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Role of atypical chemokine receptor ACKR2 in experimental oral squamous cell carcinogenesis

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ABSTRACT

Background: Chemokines and chemokine receptors are critical in oral tumourigenesis. The atypical chemokine receptor ACKR2 is a scavenger of CC chemokines controlling the availability of these molecules at tumour sites, but the role of ACKR2 in the context of oral carcinogenesis is unexplored.
Methods: In this study, wild-type (WT) and ACKR2 deficient mice (ACKR2$^{-/-}$) were treated with chemical carcinogen 4-nitroquinoline-1-oxide (4NQO) for induction of oral carcinogenesis. Tongues were collected for macro and microscopic analysis and to evaluate the expression of ACKRs, CC chemokines and its receptors, inflammatory cytokines, angiogenic factors, adhesion molecules and extracellular matrix components.
Results: An increased expression of ACKR2 in squamous cell carcinoma (SCC) lesions of 4NQO-treated WT mice was observed. No significant differences were seen in the ACKR1, ACKR3 and ACKR4 mRNA expression comparing SCC lesions from WT and ACKR2$^{-/-}$ treated mice. Significantly higher expression of CCL2, IL-6 and IL-17 was detected in ACKR2$^{-/-}$ treated mice. In contrast, the expression of other CC-chemokines, and receptors, angiogenic factors, adhesion molecules and extracellular matrix components were similarly increased in SCC lesions of both groups. Clinical and histopathological analysis revealed no differences in inflammatory cell recruitment and in the SCC incidence comparing WT and ACKR2$^{-/-}$ treated mice.
Conclusion: The results suggest that ACKR2 expression regulates inflammation in tumour-microenvironment but the absence of ACKR2 does not impact chemically-induced oral carcinogenesis.

1. Introduction

Oral squamous cell carcinoma (OSCC) is the most common oral neoplasm worldwide and frequently presents an unfavorable prognosis [1,2]. The locally infiltrative and metastasizing behavior of OSCC is linked to neoplastic cells and their surrounding stroma. The stroma comprises a wide range of molecules that may influence angiogenesis, tumour growth, invasion and metastasis [3]. In this regard, chemokines present critical functions in oral tumourigenesis [4–9]. The chemokines and/or chemokine receptors are expressed by neoplastic and stromal cells (i.e fibroblasts, inflammatory, endothelial cells) [10]. The chemokine/chemokine receptor signaling between neoplastic and stromal cells in tumour microenvironment may result in anti- or pro-tumour functions, where recruited immune effector cells may be albe to kill tumour cells [10] or where tumour proliferation, cell survival, induction of angiogenesis, increase of motility and invasiveness of neoplastic cells may be observed [4–8]. In this regard, increased expression of CC chemokines were observed in OSCC samples [8,9] and in experimentally-induced oral carcinomas [5,11] and associated with worse clinical and microscopic parameters of these tumours.

The availability of chemokines in tumour microenvironment might be regulated by the different atypical chemokine receptors, recently characterized, ACKR1 (DARC), ACKR2 (previously named D6 or CCBP2), ACKR3 (CXCR7) and ACKR4 (CCRL1) [12]. The ACKR2 binds

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with high affinity to CC chemokines acting as “scavenger” (i.e. internalizing, but without triggering intracellular signaling function) and therefore, controlling tumour-associated inflammatory response *in vivo* [12,13]. This receptor is expressed in tissues and cell types from distinct sources, as in lymphatic cells [14], skin [15] and neoplastic cells [16,17].

Protective effects of ACKR2 were demonstrated in chemically skin-induced tumours [15], Kaposi Sarcomas [16], colon adenocarcinomas [17], and breast cancer [18]. The lack of ACKR2 resulted in accumulation of CC chemokines and infiltration of leukocytes at tumour sites and these effects were associated with tumour growth and progression [14]. To date, no studies have focused in ACKR2 in oral cancer. In the current study, the involvement of ACKR2 in oral tumourigenesis was investigated employing a chemically-induced model of squamous cell carcinoma in mice.

2. Materials and methods

2.1. Animals

C57Bl/6 (wild type - WT) 6–8 weeks male mice were obtained from Centro de Bioterismo, Instituto de Ciências Biológicas (CEBIO), Universidade Federal de Minas Gerais, Brazil. ACKR2$^{-/-}$ mice were bred and obtained from Laboratory of Pulmonary Immunology and Mechanics, Department of Physiology and Biophysics, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Brazil. Mice were maintained in the Immunopharmacology laboratory’s animal facility under standard conditions with a 12 h light/dark cycle, controlled temperature (24 ± 2 °C) and free access to commercial chow and water *ad libidum*. All experimental procedures described in the current study were approved by the institutional Ethics Committee in Animal Experimentation (protocol 12/2011).

2.2. Experimental OSCC induction

The induction of oral carcinogenesis was performed as previously described [19,20]. 4-nitroquinoline-1-oxide (4NQO) (Sigma Aldrich, St. Louis, MO, USA) was obtained and dissolved in ethylene glycol (Sigma-Aldrich) and stored at 4 °C. Weekly, a stock solution was prepared and diluted in mice drinking water at 50 μg/mL or 200 μg/mL concentration. Experimental mice received 4NQO daily during 28 weeks and control mice received drinking water for the same period. After 28 weeks, mice were euthanized and tongue, cervical lymph nodes, liver, lungs, stomach, duodenum, jejunum, ileum and large intestine were collected for microscopic analysis. Additionally, tongues were photographed for macroscopic analysis.

2.3. Real time PCR array

Real Time PCR array reactions were performed as previously described [21]. Briefly, the total RNA was obtained from SCC lesions and clinically normal tongue samples (n = 4 per group). Extraction was performed with the RNeasyFFPE kit (Qiagen Inc, Valencia, CA) following the manufacturer instructions. The complementary DNA was synthesized by reverse transcription reaction (Superscript III, Invitrogen Corporation, Carlsbad, CA, USA) from 3 μg of total RNA and Real Time PCR array was performed employing a custom panel containing targets “Wound Healing” (PAMM-121) and “Inflammatory cytokines and receptors” (PAMM-011) (SABiosciences, Frederick, MD). For analysis of ACKR’s expression RealTime PCR was performed with Viia7 (Life Technologies, Pittsburgh, PA, USA) with TaqMan primers/probes sets according to the manufacturers' instructions (ACKR1, cat. 4331182; ACKR2, cat. 4331182; ACKR3, cat. 4351372; ACKR4, cat. 4331182 – Thermo Fisher Scientific, Waltham, MA USA), with standard amplification protocols. Data were analyzed by the RT2 profiler PCR Array Data Analysis online software (SABiosciences) for normalizing the initial geometric mean of the constitutive genes GAPDH, ACTB and Hprt1 and subsequently normalized by the control group, and expressed as fold change relative to the control group.

2.4. Histopathological analysis

The tongues, cervical lymph nodes, liver, lungs, stomach, duodenum, jejunum, ileum and large intestine of 11 wt and 11 ACKR2$^{-/-}$ mice were fixed in 10% buffered formalin, embedded in paraffin wax, longitudinally cut (3 μm sections) and stained with H&E. Histopathological analysis of tongue sections was performed by two oral pathologists (J.M.S and T.A.S) blinded from the group status. The Intraclass Correlation Coefficient test was performed to validate the reliability inter- and intra-examiner (ICC = 0.88). Ten representative fields were considered and the histopathological grading of tongue lesions was performed following the scores (adapted from [22]): 0 – normal epithelial architecture, 1 - mild dysplasia (changes limited at basal third of lining epithelium), 2 - moderate (changes in two-thirds of lining epithelium), 3 - severe (more than two-thirds), 4 - carcinoma *in situ* (full thickness of epithelium without involvement of connective tissue) and 5 - invasive carcinoma (carcinomatous islands into the connective tissue). Three sections of each organ were evaluated by a general pathologist (M.A.R) blinded of group status in order to evaluate the possible 4NQO effects at distant sites.

2.5. Eosinophils and mast cells staining

The Sirius Red staining was performed as previously described [5,11] in order to evaluate eosinophilic infiltration in SCC samples. Slides (n = 5 per group) were deparaffinized, subsequently incubated in Harris hematoxylin for two minutes, rinsed in tap water and in 100% ethanol. An alkaline (pH 8–9) Sirius Red solution (CI 35780, Sigma Aldrich) was used to incubate slides for two hours, 25 °C. Finally, slides were rinsed, dehydrated and covered. Mast cells infiltration was accessed using Toluidine Blue staining. Briefly, after deparaffinization and hydratation steps, slides were incubated in toluidine blue solution (saline phosphate buffer, pH 5.7 in 0.4 g of toluidine blue – cat. CI 52040, Vetec, São Paulo, SP, Brazil) for 2 min. Then, slides were rinsed, dehydrated in absolute ethanol and covered. The stained cells were counted in 20 consecutive fields of epithelium lining area and neoplastic invasive front areas (if present), at 400× magnification. Results were expressed as the mean of total number of eosinophils and mast cells, per sample.

2.6. Immunohistochemistry

The immunohistochemical staining was performed as previously reported [5]. Briefly, sections of tongue tumours were deparaffinized, dehydrated and rinsed in distilled water. The antigen retrieval was performed at 96 °C, 25 min in citric acid buffer (pH = 6.0), then, slides were incubated with 0.3% hydrogen peroxide 15 min twice and with Avidin/Biotin blocking system (Dako, Carpinteria, CA, USA) following the manufacturer instructions. Then, sections were incubated at 4 °C overnight with rabbit polyclonal anti-mouse Ki-67 (cat.15580, Abcam, Cambridge, England) 1:50; rat anti-mouse Ly-6G (cat. 562737, BD Biosciences, San Jose, CA, USA) 1:300. For F4/80, we used the APC/Cy7 rat anti-mouse F4/80 (clone BM8, Biolegend, San Diego, CA, USA) 1:100, overnight, followed by incubation with goat anti-rat IgG Alexa 546 (cat. A11081, Thermo Fisher Scientific, Rockford, IL, USA) 1:100, for one hour, room temperature. Tissues were incubated with LSAB secondary antibody kit (cat. K0675, Dako, Carpinteria, CA, USA) following the manufacturer’s instructions. Negative controls were obtained by omission of primary antibody, which were substituted by 1% PBS-BSA. The immunostained cells were analyzed by light microscope (Axioskop 40 ZEISS; Carl Zeiss, Gottingen, Germany) and counted in 20 consecutive fields in two sections. Results are expressed as the total

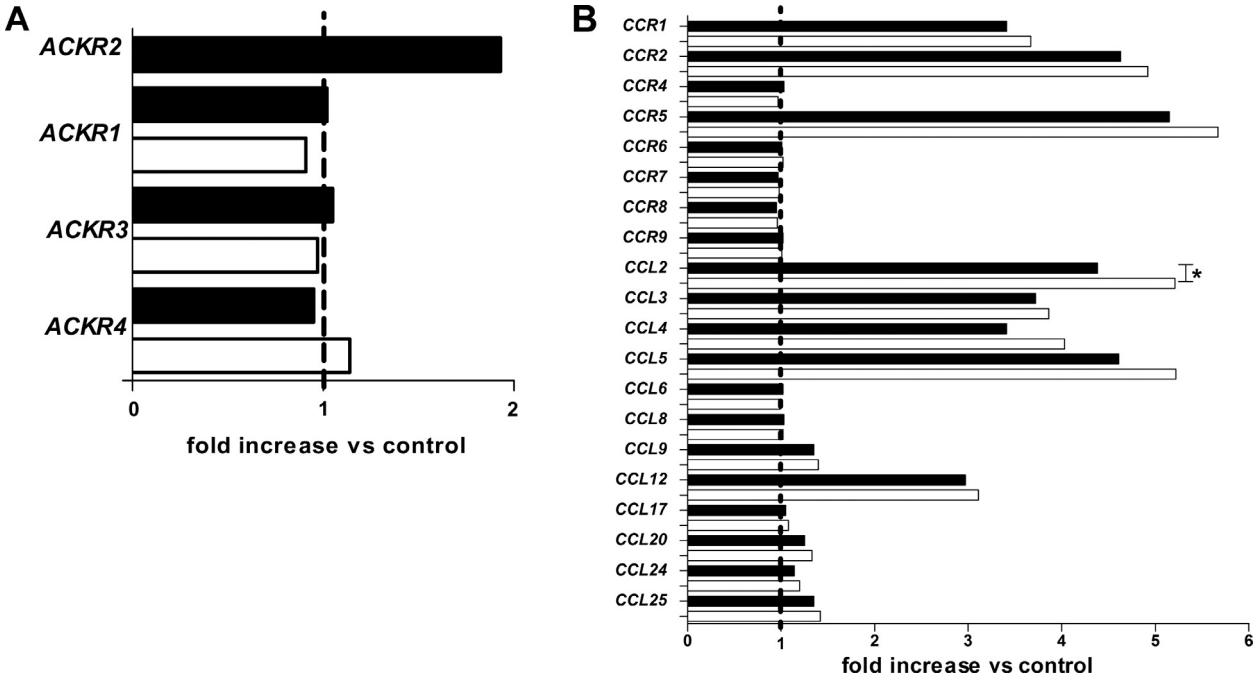

Fig. 1. Expression of atypical chemokine receptors (ACKR's), CC chemokines and receptors after 4NQO treatment. The expression was evaluated in SCC-induced tumours comparing WT (in black) and ACKR2$^{-/-}$ control and treated mice (white). Results were expressed as fold change relative to the control group after being normalized by constitutive genes (GAPDH, ACTB, Hprt1). A – ACKR's expression. B Expression of CC chemokines and its receptors.* $P < 0.05$ fold increase relative to the control.

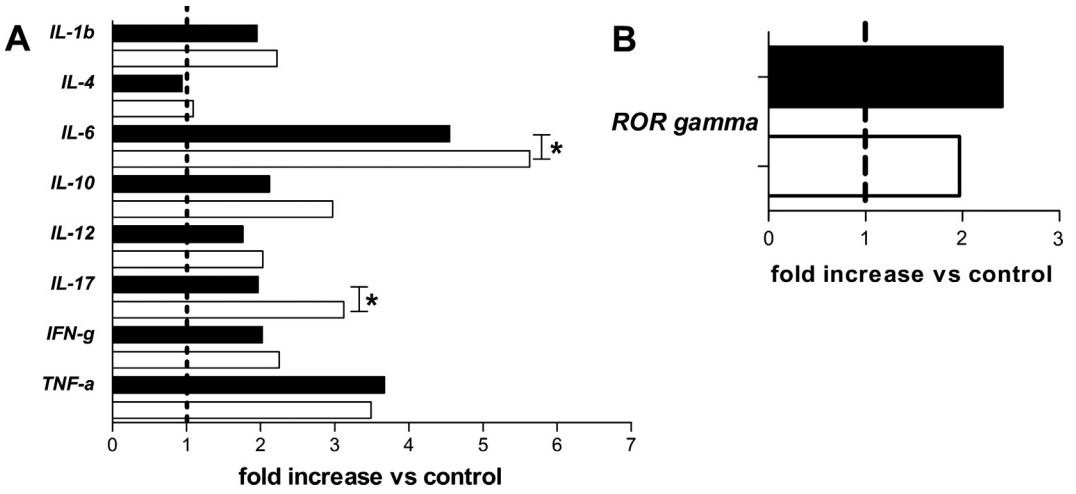

Fig. 2. Expression of inflammatory cytokines and the transcriptional factor RORγ after 4NQO treatment. The expression was evaluated in SCC-induced tumours comparing WT (in black) and ACKR2$^{-/-}$ control and treated mice (white). Results were expressed as fold change relative to the control group after being normalized by constitutive genes (GAPDH, ACTB, Hprt1). A – Inflammatory cytokines and B – RORγ mRNA expression. * $P < 0.05$ fold increase relative to the control.

number of Ki67^{+} cells in all epithelial layers and in neoplastic invasive front, per field and the total number of Ly-6G^{+} and F4/80^{+} cells under epithelial lining area and in peri or intratumoural areas, per sample.

2.7. Statistical analysis

Statistical analysis was performed using the software GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). The Student *t*-test was performed after checking for data normality. The results are reported as the means ± SD. P values < 0.05 are considered to be statistically significant. For PCR array data, statistical significance was evaluated by the Mann-Whitney test, and the values tested for correction by the Benjamini - Hochberg Procedure.

3. Results

3.1. Molecular analysis of SCC lesions

The expression of ACKR2 was analyzed in control untreated tongues and in SCC tongue lesions of WT mice. Results showed an increase of ACKR2 expression after 4NQO treatment (Fig. 1A). The expression of other ACKR's (i.e. ACKR1, ACKR3 and ACKR4) was similar when comparing WT and ACKR2$^{-/-}$ treated mice (Fig. 1A).

Taking into account the CC chemokine "scavenging" ability reported to ACKR2 in controlling tumour-associated inflammatory response, the second step consisted in evaluate the expression of CC-chemokines and their receptors and inflammatory markers. Interestingly, results showed an increase of chemokines CCL2, CCL3, CCL4, CCL5 and CCL12 - and the receptors CCR1, CCR2 and CCR5 in WT and ACKR2$^{-/-}$ treated with 4NQO when compared with non-

treated controls (Fig. 1B). In addition, the mRNA expression of chemokine CCL2 was found -one fold increase higher in SCC -induced lesions from ACKR2$^{-/-}$ in relation to WT (Fig. 1B).

Further, the expression of Interleukin (IL)-1β, IL-4, IL-6, IL-10, IL-12, IL-17, Interferon gamma - IFN-γ, and Tumour Necrosis Factor-alpha – TNF-α were also analyzed in tongue lesions. Consistently, all inflammatory cytokines analyzed showed increase in WT and ACKR2$^{-/-}$ treated groups in relation to controls, except for IL-4. The expression of IL-6 and IL-17 were significantly enhanced in ACKR2$^{-/-}$ treated when compared to WT treated mice (Fig. 2A). In view of the IL-17 increase in SCC lesions from ACKR2$^{-/-}$ group, we next evaluated mRNA expression of Retinoic acid receptor-related orphan receptor gamma (RORγ), a transcriptional factor involved in Th17 cell differentiation [23]. However, no differences were seen when comparing RORγ expression in WT and ACKR2$^{-/-}$ treated groups (Fig. 2B).We also analyzed the expression of angiogenic factors, cellular adhesion molecules and extracellular matrix components. Results showed significant similar increased expression of angiogenic (Epidermal Growth Factor – EGF, Fibroblast Growth Factor 1 – FGF1and FGF2, Vascular Endothelial Growth Factor a – VEGFa and VEGFb), the adhesion proteins (Collagen 1a1 – COL1a1, COL1a2, Integrin A4 – ITGA4, and Vitronectin – VTN) and extracellular matrix components (Matrix Metalloproteinase 1a – MMP-1a, MMP-8, MMP-9, Tissue inhibitor of metalloproteinase1 - TIMP1 and TIMP3 and SERPINE) in induced lesions of both groups after 4NQO treatment in relation to the controls (Fig. 3). No significant differences were seen in the expression of these matrix molecules when comparing SCC lesions from ACKR2$^{-/-}$ and WT treated mice (Fig. 3).

3.2. Leukocyte infiltration in SCC lesions

ACKR2 deficiency was previously associated with increased infiltration of leucocytes at tumour sites [14,15]. We evaluated the presence of infiltrating eosinophils in lesions from WT and ACKR2$^{-/-}$ mice. Results showed significant increase in the number of Sirius Red stained eosinophils when comparing WT and ACKR2$^{-/-}$ in relation to respective non-treated controls. On the other hand, no significant difference when comparing induced lesions from WT and ACKR2$^{-/-}$ mice were observed (Fig. 4A, 4E and 4I). Mice submitted to treatment with 4NQO at 200 μg/mL also did not present significant difference of infiltrating eosinophils at tumour sites when comparing WT and ACKR2$^{-/-}$ groups (Fig. 4I).

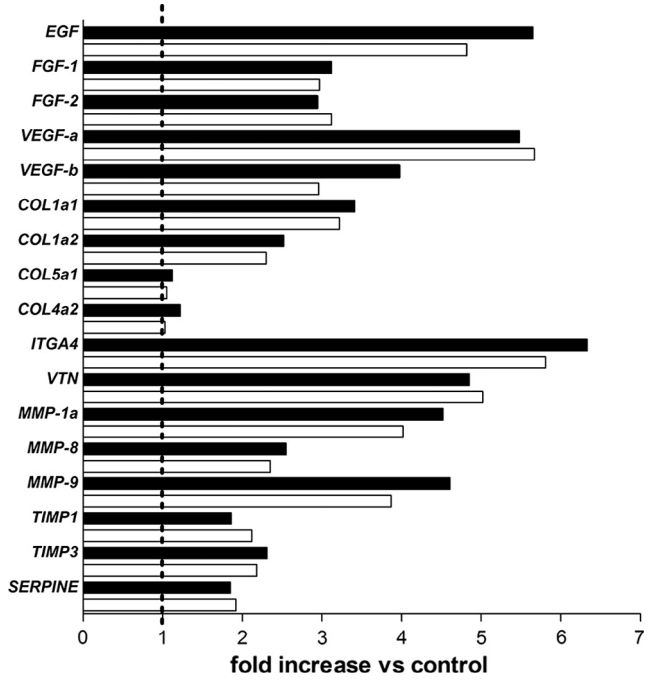

Fig. 3. Expression of angiogenic, adhesion proteins and extracellular matrix remodeling markers after 4NQO treatment. The expression was evaluated in SCC-induced tumours comparing WT (in black) and ACKR2$^{-/-}$ control and treated mice (white). Results were expressed as fold change relative to the control group after being normalized by constitutive genes (GAPDH, ACTB, Hprt1). * $P < 0.05$ fold increase relative to the control.

The number of infiltrated neutrophils, mast cells and macrophages were also accessed in samples of WT and ACKR2$^{-/-}$ mice. Consistent with the eosinophil cell counting, 4NQO treatment induced a significant increase in Ly-6G^{+} neutrophils in WT and ACKR2$^{-/-}$ mice in relation to respective non-treated controls, but no differences were found when comparing WT and ACKR2$^{-/-}$ treated mice (Fig. 4B, F and J). Similarly, mast cells and F4/80^{+} macrophages counting revealed a similar pattern between the groups (Fig. 4C, G and K and D, H and L, respectively).

3.3. Clinical and histopathological analysis

Clinical examination of SCC-induced lesions showed apparent similarity in tumour formation in WT and ACKR2$^{-/-}$ treated mice (Fig. 5C and D). The lesions were mostly exophytic (protruding from tongue surface) or papillomatous, whitish and base attached (Fig. 5C and D). No macroscopic changes in tongue surface were observed in the control groups (Fig. 5A and B). Microscopic analysis was consistent with the clinical findings and results showed increased cytomorphological atypia and similar scores of epithelial dysplasia in WT and ACKR2$^{-/-}$ treated groups (Fig. 6C, D and G). When WT and ACKR2$^{-/-}$ mice were submitted to a “more aggressive” OSCC model by using four times greater concentration of 4NQO (200 μg/mL), increased cytomorphological atypia was observed with 100% of lesions scored as invasive carcinoma in both treated groups (Fig. 6E, F and G). No differences were observed comparing WT and ACKR2$^{-/-}$ mice between the different 4NQO concentrations tested (Fig. 6G). In addition, no signs of tumour spreading were observed in our conditions.

The immunohistochemical staining revealed significant increased percentage of Ki67 immunopositivity of neoplastic cells in WT and ACKR2$^{-/-}$ treated mice in relation to the controls (Fig. 6H). However, no significant difference was seen when comparing Ki67^{+} cells between WT and ACKR2$^{-/-}$ treated mice (Fig. 6H).

4. Discussion

The aim of this study was to investigate the role of the atypical chemokine receptor ACKR2 in oral tumourigenesis. Our results showed that: (1) Chemically-induced SCC exhibit augmented ACKR2 expression, (2) ACKR2 deletion resulted in significant augment of the inflammatory molecules CCL2, IL-6 and IL-17 at tumour sites; (3) The absence of ACKR2 did not modify eosinophil, neutrophil, mast cells or macrophage infiltration to the tumour microenvironment; (4) Macro and microscopical analysis confirmed that ACKR2 did not affect SCC formation, neither histopathological grading or tumour proliferation. Consequently, ACKR2 seems to play a minor role during chemically-induced oral tumourigenesis.

The atypical chemokine receptor ACKR2 is characterized as regulator of the chemokine system, specifically promoting clearance of CC chemokines and controlling tumour-associated inflammatory responses [14,15,18]. In this regard, protective roles of ACKR2 were observed in malignancies as in Kaposi Sarcomas [16], colon adenocarcinomas [17], breast cancer [18], and in chemically skin-induced tumours [15] were the absence of ACKR2 resulted in accumulation of CC chemokines and infiltration of leukocytes and promoted tumour growth and progression. The axis CCL3/CCR5 was described to display pro-tumourigenic activities in 4NQO-induced oral tumours, for example, where it was demonstrated to influence SCC formation, severity of induced lesions, infiltration of eosinophils, expression of angiogenic, inflammatory cytokines and matrix metalloproteinases *in vivo* and neoplastic cell

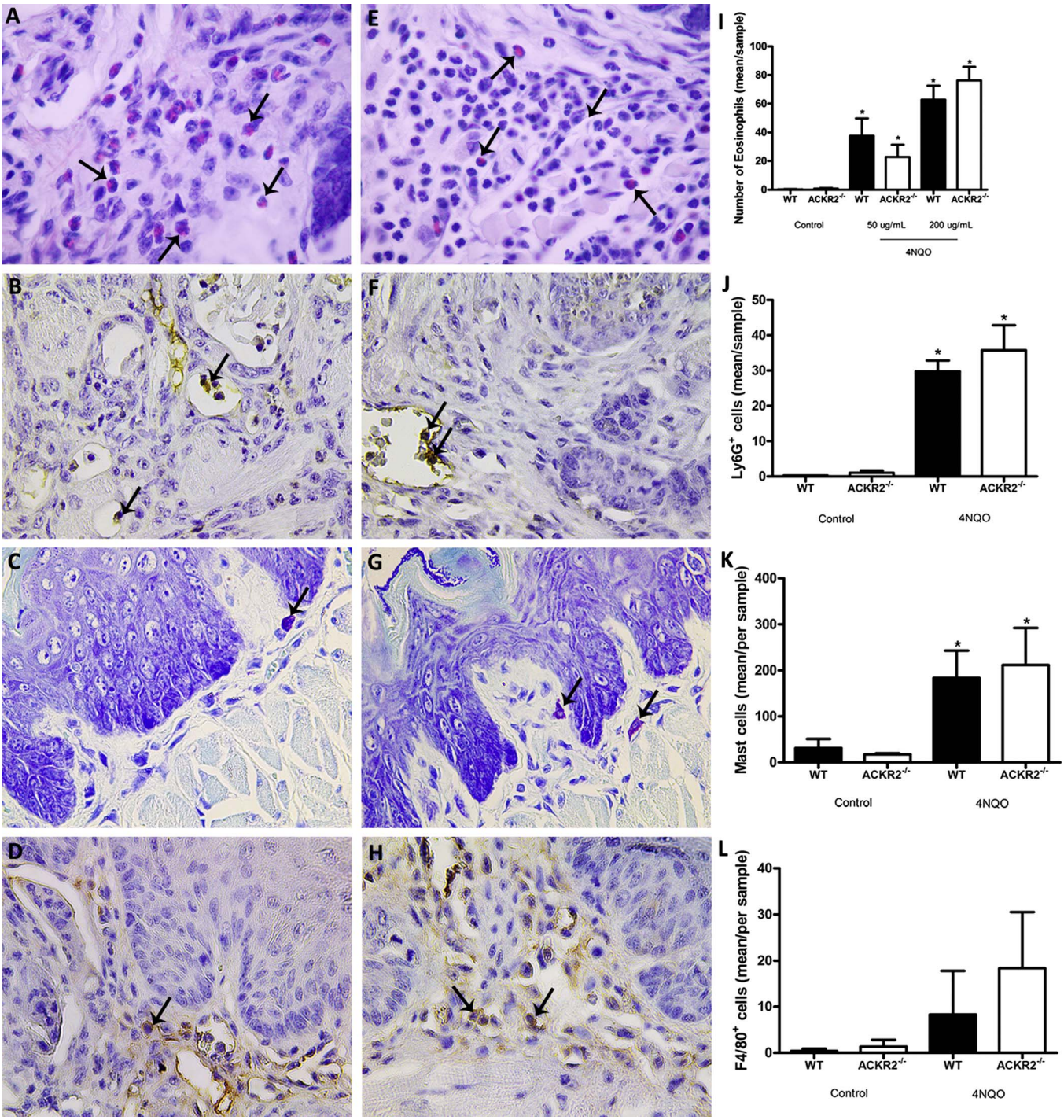

Fig. 4. Leukocytes recruitment to SCC-induced lesions. A and E – Represent respectively the Sirius Red stained eosinophils (arrows) in tongue lesions of WT and ACKR2$^{-/-}$ treated mice (4NQO dose of 50 μg/mL) 1000× original magnification. I - Quantification of Sirius Red stained eosinophils in WT and ACKR2$^{-/-}$ control and treated groups with different doses of 4NQO. B and F – Representative images of recruited Ly-6G^{+} neutrophils (arrows) in induced-SCC from WT and ACKR2$^{-/-}$ mice submitted to 200 μg/mL dose, respectively. J – Quantification of Ly-6G^{+} neutrophils in control and treated groups. C and G – Stained mast cells in WT and ACKR2$^{-/-}$ (4NQO dose of 200 μg/mL), and, K - Quantification data. D and H – Represent respectively the F4/80^{+} macrophages in WT and ACKR2$^{-/-}$ mice (4NQO dose of 200 μg/mL) and L - Quantification data. (B, C, D, F, G and H at 600× original magnification). *$p < 0.05$ relative to the respective control.

invasion *in vitro* [5]. Consistently, there are many evidences linking the elevated expression of CC chemokines in oral cancer samples, lymph node metastasis and worse clinical parameters of tumours [4]. Thus, because tumour milieu is a rich environment that comprises a wide range of molecules as chemokines, growth factors, inflammatory mediators that may interfere with tumourigenesis [24] and as ACKR2 was demonstrated to efficiently control inflammatory response *in vivo*, we first evaluated ACKR2 expression in WT mice treated or not with 4NQO. The augmented ACKR2 expression in 4NQO-treated mice, lead us to explore the ACKR2 functions in this model. We next analyzed the β-chemokines expression and did find an increase after 4NQO treatment, but only CCL2 was significantly expressed in SCC lesions in the absence of ACKR2. These findings are in accordance with previous reports [5,13–15,25,26] and may corroborate the CC-chemokine scavenging abilities of ACKR2.

The expression of inflammatory cytokines showed significant two-

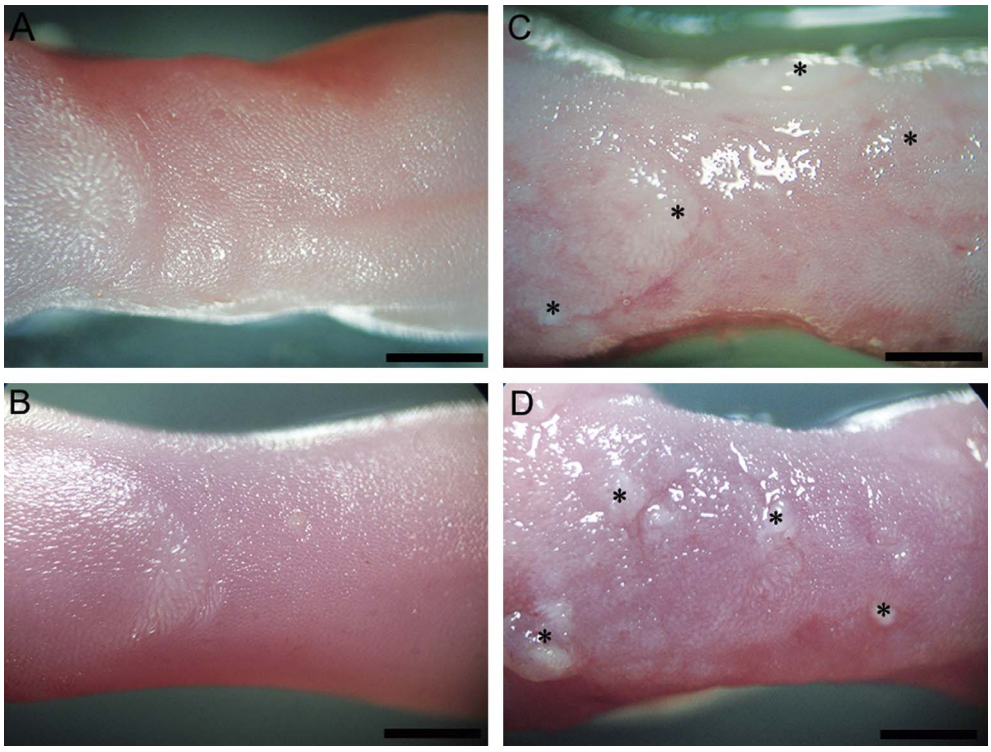

Fig. 5. Clinical findings of SCC-induced lesions in WT and ACKR2$^{-/-}$ mice after 4NQO treatment. A and B - Macroscopic tongue appearance of WT and ACKR2$^{-/-}$ control mice. C and D - Represent respectively the SCC-induced lesions in WT and ACKR2$^{-/-}$ mice. Asterisks indicate clinical SCC lesions (Barr = 0.1 cm).

to six fold increase in relation to controls in most of evaluated molecules - IL-10, IFN-γ and TNF-α - which was similar when comparing WT and ACKR2$^{-/-}$ mice. Interestingly, IL-6 and IL-17 were significantly increased in ACKR2$^{-/-}$ compared to WT. Consistently, increased production of TNF-α and IL-17A was seen in inflamed joints of ACKR2$^{-/-}$ arthritic mice compared to WT [25]. In this latter study ACKR2 deletion resulted in enhanced TNF-α, IFN-γ and IL-17A levels in late stages of DSS-induced colitis, whereas CC chemokines and IL-2, IL-10, IL-12 or IL-23 remained unchanged, regardless of better clinical symptoms and histological score [26]. They also demonstrated an increased IL-17A production in tumours from ACKR2 deficient mice that was correlated with significant infiltration of γδ T cells into inflamed colon [26]. Despite the augmented levels of IL-17 in SCC of ACKR2$^{-/-}$ mice, we could not detect differences in mRNA expression of RORγ, a transcriptional factor related with Th17 cell differentiation [23] when comparing 4NQO-induced tumours from WT and ACKR2$^{-/-}$ mice.

Taking into account the increased activation of some inflammatory pathways in SCC-induced lesions in absence of ACKR2, we next evaluated the expression of mediators involved with tumour proliferation, angiogenesis and tissue remodeling. Herein, we did observe that 4NQO treatment induced coherent increase of proliferative (EGF), angiogenic (FGF1 and FGF2, VEGFa and VEGFb) and tissue remodeling (collagen, adhesion proteins, MMP's and TIMP's) markers in SCC-induced lesions, although no significative differences were achieved when comparing

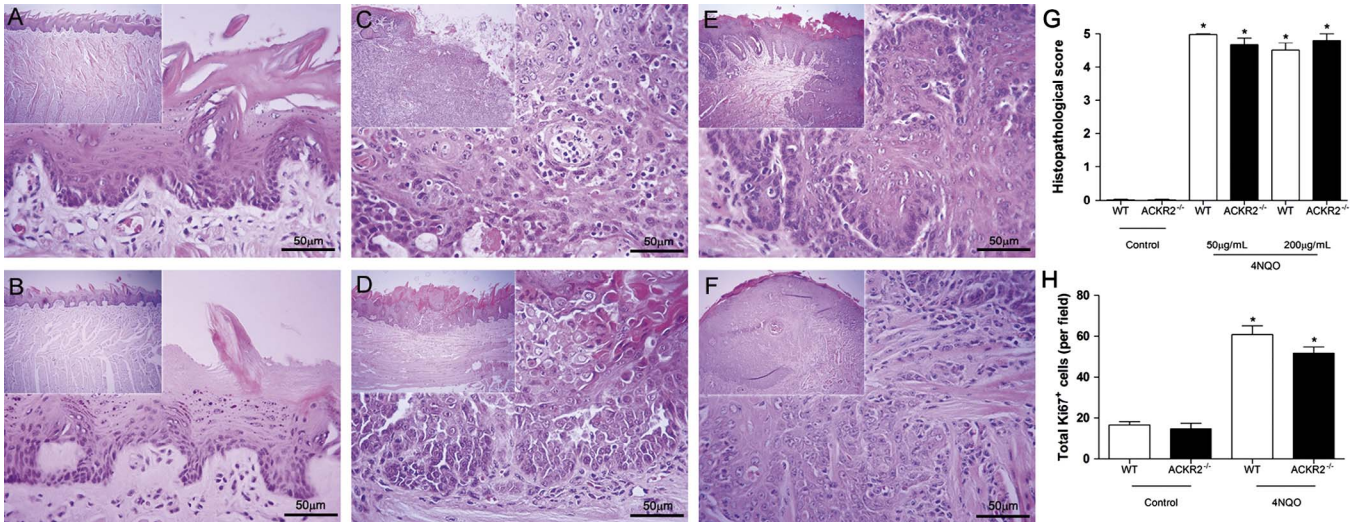

Fig. 6. Representative histopathological findings in controls and 4NQO treated mice. A and B - Absence of epithelial abnormality in WT and ACKR2$^{-/-}$ control mice. C and D - Represent the microscopic aspect of induced lesions of WT and ACKR2$^{-/-}$ treated with 4NQO at 50 μg/mL, and E and F - WT and ACKR2$^{-/-}$ at 200 μg/mL. G - Histopathological scoring of tongue lesions. H - Quantitative analysis of Ki67 immunoexpression in basal and suprabasal epithelial layers in control and treated groups $^{*}p < 0.05$ in relation to respective control.

ACKR2$^{-/-}$ with WT. In contrast, a study showed increase of VEGFa and CD31 in transfected Kaposi sarcoma sections that did not express the receptor ACKR2 (KS^{-}) [16]. The significance of these mediators triggering cell proliferation, angiogenesis and extracellular matrix remodeling were reported in oral tumourigenesis [27,28]. Current results suggest that ACKR2 does not affect proliferation/angiogenic and matrix remodeling pathways in our conditions.

The suggested protective effect of ACKR2 is related to its anti-inflammatory properties due to reduced availability of inflammatory mediators and infiltration of leukocytes [14–16,18]. We observed that 4NQO induced increased eosinophil, neutrophil and mast cell infiltration in tumours of WT and ACKR2$^{-/-}$ groups in relation to controls, but ACKR2 deficiency did not trigger significant changes in recruitment of these subtypes of leukocytes in relation to tumours induced in WT mice. One limitation of this chronic model of study was that in our conditions only one time-point was evaluated (28 weeks) and this may preclude drawing inferences of ACKR2 along neoplastic events. Some other preclinical models, on the other hand, have demonstred that mice lacking ACKR2 were more susceptible to tumour formation when compared to WT in models with constant induction of inflammation (i.e. DSS-induced colon cancer and DMBA/TPA skin-induced tumours) [14,15].

The next step consisted in evaluate whether ACKR2 deficiency would impact in tumour formation. Herein, both ACKR2$^{-/-}$ and WT treated mice had similar characteristics in SCC-induced lesions, regarding macroscopic and histopathological parameters. The current findings are in accordance with a previous study showing that mice lacking ACKR2 did not differ for hepatic tumour formation when compared with WT, although diethylnitrosamine (DEN)-induced hepatocarcinogenesis increased intrahepatic expression of CC chemokines and recruitment of macrophages in ACKR2$^{-/-}$ mice [25]. Despite these findings, authors suggested that these changes were not sufficient to influence tumour formation and progression [25]. Because we did not observe significant difference in SCC induction between WT and ACKR2$^{-/-}$, we submitted mice to a high-dose of 4NQO, as previously reported [20]. Nevertheless, no difference in clinical or microscopic parameters was detected between the groups. Also, high-dose of 4NQO did not induce lymph node or distant metastasis. These results are corroborated by previous studies where 4NQO treatment at 50 or 200 μg/mL did not induce metastatic lesions [5,11]. Only one previous study did observe the metastasis in cervical lymph nodes [20] but these findings could not be reproduced in our experimental conditions. A recent report described paradoxical functions of ACKR2 regarding anti-metastatic potential [29]. In this study, ACKR2 absence resulted in increased growth of primary mammary tumours, as observed in previous studies, but ACKR2 deficient mice were protected from metastasis by influx of bone-marrow-derived neutrophils in mammary carcinoma and melanoma models by producing increased levels of reactive oxygen species (ROS) [29].

In addition, the proliferative index at tongue lesions was consistent with our clinical and histopathological findings. An increased index of brown nuclear staining in neoplastic epithelial cells were similarly seen in WT and ACKR2$^{-/-}$ treated mice suggesting that ACKR2 does not affect cell proliferation. However, a previous study did observe a significant increase of epidermal proliferation in basal and suprabasal layers after skin topic application of phorbol-ester in ACKR2$^{-/-}$ mice [13].

Considering that other ACKR's are implicated in cancer progression [12], the expression of ACKR1 (previously called DARC), ACKR3 (termed CXCR7) and ACKR4 (CCRL1) was also evaluated in this study. ACKR1 and ACKR4 showed protective roles in tumours. For example, ACKR1 which is expressed by red blood cells promoted scavenging of angiogenic chemokines and suppressed blood vessel density and prostate tumour growth [30]. ACKR4 was demonstred to control tumour growth and metastasis because its properties of scavenging homeostatic chemokines CCL19/CCL21 in hepatocellular carcinomas [31]. On the other hand, ACKR3 exhibited dual functions in breast cancer once its expression by cancer cells promoted tumour proliferation and angiogenesis *in vitro*, whereas it was able to impair cell invasion in response to metastatic chemokine CXCL12 *in vivo* and *in vitro* [32]. Regardless of evidence supporting the role of these receptors in carcinogenesis, we did not detect differences in ACKRs expression comparing control tongues and SCC lesions.

In conclusion, despite similarity of histopathological grade of SCC lesions, the expression of inflammatory molecules CCL2, IL-6 and IL-17 was significantly increased in lesions of ACKR2$^{-/-}$ mice. Our results demonstrated that ACKR2 modify inflammatory pathways in SCC, but these changes are not sufficient to modify the course of tumourigenesis.

5. Conflict of interest statement

None declared.

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Conflict of interest

None declared.

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