

# In Vivo Downregulation of Innate and Adaptive Immune Responses in Corneal Allograft Rejection by HC-HA/PTX3 Complex Purified From Amniotic Membrane

Hua He,<sup>1</sup> Yaohong Tan,<sup>2</sup> Stephanie Duffort,<sup>2</sup> Victor L. Perez,<sup>2,3</sup> and Scheffer C. G. Tseng<sup>1,4</sup>

<sup>1</sup>TissueTech, Inc., Miami, Florida

<sup>2</sup>Department of Ophthalmology, Bascom Palmer Eye Institute, University of Miami Miller School of Medicine, Miami, Florida

<sup>3</sup>Department of Microbiology and Immunology, University of Miami Miller School of Medicine, Miami, Florida

<sup>4</sup>Ocular Surface Center, and Ocular Surface Research Education Foundation, Miami, Florida

Correspondence: Scheffer C. G. Tseng, Ocular Surface Center, 7000 SW 97th Avenue, Suite 213, Miami, FL 33173; [stsen@ocularsurface.com](mailto:stsen@ocularsurface.com).

HH and YT contributed equally to the work presented here and should therefore be regarded as equivalent authors.

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**PURPOSE.** Heavy chain-hyaluronic acid (HC-HA)/PTX3 purified from human amniotic membrane (AM) was previously observed to suppress inflammatory responses in vitro. We now examine whether HC-HA/PTX3 is able to exert a similar effect in vivo, using murine models for keratitis and corneal allograft rejection.

**METHODS.** The in vitro effect of HC-HA/PTX3 was tested using OTII ovalbumin (OVA) transgenic, purified CD4<sup>+</sup> T cells, or IFN- $\gamma$ /lipopolysaccharide (LPS)-stimulated RAW264.7 cells. Cytokine production was measured by ELISA, while cell surface markers and cell proliferation were determined by flow cytometry. In vivo effects of HC-HA/PTX3 were analyzed by quantifying the recruitment of enhanced green fluorescence-labeled macrophages and by measuring the expression of arginase 1 (Arg-1), IL-10, and IL-12 in LPS-induced keratitis in the macrophage Fas-induced apoptosis (Mafia) mouse. The effect of corneal allograft survival in a complete major histocompatibility complex (MHC) mismatched mouse model was assessed by grading corneal opacification.

**RESULTS.** In vitro studies demonstrated that HC-HA/PTX3 significantly enhanced the expansion of FOXP3 T cells and suppressed cell proliferation and protein expression of IFN- $\gamma$ , IL-2, CD25, and CD69 in activated CD4<sup>+</sup> T cells. Furthermore, immobilized HC-HA/PTX3 significantly upregulated IL-10 gene expression but downregulated that of IL-12 and IL-23 in activated RAW264.7 cells. Finally, in vivo subconjunctival injection of HC-HA/PTX3 significantly prolonged corneal allograft survival, suppressed macrophage infiltration, and promoted M2 polarization by upregulating Arg-1 and IL-10 but downregulating IL-12.

**CONCLUSIONS.** HC-HA/PTX3 can suppress inflammatory responses in vivo by modulating both innate and adaptive immunity of macrophages and CD4<sup>+</sup> T cells.

**Keywords:** amniotic membrane, CD4<sup>+</sup> T cell, macrophage polarization, corneal allograft rejection

Corneal allotransplantation has widely been used to treat blindness caused by a number of corneal diseases.<sup>1</sup> Despite the immune privilege of the cornea, immune rejection remains the major cause of corneal allograft failure.<sup>2,3</sup> Similar to the immunologic rejection of other solid organ allotransplants, this process is mainly mediated by adaptive immune responses such as the infiltration of T cells. Upon activation, CD4<sup>+</sup> T cells produce a wide array of effector molecules such as IFN- $\gamma$ , nitric oxide (NO), superoxide radicals, and TNF- $\alpha$ , all of which are detrimental to the corneal allograft.<sup>4</sup> In fact, T-cell depletion has been shown to result in a marked reduction of corneal graft rejections.<sup>5-8</sup> Contrary to adaptive immune response, innate immune responses characterized by monocytes and macrophages have also been shown to play a crucial role in allograft rejection by serving as antigen presenting cells (APCs) to activate the adaptive immune response. For example, IL-12 and IL-23 secreted by "pro-inflammatory" M1 macrophages can activate Th1 and Th17 CD4<sup>+</sup> lymphocytes, respectively.<sup>9-11</sup> Furthermore, IL-10 and TGF- $\beta$  produced by anti-inflammatory

M2 macrophages promote Th2 and regulatory T cells (Tregs), respectively.<sup>12-14</sup> Hence, a therapeutic strategy aimed at both macrophages and CD4<sup>+</sup> T cells should be appealing in curtailing corneal allograft rejection.

Cryopreserved human amniotic membrane (AM) has been applied to surgical or injury sites to reduce inflammation, angiogenesis, and scarring on the ocular surface (for reviews see Refs. 15-17). Transplanted AM induces apoptosis of neutrophils,<sup>18,19</sup> monocytes, and macrophages;<sup>20</sup> reduces infiltration of neutrophils,<sup>18,19</sup> macrophages,<sup>21,22</sup> and lymphocytes<sup>23</sup>; and promotes polarization of M2 macrophages.<sup>24</sup> These in vivo findings are further supported by in vitro results. Specifically, human AM and soluble AM extract (AME) can induce apoptosis of IFN- $\gamma$ , LPS, and IFN- $\gamma$ /LPS-activated but not resting macrophages.<sup>25,26</sup> AME also downregulates expression of M1 macrophage markers such as TNF- $\alpha$ , IL-6, CD86, and MHC II while upregulating M2 macrophage markers such as cytokine IL-10.<sup>26</sup> Nonetheless, the effects of AM and AME on adaptive immune responses, more specifically T cells, have

never been demonstrated. Recently, we have purified heavy chain-hyaluronic acid (HC-HA)/PTX3 complex from AME and demonstrated that this complex is formed by a covalent linkage between HA, heavy chain-1 (HC1) of inter- $\alpha$ -trypsin inhibitor (I $\alpha$ I),<sup>27,28</sup> and PTX3.<sup>29</sup> We have further shown in vitro that HC-HA/PTX3 is the active component responsible for AM's known therapeutic actions because it induces apoptosis of activated but not resting neutrophils and macrophages, enhances phagocytosis of apoptotic neutrophils by macrophages, and promotes polarization of RAW264.7 cells to an M2 phenotype.<sup>29</sup> All these data prompt us to speculate that HC-HA/PTX3 may also modulate both innate and adaptive immune responses in vivo. To prove this concept, we have examined whether in vivo subconjunctival injection of HC-HA/PTX3 is able to reduce corneal allograft rejection in a complete MHC mismatched murine model, a model in which both macrophages and CD4<sup>+</sup> lymphocytes play an important role.<sup>4,30</sup> Herein, for the first time, we are able to demonstrate that HC-HA/PTX3 suppresses inflammatory responses in vivo by directly suppressing CD4<sup>+</sup> Th1 cells and macrophages.

## MATERIALS AND METHODS

### Materials

Female BALB/c (H-2<sup>d</sup>) and C57BL/6 (H-2<sup>b</sup>) mice between 4 and 8 weeks of age and macrophage Fas-induced apoptosis (Mafia) mice were purchased from Jackson Laboratory (Bar Harbor, ME) and used for corneal transplantation and macrophage recruitment into the cornea, respectively.<sup>31,32</sup> Ovalbumin (OVA) TCR-Tg mice (OTII) and C57BL/6 mice were used for in vitro T-cell studies and were routinely maintained in our animal facility.<sup>33</sup> All of the animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the procedures were approved by the University of Miami Animal Care and Use Committee. Mouse monocyte/macrophage cell line RAW264.7 was purchased from ATCC (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), PBS, ampicillin/streptomycin, and 0.25% trypsin/EDTA were obtained from Invitrogen (Carlsbad, CA), while Ultrapure LPS was obtained from InvivoGen (San Diego, CA). High molecular weight HA, Healon (4000 kDa, medical grade), was purchased from Advanced Medical Optics (Santa Ana, CA), and the HA Quantitative Test Kit was purchased from Corgenix (Broomfield, CO). Plastic culture dishes were from Becton Dickinson (Lincoln Park, NJ), and CovaLink NH 96-well plates were from Nunc (Thermo Scientific, Rochester, NY). Sulfo-NHS, BCA Protein Assay Kit, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) were purchased from Pierce (Rockford, IL). Murine recombinant proteins (IFN- $\gamma$  and IL-4) and ELISA kits (murine IFN- $\gamma$ , IL-2, and IL-17) were from R&D Systems (Minneapolis, MN), and the other ELISA kits (murine IL-10, IL-12, and IL-23) were from Biologend (San Diego, CA). Ovalbumin was purchased from Worthington (Lakewood, NJ), and OVA peptide<sub>323-339</sub> (ISQAVHAAHAEINEAGR) and Dby HY peptide (NAGFNSNRANSSRSS) were purchased from Anaspec (Fremont, CA). Rabbit anti-OVA IgG was from Cappel (Durham, NC), and monoclonal antibodies against CD3, CD4, CD25, CD28, CD69, FOXP3, Ki67, and bromodeoxyuridine (BrdU) were from BD Biosciences (Franklin Lakes, NJ). Bromodeoxyuridine was from Roche Applied Science (Indianapolis, IN). CD4<sup>+</sup> T-cell Negative Isolation Kit was purchased from Miltenyi Biotec, Inc. (Auburn, CA), and primers (Arg-1

[Mm00475988\_m1], IL-10 [Mm00439614\_m1], IL-12p35 [Mm00434165\_m1], and IL-12p40 [Mm00434174\_m1] for quantitative PCR [q-PCR]) were from Applied Biosystems (Carlsbad, CA).

### Methods

**Preparation of HC-HA/PTX3.** As reported,<sup>26,27</sup> HC-HA/PTX3 was prepared from frozen human placentas provided by Bio-Tissue, Inc. (Miami, FL). After separation from other placental tissues under aseptic conditions, AM was sliced into small pieces and frozen at -80°C or briefly in liquid nitrogen, followed by homogenization in a Cuisinart CBT-700 blender (East Windsor, NJ) at a ratio of 1:1 (AM weight [g]/PBS volume [mL]). The homogenate was mixed at 4°C for 1 hour, and the supernatant, termed AME, was collected by centrifugation at 48,000g at 4°C for 30 minutes. Amniotic membrane extract was further subjected to two runs of ultracentrifugation at 125,000g in CsCl/4 M guanidine HCl at a density of 1.35 g/mL (the first run) and 1.40 g/mL (the second run) for 48 hours at 15°C. Fractions containing HA (measured by HA Quantitative Test Kit) but no detectable amounts of proteins (measured by BCA assay) were designated as HC-HA/PTX3. Therefore, the amount of HC-HA/PTX3 was expressed based on the HA amount present in the complex.

**Immobilization of HA and HC-HA/PTX3.** The covalent coupling of HA or HC-HA/PTX3 on the surface of CovaLink NH 96 wells was performed as previously reported.<sup>27,29</sup> Briefly, 100  $\mu$ L of 20  $\mu$ g/mL HA or HC-HA/PTX3 in distilled water was added into each CovaLink NH 96 well with 0.184 mg/mL Sulfo-NHS and 0.123 mg/mL EDAC. This coupling mixture was incubated at 4°C overnight. After the coupling solution was removed, the wells were washed three times with PBS containing 2 M NaCl and 50 mM MgSO<sub>4</sub>; the wells were then washed three times with PBS.

**Flow Cytometry.** Cells were collected and stained with already-labeled antibodies (CD4, CD25, and CD69, 1:50 dilution) for 15 minutes at room temperature in the blocking buffer (3% BSA and 0.05% Tween-20 in PBS), followed by intracellular staining of FOXP3 and Ki67 (1:50 dilution) with FOXP3 staining buffer (eBiosciences, San Diego, CA). Fluorescence-activated cell sorting (FACS) analysis was performed using Becton Dickinson LSRII, FACS Diva software (BD, San Jose, CA) and FlowJo software (Tree Star, Ashland, OR). For each sample, 50  $\times$  10<sup>3</sup> events were recorded, and live lymphocytes were gated and analyzed.

**Cytokine ELISA.** RAW264.7 cells were first cultivated in DMEM/10% FBS and then stimulated with IFN- $\gamma$  (200 units/mL), LPS (1  $\mu$ g/mL), IFN- $\gamma$ /LPS, LPS/immune complex (IC or IgG-opsonized OVA, 150  $\mu$ g/mL), or IL-4 (10 ng/mL) for 24 hours. Immunoglobulin G-OVA was made by mixing a 10-fold molar excess of IgG to OVA for 30 minutes at 25°C.<sup>34,35</sup> Splenocytes were isolated from OTII mice B6.lg-tg Tcr $\alpha$ Tcr $\beta$  425Cbn/5 (4-8 weeks old from Jackson Laboratory) and seeded at a density of 1  $\times$  10<sup>6</sup> cells with OVA peptide<sub>323-339</sub> (ISQAVHAAHAEINEAGR) (0-10  $\mu$ M) or the control HY peptide (NAGFNSNRANSSRSS) for 48 hours in a 12-well plate. Cultures were then treated with PBS, 25  $\mu$ g/mL HA, or 25  $\mu$ g/mL HC-HA/PTX3. Alternatively, CD4<sup>+</sup> T cells were isolated by a negative CD4<sup>+</sup> T-cell isolation kit and costimulated for 48 hours with 1  $\mu$ g/mL  $\alpha$ -CD3/ $\alpha$ -CD28. The cell supernatants were collected, and cytokine production was quantified by the respective ELISA according to manufacturers' instructions.

**T-Cell Proliferation Assay.** Splenocytes and purified CD4<sup>+</sup> T cells isolated and stimulated for 2 to 4 days, as described above, were labeled with BrdU (10  $\mu$ M) by adding BrdU to the cell culture medium 12 hours before the termination of the culture. CD4 expression, BrdU labeling, or

Ki67 expression were analyzed by flow cytometry as described above.

**Influx of Enhanced Green Fluorescent Protein (EGFP<sup>+</sup>) Macrophages to LPS-Elicited Keratitis Mouse Corneas.** Two microliters LPS (2 mg/mL) was injected into the corneal stroma of each Maf/a mouse followed by a subconjunctival injection of PBS or HC-HA/PTX3 (1 mg/mL) at one quadrant (10  $\mu$ L) or four (5  $\mu$ L each) quadrants. Influx of EGFP<sup>+</sup> macrophages was imaged as previously described<sup>31-33,36-38</sup> at day 1, 2, 3, and 6. Fluorescence confocal imaging was performed using an upright Leica DMLFSA microscope with long-distance water-dipping lens (Leica HXC APO 20  $\times$  0.5 W; Leica Microsystems, Inc., Buffalo Grove, IL). At Day 4, corneas were harvested and incubated in PBS (pH 7.2-7.4) supplemented with EDTA (20 mM) for 15 minutes at 37°C. After a wash with DMEM containing 10% FBS, corneas were cut into small pieces and incubated with collagenase (82 U/cornea; Sigma-Aldrich, St. Louis, MO) for 60 minutes at 37°C. After the digestion, the corneas were broken up, using a P200 pipette, into a single-cell suspension and filtered through a cell strainer (BD Biosciences, San Jose, CA). After one wash with the staining buffer (PBS supplemented with 1% FBS and 0.1% Sodium Azide (Sigma-Aldrich)), EGFP<sup>-</sup> and EGFP<sup>+</sup> cells were sorted by FACS as described above.

**Quantitative Real-Time PCR (qRT-PCR).** Total RNAs were extracted using RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed into cDNA by High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA). cDNAs were then amplified by qRT-PCR using specific primer-probe mixtures and *Taq* DNA polymerase in a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). The amplification program consisted of an initial activation for 10 minutes at 95°C followed by 40 cycles of denaturation for 15 seconds at 95°C and an annealing and extension cycle for 1 minute at 60°C. The relative gene expression data were analyzed by the comparative CT method ( $\Delta\Delta$ CT). All assays were performed in triplicate. The results were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control.

**Orthotopic Corneal Transplantation.** Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Corneal transplantations were performed by transplanting corneas with a 2-mm central full-thickness from BALB/c mice to wild-type C57BL/6 mice using eight interrupted 11-0 nylon sutures (SharpPoint, Reading, PA), as previously described.<sup>39-41</sup> After completion, subconjunctival injection of PBS (as a control) or HC-HA/PTX3 (1 mg/mL) was performed near the fornix at one quadrant (10  $\mu$ L) or four quadrants (5  $\mu$ L for each quadrant), and erythromycin ointment was then applied. This injection procedure was then performed twice a week thereafter. All corneal sutures were removed at postoperative day 7. After the suture removal, corneal grafts were evaluated twice a week using slit-lamp biomicroscopy. Mice with corneal graft complications such as flat anterior chamber, ulceration, or other complications related to surgical difficulties were excluded. A standard scoring system of 0 to 4 was used for grading the corneal opacification: 0 = clear, 1 = slight haze, 2 = increased haze but with visible anterior chamber structures, 3 = advanced haze with difficult view of anterior chamber structures, and 4 = opaque cornea with invisible anterior chamber structures. Grafts that received two consecutive scores  $\geq 3$  without resolution were considered to be rejected.<sup>39</sup>

**Statistical Analysis.** The unpaired Student's *t*-test or nonparametric one-way ANOVA was used to test for differences of cell proliferation, cytokine production, infiltrated macrophages, and relative mRNA copy numbers. Experiments were repeated two times or more. Summary data were reported as means  $\pm$  SD. The corneal transplant survival data were plotted

with a Kaplan-Meier curve, and differences in the survival rate were compared with the log-rank test using PRISM software (GraphPad Software, San Diego, CA). A calculated probability (*P*) value less than 0.05 (*P* < 0.05) was considered statistically significant.

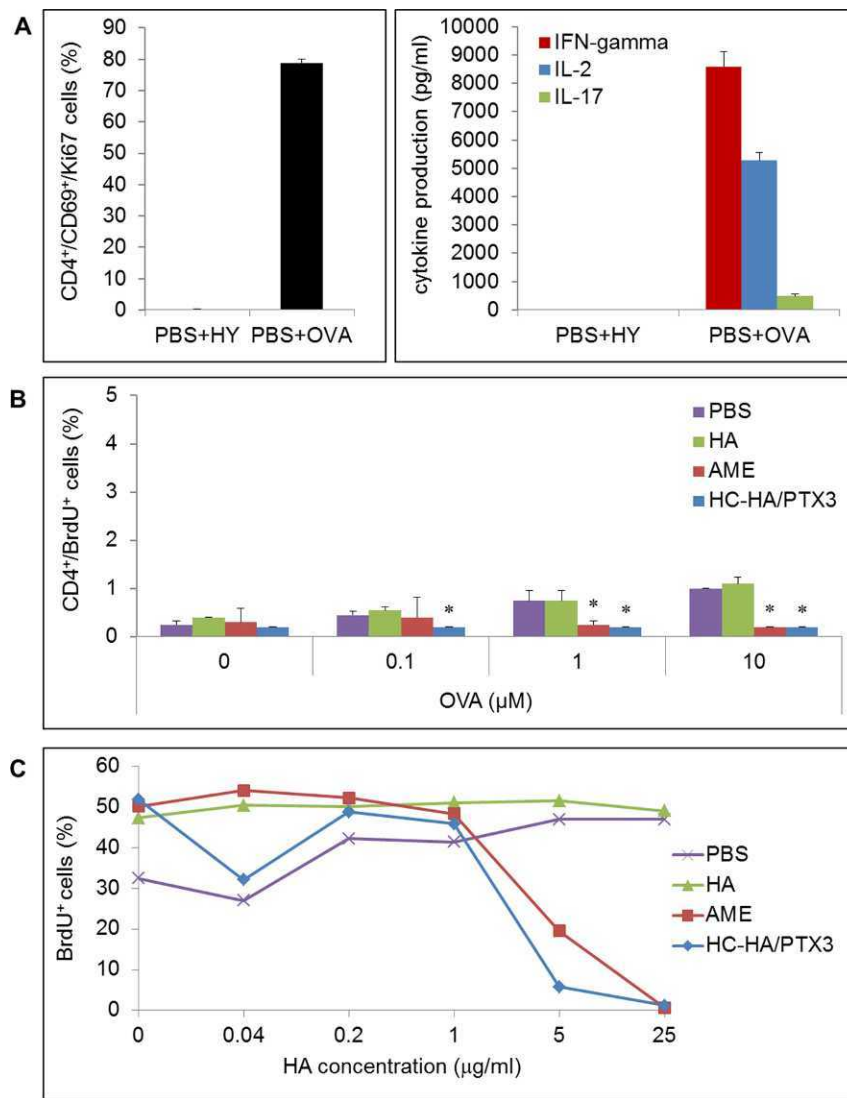
## RESULTS

### HC-HA/PTX3 Directly Suppresses Activation of CD4<sup>+</sup> T Cells

In the course of corneal allograft rejection, CD4<sup>+</sup> T cells become activated when APCs present the peptide antigen through MHC II.<sup>4-6,8</sup> Once activated, CD4<sup>+</sup> T cells proliferate rapidly and differentiate into one of several subtypes, including Th1, Th2, Th17, or Tregs, which secrete different cytokines to facilitate a different type of immune response. Specifically, Th1 cells secrete IFN- $\gamma$  and IL-2 to enhance pro-inflammatory responses, while Th2 cells secrete IL-4 and IL-10 to promote anti-inflammatory responses.<sup>42,43</sup> To verify HC-HA/PTX3 suppresses antigen-specific T-cell activation, we measured cell proliferation, cytokine production, and expression of T-cell activation markers in splenocytes isolated from OTII OVA TCR mice<sup>44</sup> by flow cytometry or ELISA. Initially, we found the OVA peptide (OVA), but not a nonspecific HY peptide (Fig. 1A), triggered antigen-specific proliferation of CD4<sup>+</sup> T cells and production of IFN- $\gamma$ , IL-2, and IL-17 in the culture supernatants. Subsequently, we noted that the percentage of CD4<sup>+</sup>/BrdU<sup>+</sup> cells were dose-dependently increased by OVA stimulation with or without HA (Fig. 1B). In contrast, the percentage of CD4<sup>+</sup>/BrdU<sup>+</sup> cells in AME and HC-HA/PTX3 was kept at the same baseline level as the control without addition of OVA (Fig. 1B). Because 10  $\mu$ M OVA achieved the highest induction of CD4<sup>+</sup>/BrdU<sup>+</sup> cells (Fig. 1B), we used this concentration to examine the dose-dependent suppression of AME and HC-HA/PTX3. Compared with the PBS control, the percentage of BrdU<sup>+</sup> cells was not changed by increasing concentrations of HA, up to 25  $\mu$ g/mL, but notably reduced to nil by 5 and 25  $\mu$ g/mL of AME and HC-HA/PTX3 (Fig. 1C). In contrast, we noticed the cell viability was not affected by HA, AME, and HC-HA/PTX3 (data not shown). In all, these results clearly show that HC-HA/PTX3 suppresses the cell proliferation of CD4<sup>+</sup> T cells.

The aforementioned changes of cell proliferation were also coupled with suppression of antigen-specific CD4<sup>+</sup> T-cell activation. For example, secretion of both IFN- $\gamma$  (Fig. 2A) and IL-2 (Fig. 2B) was dose dependently upregulated by OVA (0-10  $\mu$ g/mL) in the control with or without 25  $\mu$ g/mL HA, but was abolished by 25  $\mu$ g/mL AME or HC-HA/PTX3. The same finding was also noted in the expression of CD69 (Fig. 2C), an early T-cell activation marker involved in lymphocyte proliferation,<sup>45</sup> and CD25 (Fig. 2D), the alpha chain of the IL-2 receptor.<sup>46,47</sup> These data collectively support the notion that HC-HA/PTX3 was the active component in AME to exert dose-dependent suppression of antigen-specific CD4<sup>+</sup> T-cell activation.

To confirm that HC-HA/PTX3 could directly suppress the overall activation of CD4<sup>+</sup> T cells, we isolated CD4<sup>+</sup> cells from cell suspensions of lymph nodes and spleens of 4- to 8-week-old C57BL/6 mice, stimulated these cells with  $\alpha$ -CD3/ $\alpha$ -CD28 along with treatment of 25  $\mu$ g/mL HA or HC-HA/PTX3 for 48 hours in 12-well plates coated with anti-hamster IgG. Consistent with the aforementioned results of antigen-specific stimulated CD4<sup>+</sup> OVA TCR T cells (Figs. 1, 2), the proliferation of these  $\alpha$ -CD3/ $\alpha$ -CD28-stimulated CD4<sup>+</sup> cells was significantly lowered (*P* < 0.05) by HC-HA/PTX3 but not HA (Fig. 3A). There was also a significant expansion of CD25<sup>+</sup>/FOXP3<sup>+</sup> T cells by HC-HA/PTX3 (Fig. 3B). The production of IFN- $\gamma$  (Fig. 3C) and IL-2 (Fig. 3D), both critical Th1 cytokines, was also significantly downregulated by HC-HA/



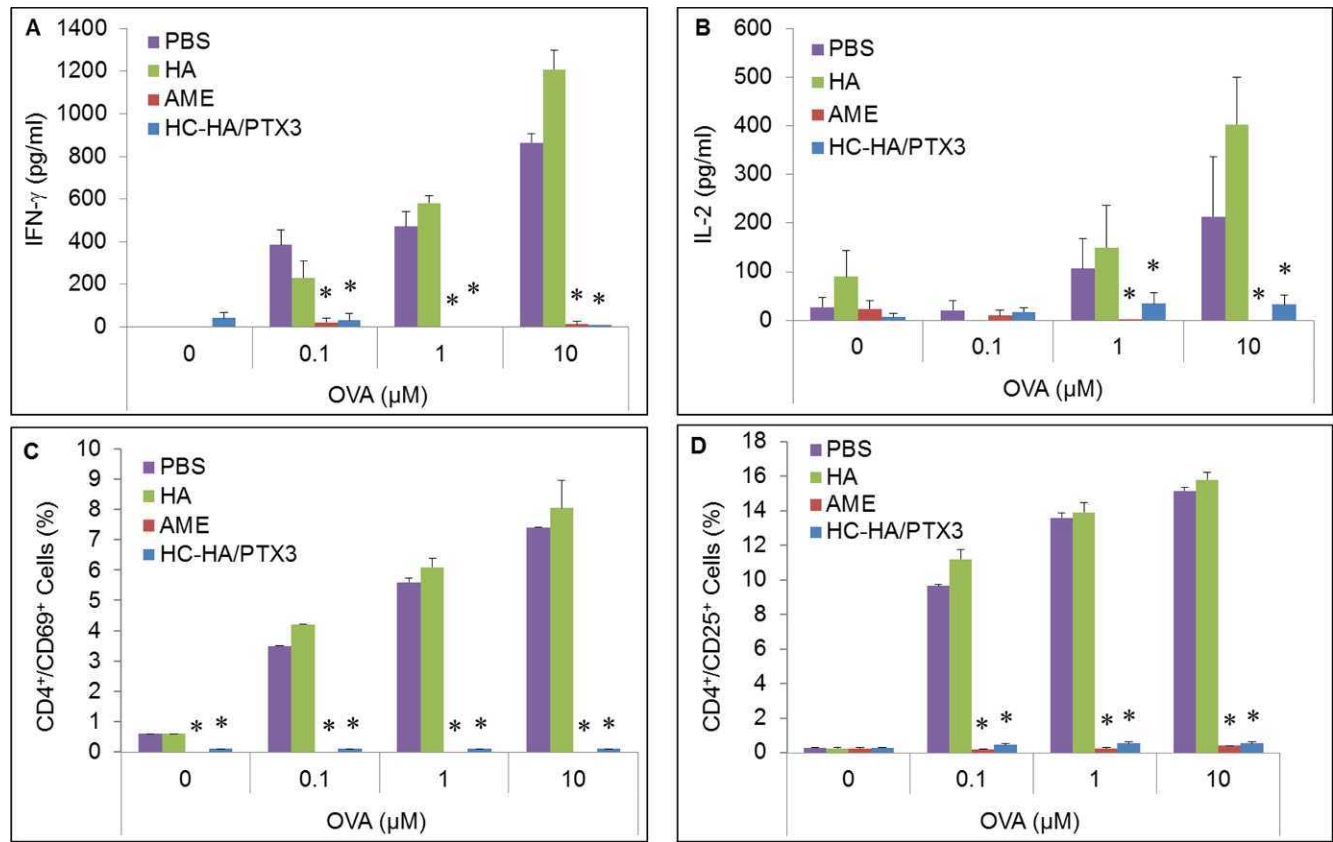
**FIGURE 1.** HC-HA/PTX3 inhibits CD4<sup>+</sup> T-cell proliferation. Splenocytes isolated from OVA T-cell receptor transgenic mice were cultivated at DMEM/10% FBS and stimulated by a nonspecific HY peptide (NAGFNSNRANSRSS, 10 μM) or OVA peptide<sub>323-339</sub> (ISQAVHAHAHAINEAGR, 0–10 μM) for 4 days before flow cytometry with specific antibodies to CD4, CD69, and Ki67, and ELISA of IFN-γ, IL-2, and IL-17 (*n* = 3) (A). Subsequently, splenocytes were treated with or without 25 μg/mL HA, AME, or HC-HA/PTX3 and stimulated with OVA peptide<sub>323-339</sub> (0–10 μM) for 4 days. Cells were labeled with BrdU (10 μM) during the last 12 hours of the cell cultivation and CD4<sup>+</sup> T-cell proliferation was analyzed similar to A (*n* = 3) (B). Alternatively, splenocytes were treated with a serial of concentrations (0, 0.04, 0.2, 1, 5, and 25 μg/mL) of HA, AME, or HC-HA/PTX3 and stimulated with 10 μM OVA peptide<sub>323-339</sub> for 4 days, and CD4<sup>+</sup> T-cell proliferation was determined by flow cytometry (*n* = 3) (C).

PTX3 (*P* < 0.01) but not HA. The production of IL-17 in pan-stimulated CD3<sup>+</sup>/CD28<sup>+</sup> cells was suppressed by HA (*P* = 0.05) but not by HC-HA/PTX3 (*P* = 0.68) (Fig. 3E), while the production of IL-17 in antigen-specific TCR CD4<sup>+</sup> OVA T cells was lowered, though not significantly, by HC-HA/PTX3 (*P* = 0.20) and HA (*P* = 0.32) (Fig. 3F). Taken together, these data support the novel notion that HC-HA/PTX3 directly suppresses Th1 cells while promoting the expansion of Tregs without significantly affecting Th17 cells.

**HC-HA/PTX3 Upregulates IL-10 but Downregulates IL-12 and IL-23 in IFN-γ/LPS-Activated Macrophages**

Recently, we have reported that HC-HA/PTX3 purified from human AM promotes an M2 phenotype by downregulating IL-12 and upregulating IL-10 upon LPS/toll-like receptor (TLR)

ligation.<sup>29</sup> We thus would like to examine whether HC-HA/PTX3 could suppress LPS- and INF-γ- activated M1 macrophage and, therefore, potentially regulate T-cell adaptive immune responses. M1 macrophages activated by IFN-γ/LPS secrete pro-inflammatory cytokines such as IL-12 and IL-23 and upregulate expression of MHC II,<sup>48</sup> whereas M2 macrophages, typically induced by IL-4, secrete anti-inflammatory cytokines such as IL-10 and Arg-1.<sup>13,49</sup> Our results showed that HC-HA/PTX3 was able to upregulate expression of IL-10 protein and downregulate expression of IL-12 protein in RAW264.7 macrophages activated by LPS and INF-γ (Figs. 4A, 4B). Because IL-23 is also produced by activated macrophages and dendritic cells to activate Th17 cells,<sup>50,51</sup> we then examined whether IL-23 protein was produced by RAW264.7 cells in response to different prototypic stimuli. Consistent with earlier studies,<sup>49,52</sup> IL-23 protein was not induced by LPS, LPS/IC, or IL-4. However, IL-23 protein was induced by IFN-γ alone and



**FIGURE 2.** HC-HA/PTX3 downregulates IFN- $\gamma$ , IL-2 production and CD4<sup>+</sup>/CD69<sup>+</sup>, CD4<sup>+</sup>/CD25<sup>+</sup> expression. Splenocytes were treated with or without 25  $\mu$ g/mL HA, AME, or HC-HA/PTX3 and stimulated with 0 to 10  $\mu$ M OVA peptide<sub>323-339</sub> for 4 days. The culturing medium was subjected to the respective ELISA for IFN- $\gamma$  (A) and IL-2 (B). Cells were analyzed with flow cytometry with CD4 antibody combined with CD69 antibody (C) or with CD25 antibody (D). *n* = 3; \**P* < 0.05 compared with the PBS control and the HA group.

was markedly upregulated by IFN $\gamma$ /LPS (Fig. 4C). Of importance, upregulation of IL-23 under IFN $\gamma$ /LPS was abolished when RAW264.7 cells were seeded on immobilized HC-HA/PTX3, but not on the plastic control with or without HA (*P* = 0.02 and 0.01, respectively) (Fig. 4D). Collectively, these data suggest that HC-HA/PTX3 can polarize M2 macrophages not only under LPS stimulation but also under LPS/IFN $\gamma$  stimulation. Such M2 polarization might also affect the outcome controlled by downstream T cells through downregulation of IL-12 and IL-23 as well as upregulation of IL-10.

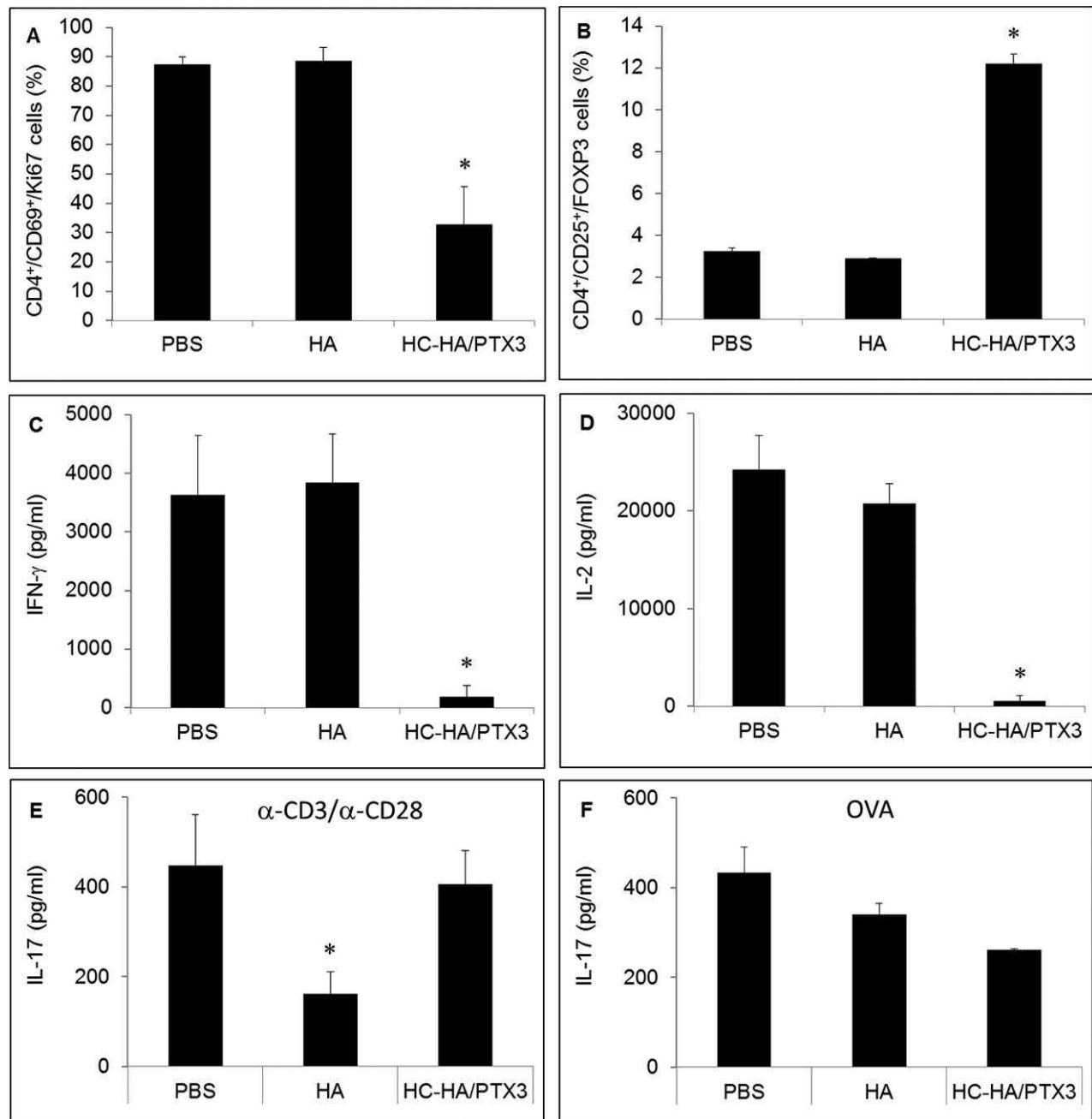
### HC-HA/PTX3 Reduces the Infiltration of Macrophages Into LPS-Elicited Keratitis Mouse Corneas

Since pro-inflammatory M1 macrophage and CD4<sup>+</sup> T-cell activation were suppressed by HC-HA/PTX3 in vitro (Figs. 1–4), we wanted to examine whether such an effect could also be observed in vivo. To do so, we adopted a Mafia mice<sup>32,33</sup> model of LPS-induced keratitis to monitor the trafficking of EGFP macrophages in the cornea using stereofluorescence vital microscopy.<sup>33</sup> Mafia mice express EGFP under the control of the *c-fms* promoter resulting in EGFP expression in CD11b<sup>+</sup> macrophages.<sup>31</sup> As expected, there were no EGFP<sup>+</sup> macrophages in the cornea (Fig. 5A, D0), but intrastromal injection of 2 mg/mL LPS (2  $\mu$ L) elicited a notable influx of EGFP<sup>+</sup> macrophages to the corneal periphery at day 1, consistent with our previous finding.<sup>33</sup> Such influx continued to increase dramatically from day 2 to day 3 but eventually died down at

day 6 (Fig. 5A). To optimize the injection regimen, we had predetermined that the injection volume of 5 to 10  $\mu$ L at the location close to the fornix was ideal to avoid the inadvertent complication of subconjunctival injection, which often causes untoward conjunctival or globe congestion and leads to inflammatory responses. Using this injection method, we observed HC-HA/PTX3 injected at four quadrants, as opposed to one quadrant (data not shown), decreased the infiltration of macrophages into the central cornea at day 2, day 3, and day 6 (Fig. 5A). To quantitate the influx of macrophages, we harvested EGFP<sup>+</sup> and EGFP<sup>-</sup> cells, by collagenase digestion and FACS, from three corneas injected with PBS or HC-HA/PTX3 at day 4. The ratio of EGFP<sup>+</sup>/EGFP<sup>-</sup>, as an indicator of the macrophage infiltration, was significantly lower in HC-HA/PTX3-injected mouse corneas than that of PBS-injected control ( $9.1 \pm 0.3$  vs.  $12.3 \pm 0.4$ , *P* = 0.02) (Fig. 5B). Taken together, our data indicate that HC-HA/PTX3 can reduce in vivo recruitment of macrophages from the bone marrow to the mouse cornea stimulated by LPS.

### HC-HA/PTX3 Polarizes Infiltrated Macrophages Toward M2 Phenotype

Since HC-HA/PTX3 reduced the macrophage influx to the cornea under LPS stimulation (Fig. 5), we speculated whether infiltrated macrophages were geared toward an M2 phenotype as noted in vitro (Fig. 4).<sup>29</sup> We extracted total RNAs from FACS-sorted EGFP<sup>+</sup> macrophages for qRT-PCR analysis. Compared with the control with PBS injection, injection of HC-HA/PTX3

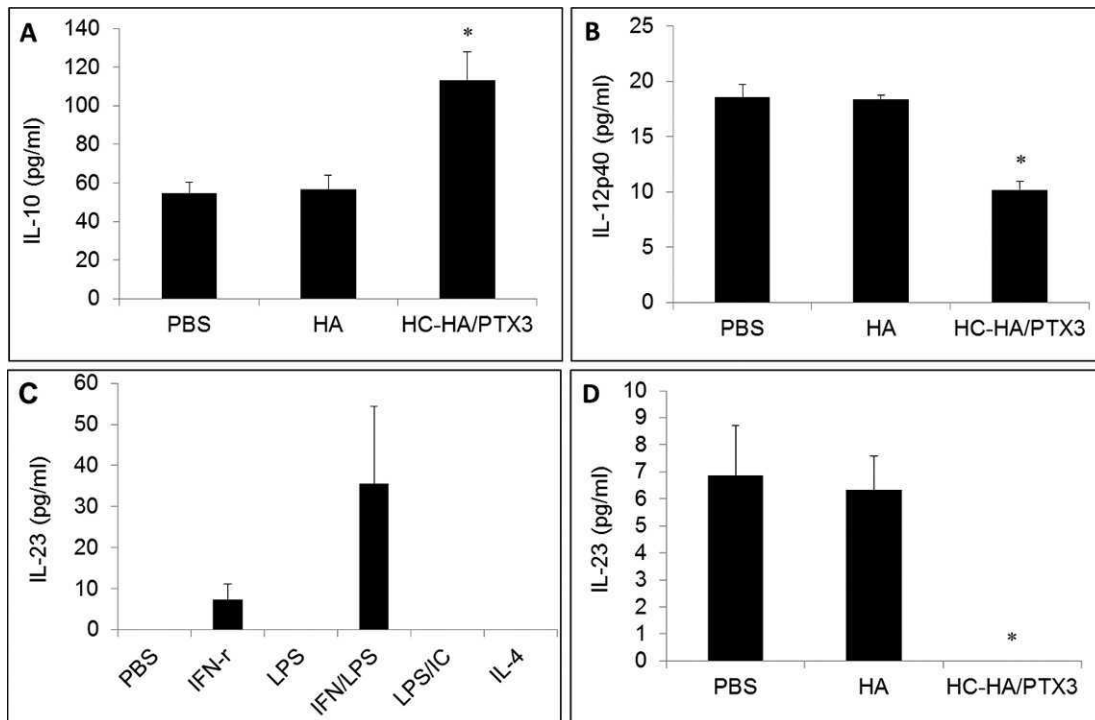


**FIGURE 3.** HC-HA/PTX3 directly inhibits activation of purified CD4<sup>+</sup> T cell. CD4<sup>+</sup> T cells were isolated from cell suspensions of lymph nodes and spleens from 4- to 8-week-old C57BL/6 mice. Purified CD4<sup>+</sup> T cells (at  $1 \times 10^6$  cells/mL) were costimulated with 1  $\mu$ g/mL  $\alpha$ -CD3/ $\alpha$ -CD28 and treated with or without 25  $\mu$ g/mL HA or HC-HA/PTX3 for 48 hours in 12-well plates coated with anti-hamster IgG. CD4<sup>+</sup> T-cell activation was analyzed by flow cytometry with specific antibodies to CD4, CD69, and Ki67 (A), and CD4, CD25, and FOXP3 (B), as well as ELISA of IFN- $\gamma$  (C), IL-2 (D), and IL-17 (E). In addition, ELISA of IL-17 was also performed on OVA peptide-stimulated splenocytes (F).  $n = 3$ ; \* $P < 0.05$  compared with the PBS and the HA group.

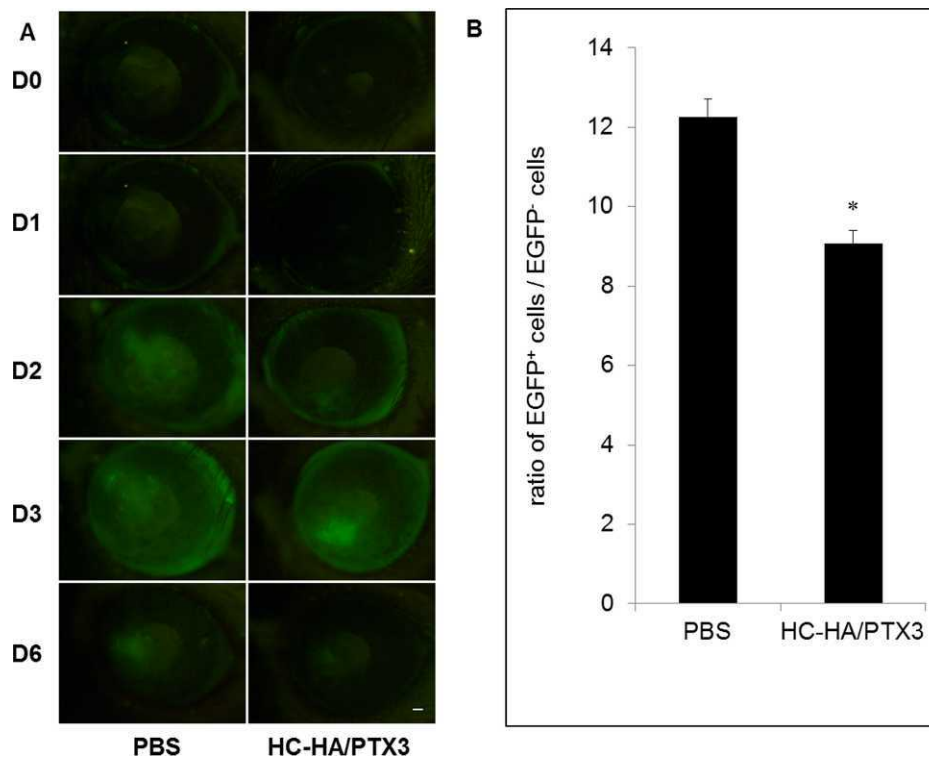
upregulated Arg-1, an M2 marker induced by IL-4 and/or IL-13,<sup>49</sup> and IL-10, another M2 marker,<sup>12,52</sup> by 7-fold and by 1.5-fold, respectively (Fig. 6). In contrast, expression of IL-12p40 (also as a subunit of IL-23)<sup>53</sup> and IL-12p35, which normally produce the M1 marker IL-12,<sup>12</sup> was significantly decreased by 1.7-fold and 3.4-fold, respectively (Fig. 6). These data suggest that subconjunctival injection of HC-HA/PTX3 not only reduces the overall macrophage influx but also polarizes the infiltrated macrophages to an M2 phenotype in vivo when the cornea is challenged by LPS.

### Subconjunctival Injection of HC-HA/PTX3 Prolongs Corneal Allograft Survival

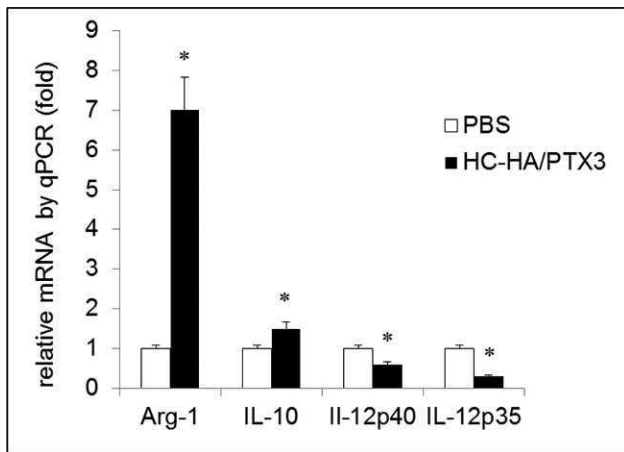
To test the hypothesis that HC-HA/PTX3 downregulates innate and adaptive immune responses, we performed corneal allograft transplantation in animals treated with HC-HA/PTX3 and assessed the allograft survival. To do so, we adopted a murine orthotopic corneal transplantation model, in which virtually all allogeneic corneal grafts are rejected by postoperative day (POD) 21.<sup>39-41,54</sup> Consistent with our earlier findings,



**FIGURE 4.** HC-HA/PTX3 upregulates IL-10 but downregulates IL-12 and IL-23 in IFN- $\gamma$ /LPS-stimulated macrophages. RAW264.7 cells on immobilized PBS, HA, or HC-HA/PTX3 were stimulated with IFN- $\gamma$ /LPS for 24 hours. IL-10 (A) and IL-12p40 (B) in cell supernatants were determined by respective ELISA ( $n = 3$ ). RAW264.7 cells on immobilized PBS, HA, or HC-HA/PTX3 were stimulated with IFN- $\gamma$ , LPS, IFN- $\gamma$ /LPS, LPS/IC, or IL-4 for 24 hours. IL-23 in cell supernatants was determined by ELISA ( $n = 3$ ) (C). IL-23 ELISA was determined as in C, except cells were stimulated with IFN- $\gamma$ /LPS ( $n = 3$ ) (D). \* $P < 0.05$  compared with the PBS control and the HA group.



**FIGURE 5.** HC-HA/PTX3 reduces the macrophage influx in LPS-elicited inflammatory mouse corneas. Following intrastromal injection of LPS in Mafia mice and subconjunctival injection of PBS or HC-HA/PTX3 at four quadrants, the EGFP macrophage influx in mouse corneas was monitored by in vivo confocal microscopy. The scale bar represents 200  $\mu$ m (A). Total cells released from three corneas by collagenase digestion were sorted by FACS into EGFP<sup>+</sup> and EGFP<sup>-</sup> cells ( $n = 3$ ) (B). \* $P < 0.05$  compared with the PBS group.

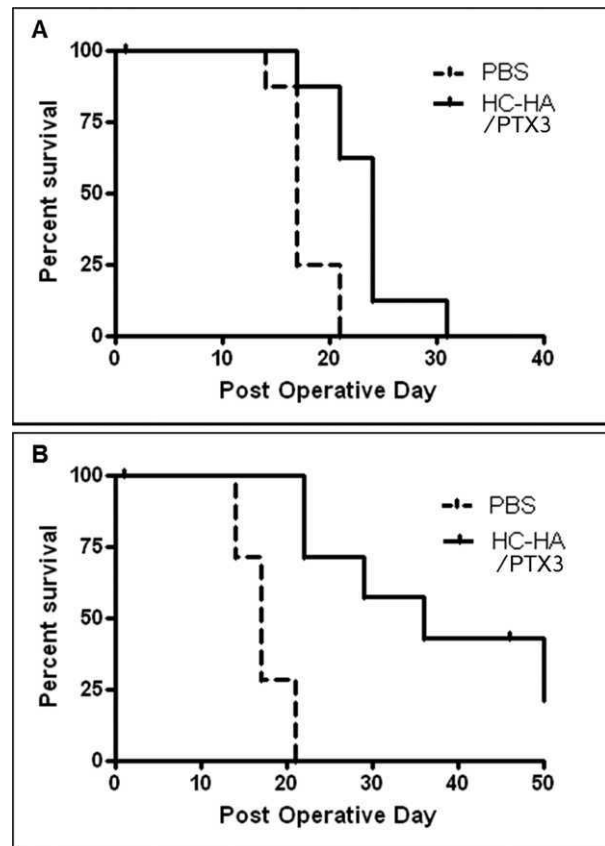


**FIGURE 6.** HC-HA/PTX3 polarizes infiltrated macrophages toward an M2 phenotype. Quantitative PCR was performed for Arg-1, IL-10, IL-12p40, and IL-12p35, using total RNAs extracted from FACS-sorted EGFP<sup>+</sup>-infiltrated macrophages into the corneas. The relative mRNA expression was normalized by GAPDH expression as an internal control (*n* = 3). \**P* < 0.05 when compared with the PBS control.

subconjunctival injection (10 μL at one quadrant) of PBS in the control (each time with 10 μL at one quadrant, twice a week) resulted in 20% allograft survival (2 out of 10 allografts) at postoperative day 16 (POD16) but 0% allograft survival by POD21 (Fig. 7). In contrast, subconjunctival injection of HC-HA/PTX3 (1 mg/mL) resulted in 60% allograft survival at POD21, and one allograft even survived to POD31 (*P* < 0.05, *n* = 10, Fig. 7A). When we changed the injection regimen to 5 μL per injection at four quadrants, the PBS control group still exhibited 0% allograft survival by POD21, while 60% of allografts in the HC-HA/PTX3 group survived at POD31, and 40% of allografts survived at POD50 (*P* < 0.001, *n* = 10, Fig. 7B). These data suggest that subconjunctival injection of HC-HA/PTX3 prolongs allograft survival, likely by modulating both macrophage and T-cell activation.

**DISCUSSION**

Cumulative evidence supports the notion that HC-HA/PTX3 is responsible for the clinical efficacy observed in transplantation of cryopreserved AM. Mirroring AM's anti-inflammatory actions, HC-HA/PTX3 regulates macrophages in multiple ways. As a soluble form, HC-HA/PTX3 induces apoptosis of LPS-activated macrophages by inhibiting cell adhesion and cell proliferation; while as an immobilized form, it polarizes LPS-activated macrophages toward an anti-inflammatory M2 phenotype, leading to effective phagocytosis of apoptotic neutrophils.<sup>29</sup> Herein, we provide further evidence supporting immobilized HC-HA/PTX3, but not HA, polarized an anti-inflammatory M2 phenotype of RAW264.7 cells to upregulate IL-10 and downregulate IL-12 under pro-inflammatory stimulation of LPS and IFN-γ (Fig. 4). Because IFN-γ is a prototypic cytokine derived from activated Th1 CD4<sup>+</sup> cells,<sup>55,56</sup> IL-12 activates naïve T cells into IFN-γ-producing Th1 cells,<sup>57,58</sup> and IL-10 helps activate Tregs to curtail activation of Th1 cells<sup>59,60</sup>; thus, we conclude that HC-HA/PTX3 is a novel matrix component that exerts not only an anti-inflammatory action in innate immune responses but also an anti-immune action in adaptive immune responses. In other words, our findings depict a novel action to intercede cytokine dialogues between macrophages and lymphocytes.



**FIGURE 7.** HC-HA/PTX3 prolongs the corneal allograft survival. Following allogeneic corneal transplantation, HC-HA/PTX3 (1 mg/mL) or PBS was injected subconjunctivally at one or four quadrants twice a week. The allograft survival was scored by the graft clarity in a range of 0 to 4 twice a week using slit-lamp biomicroscopy. Compared with the PBS control, HC-HA/PTX3 prolonged the allograft survival by injecting 10 μL at one quadrant (A) (*P* < 0.05, *n* = 10) and further improved the graft survival by injecting 5 μL at each of the four quadrants (B) (*P* < 0.001, *n* = 10).

Our data also correspond well with the notion that the HC-HA/PTX3 complex suppresses Th1 CD4<sup>+</sup> cells not only in a mixed population of naïve T and B lymphocytes, dendritic cells, and macrophages (Figs. 1, 2), but also in purified T lymphocytes from lymph nodes and spleens (Fig. 3). HC-HA/PTX3 also promotes expansion of Tregs from CD4<sup>+</sup> T cells (Fig. 3). In contrast, HA reduced the level of IL-17 expressed by CD3<sup>+</sup>/CD28<sup>+</sup> CD4<sup>+</sup> cells (Fig. 3), a finding that explains why intraperitoneal injection of high-molecular HA reduces IL-17 protein production in the cartilage of mice with collagen-induced arthritis (CIA).<sup>61</sup> Nonetheless, the IL-17 level was not significantly downregulated by HC-HA/PTX3 in α-CD3/α-CD28 pan-stimulated CD4<sup>+</sup> cells or antigen-specific-stimulated CD4<sup>+</sup> T cells (Fig. 3). Collectively, HC-HA/PTX3 could be a novel matrix that suppresses M1 polarization and promotes M2 polarization of LPS-stimulated macrophages in the innate immune responses,<sup>29</sup> as well as suppresses Th1 cell activation and Treg expansion without affecting Th17 cells to downregulate alloreactive responses. This notion is supported in vivo by dose-dependent suppression of corneal allograft rejection following subconjunctival injection of HC-HA/PTX3 (Fig. 7) and is also consistent with a previous study showing IL-17 is not required for corneal allograft rejection and may instead contribute to the immune privilege of corneal allografts by promoting CD4<sup>+</sup>/CD25<sup>+</sup> Tregs.<sup>62,63</sup> An additional benefit in



achieving this outcome might be attributed to the anti-angiogenic action exerted by HC-HA/PTX3,<sup>64</sup> as it is known disruption of anti-angiogenic signals by recruited allospecific T cells leads to corneal allograft rejection.<sup>54</sup> Further studies into the mechanism of how extracellular HC-HA/PTX3 might render macrophages and lymphocytes into a noninflamed state will not only be useful to unravel a new strategy of controlling allograft rejection but also shed a new light on how this novel matrix, present from ovulation<sup>65,66</sup> to pregnancy,<sup>27-29</sup> might be deployed to treat a number of diseases by strengthening immune privilege and tissue tolerance.

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