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**Introduction**

Oral squamous cell carcinoma (OSCC) is a major subcategory of head and neck squamous cell carcinomas (HNSCC) and is currently the 6<sup>th</sup> highest cause of cancer mortality worldwide (Bray et al., 2018; Siegel, Miller, & Jemal, 2019). Despite current treatments such as surgery with adjunctive radiation and chemotherapy, the 5-year survival has remained at 50% for decades (Kumar, Nanavati, Modi, & Dobariya, 2016; Rivera, 2015). Most human solid cancers are believed to be driven by chronic inflammation that instigates nascent tumour proliferation (dysplasia), transformation and metastasis while conversely suppressing anti-tumour immune responses (Taniguchi & Karin, 2018). Inflammation promoted by dysregulated NF- $\kappa$ B (nuclear factor of kappa B) is also a hallmark of early OSCC (Cancer Genome Atlas, 2015; Tang et al., 2015). Lack of knowledge of this molecular driver is a key feature limiting curative outcomes.

Carcinogens and pro-inflammatory agents such as tobacco and alcohol are known risk factors for the development of OSCC, while human papillomavirus has been identified as a driver etiological agent in only a small number of oral cavity SCC, up to 5%. Further, the potentially malignant oral mucosal conditions leukoplakia (Warnakulasuriya &

Ariyawardana, 2016) and oral lichen planus (Idrees, Kujan, Shearston, & Farah, 2020) enhance the likelihood of OSCC development. Pertinently, the chronic inflammatory status of oral lichen planus is strongly associated with increased risk for OSCC development. Thus, chronically inflamed oral mucosal as well as the inflammatory response to oral carcinogens, has been proposed to contribute to the accumulation of genetic lesions and epithelial keratinocyte transformation (Rivera, 2015).

The transcription factor NF- $\kappa$ B is a dimer generated from any of the five subunits: RelB, RelA (p65), c-Rel, p50/p105 (NF- $\kappa$ B1) and p52/p100 (NF- $\kappa$ B2), (Figure 1A) (Gerondakis et al., 2006; Zhang, Lenardo, & Baltimore, 2017). Some of the heterodimers, such as RelA:p50 and c-Rel:p50, are primary drivers of inflammation and promote transcription of hundreds of target genes. Other dimers, for example p50:p50 homodimer, may act as transcriptional repressors and this complexity makes it difficult to determine the role of NF- $\kappa$ B in any given situation. Thus, NF- $\kappa$ B has been shown to be required for survival and effector functions of lymphoid cells (Gerondakis et al., 2006), while conversely sustained NF- $\kappa$ B activation is implicated in lymphoid and epithelial malignancies (Burkitt et al., 2015; Concetti & Wilson 2018; O'Reilly et al., 2018). Similarly, NF- $\kappa$ B has been implicated in both promoting and inhibiting OSCC (Cancer Genome Atlas, 2015; Chen et al., 2018A; Chen et al., 2018B; Kong et al., 2020; Ye et al., 2019). To obtain a clearer picture of the role of individual NF- $\kappa$ B subunits in OSCC development, we took a genetic approach and investigated the effects of NF- $\kappa$ B1 and c-Rel subunit loss in the oral carcinogen 4-nitroquinoline-1 oxide (4-NQO) model of OSCC.

## **Materials and Methods**

### *Mice*

Adequate measures were taken to minimize pain and discomfort. Experiments were carried out in accordance with the Guidelines laid down by the National Institute of Health (NIH), USA regarding the care and use of animals for experimental procedures and in accordance with The Walter and Eliza Hall Institute of Medical Research (WEHI) Animals Ethics Committee (AEC 2018.042 approved 28/11/2018). *Nfkb1*<sup>-/-</sup> (Sha, Liou, Tuomanen, & Baltimore, 1995) and *c-Rel*<sup>-/-</sup> mice (Gerondakis et al., 2006; Kontgen et al., 1995) were generated on a mixed C57BL/6x129SV background and backcrossed onto a C57BL/6

background for >10 generations. Control wt (C57BL/6) mice were housed in adjacent boxes within the same room.

#### *4-NQO induction of oral cavity lesions*

Oral cavity lesions were induced as previously described (Li et al., 2020; Tang, Knudsen, Bemis, Tickoo, & Gudas, 2004). Briefly, 10 week old male and female wild type (C57BL/6), *Nfkb1*<sup>-/-</sup> or *c-Rel*<sup>-/-</sup> mice were treated with regular drinking water ± 100 µg/ml 4-NQO (Sigma) for 14 weeks. Wt n= 6 mice per treatment (control [3M, 3F] or 4-NQO [3M, 3F]), *Nfkb1*<sup>-/-</sup> n= 4 mice per treatment (control [3M, 1F] or 4-NQO [2M, 2F]) and *c-Rel*<sup>-/-</sup> n= 5 (4-NQO [2F, 3M]) and n= 3 (control [1F, 2M]). From 14 weeks on, all mice were given regular drinking water until sacrifice 3 weeks later at week 17 (Figure 1B).

#### *Oral lesion grading*

Seventeen weeks post 4-NQO commencement, the incidence, development, and extent of oral lesions were assessed clinically *in vivo*, while mice were anesthetized with isoflurane. The oral cavity examination, including the anteroventral and dorsal tongue, palate, floor of the mouth, lips, and right and left buccal mucosa, was examined and scored in anaesthetized mice using specialized tailored oral cavity inspection tools (Figure 1C, Figure 2A) and a visual gross tongue lesion grading system, consisting of five grades that was adapted from Tang et al. (Tang et al., 2004) (Figure 2). Oral lesion number and size were also quantitated. The histological diagnosis was confirmed by two pathologists (Huang and Zhang, from Nanjing Stomatological Hospital, Medical School of Nanjing University, China) on the hematoxylin/eosin stained, sectioned tissue samples.

#### *Statistical analysis*

All statistical analyses were performed using GraphPad Prism 7 software. Means comparisons were performed using an ANOVA with post hoc Tukey's t-test and Fisher's exact test for categorical data with significance determined as p<0.05. Results are graphically presented as the mean ± standard deviation.

## **Results**

The transcription factor NF-κB plays a pivotal role in the response to noxious agents (Figure 1A). 4-NQO is a potent oral carcinogen, that when administered in drinking water, induces

inflammatory and proliferative lesions of the tongue, lips and buccal mucosae in mice (Li et al., 2020; Tang, Knudsen, Bemis, Tickoo, & Gudas, 2004). To assess the role of NF- $\kappa$ B subunits in this response we treated *Nfkb1*<sup>-/-</sup> *c-Rel*<sup>-/-</sup> and C57BL/6 wild type with 4-NQO (100 $\mu$ g/mL, *ad libitum*; Figure 1B). Three weeks post cessation of 4-NQO treatment oral pathology was readily observed (Figure 1C-F) and its grade was assessed (Figure 2A). *Nfkb1*<sup>-/-</sup> mice displayed a marked trend in disease acceleration. We observed an increased severity of lesions, increased mucosal alterations, including erythema, diffuse erosions, more leukoplakias, as well as increased number and area of oral lesions (Figure 2B-E) compared to untreated *Nfkb1*<sup>-/-</sup> and a trend to increased severity and number of lesions compared to wt mice treated with 4-NQO.

In a similar fashion, genetic deletion of c-Rel (*c-Rel*<sup>-/-</sup>) resulted in mice being more susceptible to 4-NQO induced OSCC with a statistically significant increase in the severity, number and area of lesions observed compared to wt control mice treated with 4-NQO (Figure 1F, Figure 2B-E). These results clearly demonstrate that both the NF- $\kappa$ B subunits NF- $\kappa$ B1 and c-Rel, play an important tumor suppressor role in the 4-NQO model of OSCC.

## Discussion

We investigated the effect of individual NF- $\kappa$ B subunit (NF- $\kappa$ B1/p50 and c-Rel) loss on the early inflammatory stages of oral carcinogenesis, using the 4-NQO mice model. This model reflects the clinical setting quite closely, inducing a heterogeneous range of lesions (hyperplasia, varying degrees of squamous cell dysplasia, carcinomas) with multiple genetic modifications (Tang, Knudsen, Bemis, Tickoo, & Gudas, 2004). Carcinogens and inflammatory agents contribute to the accumulation of genetic and epigenetic lesions throughout the oral mucosa, affecting cell cycle, DNA repair mechanisms, cell differentiation and apoptosis, ultimately resulting in the transformation of normal keratinocytes (Rivera, 2015). Many of these pathways are influenced by genes induced or repressed by NF- $\kappa$ B activation (Taniguchi & Karin, 2018; Zhang et al., 2017).

In contrast to other NF- $\kappa$ B proteins that are ubiquitously expressed, c-Rel is mainly expressed in mature haematopoietic cells (Grumont & Gerondakis, 1990). Accordingly, mice lacking c-Rel exhibit defects in the activation of lymphocytes, manifesting in reduced cytokine production (Gerondakis et al., 2006; Gilmore & Gerondakis, 2011). Despite these defects in

immune cell activation, c-Rel loss is not detrimental to the survival or overall immune function in mice (Kontgen et al., 1995; O'Reilly et al., 2015). Loss of c-Rel may have exacerbated early pathology in the 4-NQO OSCC model due to loss of c-Rel-mediated signalling and regulation of cell turnover following DNA damage, as previously reported for c-Rel loss in another DNA damage induced epithelial cancer model (Burkitt, et al., 2015). On the other hand, mice lacking NF- $\kappa$ B1 exhibit a range of defects; aberrant lymphocyte and myeloid cell activation, abnormalities in epithelial cell proliferation and survival (de Valle et al., 2016; O'Reilly et al., 2018; Sha et al., 1995). Given the increased severity of the OSCC lesions, and the similarities to the previously shown accelerated development of gastric cancer in *Nfkb1*<sup>-/-</sup> mice (O'Reilly et al., 2018), we hypothesise that NF- $\kappa$ B heterodimers containing these subunits act to limit inflammation in this model. Interestingly, NF- $\kappa$ B has been designated as both a promoter and inhibitor of OSCC (Cancer Genome Atlas, 2015; Chen et al., 2018A; Chen et al., 2018B; Kong et al., 2020; Ye et al., 2019) and NF- $\kappa$ B (NF $\kappa$ B1) transcriptional networks have been demonstrated to be modified at early stages in the 4-NQO model (Tang et al., 2015).

Tumour cells interact with their milieu and infiltrating immune cells in a unique manner and our data shows that constitutive loss of NF- $\kappa$ B1 or c-Rel accelerates the severity of OSCC in the 4-NQO mouse model. These NF- $\kappa$ B subunits have been implicated in both promoting and inhibiting the human disease (Cancer Genome Atlas, 2015; Chen et al., 2018B; Gupta et al., 2018). Considering that NF- $\kappa$ B sub-units play different roles in unique cell types, it would be of considerable interest to now understand the cell types within which these NF- $\kappa$ B components play a role in the 4-NQO model by using mice where the *Nfkb1* or *c-Rel* genes are conditionally deleted. Using our genetic models, we will now be able to investigate OSCC development at the molecular level. These investigations will also allow us to model new treatments that may have translational potential.

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## Figure Legends

### Figure 1. NF- $\kappa$ B dysregulation and 4-NQO induction combine to accelerate OSCC.

(A) An inflammatory response activates IKK2 kinase to phosphorylate and inactivate the inhibitory I $\kappa$ B proteins that restrain NF- $\kappa$ B dimers (e.g. NF- $\kappa$ B1:c-Rel and NF- $\kappa$ B1:RelA) in the cytosol of unstimulated cells. Inactivation of I $\kappa$ B inhibitory proteins liberates NF- $\kappa$ B dimers allowing them to enter the nucleus and regulate gene transcription. (B) Schematic of the 4-NQO regimen. Mice were given 4-NQO or regular drinking water for 14 wks then regular water for a further 3 weeks. Triangles = time points for harvest (red =scoring). (C-F) Representative photographs of tongues, *in situ*, of control mice and those treated with 4-NQO of the indicated genotypes.

### Figure 2. Loss of NF- $\kappa$ B1 or c-Rel accelerates onset and severity of 4-NQO induced oral lesions.

(A) Representative photographs of grading and measurement of gross tongue lesions. (B) Gross tongue lesion grading system: Grade 0 (Normal): no visible lesions; Grade 1 (Mild): discoloration/ erythema/ erosions; Grade 2 (Intermediate): mild hyperplastic lesions; Grade 3 (Severe): small berry-like elevation lesions; Grade 4 (Most severe): ill-defined/indurated/exophytic lesions, cauliflower-like surface, aggressive behavior. (C) Oral lesion number and (D) size. Means comparisons were performed using an ANOVA with post hoc Tukey's t-test and Fisher's exact test for categorical data with significance determined as \* $p < 0.05$ . Results are graphically presented as the mean  $\pm$  standard deviation. C = control, N = 4-NQO treated. (E) Representative histopathology photomicrographs from control (normal water) and 4-NQO treated mice of the indicated genotypes, top row magnification x50, bottom row magnification x50 or x100. Arrowheads indicate inflammation.

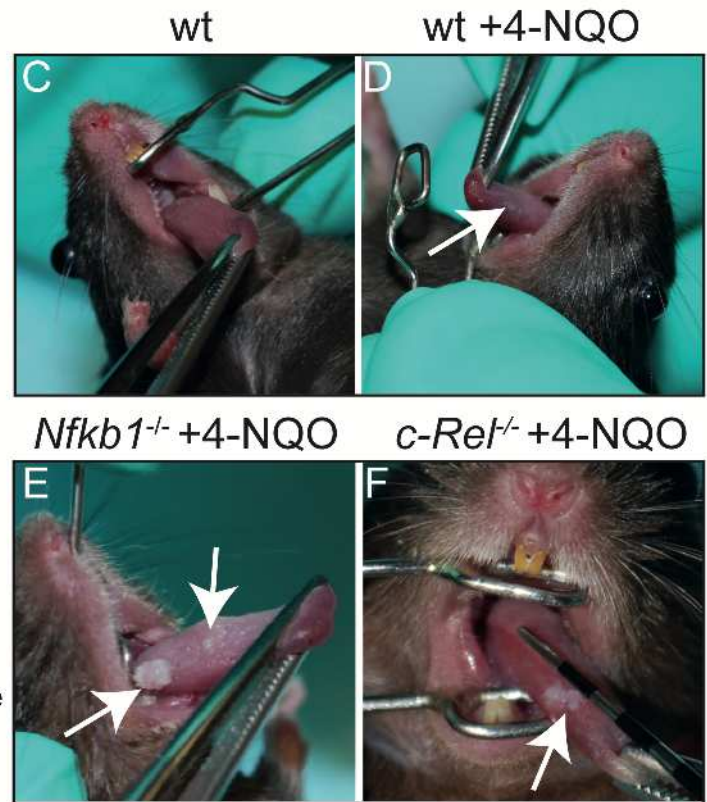
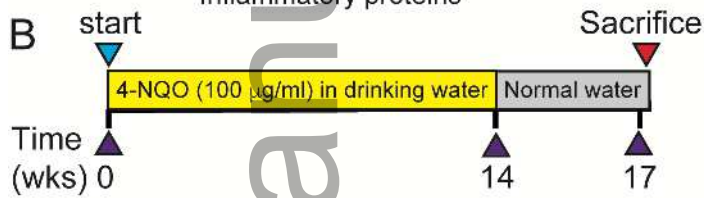
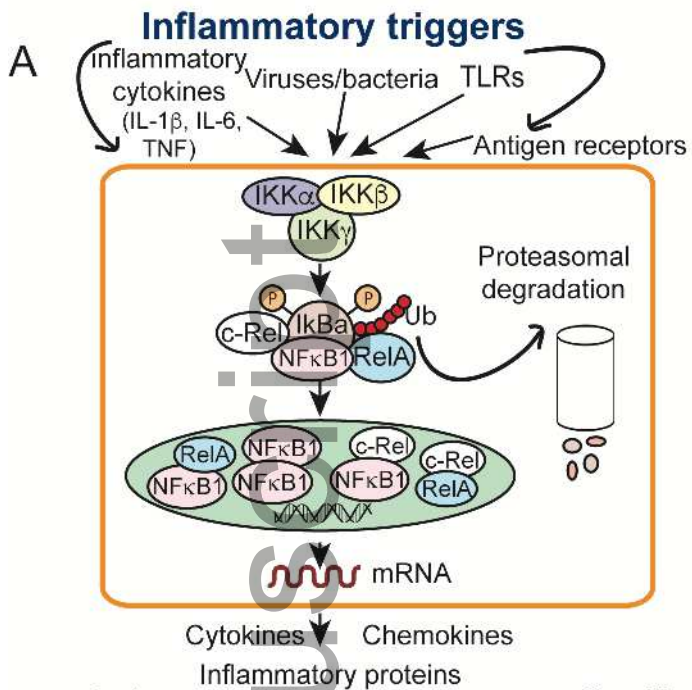
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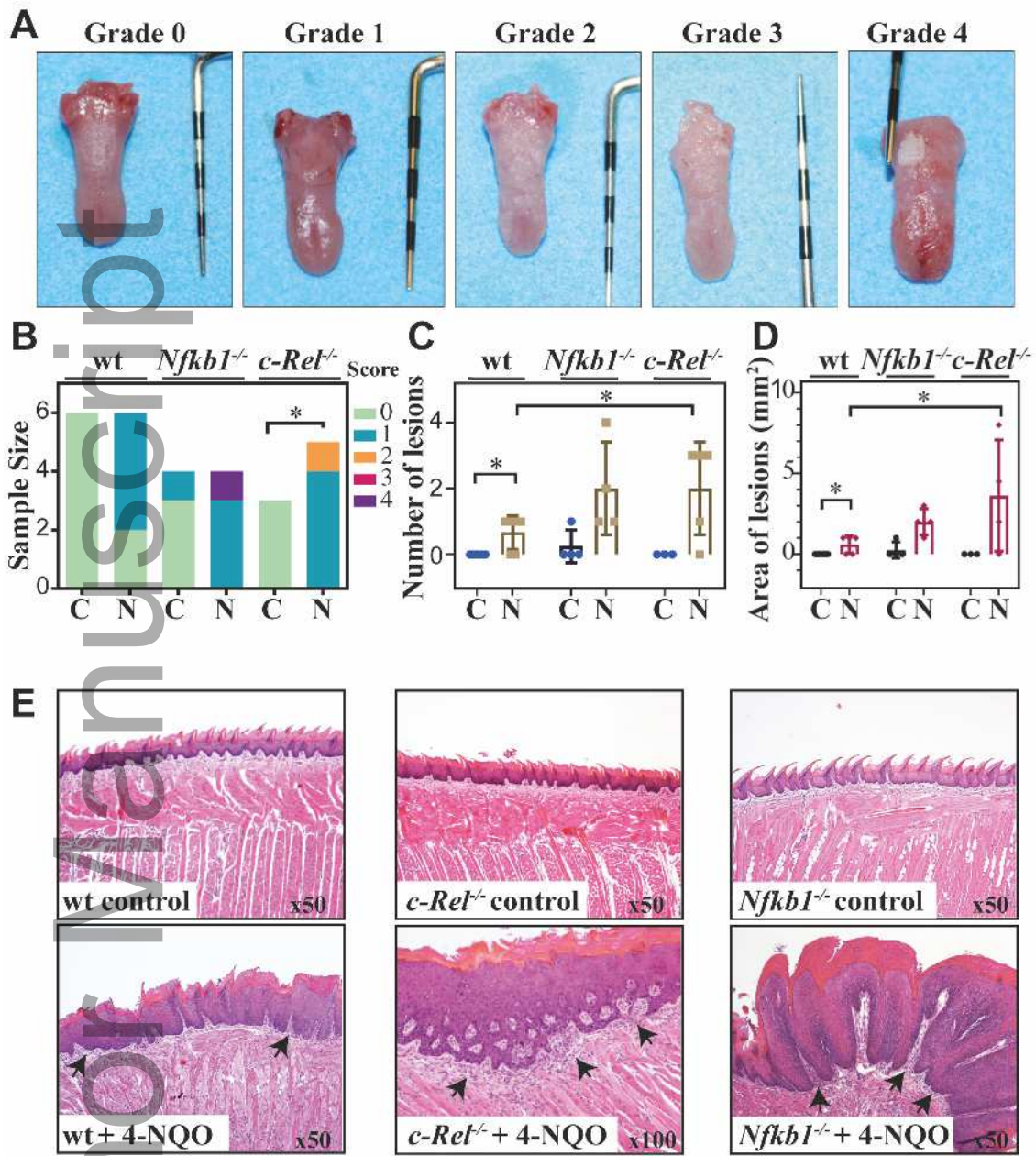


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