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T E S I S

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Internship report

PERIPHERAL AND CENTRAL NEUROINFLAMMATORY RESPONSES IN CX3CR1 - eGFP KNOCKOUT MICE SUBJECTED TO EXPERIMENTAL DRY EYE

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ABREVIATION LIST

ATF3	*Activating transcription factor 3*
BAC	*Benzalkonium chloride*
CCL2	*C-C Motif Chemokine Ligand 2*
CD68	*Cluster of Differentiation 68*
cDNA	*Complementary deoxyribonucleic acid*
CX3CL1	*C-X3-C Motif Chemokine Ligand 1*
CX3CR1	*C-X3-C Motif Chemokine Receptor 1*
DAPI	*4',6-diamidino-2-phénylindole*
DED	*Dry eye disease*
eGFP	*Enhanced green fluorescent protein*
FOS	*Fos Proto-Oncogene*
GAPDH	*Glyceraldehyde 3-phosphate dehydrogenase*
GFAP	*Glial fibrillary acidic protein*
HRT	*Heidelberg Retina Tomograph*
Iba1	*Ionized calcium binding adaptor molecule 1*
IDV	*Institut de la Vision*
IL	*Interleukin*
IFN	*Interferon*
IVCM	*In vivo confocal microscopy*
KO	*Knock Out*
mRNA	*Messenger ribonucleic acid*
NGS	*Normal goat serum*
NK	*Natural killer*
PBS	*Phosphate-buffered saline*
RT-qPCR	*Quantitative Reverse transcription polymerase chain reaction*
SP5	*Spinal trigeminal nucleus*
TCEP	*Tris-(carboxyethyl) phosphine hydrochloride*
TG	*Trigeminal ganglion*
TNF	*Tumor necrosis factor*
TRP	*Transient receptor channels*
Vi/Vc	*Trigeminal subnucleus interpolaris/caudalis*
Vc/C1	*Subnucleus caudalis/upper cervical cord*
WT	*Wild type*

ABSTRACT

Chronic corneal pain related to dry eye disease (DED) is a debilitating condition linked to inflammation, corneal nerve damage that can cause peripheral and central sensitization mechanisms and significantly affect quality of life of patients. It has been reported that components of inflammatory response such as chemokines are increased in tears from DED patients and correlates with pain and DED severity. Previous work from the team reported that CX3CL1 expression is up regulated in the conjunctiva of patients receiving benzalkonium chloride (BAC)-containing medication and was correlated with clinical inflammation. CX3CL1 has a role in the maintenance of homeostasis and any disturbance lead to inflammatory reactions that exacerbate the transmission of nociceptive information. Nonetheless, no studies have evaluated the role of CX3CL1 and its receptor CX3CR1 in the inflammatory mechanisms in a preclinical model of dry eye associated with corneal pain.

The aim of this project was to analyze the peripheral and central neuroinflammatory process in the corneal trigeminal pathways in a model of chronic ocular inflammatory dry eye pain induced by topical instillations of 0.2% BAC in *CX3CR1* $^{+/+}$ (WT) and *CX3CR1*$^{GFP/GFP}$ (KO) mice. WT mice treated with 0.2% BAC exhibited ocular inflammation and mechanical corneal hypersensitivity in comparison with control mice receiving topical PBS. This inflammation was associated with the activation of Iba1 positive cells in trigeminal ganglion and sensory trigeminal complex. Meanwhile, *CX3CR1*$^{GFP/GFP}$ KO mice treated with 0.2% BAC exhibited less corneal inflammation in comparison with BAC-treated WT mice. Additionally, we noted that BAC-treated *CX3CR1*$^{GFP/GFP}$ KO mice have less activated Iba1 positive cells in trigeminal ganglion and sensory trigeminal complex as compared to BAC-treated WT mice. Further investigations may contribute to a better understanding of the mechanisms of dry eye pain by the identification of the role of CX3CL1/CX3CR1. Specifically, to translate results into solutions for the benefit of patients suffering from ocular pain because targeting chemokines or chemokine receptors may have therapeutic potential for tackling DED

I. INTRODUCTION

I. 1. Anatomy of the corneal nociceptive pathways

The cornea is part of the ocular surface and has two main functions: the protection of the inner contents of the eye and providing two thirds of the eye´s refractive power [1]. Five sublayers compose the human cornea. The superficial epithelium, the Bowman´s layer, the stroma, and the Descemet´s membrane that gives support to the single layer of endothelial cells [1].

The cornea receives the densest nociceptive innervation of the body. It has a nerve density 40 times bigger than the tooth pulp and 300 times more than the skin. Its nociceptive pathway is comprised by three main structures: the cornea, trigeminal ganglion and trigeminal brainstem sensory complex (SP5) [2]. The nerve endings enter the cornea radially from the periphery to form the sub-basal nerve plexus and then intraepithelial nerve fibers extend towards the epithelium. The cellular bodies of the corneal neurons are located in the dorsomedial portion of the ophthalmic region of trigeminal ganglion. The central axons of corneal sensory neurons terminate in two main regions of SP5, involved in recognizing specific stimulus coming from the cornea [3, 4]. The region of subnucleus caudalis /upper cervical cord integrates irritation and noxious stimuli of the cornea and the region of trigeminal sub nucleus interpolaris/ caudalis and facial motor nucleus are more implicated in the control of lacrimation and the blinking reflex [4], [5]. (Fig.1)

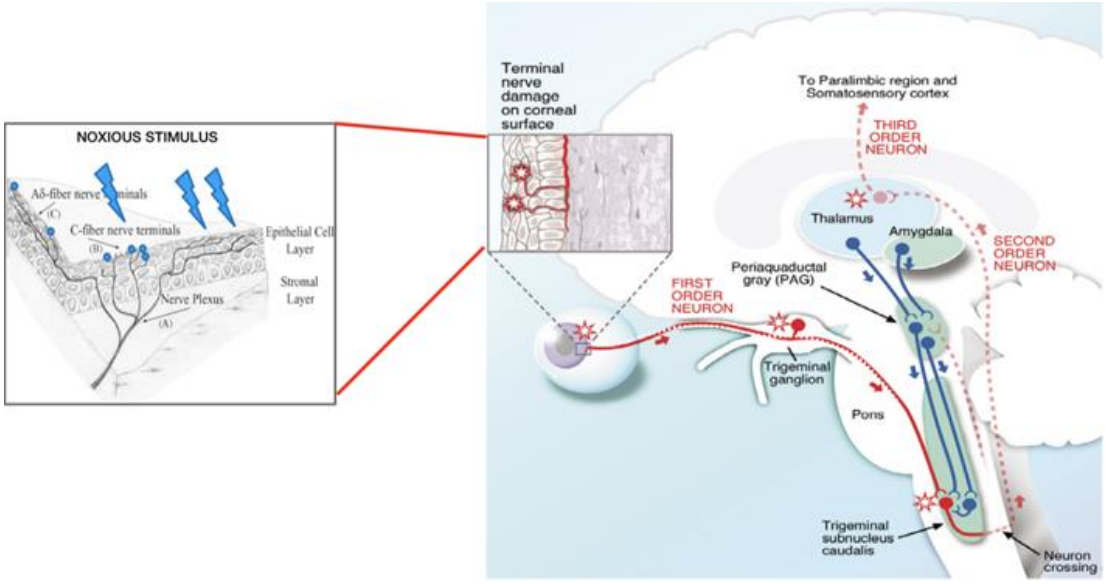

Fig.1. After a noxious stimulus in the cornea there will be a terminal nerve damage on corneal surface, this signal is then transmitted through the ophtamic branch of the trigeminal nerve to the first order neuron in the trigeminal ganglia, from here the stimulus is transmitted to the brainstem sensory complex, where will be a fiber decussation to continue to the thalamus and finally to the somatosensory cortex [27].

II. 2. Ocular pain

The most frequent origin of pain is a noxious stimulus that could be from a different origin, this leads to a cell damage and the consequent stimulation of peripheral sensory nerve fibers, called nociceptors. Nociceptors in the cornea are of type C (non myelinated) and type Aδ (mildly myelinated) and they respond to different stimuli based on the expression of specialized transient receptor channels that send nerve impulses towards the brain and the sensory regions [5]. Three main classes of nociceptor have been identified in the human and rodent cornea. The **mechano-nociceptors**, which represent the 20% of the total density, responsible of sensing physical perturbations and mechanical distress. **Polymodal nociceptors**, are the most numerous types, they represent 70% and are responsible of the detection of temperature flux, endogenous mediators and exogenous chemicals. The **thermo-receptors** represent 10% of nociceptors, responsible of detecting temperature modifications due because of tear film evaporation [5, 6].

Different ocular surface pathologies are responsible for ocular pain development such as injury, infection, systemic diseases, which affect the nerve functionality and could lead to neuropathic corneal pain. Within them, one of the more common pathology characterized by neuropathic pain is dry eye disease (DED) [7].

I. 3. Dry eye disease

DED is a "multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles" [7]. DED is the most common ocular disease, affecting >5% of the world's population and increases sharply with age, reaching prevalence values of 25–30% in individuals over sixty [8]. In DED, unpleasant dryness sensation, the most precocious and main symptom of the disease, implies activation of sensory nerves subserving nociception at the ocular surface and the subsequent sensory processing of this information. Corneal inflammation and nerve damage are the start point to develop pain sensation and a permanent peripheral (cornea) and central (trigeminal brainstem sensory complex) sensitization mechanisms could be developed [9]. However, the cellular and molecular mechanisms involved in dry eye pain are still not well understood. Accumulative evidence has still recognized that inflammation plays an important role in the pathogenesis of dry eye pain [8]. Among inflammatory factors, chemokines and cytokines such as IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, interferon gamma (IFN-γ), tumor necrosis factor (TNF-α), CXCL10 and CX3CL1 are increased in tears from DED patients [9, 10].

CX3CL1 is unique among chemokines because it is synthesized as a transmembrane molecule consisting of an extracellular N-terminal domain, a mucin-like stalk, a transmembrane α helix, and a short cytoplasmic tail. The soluble form consists of the chemokine domain and the extracellular mucin-like stalk and is generated *via* its cleavage by the disintegrin-like metalloproteinases ADAM 10 and ADAM 17 [12, 13]. The CX3CL1 soluble form has chemoattractive activity for monocytes, natural killer (NK) cells, and T cells. Meanwhile, membrane-bound CX3CL1 supports integrin-independent leukocyte adhesion and can be induced on primary endothelial cells by inflammatory cytokines such as TNF-α, interferon-γ (IFN-γ), and interleukin-1 (IL-1) [14]. CX3CL1, produced by neurons, also plays an important role in the transmission of nociceptive information, and mediates neuron-immune cells communication through its receptor CX3CR1. CX3CL1/CX3CR1 signaling is necessary in the maintenance of homeostasis and any disturbance of this axis will lead to inflammatory reactions that exacerbate the transmission of nociceptive information [15].

Moreover, previous work from the team has reported that CX3CL1 expression is increased in the conjunctiva of patients receiving BAC-containing medication and correlates with clinical inflammation [11]. *In vitro* experiments also demonstrated that 0.01% BAC enhances the production of CX3CL1 in conjunctival epithelial cell line IOBA-NHC. In addition, *in vivo* experiments have shown that topical BAC induced macrophage infiltration and subsequent inflammation of the conjunctiva is decreased in CX3CR1-deficient mice as compared with $CX3CR1^{+/+}$ controls [11].

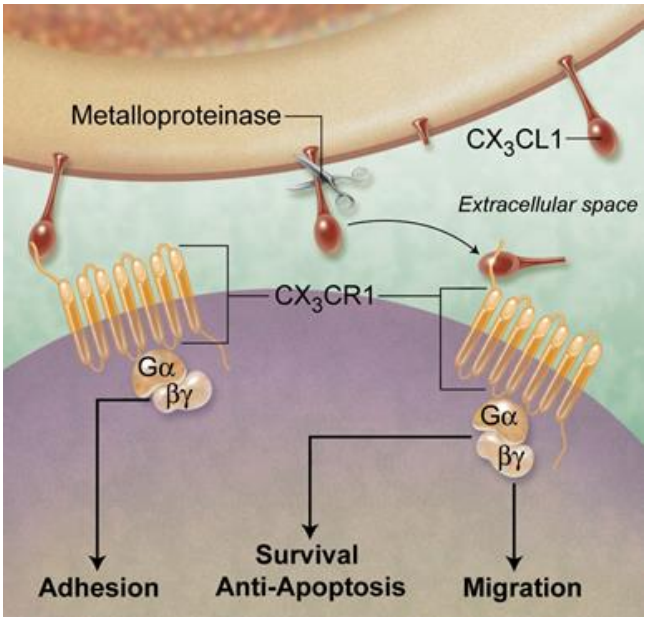

Fig.2. Interactions between CX3CL1 and CX3CR1. CX3CL1 is synthesized as a membrane-bound molecule, in inflammatory conditions it is cleaved by a metalloproteinase to generate a soluble chemokine that binds to the CX3CR1 and acts as a chemoattractant [28].

Several studies have also suggested that CX3CL1 expression at the site of inflammation can attract and activate NK cells, resulting in the consequent lysis of neighboring endothelial cells [16]. CX3CL1 expressed on inflamed endothelium may play a role as a vascular gateway for cytotoxic effector cells by rapidly capturing them from the blood and promoting migration into tissue [13]. Furthermore, CX3CL1 is also implicated in mediation of migration of CX3CR1-bearing cells during neuronal injury, in migration of resident immune cells in the cornea and in the genesis of neuropathic pain by regulating neuronal-glial interactions [17, 18]. **However, no work has been carried out yet on the role of this CX3CL1 chemokine and its receptor CX3CR1 in DED animal models.**

The complexity of DED and the great need for novel therapy for patients suffering from corneal pain impose a deepening of our fundamental knowledge on the anatomy of the nociceptive system and better clinical characterization of the inflammatory mechanisms involved in the initiation and the persistence of the ocular pain during DED. Thus, the aim of this project was to analyze the peripheral and central neuroinflammatory process in the trigeminal pathways in a model of chronic ocular inflammatory dry eye pain in *CX3CR1* $^{+/+}$ (WT) and *CX3CR1*$^{eGFP/eGFP}$ (KO) mice. Overall, this project will contribute to a better understanding of the mechanisms of dry eye pain by the identification of new mechanisms linking inflammatory cells and corneal peripheral nerves. Specifically, to determine the role of CX3CL1/CX3CR1 in the inflammation and pathogenesis of DED would help to translate results into solutions for the benefit of patients suffering from ocular pain because targeting epigenetic alterations may have therapeutic potential for chronic ocular pain.

II. MATERIALS AND METHODS

II. 1. Animals

Wild type and transgenic *CX3CR1*$^{eGFP/eGFP}$ mice on a C57BL/6J background were used in the present study. *CX3CR1*$^{eGFP/eGFP}$ mice are CX3CR1 deficient, with both alleles of the *CX3CR1* gene replaced by an enhanced green fluorescence protein (eGFP) reporter gene. Following an internal IDV collaboration with the team of Florian Sennlaub and Xavier Guillonneau, adult male *CX3CR1*$^{eGFP/eGFP}$ mice were already available at the team. Adult mice were maintained under controlled conditions (22 ± 1 °C, 60 ± 10% relative humidity, 12/12 h light/dark cycle, food and water ad libitum). All procedures were in strict accordance with the guidelines for the care and use of experimental animals approved by the French National Institute of Medical Research (INSERM), national and international laws and with policies for the use of animals in Research in Vision and Ophthalmology (European Communities Council Directive 86/609/EEC, Authorization No. 75-1235 Granted to A. R-LG).

II. 2. Drugs

50% BAK solution was purchased from Sigma–Aldrich (Saint-Quentin Fallavier) and was prepared in 0.1 M phosphate-buffered saline (PBS; pH 7.4).

II. 3. Topical instillation

The preclinical model of dry eye pain was performed by chronic topical instillations of BAC as previously reported (Launay et al, 2016). Briefly, mice were gently restrained and 10 µl of 0.2% BAC or 0.1 M PBS (control group) solutions were topically applied on the right eye. Mice received the topical instillation for seven days two times per day separated by a 4 h interval. Eyes were held open for 20 s to prevent aggressive blinking during application of the drug, which may cause variability in the ocular surface contact time of the solution. The experimental groups included: *CX3CR1*$^{eGFP/eGFP}$ mice, referred to as "KO" and CX3CR1$^{+/+}$ mice, referred to as "WT" were used as control animals.

II. 4. In vivo characterization of the preclinical model of BAC-induced dry eye pain

Weight measurement: In order to evaluate whether topical 0.2% BAC lead to changes in weight gain, animals were weighed on day (D) 0 (D0) and D7.

Measurement of the spontaneous aqueous tear production: Tear production was measured with the phenol red thread test (Zone-Quick; Lacrimedics, Eastsound,WA). The threads were held with jeweler forceps and placed in the lateral canthus of the conjunctival fornix of the eye for 30 s as previously described (Lin et al., 2011; Launay et al, 2016). Tear production was monitored on D0, D3, and D7. The thread is yellow in color (acidic) and when exposed to tears, it changes to a light red color. After 30 s, the tear distance (in millimeters) is determined using a scale.

Slit lamp biomicroscopy: Fluorescein staining of the corneal epithelium was used to evaluate corneal integrity as previously described (Liang et al., 2012). On D7 (1 h after the last instillation), ocular surface was evaluated by the fluorescein test as described below. A drop of 1% fluorescein sodium (Fluoresceine Faure 0.5%; Novartis France, Rueil-Malmaison, France) was placed in the right eye of anaesthetized mice after being rinsed with sterile 0.9% NaCl. Ocular surface staining was evaluated using a SteREAO Lumar microscope (Zeiss) under cobalt blue light, and images were captured by a digital camera through EyeSuite™ software (Koeniz, Switzerland).

***In vivo* confocal microscopy:** A laser-scanning *in vivo* confocal microscopy (IVCM, Heidelberg Retina Tomography (HRT) II/Rostock CorneaModule (RCM; Heidelberg Engineering GmbH, Heidelberg, Germany) was used to examine the entire cornea of anesthetized mice. The first layer of the superficial epithelium, the sub-basal plexus and the stroma were considered. A minimum of 150 serial TIFF images (400 × 400 µm) were acquired per animal.

Mechanical corneal sensitivity (behavioural study): von frey filaments test was used to evaluate the mechanical corneal sensitivity at D0 to obtain the basal threshold, D3 and D7. Mechanical threshold response was determined by assessing the first treated-eye blinking response evoked by calibrated von Frey filaments of increasing force (0.08–0.4 mN) applied in the center of the cornea of immobilized mice.

II. 5. RT-qPCR analysis

Tissue preparation: All mice groups were deeply anesthetized with a 300 µL mixture of Ketamine 1000 U (80 mg/kg body weight) and xylazine (8 mg/kg body weight, Virbac, France) injected intraperitoneally. Animals were then perfused with cold 10 mL 0.9% NaCl solution and the ipsilateral trigeminal ganglia and ipsilateral trigeminal brainstem complex were dissected out and placed in liquid azote until the extraction procedure.

RNA extraction and RT qPCR reaction: RNAs were extracted from trigeminal ganglia and trigeminal brainstem complex using the Macherey-Nagel NucleoSpin RNA extraction kit according to the manufacturer's protocol. Reverse transcription was performed with 330 (trigeminal ganglia) or 660 ng (trigeminal brainstem complex) of RNA using the High Capacity cDNA Reverse Transcription (Applied Biosystems) according to the manufacturer's instructions. Finally, cDNA was diluted in DNAse/RNAse free water to a final concentration of 5 ng/µL. Realtime quantitative PCR was performed with 25 ng of cDNA added to a 15 µL solution of Applied Biosystems mastermix (TaqMan Universal PCR Master Mix) and primers to a final volume of 20 µL. All primers and reagents were purchased from Applied Biosystems. The primers used were: CCL2 (Mm99999056_m1), CCR2 (Mm00438270_m1), CX3CL1 (Mm00436454_m1), CX3CR1 (Mm00438354_m1), TNF (Mm99999068_m1), CD68 (Mm03047343_m1), GFAP (Mm01253033_m1), FOS (Mm00487425_ml) and ATF3 (Mm00476032_ml). Specific mRNA levels were calculated after normalization of the results for each sample with those for GADPH mRNA. The data are presented as relative mRNA units with respect to control values (expressed as a fold-over PBS value).

II. 6. Immunohistological studies

Tissue preparation: All mice groups were deeply anesthetized with a 300 µL mixture of Ketamine 1000 U (80 mg/kg body weight) and xylazine (8 mg/kg body weight, Virbac, France) injected intraperitoneally. Eyes were dissected out just before the perfusion. Animals were then perfused with cold 10 mL 0.9% NaCl solution and immediately with cold 40 mL 4% PFA solution. After fixation, trigeminal ganglia and brainstem were carefully dissected out and post-fixed 48 h in the same fixative. Free-floating sections (40 µm) of the trigeminal subnucleus complex (trigeminal nucleus interpolaris to the spinal cord level C1) were performed using a vibratome (Leica Microsystems, Germany). The trigeminal ganglia were placed in 10% sucrose solution in PBS (overnight), then in a 30% sucrose solution before freezing at −20 °C in 7.5% gelatin and 10% sucrose. Cryostat sections (14 µm) were performed for trigeminal ganglia and eyes, then mounted on Superfrost slides and keep at −20 °C until use.

Iba1 immunofluorescence labeling: After three washes in PBS, the sections were placed in a blocking solution containing normal goat serum (5% for brainstem) (3% for trigeminal ganglia and eyes) and 0.1% triton X-100 for 1 h, then incubated with primary antibody at 4 °C (48 h for floating sections) or 24 h for cryostat sections. The primary antibody used in this study was rabbit - anti Iba1 (1:1000; Wako) and was revealed using Alexa Fluor 594-conjugated goat anti - rabbit (1:500; Invitrogen) for 1 h. DAPI was applied for 10 minutes. After three washes with PBS 1X the sections were mounted with Fluoromont Aqueous Mounting (Sigma-Aldrich). Tissue sections were examined with a Zeiss M1 epifluorescence microscope equipped with a digital camera (Axio Cam HRC; Carl Zeiss) and image acquisition software (Zen; Carl Zeiss). TIFF images were obtained.

II. 6. Statistical analysis

All data are presented as mean ± SEM. Data were evaluated by one-way analysis of variance with the non-parametric ANOVA test using the GraphPad Prism 4.0 statistical software. The level of significance was set at $P < 0.05$.

III. RESULTS

II. 1. Clinical evaluation of dry eye pain model

In order to evaluate whether topical 0.2% BAC lead to alterations in weight gain, animals were weighed before treatment and at the end of the experiments. After 7 days of 0.2% BAC instillation, the body weight gain was significantly decreased in WT mice in mice in comparison with PBS-treated WT mice (1.20 ± 0.56 g *versus* 0.30 ± 0.14 g; P<0.05). Instead, KO mice exhibited less body weight gain and no significantly difference was observed between groups (0.61 ± 0.18 g *versus* 0.50 ± 0.21 g) (Fig. 3A). Moreover, tear production was reduced in WT BAC-treated mice (3.33 ± 0.57 mm compared with PBS group: 5.00 ± 1.41 mm). In contrast, tear production was quite similar between BAC and PBS-treated KO mice (4.5 ± 0.5 mm and 3.6 ± 0.57 mm, respectively) (Fig. 3B). Then, the corneal integrity was evaluated with slit lamp biomicroscopy. As expected in WT, we observed that fluorescein staining of the cornea was greater after 0.2% BAC as well as in KO animals (Fig. 3C).

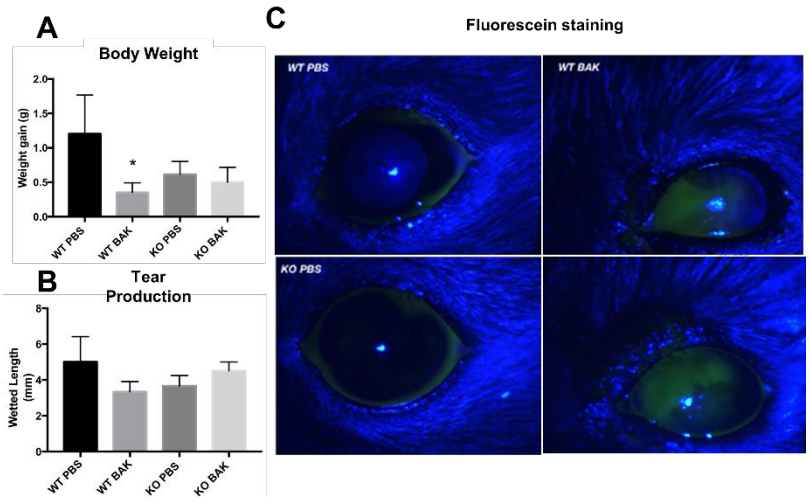

Fig. 3. Evaluation of corneal integrity. (A) Animal body weight gain, BAK significantly decreased weight gain in WT group. WT PBS (n=6), WT BAK (n=5), KO PBS (n=5) and KO BAK (n=4). (B) Tear production was measured (n=4 mice per group) using the phenol red thread test, over a period of 7 days, no significant difference was found (C) Corneal fluorescein staining at day 7, fluorescein staining was greater in the BAK treated group. * P < 0.05

In vivo confocal microscopy was then used to image the different layers of the cornea. WT and CX3CR1 KO PBS-treated mice exhibited a healthy corneal epithelium with pavimentous cells, a sub-basal plexus with numerous thin nerve fibers, a stroma with classical square cells (keratocytes), and an endothelium with regular dark reflective cells (Fig. 4). We noted that 7 days 0.2% BAC instillation induced a complete destruction of the superficial epithelium (not detected), and immune cell infiltration (numerous bright and reflective immune cells, orange arrows) in the sub-basal plexus and the stroma (Fig. 4). However, it seems to be less inflammatory cells in Bac-treated KO mice compared to BAC- treated WT mouse. Furthermore, corneal nerves (Fig.4 blue arrows) were less abundant in both layers as compared to PBS groups but not alteration was observed in the endothelium (Fig. 4).

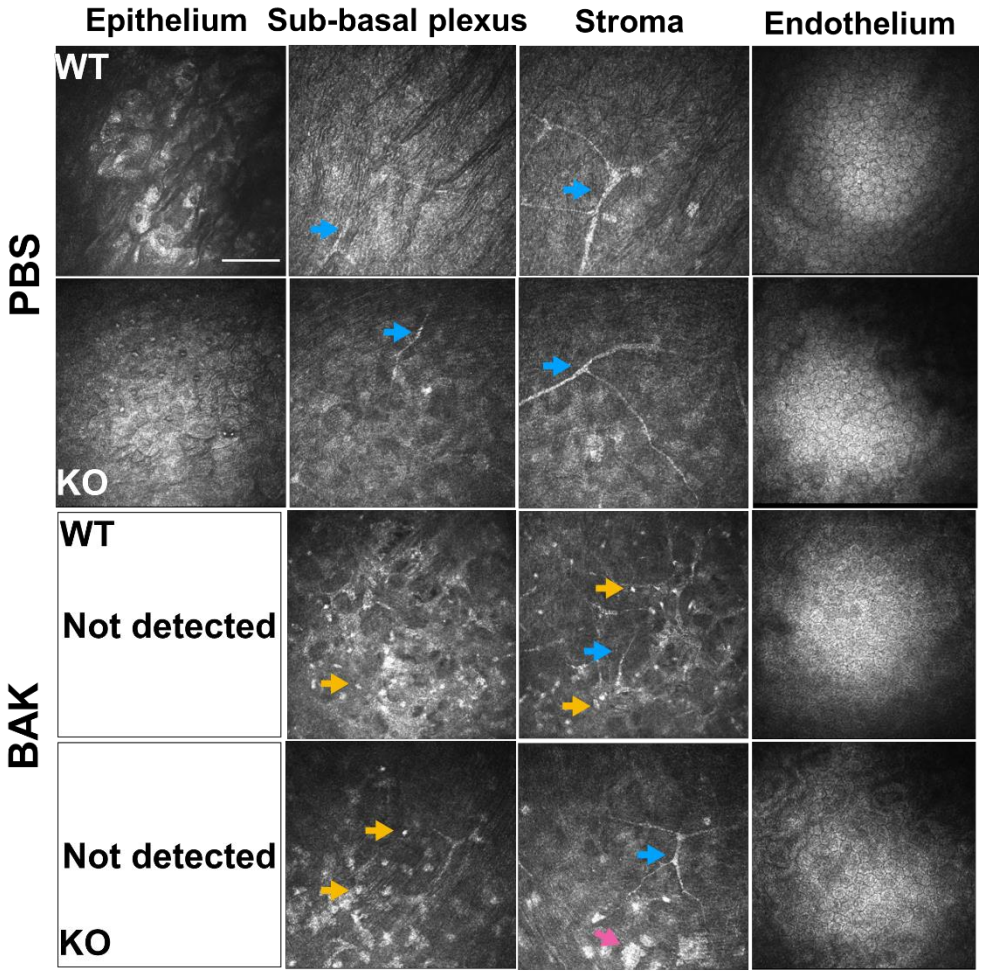

Fig. 4. Noninvasive imaging of the cornea with the in vivo confocal microscopy device (IVCM). Images from *in vivo* confocal microscopy show the epithelium (0–30 µm), sub-basal plexus (30–40 µm), stroma (40–140 µm) and endothelium (140– 150 µm) from PBS- and BAK-treated animals. Note the decreased numbers of corneal nerves (blue arrows) and increased numbers of inflammatory cells, Round hyper reflective macrophages are present (yellow arrows) Activated keratocytes (pink arrows) Scale bar = 100 µm.

We next evaluated whether the absence of CX3CR1 modified the BAC-induced dry eye pain behaviour. In this context, von frey filaments were used to evaluate the mechanical corneal sensitivity. At D0, basal mechanical corneal sensitivity was not significantly different between all the groups.

After 3 days of 0.2% BAC treatment, the corneal mechanical threshold was significantly decreased both in BAC-treated WT and KO mice in comparison with PBS -treated WT and KO controls. For WT mechanical corneal sensitivity was 0.008 ± 0 *versus* 0.022 ± 0.002 g ($P<0.001$). Meanwhile, for the KO mechanical corneal sensitivity was 0.015 ± 0.002 *versus* 0.02 ± 0 g ($P<0.05$).

This corneal hypersensitivity remained significant at D7 in both groups (WT and KO) after 7 days of 0.2% BAC treatment. For WT corneal sensitivity was 0.008 ± 0 *versus* 0.02 ± 0.002 g ($P<0.001$). Instead, for the KO corneal sensitivity was 0.01 ± 0.002 versus 0.024 ± 0.004 ($P<0.01$) (Fig. 5).

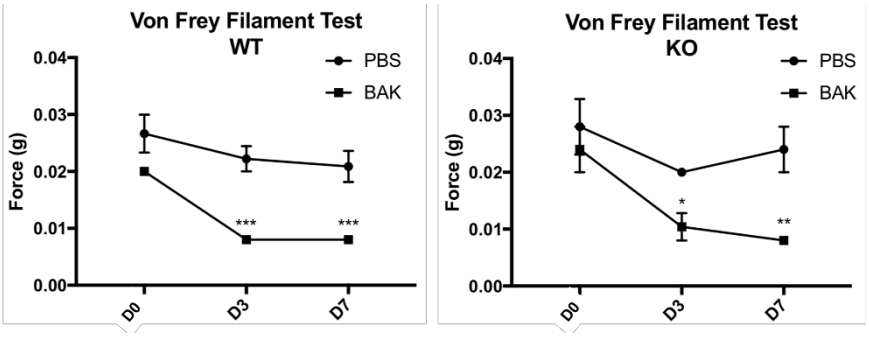

Fig.5. Evaluation of mechanical corneal sensitivity. Using von Frey filaments, three evaluations were performed along the PBS and BAC topical administrations (D0, D3, D7). Here are shown the forces that each filament applies 0.04 g, 0.02g and 0.008 (g). Corneal mechanical threshold was significantly decreased in WT and KO mice BAC-treated (n=9), WT BAC (n=9), KO PBS (n=5) and KO BAC (n=5). Results are expressed as mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

II. 2. Analysis of Iba1 staining in the cornea

We then monitor the corneal alteration and inflammation by evaluating DAPI staining and Iba1 (monocytes/macrophages) immunostaining on ipsilateral eye from all groups of mice after 7 days of PBS and 0.2% BAC administration. DAPI staining of corneal sections confirmed that 0.2% BAC induced corneal damage in superficial corneal epithelium. In addition, numerous DAPI stained cells (probably infiltrating cells) were observed in the corneal stroma in both BAC-treated WT and KO mice. Moreover, chronic 0.2% BAC treatment induced an increase in the number of Iba1 positive cells (Fig. 6, red cells) in the corneal stroma of BAC-treated WT animals and and although they were found also in BAC-treated KO animals presence was less evident, showing a less inflamatory pattern in BAC-treated KO mice. Interestingly, CX3CR1-eGFP positive cells were difficult to find in the center of the cornea in KO animals, but were present in the limbus of the cornea (not shown). (Fig. 6). In this study, we also observed a colocalization between Iba 1 positive cells and CX3CR1-eGFP positive cells.

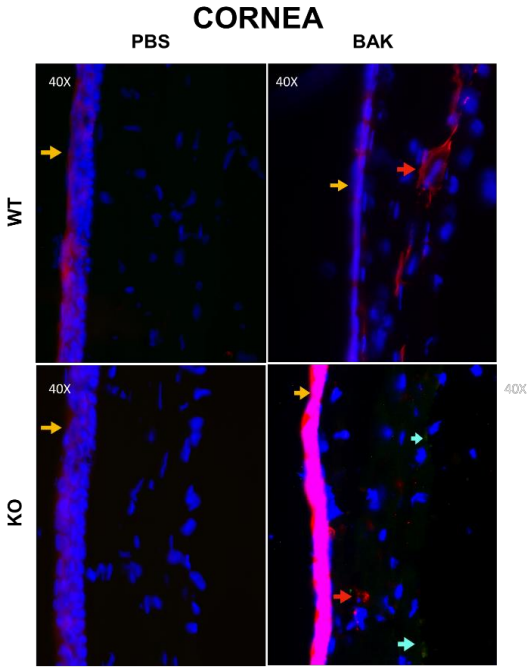

Fig. 6. Microscopic images illustrating DAPI (blue) and Iba 1 (red) staining in corneal sections from WT and KO animals subjected to PBS and BAC treatment. Note the decrease of the epithelial layers in both groups treated with BAC (yellow arrows). CX3CR1-EGFP cells were difficult to find in the center of the cornea, and a colocalization between Iba 1 positive cells (blue arrows) and CX3CR1-EGFP cells was noted. Iba 1 positive cells were only detected in BAC treated animals.

II. 3. Analysis of Iba1 staining in ophthalmic branch of trigeminal ganglion

We next compared the Iba1 (marker of monocytes/macrophages) immunostaining in the ipsilateral trigeminal ganglion from all groups of mice to determine if the corneal inflammation spread to the TG. BAC-treated WT mice exhibited activated (amoeboid morphology with a large soma and short processes) monocytes/macrophages, while PBS-treated control mice exhibited resting microglial cells with thin ramifications and small cellular bodies (Fig. 7.). On the other hand, we did not observe any morphological changes of Iba1-positive cells in the ipsilateral trigeminal ganglion in BAC-treated KO and PBS-treated WT mice. WT PBS (n=2), WT BAC(n=2), KO PBS (n=2), KO BAC (n=3). Quantification of the number of Iba1 positive cells in all groups of mice are under investigation.

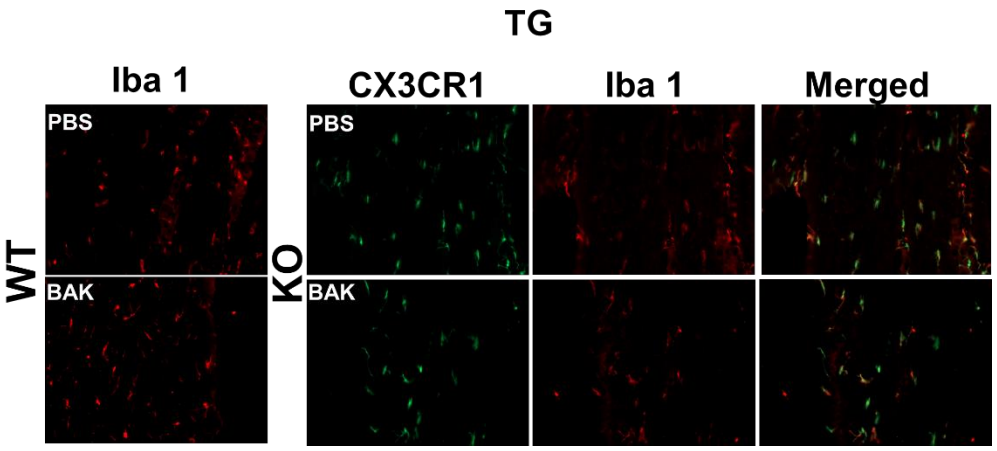

Fig. 7. Microscopic images illustrating the immunostaining of Iba 1 (red) and CX3CR1-eGFP cells in green in the ophthalmic branch of the ipsilateral trigeminal ganglia from WT and KO mice receiving either PBS and BAC instillations. A common trend in morphological changes was found in Iba 1 positive cells in WT BAC animals with characteristics of activated monocytes/macrophages (amoeboid morphology with a large soma and short processes). KO animals have similar morphology compared to WT BAC. WT PBS (n=2), WT BAC (n=2), KO PBS (n=2), KO BAC (n=3).

II. 4. Analysis of Iba1 staining in the sensory trigeminal complex

We then investigated the distribution of microglial cells (Iba 1 staining) in the ipsilateral sensory trigeminal complex (Vi/Vc and Vc/C1 transition regions) to determine whether this cell population increases in number or if an activation (morphological changes) occur. We observed that there was a trend in BAC-treated WT mice that exhibited activated microglia, (amoeboid morphology with a large soma and short processes) in comparison with the control PBS mice with resting thin ramifications and small cellular bodies microglial cells. Meanwhile, the KO BAC-treated and PBS-treated control mice exhibited Iba1 positive cells with resting morphology (Fig. 8).

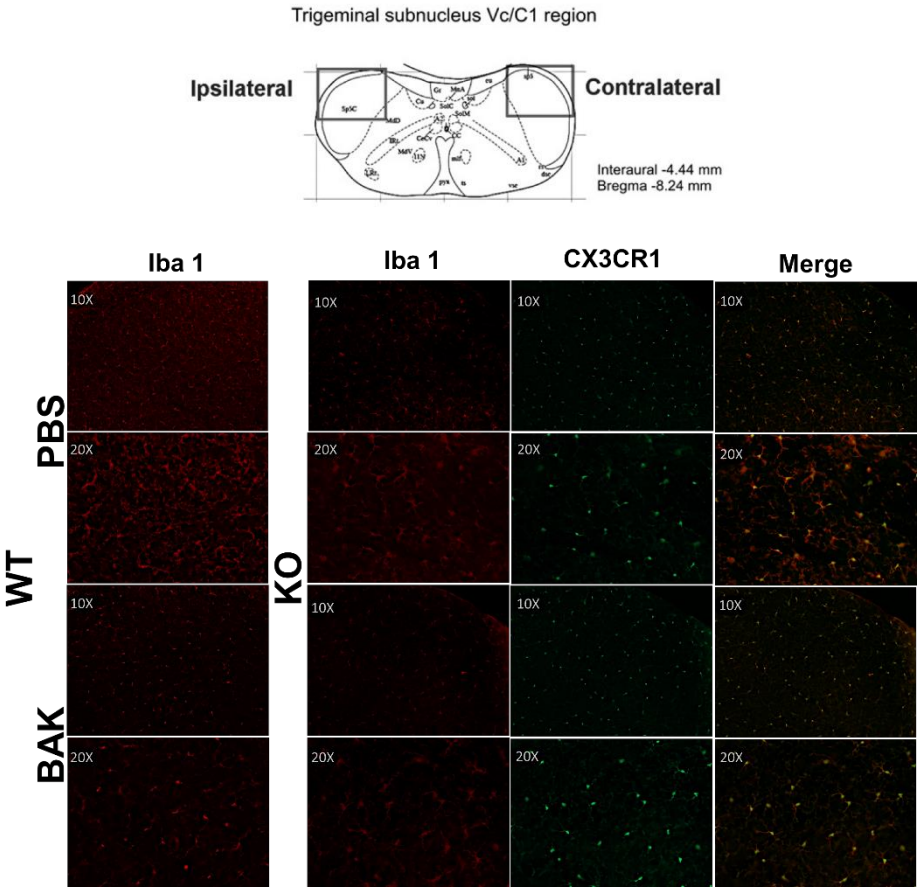

Fig. 8. Microscopic images illustrating the immunostaining of Iba 1 (red) in the ipsilatateral Sp5 region and the presence of CX3CR1-eGFP cells (green) (A) Schematic drawing from Paxinos Atlas showing the stereotaxic coordinates of SP5 region (B) Iba 1 immunostaining in WT and KO animals. A common trend in morphological changes was found in Iba 1 positive cells in WT BAK animals with characteristics of activated monocytes/macrophages (amoeboid morphology with a large soma and short processes). Most of the Iba1 positive cells merged with CX3CR1-eGFP cells. WT PBS (n=2), WT BAK (n=2), KO PBS (n=2), KO BAK (n=3). Quantification remains to be further performed.

II. 5. Analysis of gene expression in trigeminal ganglion and sensory trigeminal complex

Finally, we evaluated whether a peripheral corneal inflammation has an impact on proinflammatory gene expression in the ipsilateral trigeminal ganglion and the sensory trigeminal complex. For these preliminary results, the number of animals analyzed per group are indicated into brackets: WT-PBS (n=2), WT BAK (n=3), KO PBS (n=3), KO BAK (n=3). We wanted to address the following question: does the absence of CX3CR1 modify the neuroinflammatory responses in the TG and TBSC after BAC treatment? So, we compared the expression of CCL2, CCR2, GFAP, CD68, and FOS in ipsilateral TG from WT and KO mice. At the level of the SP5, the levels of CX3CL1, CX3CR1, FOS, ATF3, CCL2, CCR2, CD68, GFAP,TNF mRNA were quantified.

Neither in TG or SP5, there was no significant difference between WT and KO PBS. However, in the trigeminal ganglion, KO animals with BAC treatment exhibited a downregulation of pro-inflammatory markers (CCL2, CCR2, GFAP, and CD68) with respect to WT animals with BAC treatment. At the levels of the SP5, we noted that the absence of CX3CR1 did not modify the expression of CX3CL1 in PBS or BAC conditions. Furthermore, we only observed that KO animals with BAC treatment exhibited an up regulation of the neuronal marker FOS compared to BAC-treated WT animals (Fig. 9).

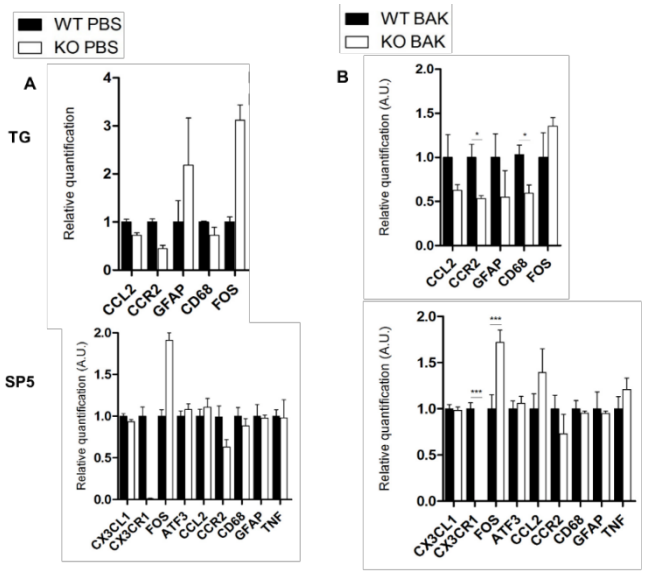

Fig.9. Molecular Analysis from the TG and SP5 regions. (CCL2, CCR2, GFAP, CD68, FOS, CX3CL1, CX3CR1, ATF3, TNF gene expression was evaluated). In TG, KO BAC treated animals showed a down regulation of CCL2, CCR2, GFAP and CD68, with a significant difference in CCR2 and CD68, compared to WT BAC. In SP5, KO BAC treated animals showed a significant down regulation of CX3CR1 compared to WT BAC. Note that the absence of CX3CR1 does not affect the expression of CX3CL1. In SP5, KO BAC showed a significant up regulation of FOS compared to WT BAC. WT PBS (n=2), WT BAC (n=3), KO PBS (n=3), KO BAC (n=3) Results are expressed as mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

IV. DISCUSSION

Dry eye disease (DED) is a multifactorial disease of the ocular surface and tears that results in symptoms of discomfort, visual disturbance, burning, pain and tear film instability). Although DED is a common disorder that affects the quality-of-life of millions worldwide, the cellular and molecular mechanisms of DED are not fully understood [3]. Therefore, attempts to find better diagnostic approaches and appropriate treatment for DED are worthy of consideration. DED has a prevalence of more than 5 % around the world and due to its chronicity has negative effects on patient's life quality. Pain sensation is one of the recurrent symptoms accompanying this affection ocular surface disease and corneal inflammation and nerve stimulation play a key role in its development [3, 4].

In the present study we analyze the peripheral and central neuroinflammatory process in the corneal trigeminal pathways in a model of chronic ocular inflammatory dry eye pain with *CX3CR1* $^{+/+}$ (WT) and *CX3CR1*$^{eGFP/eGFP}$ (KO) mice. The model was induced by 7 days topical instillations of 0.2% BAC. This treatment induces weight loss, corneal inflammation, reduction in tear production, ocular pain-related behavior and neurotoxicity in corneal nerves and neuroinflammation in trigeminal ganglion and sensory trigeminal complex [4, 19, 20]. In according with them, we observed similar results during clinical evaluation, RT-qPCR analysis and immunohistological studies in our WT BAC-treated mice.

After 0.2% BAC topical administration, we observed a reduction of body weight gain in WT mice in comparison with their PBS controls, as previously reported [4]. However, *CX3CR1*$^{eGFP/eGFP}$ KO mice exhibited less body weight gain in both BAC and PBS groups. Some studies have describe that the chemokine CX3CL1 plays an important role in the induction of obesity and inflammation. Additionally, it has been reported that these *CX3CR1*$^{eGFP/eGFP}$ mice gain significantly less weight that their heterozygote controls; which could explain our result in our model [21, 22].

Moreover, the tear production was measured using the phenol red test. We noted that after 0.2% BAC administration, WT mice have a reduced tear production in comparison with their PBS controls and we did not see difference with KO mice. These preliminary results were obtained from 4 mice per group; thus, it is mandatory to increase the number of animals per group to confirm these data.

Corneal integrity was evaluated by fluorescein staining and we reported that BAC treatment induced corneal de-epithelialization in both WT and KO mice in comparison with their controls. Moreover, *in vivo* confocal microscopy shown that BAC treatment induces immune cell infiltration in the sub-basal plexus and the stroma (KO mice exhibited less inflammatory cells) and decreases corneal nerves in both layers in comparison with PBS WT and KO groups. All of these observations are under quantification.

Immune cells are important in the defense of the ocular surface and in basal conditions these cells are present in cornea in non-activated and immature state [23]. However, inflammatory stimuli, such as physical or chemical injury can cause direct damage to the corneal epithelium. This corneal injury induced the released of cellular contents from death cells that activate resident immune cells from corneal stroma to increase the production of cytokines and chemokines. Under the influence of inflammatory cytokines, there is up-regulation of vascular endothelial cell adhesion molecules to facilitate the extravasation of inflammatory cells from the limbal vessels exacerbating the corneal inflammation [24].

Here, we have shown by immunohistological techniques that chronic BAC treatment increases corneal inflammation by reporting higher Iba1 positive cells in the stroma of central cornea from WT and KO mice. Moreover, WT mice exhibited more inflammatory cells infiltration that the KO group. These immunohistological results corroborate with those found with IVCM analysis. This could indicate that the damage caused on superficial epithelial cells activates the resident immune cells from central and peripheral cornea such as dendritic cells or keratocytes to induce the infiltration of macrophages in corneal stroma.

In addition, BAC-treated KO mice exhibited colocalization between Iba1 positive cells with eGFP in the stroma of central cornea. Interestingly, in PBS-treated KO mice the eGFP positive cells were only found in the peripheral cornea and sclerocorneal limbus (not shown). During basal conditions, immature macrophages, activated dendritic and Langerhans cells are resident in these structures [23]. Hence, our results suggest that following BAC-induced corneal alterations, immature macrophages from the peripheral cornea migrate to the central cornea to exacerbate the inflammation. It is well known that the chemokine CX3CL1 is a specific factor that chemoattracts and activates monocytes, macrophages, and microglia. However, there are other chemokines contributing to the chemotaxis of monocytes/macrophages such as CCL2, CCL7, CCL12 and CCL13 [25]. These other chemokines may also contribute to the chemotaxis and infiltration of Iba1 positive cells in cornea from KO mice receiving topical BAC.

A previous study using a corneal alkali burn model, has demonstrated leukocyte infiltration (macrophages and T lymphocytes) and inflammatory markers expression in the ipsilateral trigeminal ganglion [26]. We also previously reported gene alteration in the TG and SP5 following 7 days of topical BAC. In order to know whether the inflammatory response observed in cornea induces activation of microglial cells in the trigeminal ganglion , immunohistological analysis of was performed.

We reported that during BAC-induced ocular inflammation, the ophthalmic region of the trigeminal ganglion from WT mice contain more activated Iba1 positive cells (amoeboid morphology with a large some and short processes) that WT PBS control mice, as previously reported (Launay 2016). In contrast, our preliminary results showed that TG from KO mice have similar pattern of Iba1 positive cells between both BAC and PBS groups. These results suggest that the absence of the CX3CR1 receptor blunted the BAC-induced TG inflammation, or at least, inhibited the activation of Iba1 positive cells. Moreover, in these KO animals, our preliminary observation revealed that topical BAC instillation did not impact the number of eGFP and Iba1 positive cells, suggesting an absence of macrophage infiltration. Again, quantification are in progress. In addition, we next evaluated the mRNA expression of pro inflammatory genes by RTqPCR. We found a correlation with the decreased Iba1 activation and mRNA expression of inflammatory markers such as CCL2, CCR2, GFAP and CD68, which are decreased in BAC-treated KO mice compared to BAC-treated WT mice. These preliminary data revealed that ocular inflammation observed in KO BAC -treated mice do not trigger inflammatory responses in trigeminal ganglion. We have to take into account that the number of animals for the RT-qPCR analysis was limited and it is mandatory to increase the number of mice per group to corroborate these data.

On the other hand, the BAC-induced ocular inflammation provoked the activation of microglial cells in the sensory trigeminal complex (Vi/Vc and Vc/C1 transition regions) from WT mice in comparison with control mice. Interestingly, we observed that BAC-treated KO mice shown less activated microglial cells with respect to the BAC-treated WT mice. Finally, although the number of mice was limited, we found that c-FOS mRNA was upregulated in KO mice in both BAC and PBS groups. Altogether, these data suggest that the absence of the CXC3CR1 blunted BAC-induced microglial activation as well as up regulation of pro inflammatory markers in the SP5.

V. CONCLUSION

This six-month internship provides novel information about the role of CX3CL1 and its receptor CX3CR1 during sustained ocular inflammation. We observed that after BAC treatment, *CX3CR1*$^{eGFP/eGFP}$ mice exhibit less inflammatory cell infiltration in cornea, less microglial activation in trigeminal ganglion and sensory trigeminal complex, and less mRNA expression of inflammatory markers in trigeminal ganglion compared to *CX3CR1*$^{+/+}$ mice. These results suggest a role of CX3CL1/CX3CR1 on neuroinflammation in the corneal nociceptive pathway.

Further investigations are needed to better understand the involvement of the CX3CR1 receptor in dry eyes and painful symptoms. The role of CX3CL1/CX3CR1 in the inflammation and pathogenesis of DED would help to translate results into solutions for the benefit of patients suffering from ocular pain because targeting epigenetic alterations may have therapeutic potential for chronic ocular pain.

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