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Tofacitinib inhibits the development of experimental autoimmune uveitis and reduces the proportions of Th1 but not of Th17 cells

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Purpose: Tofacitinib is a pan-Janus kinase (JAK) inhibitor that suppresses cytokine signaling and in turn, the cells that participate in inflammatory immunopathogenic processes. We examined the capacity of tofacitinib to inhibit the induction of experimental autoimmune uveitis (EAU) and related immune responses.

Methods: EAU was induced in B10.A mice with immunization with bovine interphotoreceptor retinoid-binding protein (IRBP), emulsified in complete Freund's adjuvant (CFA), and a simultaneous injection of pertussis toxin. Tofacitinib, 25 mg/kg, was administered daily, and the vehicle was used for control. EAU development was assessed by histological analysis of the mouse eyes, and related immune responses were assessed by (i) the levels of interferon (IFN)-γ and interleukin (IL)-17, secreted by spleen cells cultured with IRBP; (ii) flow cytometric analysis of intracellular expression by spleen, or eye-infiltrating CD4 or CD8 cells of IFN-γ, IL-17, and their transcription factors, T-bet and RORγt. In addition, the inflammation-related cell markers CD44 and CD62L and Ki67, a proliferation marker, were tested. The proportions of T-regulatory cells expressing FoxP3 were determined by flow cytometric intracellular staining, while levels of antibody to IRBP were measured with enzyme-linked immunosorbent assay (ELISA).

Results: Treatment with tofacitinib significantly suppressed the development of EAU and reduced the levels of secreted IFN-γ, but not of IL-17. Further, treatment with tofacitinib reduced in the spleen and eye-infiltrating cells the intracellular expression of IFN-γ and its transcription factor T-bet. In contrast, treatment with tofacitinib had essentially no effect on the intracellular expression of IL-17 and its transcription factor, RORγt. The selective effect of tofacitinib treatment was particularly evident in the CD8 population. Treatment with tofacitinib also increased the population of CD44, but reduced the populations of cells producing CD62L and Ki67. Treatment with tofacitinib had no effect on the proportion of FoxP3 producing regulatory cells and on the antibody production to IRBP.

Conclusions: Treatment with tofacitinib inhibited the development of EAU, reduced the production of IFN-γ, but had essentially no effect on the production of IL-17.

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway is a major signaling cascade downstream of type I and type II cytokine receptors and has been investigated as a therapeutic target for various autoimmune and inflammatory diseases [1]. Tofacitinib is a first-generation JAK inhibitor that inhibits the enzymatic activity of JAK1, JAK3, and to a lesser extent, JAK2 [2,3]. Therefore, tofacitinib blocks signaling downstream of the common γ-chain-using cytokines, which rely on JAK1 and JAK3. Moreover, tofacinitib also blocks Gp130-using cytokines, as well as the signaling events downstream of type I and II interferons. Consequently, tofacitinib affects cytokines and cells that contribute to several inflammatory pathologies by modulating the activity of $CD4^+$ T cells, including T-helper (Th)1 and pathogenic Th17 cells, $CD8^+$ T cells, B cells as well as innate immune cells [4]. Tofacitinib has been approved by regulatory agencies for the treatment of moderate to severe rheumatoid arthritis, psoriatic arthritis, and ulcerative colitis and is currently being investigated for potential utility in the treatment of ankylosing spondylitis, alopecia, and other inflammatory pathologies.

The term "uveitis" is used to define a family of intraocular inflammatory conditions, which include diseases such as sympathetic ophthalmia, birdshot chorioretinopathy, Behcet's disease, sarcoidosis, and Vogt-Koyanagi-Harada syndrome [5,6]. Cellular autoimmune processes are thought to play major roles in the pathogenesis of these diseases, which is supported by the similarity between the pathological changes specific to these diseases and the typical ocular changes seen in experimental autoimmune uveitis (EAU) [5-7], a disease driven by immunopathogenic Th1 and Th17 cells [8,9]. In the present study, we found that treatment with tofacitinib

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inhibited the development of murine EAU. Interestingly, treatment with tofacitinib selectively inhibited the production of IFN-γ by CD4 and even more by CD8 cells, but not the production of interleukin (IL)-17.

METHODS

Mice: B10.A mice, at 7–10 weeks of age, were provided by Jackson Labs (Bar Harbor, ME). Female and male mice were used in the experiments shown in Figure 1A-I, whereas only female mice were used in all other experiments. The mice were housed in a specific pathogen-free facility, and all procedures were performed in compliance with the NIH Resolution on the Use of Animals in Research, under protocol NEI-624, approved by the National Eye Institute Animal Care. Animal care and use conformed to institutional guidelines and to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Materials: Tofacitinib was provided by Dr. Thomas Craig (National Center for Advancing Translational Sciences - National Institutes of Health). The compound was dissolved in a solvent that contained Tween-20 and methylcellulose [10]. Bovine interphotoreceptor retinoid-binding protein (IRBP) was prepared as described elsewhere [11]. Purified protein derivative (PPD) was purchased from Parke-Davis (Morris Plains, NJ).

Induction and scoring of EAU: Mice were immunized with injection of 0.2 ml emulsion containing 50 µg of bovine IRBP in complete Freund's adjuvant (CFA), administered by subcutaneous injections [12,13]. Pertussis toxin, 0.2 µg (List Biologic Laboratories, Campbell, CA) or 1 µg (Sigma, St. Louis, MO), was injected intraperitoneally, concurrently with the immunization. The mice were euthanized (CO_2; followed by decapitation) on day 14 post-immunization, and the eyes and spleen cells were collected for histological analysis, using a scale of 0–4 in half-point increments, as detailed elsewhere [12,13].

Treatment with tofacitinib: Tofacitinib, at 25 mg/kg, was given daily, administered by gavage or two intraperitoneal injections. The vehicle was similarly administered in the control mice. The treatment started on the day of immunization.

Measurements of cellular immune responses: Cytokine release: Mouse spleens were collected at euthanasia, and cell suspensions were cultured in Rosewell Park Memorial Institute (RPMI) 1640 medium containing HL-1 (Lonza, Walkersville, MD), antibiotics, L-glutamine, and 2-ME, at 50 µM. Cytokine production was measured by enzyme-linked immunosorbent assay (ELISA; DuoSet ELISA kit, R&D Systems, Minneapolis, MN) in culture supernatants collected after 48 h of incubation with IRBP at different concentrations, as indicated, as well as PPD, at 5 µg/ml. The method is detailed elsewhere [12,13]. In view of inter-experiment variability in the level of cytokine production among repeated experiments, the cytokine release measurements of these experiments were normalized by converting from picogram per milliliter to a scale of 0–1, to account for batch effects between experiments. The method and the formula are detailed in [14].

Isolation of eye-infiltrating cells: The cells were enucleated, and the external tissues and lenses were trimmed. The remaining tissue was minced in RPMI 1640 media and incubated with 1 mg/ml collagenase D treatment (Roche, Indianapolis, IN) for 45 min at 37 ˚C. Cells were then dispersed with trituration, washed, filtered, and resuspended in RPMI 1640 and 10% fetal bovine serum (FBS).

Flow cytometry: Cells from the eyes and spleen were incubated with Fc block (clone 2.4G2, BioXcell, Lebanon, NH) and stained with the following antibodies from Biolegend (San Diego, CA), Thermo Fisher Scientific (Waltham, MA), BD Biosciences (San Jose, CA): anti-CD3 (clone 145–2C11), anti-CD4 (RM4.5), anti-CD8 (53–6.7), anti-CD45 (30-F11), anti-NK1.1 (PK136), anti-CD44 (1M7), and anti-CD62L (MEL-14). For intracellular cytokine staining, the cells were pulsed for 4 h with phorbol myristate acetate (PMA; 50 ng/ml), ionomycin (500 ng/ml), and brefeldin A (GolgiPlug, BD), followed by 4% paraformaldehyde fixation and permeabilization (BD Bioscience). The permeabilized cells were stained with fluorescently conjugated antibodies against K_i-67 (SolA15), Foxp3 (FJK-16s), T-bet (clone 4B10), RORgt (AFKJS-9), IFN-g (XMG1.2), and IL-17A (TC11–18H10.1) overnight. Stained cells were collected on a MACSQuant analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Serum antibody levels: Blood samples were collected upon euthanasia (day 14 post-injection), and anti-IRBP antibody levels in the pooled sera were measured with ELISA: 96-well plates were coated with IRBP and incubated with the tested serum samples. The level of the attached antibody was measured by incubating with horseradish peroxidase (HRP)–conjugated anti-mouse immunoglobulin G (IgG) antibody (R&D, Minneapolis, MN).

Statistical analysis: Statistical significance for histological scoring was determined using an unpaired *t* test. A p value less than or equal to 0.05 was considered statistically significant. Statistical significance for the cytokine release assays was determined using two-way analysis of variance (ANOVA). Statistical significance for the histology score was

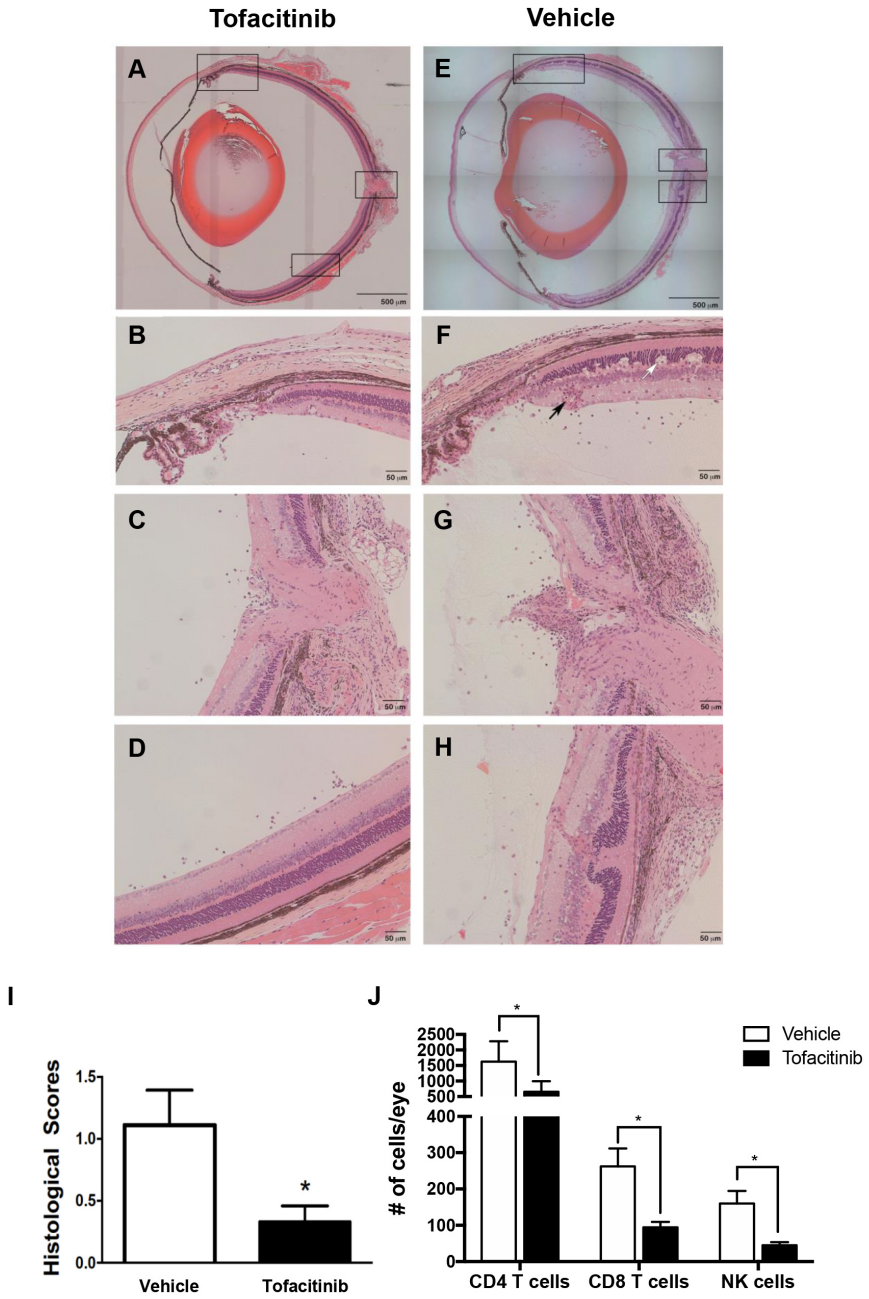

Figure 1. Treatment with tofacitinib inhibits the development of EAU. Groups of B10.A mice were immunized with interphotoreceptor retinoid-binding protein (IRBP) and treated twice daily with tofacitinib or the vehicle. On day 14, the mice were euthanized, and their eyes were analyzed histologically for pathological changes typical of experimental autoimmune uveitis (EAU). Panels **A-D** show minor pathological changes in the eye of a tofacitinib-treated mouse, scored at 0.5+. The changes include accumulation of inflammatory cells at the limbal (panel **B**) and optic nerve head (panel **C**) areas (the two entry sites for the inflammation-inducing cells), as well as small numbers of inflammatory cells in the vitreous (panel **D**). Panels **E-H** are sections of an eye of a control mouse scored at 1.5+. Panel **F** shows infiltration at the limbal area, as well as typical perivascular accumulation of inflammatory cells (black arrow) and photoreceptors loss (white arrow). Panel **G** shows intense infiltration of inflammatory cells at the optic nerve head; panel **H** shows the typical retinal folding and infiltration of inflammatory cells throughout the retina. **I**: Summary of the histological data collected in five repeated experiments, with three to five mice per group. Treatment with tofacitinib significantly inhibited the development of disease. **J**: The actual numbers of $CD4^+$ and $CD8^+$ T cells, as well as NK cells ($CD3^-NK1.1^+$) in the eyes of the immunized mice treated with vehicle or tofacitinib. The means ± standard error of the mean (SEM) from individual mice from a representative experiment; similar findings were made in three additional experiments (n = 5/group) * $p<0.05$. Student *t* test.

the Mann–Whitney test. P values were corrected for multiple comparisons (Figure 2) using the Holm-Sidak method and equation [14].

RESULTS

Treatment with tofacitinib inhibits development of EAU: Groups of B10.A mice were immunized with IRBP and treated with tofacitinib at 25 mg/kg, starting on the day of immunization. On day 14 post-immunization, the mice were euthanized, and their eyes collected and examined histologically for pathological changes. Figure 1A-H demonstrates typical histopathological changes in EAU. The left panels show sections of the eye of a mouse treated with tofacitinib, with minor pathological changes, scored at 0.5, while the eye on the right was obtained from a mouse that received the vehicle. In this eye, the changes were scored at 1.5. The changes are described in detail in the figure legend. Figure 1I summarizes data collected in five repeated experiments, with four to five mice in each group. Treatment with tofacitinib significantly reduced the severity of histopathological changes, compared with the mice treated with the vehicle. Figure 1J shows the actual numbers of inflammatory cells (CD4, CD8, and NK cells) collected from the eyes of the mice treated with tofacitinib or vehicle. In line with lower disease severity, tofacitinib-treated mice had statistically significantly lower numbers of infiltrating cells in their eyes. NK cells are a major component of the innate immune response, and the finding of their reduced number in the tofacitinib-treated mice suggests that the innate immune response was also affected in the treated mice.

Treatment with tofacitinib suppresses production of IFN-γ but not of IL-17: The major populations of lymphocytes involved in the pathogenesis of several autoimmune diseases are Th1 and Th17 cells which mostly exert their effect by secreting their signature cytokines, IFN-γ and IL-17, respectively. To better understand the effects of tofacitinib on the production of these two cytokines, splenic cells were collected from mice treated with tofacitinib or the vehicle control and cultured in the presence of IRBP, the uveitogenic antigen, as well as PPD, a component of the adjuvant (CFA). Supernatants of these cultures were then collected, and the levels of IFN-γ and IL-17 were determined with ELISA. The combined data collected in three repeated experiments are shown in Figure 2A. Notably, treatment with tofacitinib reduced the production of IFN-γ, but not the levels of IL-17.

The effect of tofacitinib treatment on the lymphocyte populations was further analyzed with flow cytometry, measuring the proportions of cells producing IFN-γ, or IL-17, as well as their transcription factors, T-bet and RORγt, respectively. Figure 2B,C shows data for the CD4 cells collected from the eyes and spleen, respectively, of mice treated with tofacitinib or vehicle. In line with the data of the released cytokines (Figure 2A), treatment with tofacitinib reduced the proportions of CD4 cells producing IFN-γ, but had essentially no effect on the IL-17-producing cells in the eyes and spleens of the treated mice.

Treatment with tofacitinib reduces the proportion of cells producing IFN-γ and Tbet among the total CD8 eye-infiltrating and spleen cells: Unlike CD4 cells, CD8 cells produce only trace levels of IL-17, and the analysis of the tofacitinib effect was determined with flow cytometry by measuring only the proportions of cells producing IFN-γ and Tbet among the CD8 cells. Figure 3 shows that treatment with tofacitinib significantly reduced the proportions of cells expressing IFN-γ and T-bet among the CD8 populations of eye-infiltrating and spleen cells.

Treatment with tofacitinib reduces the proportions of Ki67-expressing cells in mouse eyes: To further analyze the effect of tofacitinib, we compared eye-infiltrating cells from mice treated with tofacitinib or vehicle for staining with Ki67, a marker for proliferating cells [15]. As shown in Figure 4, the population of $Ki67^+$ cells was statistically significantly lower in the tofacitinib-treated mouse eyes than in the controls, in line with the inflammatory process being less severe in the tofacitinib-treated eyes.

Treatment with tofacitinib decreases the proportion of CD44 and increases the proportion of CD62L in CD8 cells but not in CD4 cells: To learn more about the effect of tofacitinib treatment on subpopulations of CD4 and CD8 cells, we analyzed with flow cytometry spleen and eye-infiltrating cells from treated mice, or vehicle control, for expression of CD44 and CD62L cell markers. Activation of T-lymphocytes induces an increase in CD44 expression but a reduction in CD62L (L-selectin) expression [16]. As shown in Figure 5, the tofacitinib treatment effect was apparent in the CD8 cells, but not among the CD4 cells. Thus, the CD8 cells collected from the eyes (Figure 5A) and spleens (Figure 5B) of the tofacitinib-treated mice expressed lower expression of CD44 and higher expression of CD62L.

FoxP3-expressing Treg cells are not involved in the immunosuppressive effects of tofacitinib: T-regulatory (Treg) cells play major roles in suppressing pathogenic immune processes [17]. The majority of Treg cells express the transcription factor Foxp3, which in mice is essentially synonymous with inhibitory function. Their involvement in immunosuppressive processes is inferred from changes in their frequency among the total $CD4^+$ population [17]. To examine the possible involvement of $FoxP3^+$ cells in the immunosuppressive

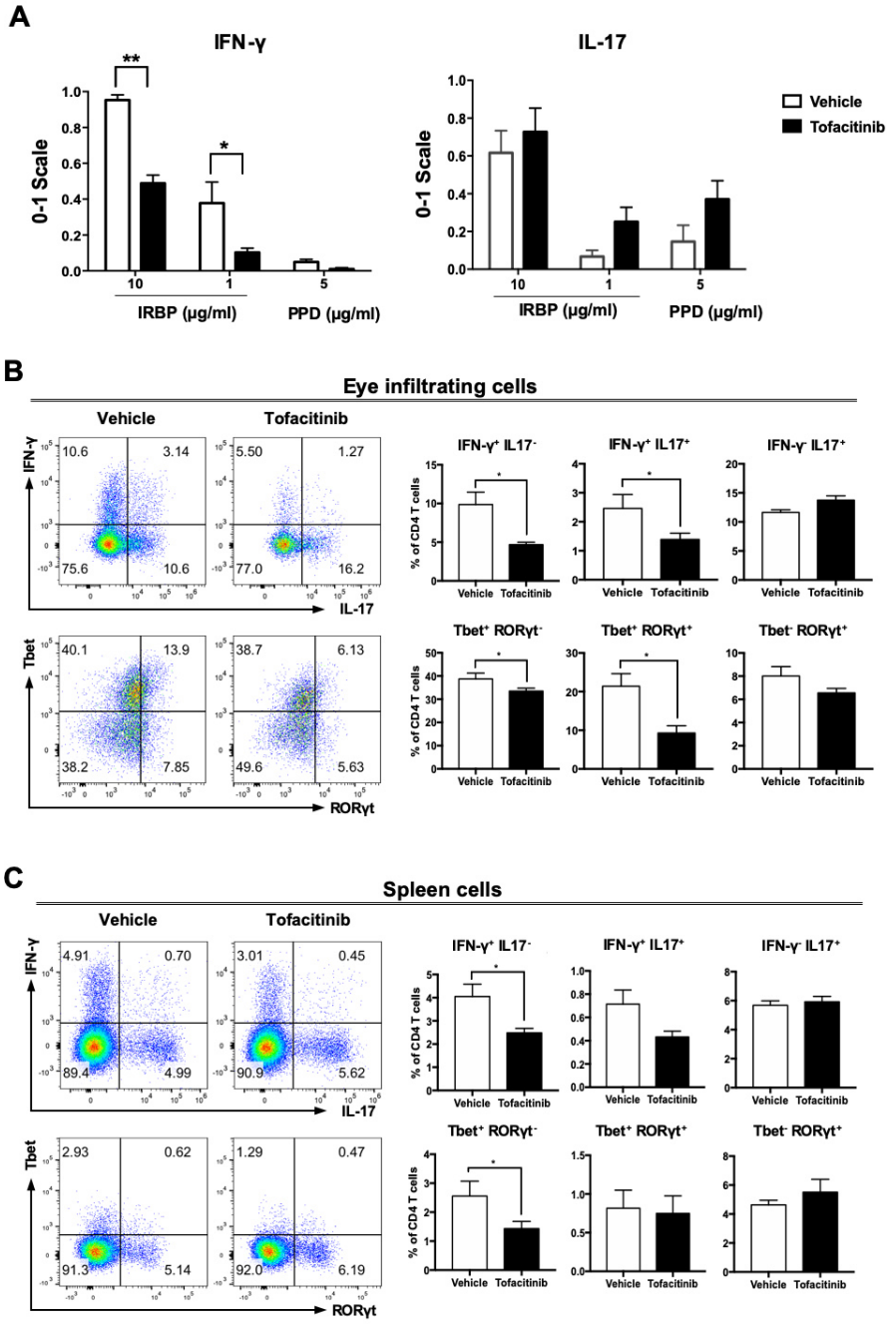

Figure 2. Treatment with tofacitinib selectively inhibits production of IFN-γ, but not of IL-17. **A**: Cytokine levels in culture supernatants. Spleen cells from B10.A mice immunized with interphotoreceptor retinoid-binding protein (IRBP) and treated with tofacitinib, or vehicle, were collected on day 14 post-immunization and cultured with IRBP or purified protein derivative (PPD) at the indicated concentrations. Supernatants collected after 48 h incubation were analyzed for the levels of interleukin (IL)-17 and interferon (IFN)-γ using enzyme-linked immunosorbent assay (ELISA) kits. The data combine actual values of three separate experiments, using the formula shown in reference 14. *p<0.05; **p<0.01. **B** and **C**: Intracellular cytokine staining for IFN-γ and IL-17, or their transcriptional factors, T-bet and RORγt, in mouse eye-infiltrating (**B**) and spleen (**C**) cells, collected on day 14 post-immunization. The methods are detailed in the Materials and Methods section. On the left are shown actual flow cytometric results of representative experiments and on the right are summaries of four independent experiments ± standard error of the mean (SEM), with five mice per group. Statistical significance was determined using an unpaired *t* test. *p<0.05, ** p<0.001, versus vehicle group.

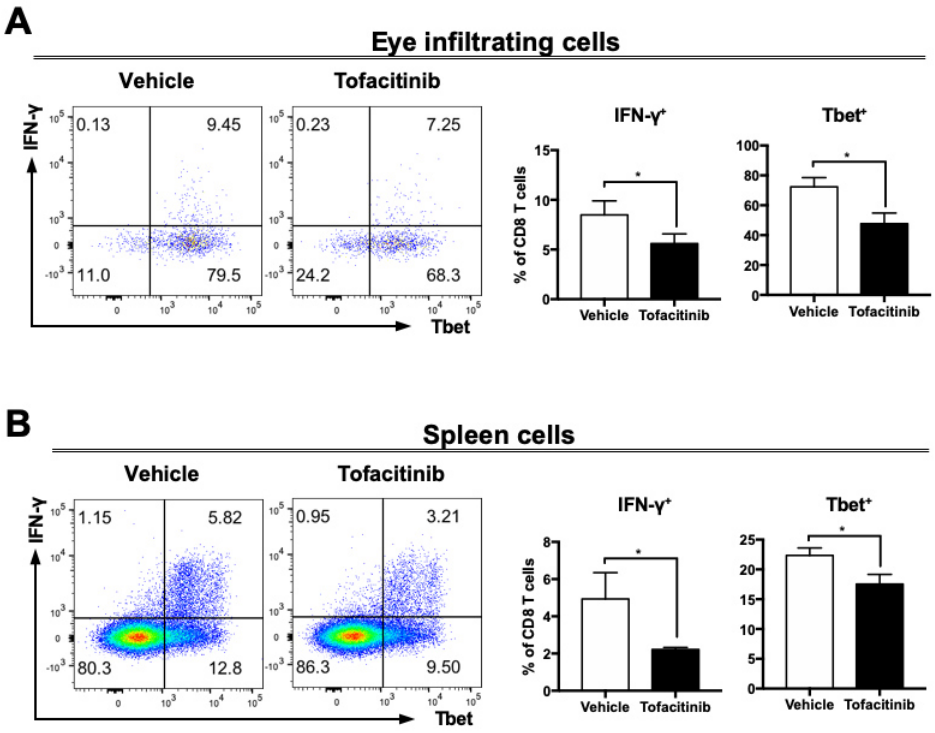

Figure 3. Treatment with tofacitinib suppresses IFN-γ production by CD8 T cells in the eyes and spleen. Intracellular cytokine flow cytometric staining for interferon (IFN)-γ or its transcriptional factor T-bet in eye-infiltrating (**A**) and spleen (**B**) cells, collected on day 14 post-immunization and gated on live CD45$^+$ and CD8$^+$ cells, after ex vivo stimulation with phorbol myristate acetate (PMA) or ionomycin and brefeldin A for 4 h. On the left, representative data of cells from a typical mouse and on the right, summaries of four independent experiments, with each group containing five mice. Data are shown as mean ± standard error of the mean (SEM). Statistical significance was determined using an unpaired *t* test. *p<0.05.

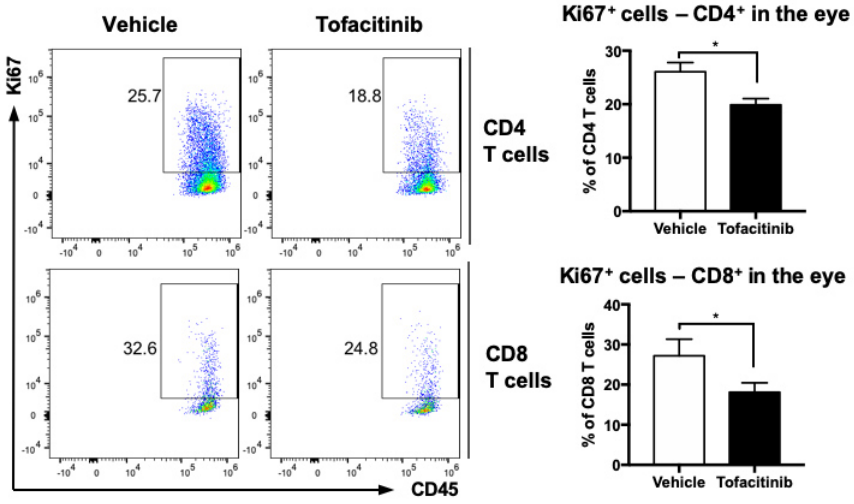

Figure 4. Treatment with tofacitinib suppresses the proportions of proliferating (Ki67$^+$) cells in the affected eyes. Cell preparations of eye-infiltrating cells were prepared as detailed in the Materials and Methods section. The percentage of Ki67$^+$ cells among the CD4 and CD8 cells was determined with flow cytometry. On the left, actual data of a representative experiment and on the right, summary of data from three independent experiments, with each group consisting of five mice. Data shown as mean ± standard error of the mean (SEM). Statistical significance was determined using an unpaired *t* test. *p<0.05.

effects of tofacitinib we determined their percentage among CD4 cells in the spleens of the treated mice, compared to the vehicle-treated control mice. Figure 6A shows on the left the data of a representative experiment with cells from the eye and spleen, and on the right, the summary of four experiments. Tofacitinib had no effect on the percentage of FoxP3^{+} cells among the cells of the inflamed eye and spleen. This observation indicates that Treg cells are not involved in mediating the immunosuppressive effects in mice treated with tofacitinib.

Treatment with tofacitinib does not affect antibody production to IRBP: To further assess the effects of tofacitinib treatment on immune response to IRBP, serum samples from individual mice were collected and pooled, and the levels of antibody against IRBP were measured. Figure 6B displays

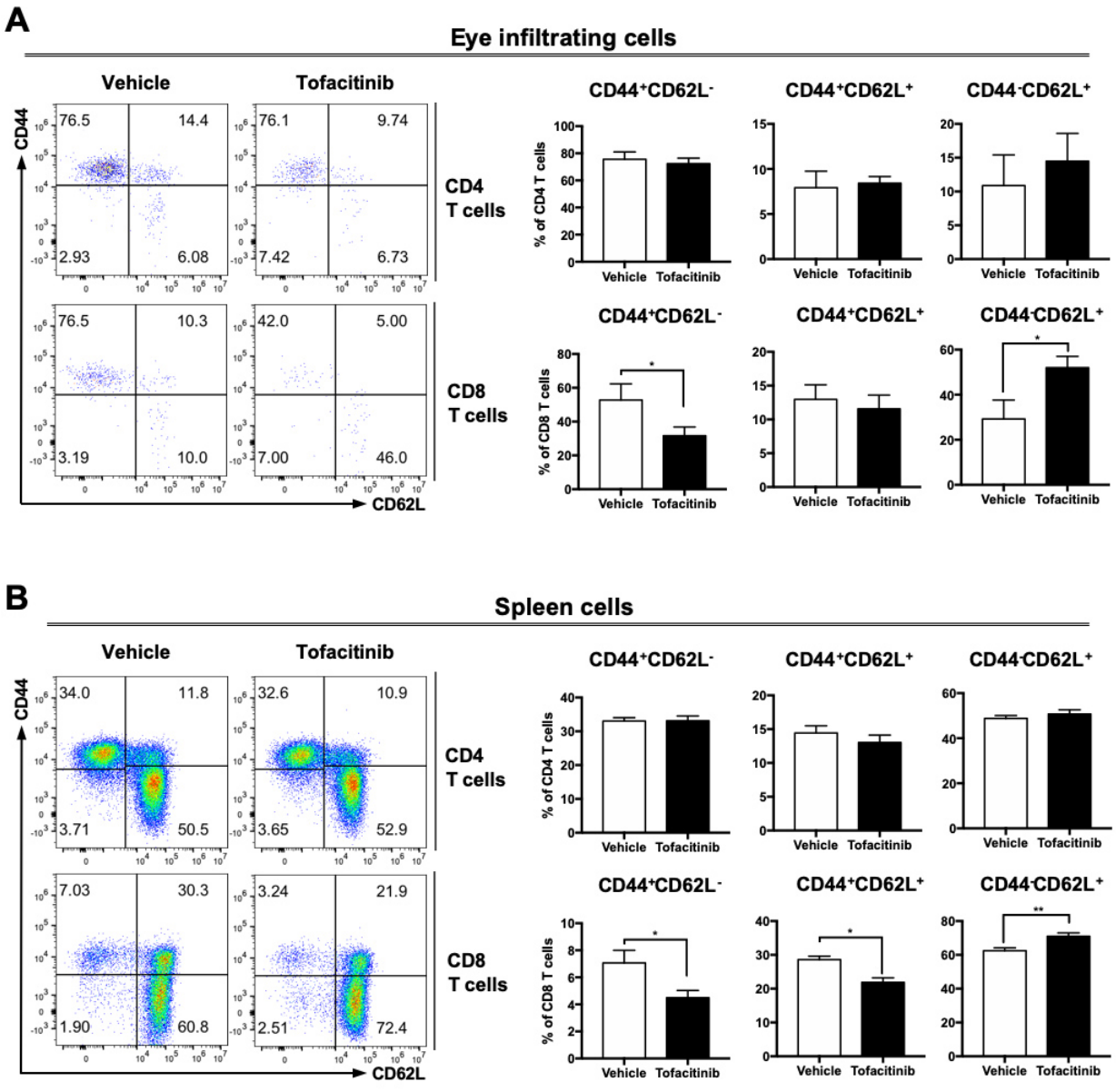

Figure 5. Treatment with tofacitinib differently affects subpopulations of CD8 cells but not of CD4 cells in the eyes and spleen of immunized mice. Cells harvested from inflamed eyes (**A**) or spleen (**B**) of immunized mice, treated with tofacitinib or vehicle were analyzed with flow cytometry for expression of CD44 and CD62L. On the left, data of representative mice and on the right, summary of four independent experiments, with four to five mice per group, presented as mean ± standard error of the mean (SEM). Statistical significance was determined using an unpaired *t* test. *p<0.05.

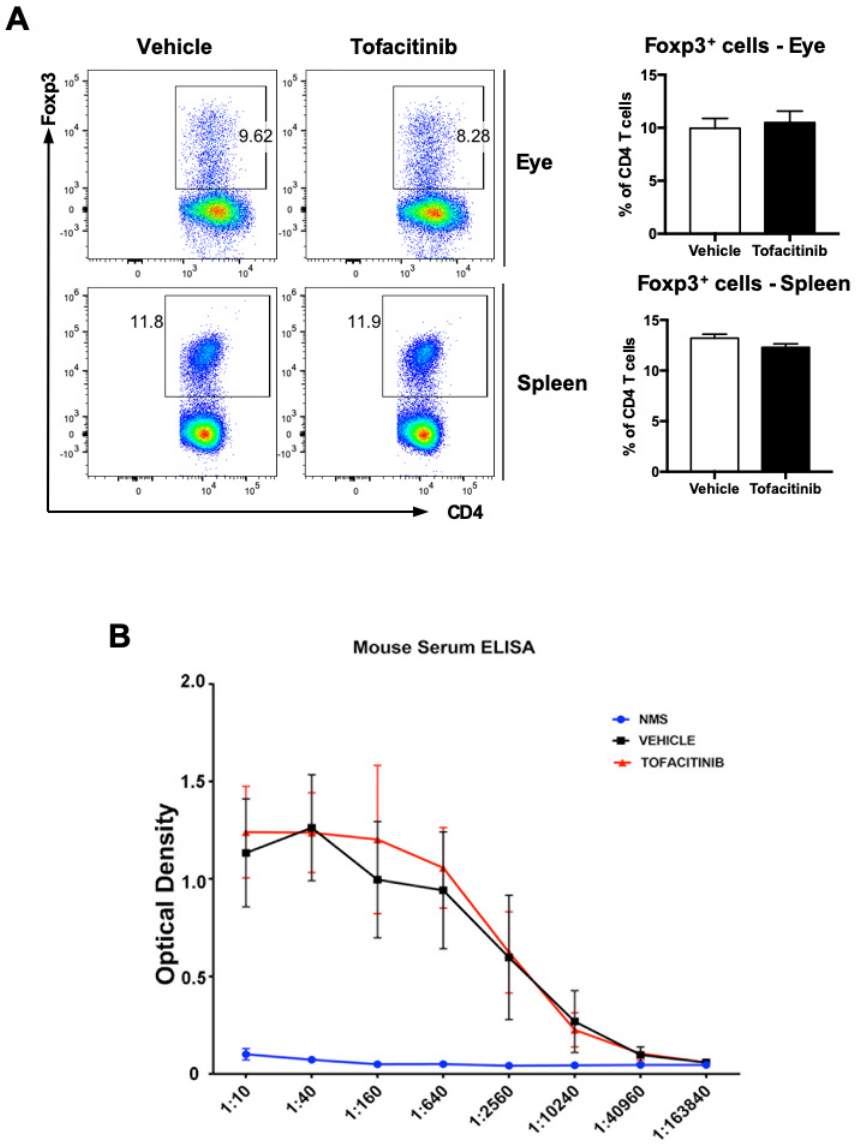

Figure 6. Treatment with tofacitinib has no apparent effect on the generation of FoxP3⁺ cells and on antibody production to IRBP. **A**: Eye-infiltrating and splenic cells from mice treated with tofacitinib or vehicle-treated controls were collected on day 14 post-immunization and analyzed with flow cytometry for intracellular FoxP3. On the left, a representative experiment and on the right, the summary data from four independent experiments with five mice per group. Minimal differences were noted between cells of the two mouse groups. **B**: Pooled sera from mice treated with tofacitinib or from vehicle-treated control mice, collected on day 14 post-immunization, were tested with enzyme-linked immunosorbent assay (ELISA) for antibody against interphotoreceptor retinoid-binding protein (IRBP). Pooled sera of normal mice (NMS) were used as controls. Essentially no differences were noted between the sera of the two groups of immunized mice. The figure combines data of three separate experiments.

the combined data of three experiments, showing that treatment with tofacitinib had essentially no effect on antibody production.

DISCUSSION

In this study, we investigated the effects of the pan-JAK inhibitor tofacitinib in a mouse model of EAU. We showed that treatment with tofacitinib statistically significantly inhibited the development of EAU. The severity of the pathology even in the control mouse eyes was found to be relatively low (mean score of 1.27 ± 0.32 in the vehicle-treated group). This low level of disease development could be attributed to the high frequency of administration of the drug (once or twice daily), which was assumed to cause stress in the experimental animals and consequently, resulted in reduced immunopathogenic response [18]. There is a large body of work assessing the efficacy of tofacitinib in clinical and experimental inflammatory and autoimmune conditions, such as graft versus host disease (GVHD), arthritis, and lupus erythematosus [10,19]. However, to the best of our knowledge, there are only two publications that investigated the effect of tofacitinib on experimental autoimmune encephalomyelitis (EAE), a prototypic experimental autoimmune disease that in many aspects resembles EAU, and the studies yielded conflicting results. Zhou et al. [20] reported that EAE can be

ameliorated by tolerogenic dendritic cells generated by exposure to tofacitinib, but Yoshida and colleagues [21] observed acceleration of the onset of EAE upon treatment with a low dose of this JAK inhibitor. The present study, which is the first in which tofacitinib was administered in a mouse model of EAU, showed amelioration of the disease without induction of regulatory T cells. Notably, in this study, the dose of the drug used was considerably higher than that used by Yoshida et al. Tofacitinib has been found to be beneficial in the treatment of dry eye disease in humans [22] and is capable of reducing ocular surface inflammation in mice [23].

The present findings concerning the effects of treatment with tofacitinib on the cellular immune response against the immunizing antigens are of particular interest. The analyses were performed with spleen and eye-infiltrating cells, and the methods we used to evaluate the effects of tofacitinib included (i) the release of the cytokines by cultured spleen cells, stimulated with the antigen, and (ii) determination of intracellular production of the cytokines, using flow cytometry. Notably, whereas we observed a statistically significant reduction in the production of IFN-γ, the IL-17 levels were essentially unaffected in culture supernatants (Figure 2) and intracellularly (Figure 2 and Figure 3). In addition, the flow cytometric methods made it possible to collect information concerning the effects of tofacitinib on CD8 cells and the expression of several inflammation-related cell markers, including CD44 and CD62L, as well as Ki67, a proliferation marker. It is also of interest that treatment with tofacitinib affected the CD8 population more than the CD4 population.

For all flow cytometric experiments, shown in Figures 2–6, we used female C57Bl/6J mice. Female mice were found to yield more consistent data than their matched males and are currently used in other studies in our laboratory. However, the trends of data collected with male mouse cells were routinely found to be similar to those collected with female mouse cells. Interestingly, analysis of gender in patients with uveitis revealed that the disease is more prevalent in women than in men [24].

The finding that treatment with tofacitinib, while strongly suppressing the adaptive IFN-γ, does not suppress the adaptive IL-17 response, may appear to be counterintuitive, given the accepted pathogenic role for Th17 cells in inflammatory and autoimmune processes [25,26], including EAU [8]. The reason for this discrepancy between the effects on IFN-γ and IL-17 is not clear. That said, IL-17 and IFN-γ produced by adaptive cells at the site of inflammation in the eye are pathogenic cytokines, and affecting only one of them may be sufficient to reduce disease scores. Furthermore, it has been suggested that the most pathogenic Th17 cells are the ones coexpressing IFN-γ and IL-17 [27]. Therefore, it is conceivable that inhibition of IFN-γ could reduce the pathogenicity of Th17 cells without reducing their IL-17 production. Finally, previous reports have shown that tofacitinib can inhibit the development of pathogenic Th17 cells (which depends on IL-1β, IL-6, and IL-23) and enhance the development of the non-pathogenic Th17 population, which requires only IL-6 and TGF-β1 [28,29].

In summary, the present data showed that treatment with tofacitinib suppresses the development of EAU, an animal model for uveitic conditions in humans. Treatment with this compound reduced the population of Th1 cells and the associated production of IFN-γ specific to the uveitogenic antigen, but had no statistically significant effect on the size of the Th17 cell population. We propose that in this model tofacitinib suppresses the development of pathogenic Th1 cells and may promote the formation of non-pathogenic Th17 cells.

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