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Anterior chamber associated immune deviation to cytosolic neural antigens avoids self-reactivity after optic nerve injury and polarizes the retinal environment to an anti-inflammatory profile

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ABSTRACT

It has been hypothesized that anterior chamber-associated immune deviation (ACAID) to neural antigens induced prior to central nervous system injury can inhibit self-reactivity and lessen secondary degeneration. This work evaluated the effect of ACAID induced to three neural tissue-derived extracts (whole extract, cytosolic extract, CE; or organelle-membrane extract) prior to optic nerve injury on retinal ganglion cell (RGC) survival. The results show that only ACAID to the CE increased RGC survival at 7 and14 days post-injury (dpi). This effect was achieved by retinal polarization towards an anti-inflammatory profile, driven by regulatory T cells and M2-type macrophages at 7 dpi.

1. Introduction

After central nervous system (CNS) injury, chronic inflammatory processes triggered at the lesion site extend tissue damage and interfere with repair (Fitch et al., 1999; Wattananit et al., 2016). Chronic inflammation is maintained by neural tissue-derived molecules released at the lesion site, which recruit T helper (Th)-1 cells that break self-antigen tolerance and promote secondary neuronal degeneration (Jones et al., 2002, 2005). Thus, post-injury secondary degeneration could be alleviated by avoiding self-reactivity to neural antigens.

Recently, we hypothesized that self-reactivity may be avoided by inducing anterior chamber-associated immune deviation (ACAID) to neural antigens prior to CNS injury (Toscano-Tejeida et al., 2016). ACAID suppresses antigen-specific Th1 responses (e.g. delayed type hypersensitivity, DTH) by inducing forkhead box P3 (Fox-P3)+ regulatory T cells (Tregs) that inhibit lymphocyte differentiation in secondary lymph tissues, as well as cytotoxic T cell function at the antigen exposure site (Niederkorn, 2006; Saban et al., 2008). Accordingly, inducing ACAID to a spinal cord extract or myelin basic protein prior to spinal cord injury improves motor neuron survival and functional recovery. ACAID to the spinal cord extract also promotes a discrete anti-inflammatory profile 60 days post-injury (dpi), likely triggered by Fox-P3 (Pineda-Rodriguez et al., 2017). However, the effect of ACAID during early post-injury phases, when massive neuronal death occurs, is unknown.

To elucidate how ACAID preserves the nervous tissue and avoids neuronal death after injury, this work used optic nerve crush injury since retinal ganglion cell (RGC) survival after damage is well characterized. In this model, about 50% of RGC die at 5–7 dpi and 75% at 14 dpi (Nadal-Nicolás et al., 2009). The effect of ACAID induced to three neural tissue-derived extracts (whole extract, WE; cytosolic extract, CE; or organelle-membrane extract, OME) was evaluated by quantifying RGC survival at 7 and 14 dpi. The results showed that although all extracts induced ACAID, only the CE preserved RGC survival after optic nerve injury; thus, all further evaluations used this group. Retinal expression of pro- and anti-inflammatory mediators, neurotrophic factors (brain-derived neurotrophic factor, BDNF; neurotrophin 3 and 4, NT-3, NT-4; Oncomodulin, Ocm), survival signals (B-cell lymphoma 2, Bcl-2; Caspase 3, Casp-3) or phenotypic markers (Treg-specific marker, Fox—P3; macrophage-specific marker CD68; alternatively-activated macrophage marker Arginase-1, Arg-1) were quantified during acute inflammation (2 and 7 dpi). The results showed that ACAID to the CE highly increased expression of all anti-inflammatory cytokines and modestly increased some inflammatory mediators at both

Abbreviations: ACAID, Anterior chamber-associated immune deviation; CE, Cytosolic extract; OME, Organelle-membrane extract; WE, Whole extract

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dpi. ACAID also increased BDNF expression at 2 dpi but decreased the expression of this neurotrophin, Ocm and Casp-3 at 7 dpi. Additionally, ACAID increased the expression of NT-4, CD68, Arg-1 and Fox-P3 at 7 dpi. Thus, ACAID to the CE improved RGC survival by avoiding self-reactivity and promoting the upregulation of anti-inflammatory cytokines and neurotrophic-survival signals. ACAID probably achieved its effects by increasing Fox−P3+ Treg recruitment at 7 dpi. Interestingly, ACAID likely also increased the recruitment or proliferation of CD68+ macrophages, notably Arg-1+ alternatively-activated macrophages (M2) into the retina. Overall, the findings extend our knowledge about the mechanisms of ACAID action after CNS injury. This includes the interaction between Fox−P3+ Tregs and other cell types to modulate inflammation. It remains to be resolved how ACAID to the CE modulated inflammation and BDNF expression at 2 dpi.

2. Materials and methods

2.1. Animals

Juvenile (postnatal day 30) and adult (≥250 g body weight; > postnatal day 60) female Wistar rats were obtained from our animal facility. They were group housed under standard controlled conditions of temperature and humidity, with a regular light cycle having free access to food and water. Animal handling, feeding and care were done by trained personnel in accordance to the National Institutes of Health *Guide for the care and use of laboratory animals.* All procedures were approved by an Animal Ethics Committee. For each experiment, age- and weight-matched rats from several litters were randomly allocated to each condition. The groups and number of rats used in each experiment are shown in Suppl. Table S1.

2.2. Spinal cord fractionation

Adult female rats were euthanized with sodium pentobarbital (100 mg/kg body weight, i.p.) to obtain neural tissue-derived self-antigens from their spinal cords (thoracic to lumbar levels). All spinal cords were pooled to obtain three different protein extracts (Gil-Dones et al., 2009). Briefly, after cleaning and rinsing with saline solution (0.9%), the tissue was frozen with liquid nitrogen and minced in a mortar. The powder was re-suspended in lysis buffer (300 µl; 10 mM Tris, 500 mM NaCl, 0.1% Triton X-100, 1% β-mercaptoetanol), treated with pre-chilled acetone and centrifuged at 19,000 rpm for 15 min at 4 °C. The supernatant, corresponding to the cytosolic extract (CE, since it had > 70% cytosolic proteins), was recovered and kept at −20 °C.

The remaining pellet was reconstituted with re-suspension buffer (7 M urea, 2 M thiourea, 5% CHAPS) and centrifuged at 19,000 rpm to obtain the supernatant. This corresponded to the organelle-membrane extract (OME), since it contained mostly (> 40%) organelle and membrane (> 20%) proteins. The whole extract (WE) was obtained by mixing the CE and OME. All extracts were treated with pre-chilled acetone and the total protein concentration was determined using the Lowry method.

2.3. Delayed type hypersensitivity (DTH) tests

DTH is a cell-mediated inflammatory reaction that depends on effector Th1 (CD4+) and cytotoxic (CD8+) lymphocytes (Hwang and Actor, 2001). The antigenic properties of the extracts, vehicle (phosphate-buffered saline, PBS) and the highly antigenic bovine serum albumin (BSA) were evaluated by DTH tests in adult rats (Farooq and Ashour, 2013). PBS, BSA, WE, CE or OME (200 µl) were intra-dermally injected in the inter-scapular region under deep anesthesia (ketamine/xylacine i.p.; 75 and 8 µg/kg, respectively); each supplemented with complete Freund's adjuvant (2.5 µg/µl, 1:1 vol). A week later, rats were challenged intra-dermally (10 µl) in the left ear with the same extract previously administered (50 µg/µl) and in the right ear with PBS (10 µl, 0.1 M). Two additional age- and weight-matched groups were used, one was needle-pricked and the other was injected with PBS (10 µl; edema), both in the left ear. Ear thickness was measured with an electronic micrometer (Mitutoyo Co., Mexico) before treatment (baseline), as well as 24 and 48 h after challenge-treatment. The difference in inflammation was calculated by subtracting baseline values from the data obtained after challenge-treatment.

2.4. ACAID induction

To induce ACAID, juvenile rats were injected (6 µg/µl, 10 µl volume; according to our previous standardization) with PBS, BSA, WE, CE or OME into the anterior chamber of both eyes after deep anesthesia. This was done at an approximate velocity of 1 µl/s using a Hamilton syringe coupled to a 31G needle with the aid of a stereoscopic microscope (Farooq and Ashour, 2013). The eyes were protected from drying throughout surgery and the postoperative period with 0.9% saline. ACAID induction was evaluated by DTH tests 14 days after anterior chamber inoculation and compared to the corresponding vehicle group. Moreover, in these rats, splenic Fox-P3 expression was quantified by RT-qPCR 48 h after challenge to confirm ACAID induction.

To measure the ability of cytosolic antigens to produce inflammation in rats tolerant to the WE, a group of rats was inoculated in the anterior chamber with the WE and then intra-dermally sensitized and challenged with the CE as previously described.

2.5. Optic nerve injury and evaluation of RGC survival

To evaluate the effect of ACAID on RGC survival, an additional group of juvenile rats was inoculated in the anterior chamber as previously described with PBS, WE, CE or OME. They were allowed to reach 250 g body weight and subjected to lateral canthotomy of the left eye to expose the optic nerve under deep anesthesia. The optic nerve was compressed for 5 s at a distance of 2–3 mm from the eye cup using auto-lock calibrated Dumont forceps (Walsh et al., 2014). After surgery, the animals were treated with local antibiotic (polytracin ophthalmic ointment, Santgar) and oral analgesic (paracetamol 12.12 mg/kg body weight) for 3 days.

The rats used for ACAID induction and optic nerve crush received a terminal over-dose of sodium pentobarbital at 7 and 14 dpi. Five additional age- and weight-matched groups were used for comparison: a group of naïve rats (basal), a group that was punctured in the anterior chamber (puncture), two groups that were inoculated into the anterior chamber with PBS or CE at postnatal day 30 and a group that was lesioned (injury).

In all groups, RGC quantification and histological analysis were performed by obtaining the eyes and optic nerves and fixing them by immersion in paraformaldehyde (4%; diluted in 0.1 M PBS) for 24 h at 4 °C. The retinas and optic nerves were dissected and post-fixed another 48 h in paraformaldehyde at 4 °C. The retinas were washed with PBS, PT (0.5% Triton X-100, prepared with PBS) and incubated with H_2O_2 (3% prepared with PBS) for 10 min, washed with PT and then incubated in inactivated horse serum (Corning, 35–030-CV; 0.5% prepared in PT) for 60 min. The retinas were then incubated with an antibody for a specific RGC marker, brain-specific homeobox/POU domain protein 3A (Brn-3a goat antibody; Santa-Cruz Biotechnology, sc-31,984. Nadal-Nicolás et al., 2009) diluted 1:100 in PT plus 0.5% inactivated horse serum for 96 h at 4 °C. The retinas were washed and incubated with biotin-coupled donkey anti-goat antibody (Invitrogen, AP180B; 1:500 in PT) at room temperature for 2 h. After washing, the tissue was incubated in ABC HRP kit (Vectastain Elite, PK-6100) at room temperature for 2 h and then incubated in diaminobenzidine as a chromogen, in the presence of peroxide and buffer to develop the enzymatic reaction for 4 min (Vector Staining Kit, SK-4100). Finally, the retinas were mounted onto slides with PBS and 80% glycerol and immediately processed for positive cell counting with ImageJ software. For this

Table 1
Primers used for RT-qPCR.

Gene	Sequence	Tm
GAPDH	Forward: 5′- CACGGCAAGTTCAACGGCACAGT-3′ Reverse: 5′- TCAGCGGAAGGGGCGGAGAT-3′	60 °C
TNF-α[a]	Forward: 5'-TGGCGTGTTCATCCGTTCTCTACC-3' Reverse: 5'-CCCGCAATCAGGCCACTACTT-3′	62 °C
IL-1β[a]	Forward: 5'-TGAGGCTGACAGACCCCAAAAGAT-3' Reverse: 5'-GCTCCACGGGCAAGACATAGGTAG-3′	60 °C
IFN-γ[b]	Forward: 5′-AGGCCATCAGCAACAACATAAGTG-3′ Reverse: 5′-GACAGCLTTGTGCTGGATCLGTG-3′	60 °C
iNOS[c]	Forward: 5′- ACACCGATTCCACTCAACTA-3′ Reverse: 5´-ACCACCTGTTAGTTCAAGCC-3	60 °C
IL-4[d]	Forward: −5′-ACCTTGCTGTCACCCTGTTC-3′ Reverse: −5′-TTGTGAGCGTGGACTCATTC-3′	60 °C
IL-6[a]	Forward: 5'-AGCCACTGCCTTCCCTACTTCA-3′ Reverse: 5'-GCCATTGCACAACTCTTTTCTCA-3′	59 °C
IL-10[e]	Forward: 5′-CAGACCCACATGCTCCGAGA-3′ Reverse: 5´-CAAGGCTTGGCAACCCAAGTA-3′	60 °C
TGF-β[f]	Forward: 5′- CTCAACACCTGCACAGCTCC-3′ Reverse: 5′-ACGATCATGTTGGACAACTGCT-3′	60 °C
Ocm[g]	Forward: 5′-ATGAGCATCACGGACATCCT-3′ Reverse: 5′-TTAAGAGTGCACCATTTCCTGG −3′	60 °C
BDNF[h]	Forward: 5′-TCCCTGGCTGACACTTTTGAG-3′ Reverse: 5′-ATTGGGTAGTTCGGCATTGC-3′	60 °C
NT-3[h]	Forward: 5′-GGTCAGAATTCCA GCCGATGATTGC-3′ Reverse: 5′-CAGCGCCAGCCTAC GAGTTTGTTGT-3′	69 °C
NT-4[h]	Forward: 5′-CTCCTGAGTGGGACCTCTTG-3′ Reverse: 5′-CACTCACTGCATCGCACAC-3′	60 °C
Bcl-2[i]	Forward: 5′- CCTGAGAGCAACCGAACGCCC-3′ Reverse: 5′- CCACAAAGGCATCCCAGCCTC-3′	69 °C
Casp-3[j]	Forward: 5´-AATTCAAGGGACGGGTCATG-3´ Reverse: 5′-TGACACAATACACGGGATCTG-3'	60 °C
CD68[k]	Forward: 5′-CTGTTGCGGAAATA CAAGCA-3′ Reverse: 5′-GGCAGCAAGAGAGATTGGTC-3′	60 °C
Arg-1[l]	Forward: 5′-AAAGCCCATAGAGATTATCGGAGCG-3′ Reverse: 5′-AGACAAGGTCAACGGCACTGCC-3′	69 °C
Fox-P3[m]	Forward: 5′-TGAGCTGGCTGCAATTCTGG-3′ Reverse: 5′-ATCTAGCTGCTCTGCATGAGGTGA-3′	60 °C

[a] Roque et al. (2016).
[b] Gu et al. (2012).
[c] Han et al. (2015).
[d] Sewell et al. (1998).
[e] Huang et al. (2010).
[f] Le Luduec et al. (2008)
[g] Hauk et al. (2008).
[h] Ming et al. (1999).
[i] Chaudhary et al. (1999).
[j] Wu et al. (2002).
[k] Zorzi et al. (2010).
[l] Klasen et al. (2001).
[m] Bai et al. (2012).

procedure, 12 photographs were obtained per retina, one for each quadrant of the central, medial and peripheral regions (Nadal-Nicolás et al., 2009). Photographs of each retina were taken with a Leica DM3000 microscope using the Leica Application Suite software.

In all cases, injury level and severity were corroborated by optic nerve histology. For this, the optic nerves were washed with PBS and then dehydrated, paraffin embedded and longitudinally sectioned (7 μm) with a microtome (Microm). The sections were rehydrated, stained with hematoxylin-eosin dyes and mounted with Cytoseal-60 (Richard-Allan Scientific, 8310–16). Photographs of the lesion site were obtained to document lesion severity.

2.6. mRNA expression analysis with RT-qPCR

The primers used are shown in Table 1. At least two independent replications of each experiment were performed in all cases. Each RNA sample was run in duplicate.

2.6.1. Spleen

After ACAID induction and DTH evaluation, animals inoculated into the anterior chamber, immunized and challenged with the three neural tissue-derived fractions and their respective vehicle groups were used to quantify Fox-P3 mRNA expression. The spleens were removed under terminal anesthesia 48 h after ear challenge (during maximum inflammation), frozen-pulverized with liquid nitrogen and homogenized with TRIzol™ reagent (Invitrogen, 15,596,026) during 5 min. The samples were incubated with chloroform, subjected to vortex and centrifuged at 12,000 ×*g* for 15 min at 4 °C. The supernatant was obtained, mixed with 2-propanol and centrifuged at 12,000 ×*g* for 15 min at 4 °C. The RNA pellet was washed with 70% ethanol and re-suspended in RNAse-free water (Invitrogen, 10977-015). RNA concentration was determined using a Nanodrop Lite spectrophotometer (ThermoFisher Scientific). cDNA was synthesized with an iScript Advanced cDNA Synthesis Kit for RT-qPCR (BioRad, SF1725038) and amplified with the CFX96 Touch™ RT-qPCR Detection System using the SsoAdvanced™ Universal SYBR® Green Supermix (BioRad, 1,725,270). Relative mRNA amounts were analyzed with the 2ΔCt method, using GAPDH as the reference gene. GAPDH primers were a generous gift from Dr. Alejandra Ochoa Zarzosa (Universidad Michoacana de San Nicolás de Hidalgo, México).

2.6.2. Retina

Rats inoculated in the anterior chamber with the CE followed by optic nerve crush were sacrificed 2 and 7 dpi. Their retinas were dissected and frozen at −80 °C. Total RNA was extracted, quantified, amplified and analyzed in the same manner as in the spleen.

2.7. Statistical analysis

Data normality and equal variance were analyzed by Shapiro-Wilk and Levene tests, respectively. Non-parametric tests were used when the data did not satisfy either of the above criteria. All analyses were performed using SigmaStat 8.0 software. The specific tests used to analyze each experiment are shown in Suppl. Tables S1 and S2.

The response of rats tested with the extracts (WE, CE, OME) for DTH and for RGC quantification at 7 dpi was compared by one-way analysis of variance (ANOVA) followed by Holm-Sidak tests. On the other hand, the response of rats inoculated with the CE was compared to that of the vehicle-treated group for ACAID, RGC quantification at 14 dpi and mRNA expression by independent *t*-tests. All data are shown as means ± SD.

3. Results

3.1. Neural tissue-derived extracts induced a differential antigenic response

Ear inflammation after immunization and challenge with each extract was compared to vehicle (PBS) or to BSA, the gold standard for DTH tests due to its high antigenic capacity. All three neural tissue-derived extracts showed inflammation indexes significantly different from vehicle, but not different from BSA. The CE induced a stronger response than the WE (Fig. 1). These results were specific since needle-prick, edema or vehicle groups showed minimal ear inflammation (Suppl. Fig. S1A).

3.2. Neural tissue-derived extracts injected into the anterior chamber induced systemic antigen-specific immune-tolerance

Once antigenicity for all neural tissue-derived extracts was determined, each extract was inoculated into the anterior chamber of juvenile rats. Two weeks (14 days) after inoculation, systemic antigen-specific immune-tolerance (ACAID) was evaluated by DTH tests. The results showed that inoculation of each extract into the anterior chamber attenuated systemic inflammation, in comparison with vehicle

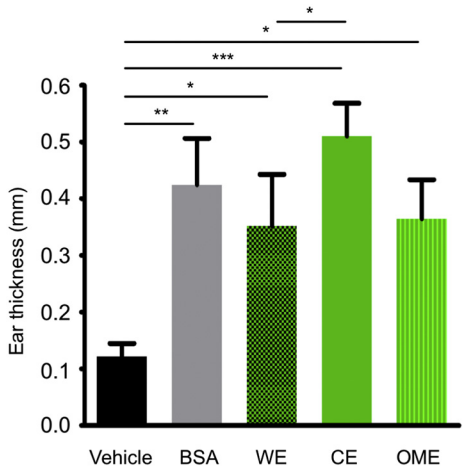


Fig. 1. Neural tissue-derived extracts induced different antigenic reactions. DTH results showing ear swelling measured 48 h after challenge with neural tissue-derived extracts (whole extract, WE; cytosolic extract, CE; or organelle-membrane extract, OME), compared to vehicle (PBS) or bovine serum albumin (BSA). $^{*}p \leq .05$, $^{**}p \leq .01$, $^{***}p \leq .001$.

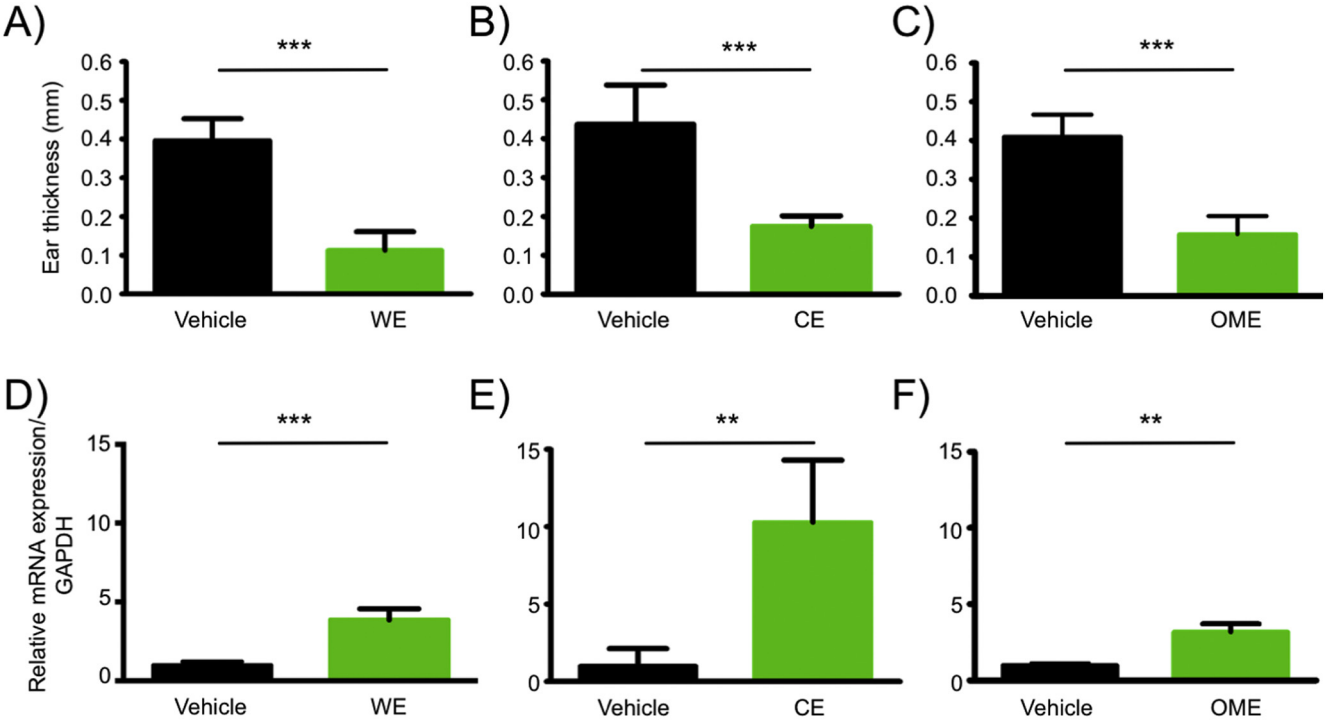
groups (Fig. 2A–C). In all cases, the reduction in inflammation was comparable with that measured in the group inoculated with BSA (Suppl. Fig. S1B).

In addition, induction of ACAID was verified by quantifying the relative expression of Fox-P3 mRNA in the spleen of rats inoculated with each fraction. Splenic Fox-P3 expression was increased in each group in comparison to the vehicle (Fig. 2D–F) and basal groups (not shown). Interestingly, ACAID to the CE increased Fox-P3 expression to a greater extent than the other fractions (Fig. 2D–F).

The ability of cytosolic antigens to be recognized by the immune system of rats tolerized with the WE was evaluated by DTH tests. The results show that inoculation of the WE into the anterior chamber failed to attenuate systemic inflammation elicited by the CE, evidenced by similar ear inflammation as in the group inoculated in the anterior chamber with PBS (Suppl. Fig. S1C).

3.3. ACAID to the CE, induced prior to optic nerve injury, improved RGC survival

Three groups of rats were inoculated with each extract, injured and sacrificed at 7 dpi to evaluate the effect of ACAID compared to PBS, basal, puncture, PBS/CE intracameral delivery and injury-only groups. The retinas from these groups were dissected and immuno-stained for Brn-3a (Suppl. Fig. S2). The results show that all injured groups were statistically different from the basal group. However, rats where ACAID was induced by the CE showed the highest RGC survival at 7 dpi, compared to all other groups (although it was not statistically different from the injury-only group, Fig. 3A–B). The number of Brn-3a+ RGC did not show significant differences among the other groups (Fig. 3A–B). The basal, puncture and PBS or CE-anterior chamber inoculated groups showed a similar number of surviving RGC (Suppl. Fig. S3). The effect of the CE was maintained at 14 dpi compared to vehicle



AC inoculation	PBS	WE	PBS	CE	PBS	OME
Interscapular immunization	WE	WE	CE	CE	OME	OME
Ear challenge	WE	WE	CE	CE	OME	OME

Fig. 2. Neural tissue-derived extracts induced ACAID. DTH data showing ear swelling for rats inoculated in the anterior chamber with neural-tissue derived extracts (whole extract, WE; cytosolic extract, CE; or organelle-membrane extract, OME) compared to vehicle inoculated rats (A–C). Relative splenic Fox-P3 mRNA expression of rats inoculated in the anterior chamber with the WE, CE or OME or their corresponding vehicle (D–F). Vehicle groups were inoculated in the anterior chamber with PBS, but immunized and challenged with the corresponding neural tissue-derived extract for DTH tests. $^{**}p \leq .01$, $^{***}p \leq .001$.

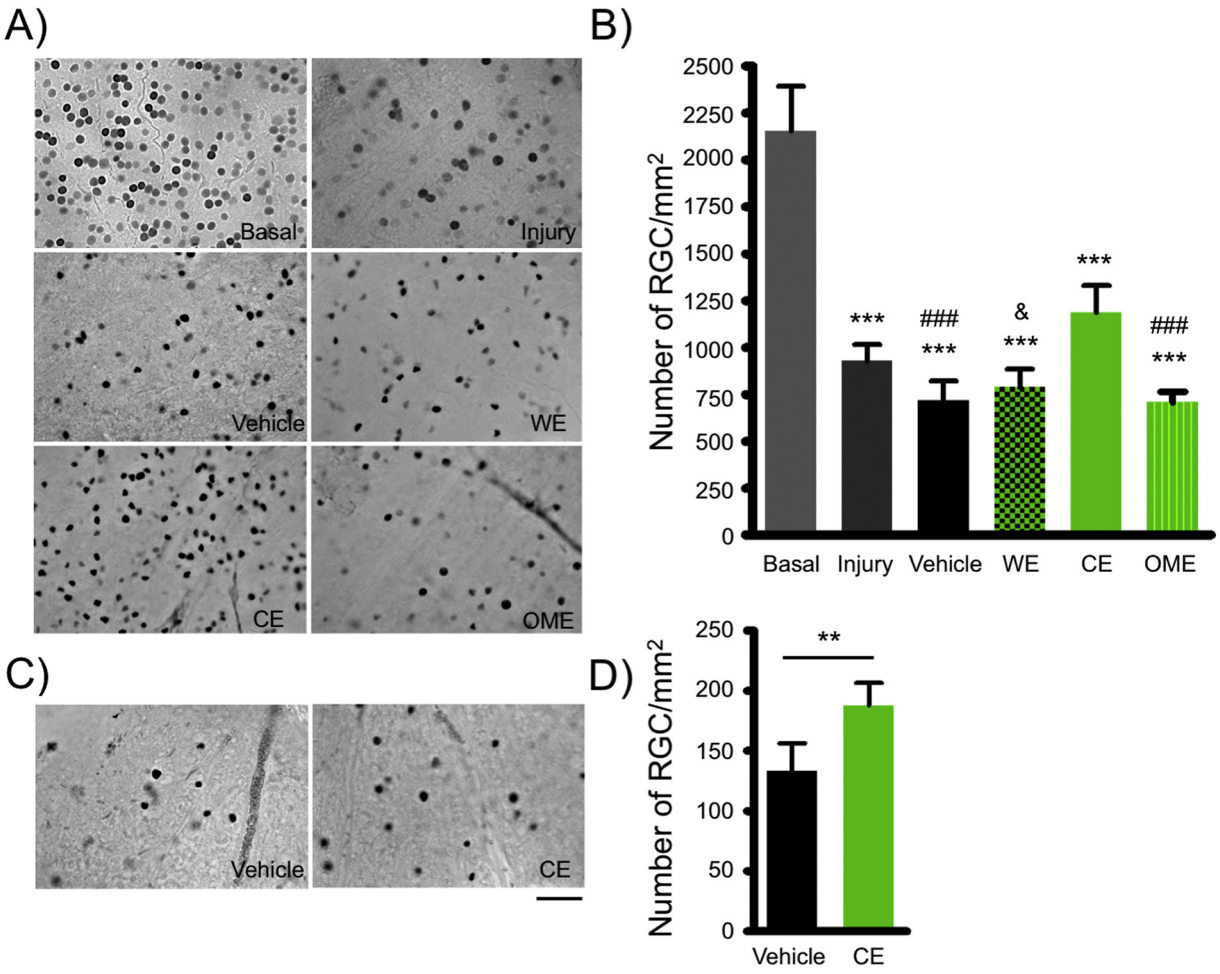


Fig. 3. ACAID to cytosolic antigens improved retinal ganglion cell survival after optic nerve crush injury. Representative photomicrographs showing Brn3a+ surviving retinal ganglion cells (RGC) at 7 (A) or 14 (C) days after optic nerve crush injury in animals inoculated with neural-tissue derived extracts (whole extract, WE; cytosolic extract, CE; or organelle-membrane extract, OME) compared to vehicle inoculated and injury-only rats, as well to the basal group. Quantification of surviving RGC at 7 (B) and 14 (D) days after optic nerve crush in basal, injury, vehicle, WE, CE or OME groups. Scale bar = 50 μm. Basal vs. all groups $^{***}p \leq .001$; CE vs. vehicle and OME, $^{\#\#\#}p \leq .001$; CE vs. WE, $^{\&}p \leq .01$.

(Fig. 3C–D) and OME (data not shown).

3.4. ACAID to CE biased the retinal microenvironment to an anti-inflammatory profile

The retinal inflammatory profile was studied at 2 and 7 dpi by quantitative RT-qPCR in rats inoculated into the anterior chamber with the CE and injured (Suppl. Fig. S2). The results showed that ACAID to the CE increased expression of the pro-inflammatory cytokines IL-1β and INF-γ and decreased iNOS expression at 2 dpi (Fig. 4A); while increasing TNF-α, INF-γ and iNOS at 7 dpi (Fig. 4B). Additionally, ACAID to the CE increased all anti-inflammatory cytokines at both dpi. Particularly notable was the highly increased IL-4 expression at both dpi and that of IL-6 at 7 dpi (Fig. 4).

The inflammatory profile induced by ACAID to the CE also differentially modified the retinal expression of neurotrophic and survival factors early after optic nerve injury, by increasing BDNF expression at 2 dpi and decreasing it at 7 dpi (Fig. 5A and B). ACAID to the CE also decreased Ocm and Casp-3 expression at 7 dpi, as well as increasing NT-4 expression, with no changes at 2 dpi. (Fig. 5C and D).

3.5. The effects of ACAID to the CE are mediated by regulatory T cells and M2 macrophages

To elucidate the cell types involved in the regulation of the anti-inflammatory profile induced by ACAID to the CE in the retina, the expression of phenotypic markers for Tregs (Fox—P3), macrophages (CD68) and alternatively-activated macrophages (Arg-1; M2 type) were quantified. The results showed that the expression of the three markers significantly increased at 7 dpi, with no changes at 2 dpi. Most notable was the increase in Arg-1 expression (Fig. 5E and F).

4. Discussion

ACAID may be used to avoid self-reactivity to neural antigens and thus reduce secondary neuronal death after CNS traumatic injury and neurodegenerative diseases (Toscano-Tejeida et al., 2016). Previous studies on multiple sclerosis (Bhowmick et al., 2011; Farooq and Ashour, 2013) and spinal cord injury (Pineda-Rodriguez et al., 2017) suggest that this may be the case. However, the mechanisms by which secondary neurodegeneration is prevented have not been fully described. In this study, ACAID was induced to three neural tissue-derived extracts prior to optic nerve injury and its effect on retinal ganglion cell (RGC) survival was evaluated. The results show that only ACAID to the CE increased RGC survival by inducing a retinal micro-environment featuring upregulation of anti-inflammatory mediators and neurotrophic/survival signals. Moreover, this study shows that the anti-inflammatory effects of ACAID to the CE are mediated by other cell populations in addition to Tregs, as evidenced by increased expression of markers for macrophages and alternatively activated macrophages

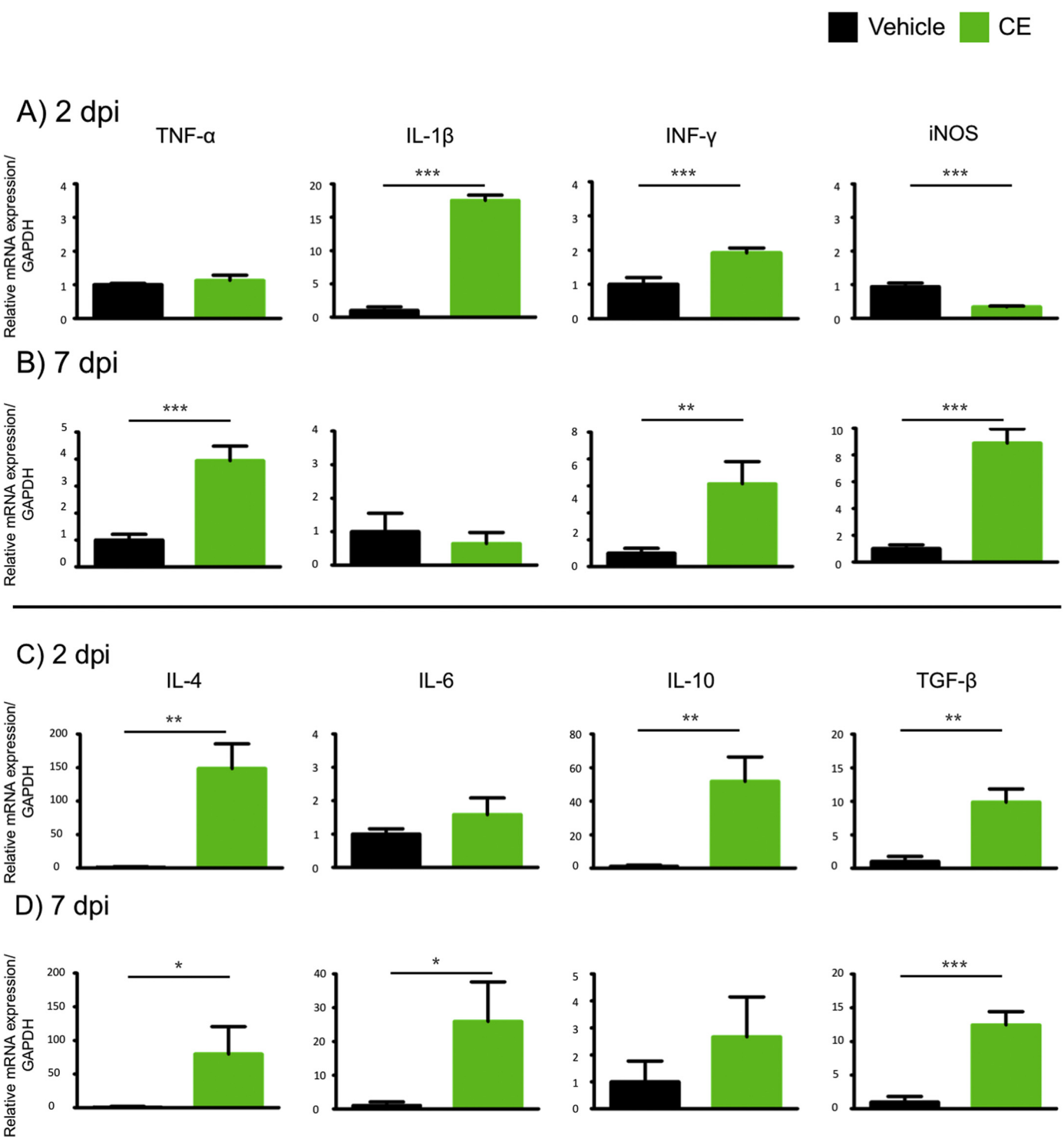


Fig. 4. ACAID to cytosolic antigens modified the retinal inflammatory profile early after optic nerve injury. Quantification of relative mRNA expression of pro-inflammatory (A-B) and anti-inflammatory mediators (C-D) in the retina at 2 and 7 days post-injury (dpi) in vehicle inoculated rats and in animals inoculated with the cytosolic extract (CE). Tumor necrosis factor, TNF; interleukin, IL; interferon, IFN; inducible nitric oxide synthase, iNOS; transforming growth factor, TGF. $^{*}p \leq .05$, $^{**}p \leq .01$, $^{***}p \leq .001$.

(M2) after optic nerve injury.

The data show that the CE was the most reliable source of immune-tolerogenic neural antigens. Accordingly, the CE was the only neural tissue-derived fraction that significantly reduced secondary neuronal death after optic nerve injury compared to all rats inoculated in the anterior chamber and lesioned. This effect was presumably related to the increased ability of ACAID to the CE to induce Tregs, as shown by increased splenic Fox-P3 expression. These actions were specific to the CE, since neither the WE nor the OME showed similar effects. Interestingly, although the WE contains both the CE and OME, tolerization with the WE did not avoid inflammation in rats sensitized and challenged with the CE. This fact suggests that cytosolic antigens are unrepresented in the WE and fail to be recognized by the immune system and to lessen the inflammatory response associated to RGC

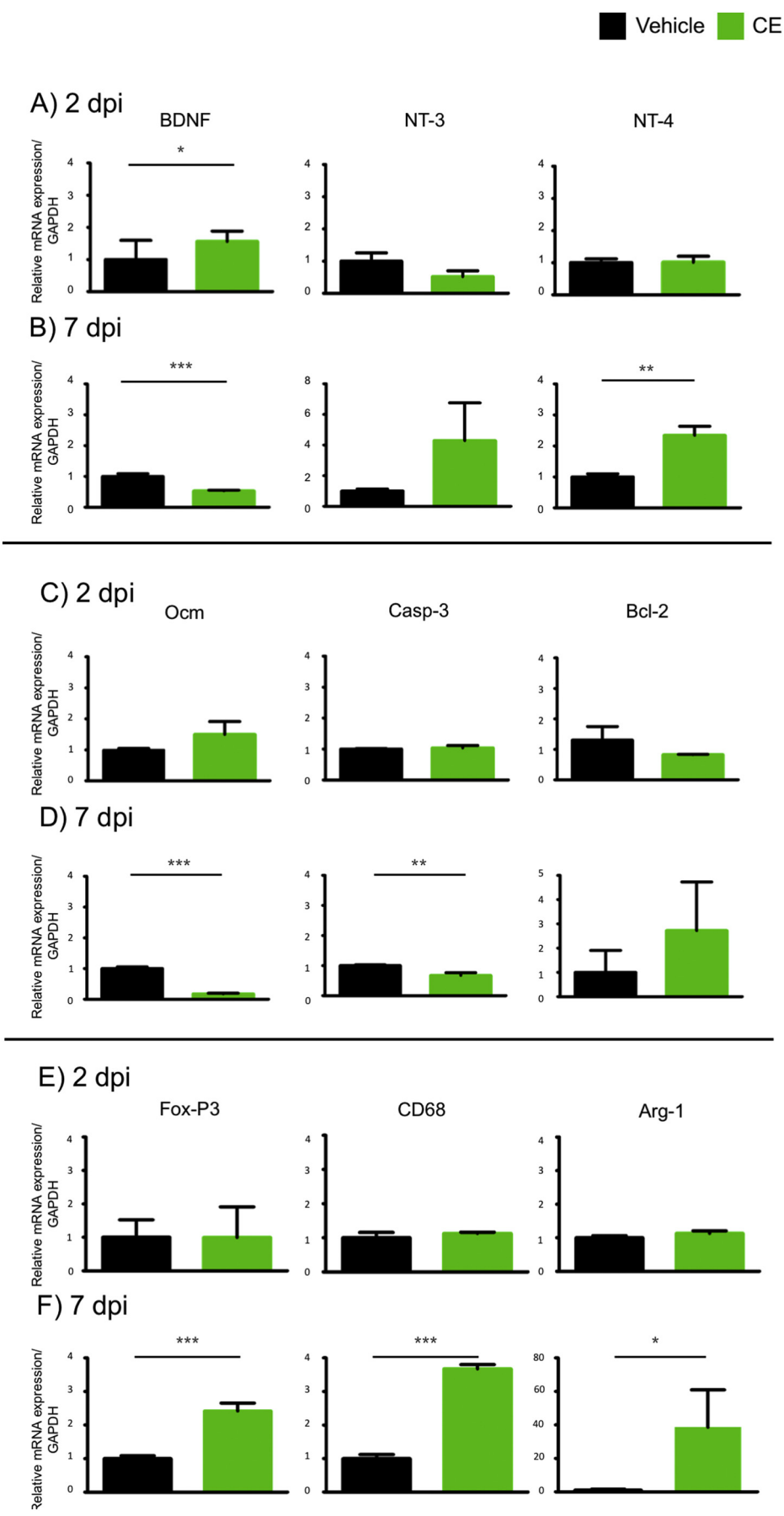


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Fig. 5. ACAID to cytosolic antigens modulated neurotrophic, survival and phenotypic marker expression after optic nerve injury. Quantification of relative mRNA expression of neurotrophic factors and survival signals (A-D) as well as phenotypic markers (*E*-F) in the retina at 2 and 7 days post-injury (dpi) in vehicle inoculated rats and animals inoculated with the cytosolic extract (CE). Brain-derived neurotrophic factor, BDNF; neurotrophin, NT; oncomodulin, Ocm; caspase 3, Casp-3; B-cell lymphoma 2, Bcl-2; Forkhead Box P3, Fox−P3; cluster of differentiation, CD; arginase, Arg. $^{*}p \leq .05$, $^{**}p \leq .01$, $^{***}p \leq .001$.

death after optic nerve injury. Although a direct comparison with prior work (Pineda-Rodriguez et al., 2017) was not done, extractions protocols suggest that their SCE corresponds to our OME-WE. The effects associated with different spinal cord-derived extracts may be related to the concentration of neural antigenic determinants present in each fraction and to the injury site. Further studies should determine the proteins found in each extract and their concentration.

ACAID to the CE preserved approximately 55% of the RGC with respect to intact animals at 7 dpi (22% more cells than vehicle, 19% more than WE and 23% more than OME groups but only 12% more cells than the injury group). The injury-only group showed a higher RGC survival than the groups where ACAID was induced with the WH, OME or even inoculated with vehicle. Previous studies showed that a single (25 µl; Benozzi et al., 2002) or chronic intracameral delivery of vehicle (20 µl, once per week; Urcola et al., 2006) did not increase intraocular pressure (IOP; Benozzi et al., 2002; Urcola et al., 2006) or decrease RGC survival in rats (Urcola et al., 2006). Moreover, although a single intracameral delivery of hyaluronic acid can transiently increase IOP in rats, chronic injections are required to sustain an elevated IOP and to trigger RGC death (Benozzi et al., 2002; Moreno et al., 2005). Thus, it is unlikely that intracameral injection (10 µl per eye) increased IOP. This evidence, together with the results presented in this study showing that a single intracameral puncture or injection of vehicle/CE in juvenile animals did not alter the number of surviving RGC in adults, suggests that although manipulation per se does not kill cells it sensitizes them to trauma. Further studies should evaluate the true impact of ACAID to the CE on RGC survival using adoptive cell transfer.

A remarkable result obtained in this work was that ACAID to the CE modulated the retinal inflammatory response. Indeed, overall ACAID to the CE promoted the expression of all anti-inflammatory cytokines while blunting or increasing the expression of some pro-inflammatory cytokines. This is not surprising since a balanced interplay of both pro- and anti-inflammatory cytokines is required to prevent secondary neuronal damage (Carlson et al., 1999; Leibinger et al., 2013; Raposo et al., 2014; Tyor et al., 2002; Wattananit et al., 2016). However, the notorious upregulation of anti-inflammatory cytokines since early post-injury stages suggests that ACAID to the CE polarized the inflammatory retinal environment towards a restorative profile. In this line, this work also evaluated the expression of some neurotrophic factors that have demonstrated neuroprotective effects in early stages after the injury.

The results showed that ACAID to the CE up-regulated the expression of neurotrophic/survival factors such as BDNF at 2 dpi and NT-4 at 7 dpi, while down-regulating the expression of BDNF and Casp-3 at 7 dpi. Together, these results suggest that ACAID to the CE modulates the expression of some proteins that promote neuronal survival after optic nerve injury, which likely supports RGC survival at 7 dpi (Cohen-Cory and Fraser, 1994; Peinado-Ramón et al., 1996; Sánchez-Migallón et al., 2016). However, given the limited RGC survival observed between 7 and 14 dpi, the results suggest that modulation of the inflammatory profile promoted by ACAID to the CE is not enough to guarantee RGC long-term survival, at least after optic nerve injury.

ACAID to the CE also decreased the expression of retinal Ocm at 7 dpi. Ocm is another neurotrophic factor that promotes axon regeneration and RGC survival; its action is closely associated to pro-inflammatory neutrophils and macrophages recruited to the retina after lens injury (Yin et al., 2006; Kurimoto et al., 2013). Down-regulation of Ocm expression after CE-induced ACAID induction is congruent with its effects.

ACAID to the CE increased the expression of Fox-P3 (Treg specific marker) at 7 dpi. Previous work has shown that ACAID reduces antigen-specific inflammatory responses mainly by inducing CD4+ and CD8+CD25+Fox-P3+ Tregs (Farooq and Ashour, 2013; Farooq et al., 2014; Saban et al., 2008; Pineda-Rodriguez et al., 2017). Since the Th1 response is triggered by neural antigens during acute inflammatory responses around 6–9 dpi, increased expression of Fox-P3 induced by ACAID to the CE was expected at 7 dpi. Our results support this hypothesis and show that ACAID to the CE accelerated Treg recruitment to the lesion site by at least a week, since it has been observed that Treg-mediated regulation starts around 14 dpi (unpublished data).

ACAID to the CE also increased CD68, Arg-1 and anti-inflammatory cytokine expression, suggesting that CE-specific Tregs induce the recruitment/differentiation of M2-type macrophages in the retina at 7 dpi. This result is supported by previous work showing that CD4+CD25+ Tregs induce the differentiation of M2 macrophages, which display Arg-1 activity and produce IL-10 (Liu et al., 2011). Since it has been shown that peripheral cells are the primary source of alternatively activated macrophages after optic nerve injury (Walsh et al., 2014), our results suggest that ACAID to the CE may modulate inflammation triggered by both adaptive and peripheral innate immune cells. The regulation of lymphoid linage cells may be mediated by Treg recruitment at 7 dpi, while the effect on myeloid cells may occur by Arg-1 dependent (at 7 dpi) and independent mechanisms (at 2 dpi). To our knowledge, these findings have not been previously reported and have important implications to the mechanism of action of ACAID.

In conclusion, ACAID to the CE induced prior to optic nerve injury reduced self-reactivity and polarized the retina to an anti-inflammatory milieu promoted by Tregs and M2-type macrophages. However, since anterior chamber inoculation aggravated RGC death after optic nerve injury, the neuroprotective potential of ACAID to the CE after optic nerve injury was limited. Nonetheless the results presented in this paper contribute to elucidate the mechanisms triggered by ACAID to decrease secondary degeneration and highlight its potential to regulate inflammation mediated by innate and adaptive immunity.

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Conflict of interest

The authors declare no conflict of interest.

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