.

Mechanism of disease reduction in combination therapy recipients. (i) **Expansion of Foxp3⁺ Tregs.** Confocal microscopy was performed to visualize the GFP-expressing Foxp3⁺ T cells in the corneas of various groups. As shown in Fig. 8A, the corneas of combination therapy recipients displayed a higher proportion of Foxp3⁺ Tregs than the corneas of the rGal-9-treated or control groups. Additionally, the number of Tregs was found to be elevated in the corneal extracts of combination therapy recipients compared with the number in the corneal extracts of the other groups (Fig. 8B and C), the results which supports the clinical findings. The frequency of CD103-positive Tregs in the corneas of the combination therapy group was also higher than that in the other groups of mice (Fig. 8D and E). Our data also showed that the total number of Tregs per Th1 cell in the DLNs of the combination therapy group was higher than that in the other groups of mice (Fig. 8F and G). Phenotypic analysis of cells recovered from the DLNs and spleens of MAbT25-treated mice revealed that both the CD4⁺ Foxp3⁻ and Foxp3⁺ T cell populations expressed higher levels of the Tim-3 marker (Fig. 8H and I) than cells from the DLNs and spleens of the control group.

(ii) **Apoptosis of Tim-3-expressing effector T cells.** The up-regulation of Tim-3 on the CD4⁺ Foxp3⁻ population could account for its higher susceptibility to inhibition by Gal-9. Therefore, we performed an *in vitro* apoptosis assay to measure the susceptibility of the CD4⁺ Foxp3⁻ T cell population to rGal-9 exposure. Our data showed that CD4⁺ T Foxp3⁻ cells isolated from MAbT25-treated mice were more susceptible to Gal-9 induced apoptosis than cells from isotype-treated mice (Fig. 9A and B). Of note, Foxp3 cells obtained from both isotype- and combination therapy-treated mice were resistant to Gal-9-induced apoptosis (Fig. 9C and D).

(iii) **MAbT25-expanded Tregs suppress CD4⁺ effector T cell proliferation.** To check the ability of MAbT25-expanded Tregs to suppress the proliferative activity of the CD4⁺ effector T cell pop-

corneas of various groups. (F) FACS plots showing CD4⁺ Foxp3⁺ Tregs in the DLNs of MAb T25-Gal-9-, Gal-9-, and isotype-treated groups. (G) Bar graphs showing the numbers of Foxp3⁺ CD4⁺ T cells and Th1 cells in DLNs. (H and I) Two groups of mice were infected with HSV-1, and on day 6 p.i., one group of mice was treated with MAbT25 and another group received isotype control. DLNs of mice were analyzed for Tim-3 expression on day 12 p.i.; (H) FACS profiles of CD4⁺ T cells expressing Tim-3 marker in the DLNs; (I) bar graphs representing the percentage of CD4⁺ Foxp3⁺ and CD4⁺ Foxp3⁻ T cells expressing the Tim-3 marker. Data represent means $\pm$ SEMs of 3 to 4 mice per group from at least two independent experiments. Statistical significance was calculated by one-way ANOVA with Tukey's multiple-comparison test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

FIG 9 MAbT25-induced upregulation of Tim-3 expression on effector T cells increased their susceptibility to Gal-9-induced apoptosis. Two groups of Foxp3-GFP mice were infected with HSV-1, and on day 6 p.i., one group of mice was treated with MAbT25 or isotype antibody. DLNs were isolated on day 12 p.i. to check the susceptibility of CD4⁺ Foxp3⁻ and CD4⁺ Foxp3⁺ T cells to Gal-9-induced apoptosis. (A) FACS profiles of CD4⁺ T cells that are Tim-3 or annexin V positive from MAbT25-treated and isotype-treated (top) and isotype-treated (bottom) groups; (B) bar graphs representing the percentage of CD4⁺ annexin V-positive cells from MAbT25-treated and isotype-treated groups; (C) FACS profiles of CD4⁺ Foxp3⁺ T cells MAbT25-treated (top) and isotype-treated (bottom) groups undergoing apoptosis; (D) bar graphs representing the percentage of CD4⁺ Foxp3⁺ T cells. Experiments were repeated 2 times with 3 to 4 mice each group. Data represent the means $\pm$ SEMs. Student's *t* test was performed to determine statistical significance, and data are expressed as means $\pm$ SEMs (***, *P* < 0.0001). (E and F) Tregs isolated from DLNs of MAbT25- and Gal-9-treated group are highly suppressive. Foxp3-GFP mice infected with HSV-1 were treated with MAbT25 on day 6 p.i., and starting from day 6, these mice also received rGal-9 (50 μg/mouse) until day 14 p.i. Another group of mice received Gal-9 alone. CD4⁺ Foxp3⁺ T cells were sorted and cultured with CFSE-labeled CD4⁺ CD25⁻ Thy1.1 responder cells (Treg/Tconv, 1:1 to 1:16) in the presence of anti-CD3 and anti-CD28 antibodies. (E) Representative histograms showing the extent of CFSE dilution at a 1:4 Treg/effector T cell (Teff) ratio. (F) Bar graphs showing the percent suppression of Tregs isolated from MAbT25-Gal-9-, Gal-9-, and isotype-treated groups. Each experiment was repeated at least two times with at least 3 mice per group. Data represent means $\pm$ SEMs of at least two independent experiments. Statistical significance was calculated by one-way ANOVA with Tukey's multiple-comparison test (**, *P* < 0.01).

ulation, an *in vitro* CFSE proliferation assay was performed. The *in vitro* suppressive activity of CD4⁺ Foxp3⁺ T cells isolated from MAbT25-Gal-9-treated mice was slightly higher than that of the Tregs isolated from the Gal-9- or isotype-treated groups (Fig. 9E and F). Taken together, our results demonstrate that Tregs isolated from MAbT25-Gal-9-treated mice were highly effective at suppressing CD4⁺ effector T cell proliferation *in vitro*.

DISCUSSION

Inflammatory disease often results from an imbalance in the activity of tissue-damaging events with that of events that counteract their function (6, 21). For example, in autoimmunity and viral immunopathology, lesions are more severe when the balance of T effectors to regulatory cells favors the former (10, 21). Addition-

ally, lesions are minimized when counterinflammatory factors produced by the host are elevated, such as the cytokines IL-10 and TGF- β (11, 16, 22) and some endogenous lectins and glycans (19), and when inhibitory receptors are expressed (5). In this report, we demonstrate that the chronic damage to the eye caused by HSV infection (stromal keratitis) can be successfully managed by a recently developed novel approach, namely, treatment with MAb to TNFRSF25, which expands and activates Tregs, combined with the administration of a glycan, Gal-9, which causes apoptosis of proinflammatory T cells.

Stromal keratitis following HSV infection is an important cause of human blindness (18). The chronic inflammatory reaction that occurs in humans is usually the consequence of virus spreading to the cornea after being reactivated from latency in nerve ganglia (23). The actual tissue damage in the cornea appears to be an immunopathological process primarily involving T cells, especially those that are autoreactive or virus specific (32). Past studies in the mouse model of SK, where the lesion occurs after primary infection, have clearly established a tissue-damaging role for CD4 T cells that are mainly of the Th1 subtype (9, 15). The extent of damage caused by proinflammatory T cells can be modulated either by expanding and activating Treg or by administering molecules that destroy or impair the function of the effector cells (28, 29, 31). As a consequence, procedures that can elevate and activate Treg responses or inhibit effector T cell function represent logical approaches to therapy. We used a convenient method recently developed by one of us (E.R.P.) that can selectively expand the Treg population by up to 3- to 4-fold (24). The approach exploits the fact that among naïve T cells, the Foxp3⁺ population is unique in its regular exposure to cognate (self) antigens and is the major subset that expresses abundant TNFRSF25. Moreover, the administration of an agonistic MAb, MAbT25, selectively expanded and activated the Treg population (24). When this procedure was used in a prophylactic setting in mice ocularly infected with HSV, the severity of subsequent SK lesions was significantly diminished and a change in balance between effectors and Tregs was evident in lesions as well as in draining lymphoid tissues. Moreover, the activation status of the Treg observed in the corneas of MAbT25 recipients was higher than that observed in control animals. Although we lacked reagents to determine the specificity of Tregs, many cells would likely be activated Foxp3⁺ T cells that had anti-self-reactivity and that previous studies had shown represented the major population expanded by MAbT25 in naïve animals (24). We can only assume that the population of Tregs in corneas was inhibitory to effector cell function either because many Tregs were activated or because many of the effectors involved were self reactive, or because of both reasons. We could not obtain sufficient numbers of cells from the cornea to measure their regulatory activity *in vitro*, but in studies with the Foxp3⁺ T cell population taken from the draining lymph nodes of infected animals, regulatory effects against the activation of CD4 T cells were marginally enhanced in the Tregs taken from MAbT25-treated animals. Whatever the mechanism by which the Tregs serve to modulate the inflammation in SK lesions, the prophylactic use of the MAbT25 was an effective means of attaining lesion control.

The outcome of treatment with MAbT25 was dramatically different when MAbT25 was used in a therapeutic setting which represents the more common clinical situation where inflammatory

disease needs to be controlled. Unfortunately, in this situation, the MAbT25 treatment increased the severity of lesions. This effect was presumed to occur because the effector T cells in lesions also express high levels of TNFRSF25 and could be stimulated following engagement of their natural ligand or the agonist MAb (3, 13, 14, 17). This hypothesis is in accordance with previous reports showing that engagement of the TNFRSF25 ligand during active disease led to increased pathology in a lung model of allergy (8), whereas therapy prior to antigen exposure resulted in diminished lesions in a similar model (24). Furthermore, mice unable to develop either the natural ligand or the TNFRSF25 molecule because of gene knockout may have less severe inflammatory disease (8, 17). Accordingly, the use of the MAbT25 approach in a therapeutic setting where antigen is still driving the inflammatory response is ineffective or even hazardous. Fortunately, other strategies exist that may selectively impair the function of proinflammatory T cells but preserve, or even expand, the representation of Tregs (27, 33). Such approaches include the use of the galectin molecules 1 and 9, which may cause apoptosis when they bind to activated effectors (2, 7). The galectins fail to cause apoptosis of Tregs even if they express Tim-3 (27). Accordingly, we and others have shown that after virus infection, activated effectors may express Tim-3, which is one of the ligands for Gal-9 (27, 33). Moreover, Tim-3-producing effectors undergo apoptosis when exposed to galectin-9, as could readily be shown by *in vitro* studies (27, 33). As a consequence, we anticipated that if the Treg expansion approach was combined with another procedure that would target and impair T effectors, inflammation should be controlled in a therapeutic setting.

As was predicted, combination therapy with MAbT25 and rGal-9 proved highly effective against SK lesions, with the residual inflammatory reactions showing a marked increase in the ratio of Foxp3-positive to Foxp3-negative T cells. The responses also contained far fewer neutrophils, the cells mainly responsible for damage in the cornea (30), and levels of proinflammatory cytokines were greatly reduced. It is conceivable that the addition of another form of therapy, such as one that effectively blunts essential events in SK pathogenesis that include neovascularization and neutrophil recruitment, could be even more effective and perhaps achieve the long-sought objective of complete lesion resolution. Such investigations are ongoing in our laboratory.

In conclusion, our data have clarified the conditions under which TNFRSF25 stimulation leads to inflammatory reactions versus regulatory immunity. The benefit of Treg expansion by TNFRSF25 stimulation is restricted to a prophylactic setting when used as monotherapy. Caution should be taken when applying TNFRSF25 agonists in a setting where effector T cells are responding to their antigens, since costimulation by TNFRSF25 ligands will increase inflammation, despite concurrent Treg expansion. Importantly, however, these studies provide proof of concept that a therapeutic window for MAbT25-driven *in vivo* Treg proliferation can be opened by targeting effector T cells with combination therapeutics that selectively inhibit such cells. We demonstrated that combining TNFRSF25 agonists with Gal-9 was one such effective therapeutic strategy to control viral immunopathology. In addition, these studies indicate that *in vivo* Treg expansion by TNFRSF25 stimulation combined with procedures that negate proinflammatory T cells may emerge as a broadly applicable approach to achieve tolerance in a wider range of inflammatory diseases.

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