**p38α MAP Kinase Controls IL-17 Synthesis in Vogt-Koyanagi-Harada Syndrome and Experimental Autoimmune Uveitis**

*Alessandra Gonçalves Commodaro,1 Cíntia Raquel Bombardieri,2 Jean Pierre Schatzmann Peron,2 Kelly Cristina Saito,3 Pedro Mancini Guedes,2 Dânia E. Hamassaki,3 Rubens N. Belfort,1 Luiz Vicente Rizzo,2,4 Rubens Belfort, Jr,1 and Maristela Martins de Camargo2*

**PURPOSE.** Interleukin (IL)-17, which is responsible for the initial influx of leukocytes into the target tissue, was recently described as the main cytokine involved in autoimmune diseases. Vogt-Koyanagi-Harada (VKH) syndrome is a significant cause of noninfectious blindness in the world. Herein the authors aimed at unraveling the involvement of IL-17 in VKH and in experimental autoimmune uveitis, focusing on the signaling pathways involved in IL-17 synthesis.

**METHODS.** Mice were immunized with 161–180 peptide and pertussis toxin. Draining lymph node cells, harvested 21 days after immunization, were cultured in the presence or absence of p38α mitogen-activated protein kinase (MAPK) inhibitor (SB203580) and assayed for cytokine production and quantification of CD4+ IL-17+ cells. Mice received intraocular injections of SB203580, and disease severity was evaluated by histologic examination of the enucleated eyes at day 21. CD4+ lymphocytes from MSK-1/2-deficient mice, human CD4+ cells silenced with MSK1 siRNA, or peripheral blood mononuclear cells (PBMCs) from VKH patients were cultured in the presence or absence of p38α MAPK inhibitor and then assayed for IL-17, IFN-γ, and IL-4 production.

**RESULTS.** The inhibition of p38α MAPK fully blocked the synthesis of IL-17 by PBMCs from VKH patients and lymphocytes from EAU mice. The absence of the msk1/2 gene resulted in failure to produce IL-17 by murine and human lymphocytes. Interestingly, intraocular injections of SB203580 in EAU mice did not suppress development of the disease.

**CONCLUSIONS.** These data show that p38α MAPK-MSK1/2 is involved in the control of IL-17 synthesis by CD4+ T cells and that inhibition of p38α MAPK in vitro suppresses IL-17 synthesis but that inhibition of this kinase in vivo did not protect from EAU. (Invest Ophthal Vis Sci. 2010;51: 3567–3574) DOI:10.1167/iovs.09-4393

*Uveitis is an inflammation of the uveal tract (iris, ciliary body, and choroid).† Experimental autoimmune uveitis (EAU) is the animal model for human uveitis, such as sympathetic ophthalmia, birdshot retinochoroidopathy, Vogt-Koyanagi-Harada (VKH) syndrome, and Behcet’s disease.‡ EAU is an organ-specific, T-cell-mediated disease that can be induced in susceptible animals (primates and rodents) after immunization with retinal antigens, such as interphotoreceptor retinoid-binding protein (IRBP) and S-antigen (arrestin), or through adoptive transfer of T cells specific for these antigens.†,‡ The disease is characterized by granuloma formation in the neural retina, destruction of photoreceptor cells, and blindness.‡,† VKH syndrome is a human autoimmune disease characterized by poliosis (decrease of melanin in hair), vitiligo (loss of melanin in the skin), alopecia (hair loss), and chronic bilateral granulomatous uveitis.§ The pathogenesis of VKH probably derives from an autoimmune response against melanocyte-derived antigens, such as tyrosinase and gp100.‖ It was recently shown that cells from the peripheral blood of VKH patients produce large amounts of IL-17,‖ ‡ IL-17-producing CD4+ T cells, so-called Th17 cells, constitute a recently identified inflammatogenic cell population that plays an important role in the pathogenesis of EAU.‖‡ In the EAU model, it was shown that uveitogenic clones might be both CD4+ and CD8+ cells that are capable of producing IL-17.‖ The pathogenic role of such cellular population was further elucidated after intraocular injections of neutralizing antibodies against IL-17, which ameliorated EAU lesions.‖

p38α Mitogen-activated kinase (MAPK) was originally described by researchers at SmithKline Beecham as the target of a specific pyridinyl-imidazole inhibitor named SB203580.‖ Since then, this kinase has been described as a key regulator of several proinflammatory cytokines, and its inhibitors have been the subject of several preclinical studies and clinical trials of a potential anti-inflammatory drug shown to be effective in a murine model of the autoimmune disease rheumatoid arthritis (RA).‖‡ In this study, we evaluated the hypothesis that the p38α MAPK pathway, which is involved in the control of several proinflammatory cytokines, including IFN-γ, would also control IL-17 synthesis. If confirmed, it would be plausible that turning off this kinase would ameliorate autoimmune uveitic lesions in the EAU model.

In fact, we found high levels of IL-17 produced by both peripheral blood mononuclear cells (PBMCs) from VKH pa-
tients and by total spleen and lymph node cells of EAU mice after in vitro stimulation. In vitro treatment of these cells with SB203580, an inhibitor of p38α MAPK, or alternatively by deleting its downstream targets, the mitogen and stress-activated kinase 1 (MSK1) and 2 (MSK2) through homologous recombination, completely abolished IL-17 production by these cells. Unexpectedly, intraocular injection of SB203580 in EAU mice did not ameliorate or suppress disease when compared with control eyes. This study shows that the p38α MAPK-MSK1/2 signaling circuit is responsible for the synthesis of IL-17 by human and murine CD4+ T cells and that intraocular inhibition of p38α MAPK is not sufficient for preventing the disease associated with inflammation in the EAU model.

**SUBJECTS AND METHODS**

**Subjects**

Thirteen patients with VKH (age range, 20–60 years) were recruited from the Ophthalmology Department of the Federal University of São Paulo. The diagnosis of VKH syndrome followed the clinical and laboratory criteria established by an international committee on nomenclature.21 Nine sex- and age-matched healthy control subjects with no history of autoimmune disease were enrolled in this study. Human cord blood was obtained from several healthy women between 18 and 38 years of age. The institutional review board approved this study, and written informed consent was obtained from all donors before cord blood collection. Both the Federal University of São Paulo and the University of São Paulo ethics committees approved the study, and written informed consent was obtained from all subjects. Sample collections were carried out in adherence to the Declaration of Helsinki.

**Animals**

Six- to 8-week-old B10.RIII mice were obtained from the animal facilities at the University of São Paulo. Mice deficient in MSK1 and MSK2, described previously,22 were kept under specific pathogen-free conditions in accordance with European Union and United Kingdom laws. Bone marrow of −/− and +/+ littermates were harvested under sterile conditions and kept frozen at −80°C until use for reconstitution of RAG−/− mice. All animals were housed under specific pathogen-free conditions and were treated according to institutional guidelines. The Animal Care and Committee at the Institute of Biomedical Sciences of the University of São Paulo approved all the procedures used in this study. The animals were handled in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Induction of EAU**

B10.RIII mice were immunized subcutaneously at the base of the tail with 40 μg of 161–180 peptide emulsified in 0.2 mL complete Freund adjuvant (CFA; vol/vol). At the same time, mice were injected intraperitoneally with 0.5 mg pertussis toxin (PTX) as an additional adjuvant. Peptide SGIIPYSHLYHPNTLHVD representing residues 161–180 of IRBP was synthesized using Fmoc chemistry on a peptide synthesizer (Pioneer; Applied Biosystems, Foster City, CA). PTX and CFA were purchased from Sigma-Aldrich (St. Louis, MO).

**In Vivo Treatment with p38 MAPK Inhibitor**

The vitreous volume of matched-age mice was estimated by cutting enucleated eyes open and collecting the vitreous with a micropipette. The obtained volume averaged 2 μL. Mice were anesthetized by intraperitoneal injection of ketamine (50 mg/kg; Vetbrands, São Paulo, Brazil) and xylazine (10 mg/kg; Syntec, São Paulo, Brazil), and pupils were dilated with topical tropicamide (1%; Alcon Laboratories Inc., Irvine, CA). SB203580 (20 μM) diluted in sterile PBS was injected into the vitreous cavity of the right eye in a total volume of 1 μL (final intraocular concentration, 6.6 μM) with a 33-gauge Hamilton microsyringe. Left eyes were injected with 1 μL sterile PBS. In addition to the immunized experimental groups, a third group of animals was immunized but did not receive the inhibitor injections, and a fourth nonimmunized group received vehicle alone (dimethyl sulfoxide) injections. Animals were treated on days 1, 4, 7, 10, 13, 16, and 19 after EAU immunization (seven injections per animal). Animals were killed on day 21 for analysis of local and systemic side effects of SB203580.

**Histopathology of EAU Lesions**

Eyes were harvested and prepared for histopathologic evaluation at the end of each experiment (day 21 after immunization). They were immersed for 1 hour in phosphate-buffered glutaraldehyde 4%, transferred to phosphate-buffered formaldehyde 10% for 24 hours, and replaced with ethanol 70% until processing. Fixed and dehydrated tissue was embedded in paraffin wax, and 4- to 6-mm sections were cut through the papillary-optic nerve plane. Sections were stained by hematoxylin and eosin. Presence or absence of disease was evaluated in a double-blinded fashion by examining six sections cut at different levels for each eye. Severity of EAU was scored on a scale of 0 (no disease) to 4 (maximum disease) in half-point increments, according to a semiquantitative system described previously2 that considers lesion type, size, and number. In brief, the minimal criterion to score an eye as positive by histopathology was inflammatory cell infiltration of the ciliary body, choroids, or retina (EAU grade 0.5). Progressively higher grades were assigned for the presence of lesions in the tissue, such as vasculitis, granuloma formation, retinal folding or detachment, and photoreceptor damage.

**Determination of Cytokine Production in Mice**

Draining lymph node cells harvested 21 days after immunization were cultured in 24-well plates (1 × 10^6 cells/well) and were stimulated with 30 ng/mL IRBP. Supernatants were collected for cytokine analysis after 48 hours and were stored at −80°C until assayed. ELISA was used to quantify the levels of IL-17 (eBiosciences, San Diego, CA), IL-4, and IFN-γ (BD PharMingen, San Jose, CA).

**Determination of Cytokine Production by MSK1 siRNA CD4+ Cells**

Supernatants were collected for cytokine analysis after 6 days of differentiation under Th0, Th1, or Th2 and were stored at −80°C until assayed. ELISA was used to quantify the levels of IL-17 (eBiosciences), IL-4, and IFN-γ (BD Biosciences, San Jose, CA).

**Determination of Ex Vivo IL-17 Production in Human PBMCs**

To analyze cytokine production, 1 × 10^6 PBMCs were plated and stimulated (or not) with phytohemagglutinin (PHA; 10 μg/mL; Invitrogen Life Technologies, Carlsbad, CA) for 72 hours. Supernatants were collected and analyzed for IL-17 by ELISA (eBiosciences).

**Intracellular Cytokine Staining and FACS Analysis**

For detection of intracellular expression of IL-17 by flow cytometry, cells isolated from EAU mice lymph nodes were collected and left unstimulated or were stimulated for 5 hours with PMA (50 ng/mL; Sigma) and ionomycin (750 ng/mL; Calbiochem, Temecula, CA) in the presence of either protein transport inhibitor (Golgiplug or GolgiStop; BD PharMingen) at the recommended concentration. Cells were first stained extracellularly with fluorescein-conjugated anti-CD4+ and fixed. Then they were permeabilized with solution (Cytotox/Cytoperm; BD PharMingen) and were stained intracellularly with phycoerythrin-conjugated anti-IL-17. Samples were acquired (FACSCalibur; BD Biosciences), and data were analyzed with software (FlowJo; TreeStar, Ashland, OR).
In Vitro Differentiation of Murine CD4+ T Cells

CD4+ T (Th) cells were negatively purified from lymph nodes of reconstituted RAG−/− mice using MACS (Miltenyi Biotec, Piscataway, NJ). Purified CD4+ cells were stimulated with plate-bound anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL) under Th1 (rIL-12p70 and neutralizing anti-IL-4 antibody) or Th2-inducing conditions (ril-4 and neutralizing anti-IFN-γ antibody). All recombinant cytokines and mAbs against CD3, CD28, IFN-γ, and IL-4 were obtained from PharMingen. Cells were stimulated in the presence of 10 μM SB203580 (p38 MAPK inhibitor; Calbiochem) for 30 minutes before addition of the differentiation media. Cells were expanded for 1 week under differentiating conditions, and the inhibitor was maintained in the culture the entire time.

In Vitro Differentiation of Human CD4+ T Cells

CD4+ T (Th) cells were isolated from cord blood (RosetteSep; Stemcell Technologies, Vancouver, Canada). Purified CD4+ cells were stimulated with plate-bound anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL) under Th0 (nonpolarizing, neutralizing anti-IL-4 and anti–IFN-γ antibodies), Th1 (rIL-12p70 and neutralizing anti-IL-4 antibody), or Th2-inducing conditions (ril-4 and neutralizing anti-IFN-γ antibody). All recombinant cytokines and neutralizing mAbs to IFN-γ and IL-4 were obtained from BD Biosciences. The cells were expanded for 6 days under differentiating conditions.

Small Interfering RNA Transfection

Small interfering RNA (siRNA) against MSK1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Naive human CD4+ cells were transfected with 20 μM siRNA using the manufacturer’s transfection medium and were stimulated 5 hours later under Th0, Th1, or Th2 differentiation conditions.

Western Blot Analysis

siRNA-silenced cells (2 × 10⁶ cells) were disrupted in 100 μL sample buffer (50 mM Tris-HCl [pH 7.0], 2.5% β-mercaptoethanol, 0.1% bromophenol blue, and 10% glycerol) supplemented with protease inhibitors (Roche Diagnostic Co., Basel, Switzerland), separated by 10% SDS-PAGE, and blotted into polyvinylidene difluoride membrane. MSK1 protein was detected using a polyclonal anti-MSK1 antibody and anti–rabbit-HRP. Membranes were reprobed with a monoclonal anti–human β-actin as internal control. All antibodies were purchased from Santa Cruz Biotechnology. An enhanced chemiluminescence system (GE Biosciences, Little Chalfont, UK) was used for visualization of bands by autoradiography. Densitometric analyses were conducted with image analysis software (ImageQuant; GE Biosciences).

Multiplex Microbead Immunoassay

Supernatant samples from triplicate cultures of cells from the reconstituted Rag-deficient mice were pooled and stored at −80°C until analyses were complete. A multiplex biometric immunoassay, containing fluorescence-dyed microspheres conjugated with a monoclonal antibody specific for a target protein, was used for cytokine measurement (IL-4, IL-17, IFN-γ) according to the manufacturer’s instructions (mouse cytokine/chemokine kit; Bio-Rad, Hercules, CA). Briefly, 25 μL standard and sample was loaded onto the 96-well filtration plate. Beads (25 μL) coated with target capture antibodies against cytokines were added to each well and incubated for 2 hours at room temperature with low-speed shaking at 300 rpm. After washing, the plate was supplied with 25 μL premixed biotin conjugate antibodies and incubated for 1 hour at room temperature with shaking at 300 rpm. Finally, streptavidin-phycocerythrin was added, and after 30 minutes of shaking at room temperature the results were read with a suspension array system (Bio-Plex Luminex; Bio-Rad). Sensitivity of the assay was 5 pg/mL for IL-4, 1 pg/mL for IL-17, and 6 pg/mL for IFN-γ.

Statistical Analysis

Data are expressed as mean ± SD. Statistical analyses were performed using GraphPad software (Prism, version 5.00 for Windows; GraphPad, San Diego, CA). Nonparametric Student’s test was used to determine statistical difference, and P < 0.05 was considered significant.

RESULTS

In Vitro Inhibition of p38α MAPK by SB203580 Reduces IL-17 Secretion by Total Splenocytes and Lymph Node Cells of EAU Mice

As described by others, IL-17–secreting CD4 cells, termed Th17, play a relevant role in the pathogenesis of several autoimmune diseases. In fact, the number of Th17 cells is widely increased in the periiphery of EAU mice.13,15 In this context, we sought to evaluate whether in vitro inhibition of p38α MAPK by SB203580 would alter the secretion pattern of this cytokine.

As demonstrated by Figure 1A, there is a significant (P < 0.05) reduction in IL-17 secretion in cultures of splenocytes stimulated with the EAU cognate antigen IRBP compared with cultures stimulated with IRBP in the presence of SB203580. The levels of IL-17 found in the presence of SB203580 were similar to those found in unstimulated cells. Interestingly, we were unable to detect significant differences on IL-17 production by lymph node cells at the time point evaluated (Fig. 1B).

In Vitro Inhibition of p38α MAPK by SB203580 Reduces the Percentage of Th17 Cells in Both Spleen and Lymph Nodes of EAU Mice

Because of the great importance of the Th17 population in autoimmune responses, we decided to evaluate whether the changes observed in IL-17 secretion induced by IRBP in the presence of SB203580 were due to specific inhibition of such a population. Despite the role of CD4+ IL-17+ (Th17) pathogenic cells, some reports have shown that other cell types are able to secrete IL-17. For example, microglia cells also present such capacity.25 We analyzed by flow cytometry the percentage of CD4+ IL-17+ cells in cultures of cells from spleen and lymph nodes from EAU mice. Our data clearly showed an approximate 50% reduction in the percentage of CD4+IL-17+ cells in those cultures subjected to p38α MAPK inhibition. Moreover, this reduction was observed in cells obtained from spleen and from lymph nodes of sick mice (Fig. 2), suggesting that the observed reduction in IL-17 protein secretion (Fig. 1) probably resulted from suppression of IL-17 production, specifically by CD4+ T cells rather than by other cell types.

In Vitro Differentiation of Naive CD4+ Cells in the Absence of p38α MAPK or MSK1/2 Activity Reduces IL-17 Secretion

Naive CD4+ T lymphocytes were obtained from wild-type and Rag−/−MSK1/2−/− mice and differentiated in vitro under proinflammatory (Th1, favors IFN-γ production) or anti-inflammatory (Th2, favors IL-4 production) conditions in the presence or absence of SB203580. After 6 days of culturing, IL-4, IL-17, and IFN-γ were measured in the supernatants by multiplex immunoassay. Figure 3A (right side, lower panel) shows that wild-type CD4+ cells produce large quantities of IL-17 when cultured in the presence of IL-4–enriched media. The same cells, when cultured in the presence of IL-4–enriched media (Th1-inducing), produce measurable amounts of IL-17 but smaller amounts than those cultured under IL-4 influence (Th2-inducing). Interestingly, no IL-17 is detected when these conditions were subjected to p38α MAPK inhibition or MSK1/2 silencing.

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same cells are differentiated in the presence of the p38/H9251 MAPK inhibitor (Fig. 3A, right side, middle panel). Furthermore, adding SB203580 to the media of MSK1/2-deficient cells had no additive effect over IL-17 production (Fig. 3A, lower panel, 4th and 8th bars). The absence of MSK1/2 resulted in increased amounts of IFN-γ production by those cells cultured under Th1 (Fig. 3A, middle panel) and increased amounts of IL-4 by those cells cultured under Th2 (Fig. 3A, upper panel) conditions. Naive CD4/H11001 T lymphocytes were obtained from human cord blood, and MSK1 was silenced by siRNA. An aliquot of cells was subjected to Western blot analysis to verify the absence of MSK1 protein expression (data not shown). Five hours after siRNA transfection, cells were differentiated in vitro under nonpolarizing (Th0), proinflammatory (Th1, favors IFN-γ/H9253 production), or anti-inflammatory (Th2, favors IL-4 production) conditions for 6 days. Interleukin-4, IL-17, and IFN-γ were measured in the supernatants by ELISA. Figure 3B shows that both wild-type and MSK1-silenced CD4/H11001 cells produce considerable quantities of IL-17 when cultured in nonpolarizing media (Fig. 3B, left side, lower panel). Wild-type cells cultured in the presence of IL-12-enriched media (Th1-inducing) produced larger amounts of IL-17, but significantly (P < 0.005) lower levels of IL-17 were detected when these same cells were differentiated after MSK1 silencing (Fig. 3B, middle graph, lower panel). Similar to what was observed with mouse cells (Fig. 3A), the absence of MSK1 did not have a strong effect over the production of IFN-γ by those cells differentiated under Th1-inducing media (Fig. 3B, middle panel). In contrast to mouse cells, in which the absence of MSK1/2 did not alter the production of IL-4 (Fig. 3A, upper panel), silencing of MSK1 in human cells increased significantly the production of IL-4 by cells differentiated under Th1 (P < 0.001) and Th2 (P < 0.005) (Fig. 3B, upper panel).

**Intraocular Administration of SB203580 to Mice with EAU Did Not Change Disease Scores**

After observing the ability of SB203580 to reduce in vitro both the secretion of IL-17 and the percentage of Th17 cells, we considered that it would be interesting to study the inhibition of p38/H9251 MAPK in vivo. The hypothesis was that inhibition of p38/H9251 MAPK in loco (i.e., inside the eye) would inhibit IL-17 production, leading to reduction in the lesions induced by EAU in mice. To address that hypothesis, we induced EAU in a group of mice and treated them with seven intraocular injections of 6.6 M (approximate final concentration inside the eye) of SB203580 in the right eye and PBS in the left eye. The treatment was performed every 3 days until the end of the experiment at day 21, when animals were killed and EAU was scored through histopathologic analysis.

**FIGURE 1.** Inhibition of p38α MAPK in vitro decreased IL-17 levels by lymph nodes and spleen cells from EAU mice. Draining lymph nodes (A) and spleen (B) cells from immunized mice were harvested at day 21 and stimulated in vitro (10⁶ cells/mL) with 30 μg/mL IRBP in the presence or absence of 10 μM SB203580. After 72 hours, IL-17 levels were determined by ELISA. (A, B) n = 5 mice/group.

**FIGURE 2.** Diminished numbers of CD4⁰ IL-17⁺ T cells after inhibition of p38α MAPK in vitro. Cells from draining lymph nodes collected at day 21 after immunization and stimulated in vitro with PMA and ionomycin in the presence (IRBP + SB) or absence (IRBP) of SB203580. Cells were then labeled with anti-CD4 mAb, fixed and permeabilized, stained intracellularly for IL-17, and analyzed by flow cytometry. The percentage of CD4⁺ IL-17⁺ cells is relative to the total CD4⁺ cell count. Results are expressed as mean ± SD. n = 9 mice/group. Continuous line: percentage of CD4⁺ cells that produce IL-17 in the lymph node without IRBP priming. Dashed line: same percentage in the spleen.
Unexpectedly, intraocular administration of SB203580 was unable to reduce the incidence of lesions in the treated (right) eyes (Fig. 4A) compared with control (left) eyes (Fig. 4A). All mice from both groups showed moderate to severe signs of EAU at histopathologic examination on day 21 (Fig. 4B). The most frequently observed histopathologic features were retinal disorganization, inflammatory cell infiltration in the vitreous, vasculitis, and formation of granuloma (Fig. 4B). At funduscopy, the percentage of animals that presented trauma to the lens (from the injections) was 30% in all groups. Seven injections (one injection every 3 days) were the maximum number of injections we were able to perform without incurring side effects of excessive trauma from the injections. We opted for maximized treatment after not obtaining positive results with only one injection on day 1 after EAU immunization (data not shown).

**DISCUSSION**

The work described in the present paper shows that inhibition of p38α MAPK in vitro by the inhibitor SB203580 suppressed the production of IL-17 by CD4⁺ T lymphocytes in EAU mice and by PBMCs in VKH patients. The absence of functional MSK1/2, downstream targets of p38α MAPK, resulted in the complete absence of IL-17 production by murine and human CD4⁺ T lymphocytes. Unexpectedly, the inhibition of p38α MAPK locally, through intraocular injection of SB203580 in EAU mice, was not sufficient to prevent the occurrence of lesions from uveitis. Taken together, our results identify p38α MAPK as the protein kinase responsible for IL-17 production by Th17 cells in EAU and human autoimmune uveitis, include
MSK1/2 as a key regulator of this cytokine, and show that local inhibition of this pathway might not be enough to prevent autoimmune damage to the eye structures.

Th17 cells were recently described as crucial for the development of several experimental autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE) and EAU.24,25 Th17 cells are also involved in the development of uveitis in patients with VKH disease.10,15,16,26 This subset of lymphocytes secretes high levels of IL-17 in response to TGF-β and IL-6.27 Active patients with multiple sclerosis present high levels of mRNA for IL-6 and IL-17 both in the blood and in the spinal fluid. A correlation between the level of these cytokines and active disease was found.28,29 In the present study, we confirmed the findings of others that mononuclear cells isolated from EAU mice and VKH patients produce high levels of IL-17 on restimulation in vitro (Figs. 1, 2).9,11,12,14–16 Taken together, these studies and ours support the idea that inhibition of IL-17 synthesis is an interesting therapeutic target for autoimmune diseases.

Several proinflammatory cytokines, such as IL-6 and IFN-γ, are regulated by the p38 MAPK pathway.25,30 Both IL-6 and IFN-γ were shown to be important for induction of disease in the EAU model.15,26 It is thus acceptable that the suppression of proinflammatory cytokines through the inhibition of p38 MAPK is an approach that deserves further investigation. It is important to point out that inhibition of Th17 clones is not original to us and that different approaches to achieve that have been pursued, with variable degrees of success, by several groups.9,15,17,31,32 What was new in our approach was the attempt to dampen the signaling pathway involved in the synthesis of IL-17 rather than interference with the end product of this pathway, IL-17 itself.

Despite the intensive research being performed on Th17 cells, the intracellular events leading to the commitment of naive CD4+ cells with the production of stable high levels of IL-17 is far from completely elucidated. STAT324 and RORγt27 are important for cell commitment because gene-targeted mice lack Th17 cells in the periphery.27 It was also described that the lack of activity of IRAK4, a kinase downstream of receptor of IL-1, enables mice to become resistant to the induction of EAE by inhibiting Th17 differentiation and reducing IL-23 production by differentiated cells.33 Using an animal model in which excessive IL-1 signaling (caused by lack of IL-1Ra) results in autoimmune arthritis, another group showed that the production of IL-17 induced by IL-23 occurs by activation of Jak2, PI3K/Akt, STAT3, and NFκB and that, under their experimental condition, inhibition of p38 MAPK (by use of SB203580) and inhibition of AP-1 do not alter the production of IL-17.34 In accordance with these data, animals deficient of SHIP, a phosphatase that regulates the activity of PI3K and that was shown to regulate the differentiation of regulatory T cells, are unable to differentiate Th17 cells both in vitro and in vivo.35 On the other hand, mice deficient of Batf, a member of the AP-1

**FIGURE 4.** Intraocular injection of SB203580 did not ameliorate EAU scores. (A) B10.RIII mice were immunized with IRBP 161–180 peptide followed by intraocular injection with SB203580. Eyes were collected for histopathology 21 days later. EAU scores were assigned on a scale of 0 to 4. Histopathologic findings: immunized mice that received intraocular injection with SB in the right eye (RE – SB); immunized mice that received intraocular injection with PBS in the left eye (LE – PBS). Representative photographs (hematoxylin-eosin staining). n = 7 mice/group. Original magnifications: 100× (left), 400× (right).

**FIGURE 5.** Inhibition of p38α MAPK in vitro decreased IL-17 levels by PBMCs from VKH patients. PBMCs were plated and stimulated with PHA in the presence or absence of 10 μM SB203580. After 72 hours, supernatants were collected, and levels of IL-17 were analyzed by ELISA.
family, show decreased Th17 differentiation and are resistant to the induction of EAE. When it comes to the specific milieu of the eye, a recent work showed that the cytokines produced in vitro by the corneal epithelium after stimulation by inflammation, hyperosmotic shock, or Toll-like receptor ligands induce the differentiation of Th17 cells, suggesting that injuries to the eye might promote cell differentiation toward an autoimmune effector phenotype. In this context, our study adds another step to the pathway(s) involved in IL-17 production by CD4+ cells. In our experiments, when cells from EAU mice were cultured in the presence of SB203580, an inhibitor of the protein kinase p38α MAPK, IL-17 secretion and the percentage of CD4+ IL-17+ cells were significantly reduced (Figs. 1, 2). Peripheral blood mononuclear cells from patients with uveitis (VKH) secreted high levels of IL-17, and culturing these cells in the presence of SB203580 significantly reduced their capacity to secrete IL-17 (Fig. 5). Taken together, these results showed that Th17 production is regulated by p38α MAPK in a conserved fashion in mice and humans.

p38α MAPK activates, among other targets, the mitogen and stress-activated kinases 1 (MSK1) and 2 (MSK2). These kinases suppress the inflammatory response by inducing IL-10 synthesis and inhibiting TNF-α. Mice doubly deficient in msk1 and msk2 present an exacerbated inflammatory response to LPS challenge. Thus, it is conceivable to expect that these mice would be good producers of IL-17 because this is also considered a proinflammatory cytokine. Our data show this is not the case. CD4+ cells derived from MSK1/2-deficient mice were unable to produce detectable IL-17 when cultured in the same conditions that triggered high levels of this cytokine by wild-type cells (Fig. 3A). The same was observed when human naive CD4+ cells were silenced by siRNA specific to MSK1. The absence of MSK1 resulted in low levels of IL-17 by cells differentiated in conditions that induced high levels of IL-17 by wild-type cells (Fig. 3B).

The use of kinase inhibitors to control degenerative and inflammatory diseases with autoimmune components and cancers has been an aim of researchers at academy and industry for decades without much advancement. The primary reason for such drawback is the toxicity of side effects of kinase inhibitors when administered systemically. With SB203580, it has been shown that systemic use leads to liver and CNS problems. Because of such problems, no studies beyond phase 1 have been performed. The eye has no drainage system and, in principle, could receive drugs that would be toxic if used systemically. We decided to test whether intraocular injections of SB203580 could inhibit in vivo and in loco the production of IL-17, thus preventing the development of autoimmune uveitis in the EAU model. At this point in our study, treatment of EAU mice with intraocular injections of SB203580 was unable to abrogate ocular lesions associated with uveitis. We found no difference in the size, number, or characteristics of the lesions when the eyes treated with the inhibitor were compared with sham-treated eyes. One plausible explanation for this unexpected result is that the bioavailability of this drug in the vitreous cavity milieu is not the same as the in vitro culture conditions. Unfortunately, many pieces of information on the pharmacokinetics of this kinase inhibitor are still missing. A study performed in 2001 by Ward et al. found that the plasma terminal half-life of SB203580 after intravenous administration in the mouse is approximately 55 minutes. The same study found a half-life of 1.5 hours when this inhibitor was injected intravenously in monkeys. This suggests that species differences are remarkable and that detailed pharmacokinetics studies should be conducted in both mice and humans (for our specific case). The plasma stability in vitro for this inhibitor was approximately 24 hours for different species, including mice and humans. On the other hand, relevant differences in the free fractions of the inhibitor were found when species were compared in vitro. The authors also demonstrate evidence for nonlinear elimination kinetics for SB203580, which complicates the interpretation of data from in vivo pharmacokinetics studies.

It is important to point out that SB203580 is active on p38α MAPK within a broad range of concentrations (50 nM-10 μM); therefore, variations on the vitreal concentrations of the drug resulting from imprecise injecting should still fall into that active concentration range. Because our expectation was that the inhibitor injections would function intraocularly with effects similar to those obtained in vitro, we collected eyes at the end of the injection cycle and proceeded immediately with histopathologic processing. Unfortunately, after paraffin blocking, it was no longer possible to assess the levels of kinase activity or IL-17 in situ, and we were unable to provide definitive evidence that p38α MAPK was inhibited in treated eyes. For future studies, we must develop a method to assess p38α MAPK activity of the infiltrating cells in a manner in which the eye is preserved for histopathologic evaluation. Thus, our observed lack of activity of SB203580 when used intraocularly must be interpreted with caution. Interestingly, administration of SB203580 to healthy eyes resulted in no apparent tissue damage, suggesting that different formulations or treatment protocols might represent an alternative for local use of kinase inhibitors.

Several studies in the literature used SB203580 as a specific inhibitor of p38α and p38β MAPK. Still, this inhibitor has been shown to inhibit other protein kinases, such as GAK, CK1, and RIP2. It is possible that our findings result from the inhibition of targets other than p38α MAPK. Performing our studies in p38α MAPK-deficient mice could provide stronger evidence for this kinase role. Unfortunately, such deletion is lethal in the embryonic stage. A recent analysis of the selectivity of protein kinase inhibitors was published by Bain et al. In this study, the authors suggested that SB203580 be used in parallel with another inhibitor, BIRB0796, which inhibits p38α/β MAPK but does not act on GAK, CK1, and RIP2. Nonetheless, BIRB0796 inhibits p38α/β MAPK and JNK2α. The combination of results with both drugs could provide a clearer picture of the involvement of p38α MAPK in the studied phenomenon. BIRB0796 became commercially available recently, and we intend to test its inhibiting role over IL-17 synthesis during autoimmune uveitis to further clarify the signaling pathways involved in this inflammatory process.

In conclusion, the present study identifies p38α MAPK as the signaling pathway involved in IL-17 production during uveitis, both experimental and human. Moreover, we were able to show that p38α MAPK downstream targets, the kinases MSK1 and MSK2, are also part of the pathways controlling IL-17. Although intraocular injections of p38α MAPK inhibitor were unable to reduce inflammation of the eye in the EAU model, systemic administration or different protocols of intraocular administration of this inhibitor might prevent that recently activated CD4+ cells commit with IL-17 before they enter the eye. This approach might provide more promising results in vivo and is a further goal for our group.

The search for anti-inflammatory and anticancer treatments through the use of protein kinase inhibitors has been a path rich in pitfalls because of their toxic side effects. The results shown here encourage us to think that the eye, a drainage-deficient organ, is worth further investigation as a target for therapy with kinase inhibitors.

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