The role of Dectin-1 signaling in altering tumor immune microenvironment in the context of aging

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Abstract

An increased accumulation of immune-dysfunction-associated $CD4^+Foxp3^+$ regulatory T cells (T_{regs}) is observed in aging oral mucosa during infection. Here we studied the function of T_{regs} during oral cancer development in aging mucosa. First, we found heightened proportions of T_{regs} and Myeloid-derived suppressor cells (MDSC) accumulating in mouse and human oral squamous cell carcinoma (OSCC) tissues. Using mouse 4-Nitroquinoline 1-oxide(4-NQO) oral carcinogenesis model, we found aged mice displayed increased propensity to epithelial cell dysplasia, hyperplasia, and accelerated OSCC development, which coincided with a significantly increased abundance of IL-1β, T_{regs}, and MDSC in tongues. Partial depletion of T_{regs} reduced tumor burden. Moreover, fungal abundance and Dectin-1 signaling were elevated in aged mice suggesting a potential role for Dectin-1 in modulating immune environment and tumor development. Corroborating to this tenet, Dectin-1 deficient mice showed diminished IL-1β, reduced infiltration of T_{regs} and MDSC in the tongue, as well as slower progression and reduced severity in tumor burden, revealing that Dectin-1 signaling plays a critical role in promoting tumor incidence and cancer progression. Taken together, these data identify an important role of enhanced Dectin-1 signaling in establishing the intra-tumoral immunosuppressive milieu and promoting OSCC tumorigenesis in the context of aging.

Introduction

Head and neck cancers (HNC) including OSCC are the sixth most common cancers, accounting for an estimated 657,000 new cases and more than 330,000 deaths globally each year. The aging population (age >65) has grown to 727 million in 2020 and will double to nearly 1.5 billion, roughly ~16% of the world population by 2050. A majority of all new cancers are diagnosed among elderly adults [1]. Therefore, the convergence of the aging population and a higher cancer incidence in this population will result in a significant rise in cancer. Aging triggers the immune system to be in a chronic hyper-inflammatory state known as inflammaging. An imbalance between pro- and anti-inflammatory mechanisms due to the combination of inflammaging and impaired immune-surveillance may contribute to increased susceptibility of elderly individuals to cancer [2-4]. However, precise cellular alterations in the tumor immune microenvironment (TIME) that contribute to tumor growth and progression are not completely defined in aging individuals. HNC are among the most highly immune-cell infiltrated cancer types, highlighting the significance of immune cells in tumor development and progression blockade. Immune-suppressive cells including T_{regs}, MDSC, and tumor-associated macrophages (TAM) that are important in immune tolerance, accumulate in tumors and are thought to contribute to poor immunologic response against tumors resulting in cancer immune evasion [5-14]. Depending on the tumor type, T_{regs} may constitute 30- 45% of $CD4^+$ T cells in solid tumors of nonlymphoid origin, with OSCC cancers having the highest degrees of T_{reg} infiltration[15; 16]. T_{regs} and MDSC have been shown to contribute to tumor evasion and even hinder the success of α-PD1 cancer immunotherapy[17-20]. Therefore there is considerable interest in understanding these cells in many cancers[21-25]. Yet, molecular mechanisms that instruct intra-tumoral T_{reg} recruitment, proliferation, stability, or functions in the context of their interactions with anti-tumor CD8 cells and other immune-

suppressive cells remain elusive. Understanding these mechanisms will lead to new combinatorial strategies in the face of PD-1/PD-L1 blockade resistance, leading to improved patient outcomes. Having linked some of the immunosenescent cytokines in T_{reg} accrual and aging previously [26], here we show that the Dectin-1 signaling is required for increases in IL-1β, MDSC, and T_{regs} during carcinogenesis, which is further enhanced in aged mice contributing to acceleration in tumor development in them.

Results

Aged mice show earlier and exacerbated dysplasia/hyperplasia, and OSCC development as well as heightened levels of immunosuppressive cells in the tongue.

To obtain mechanistic insights into immunological changes driving OSCC and to define aging dependent alterations in early immunological events during carcinogenesis, we used the 4-NQO oral carcinogenesis mouse model. This constitutes 16-weeks of 4-NQO administration in drinking water followed by another 6-weeks with regular drinking water (**Fig.1A**). We compared young (3-4 months of age) and aged (20-24 months of age) C57BL/6 mice treated with 4-NQO. Control mice received the propylene glycol vehicle in drinking water. At indicated times, we examined the tongue pathology by immuno-histochemistry using established OSCC criteria (**Fig.1B**). Thickened keratinized layer (Hyperkeratosis; HK), lesions showing enlarged nuclei with increased nuclear to cytoplasmic ratio, loss of typical epithelial cell organization (dysplasia), intrusion into the connective tissue, and presence of cell nests were used for scoring on a 0-5 scale. Aged mice demonstrated earlier and higher incidence as well as the accelerated progression of hyperkeratosis, hyperplasia, and dysplasia during early time points (**15- 22 weeks**) (**Fig.1B**). Also, more mice in aged group showed these changes at early time-points (**Fig.S1**).

These data are consistent with a previous study that elegantly showed similar results in aged mice [27]. Because aging-related differences in OSCC were not significant after 23-24 weeks, we focused on early events of carcinogenesis in defining altered and accelerated dysplasia in aging mice. Therefore, we examined the rest of the parameters at this early time (12- 16 weeks) window, which appears to be critical for defining immune events that determine altered tumor outcomes in aged mice. We isolated the tongue/gingival tissues and the oral draining cervical lymph nodes (CLN) and determined the frequency of $CD3^+CD4^+Foxp3^+$ T_{regs} by flow cytometry (n=6/ Veh. group; n=8/ 4-NQO group). 4-NQO carcinogenesis process also increased the frequency of $Foxp3^+$ T_{regs} in oral tissues and draining lymph nodes, which increased with time(**Fig.1C, S2**). Aged mice displayed significantly higher proportions of T_{regs} when compared to younger mice (**Fig.1C**). Correlating to the increase in T_{regs}, the percentage of $CD3^+CD8^+$ cells decreased, and the aged mice had a significantly higher T_{reg}: CD8 ratio than young mice (**Fig.1D, left**). $CD8^+$ T cells in tongue also upregulated PD-1 but showed a decreased expression of IFN-γ in 4-NQO treated mice in both the groups (**Fig.1D, right**). Also, tongues in 4-NQO treated mice showed increased accumulation of $CD11b^+Ly6G^+$MDSC cells and were at higher levels in aged mice(**Fig.1E**). Consistent with their MDSC phenotype, $CD11b^+Ly6G^+$ cells were Arginase-1^{high} (Arg-1), when compared to $CD11b^+Ly6G^-$ cells. Moreover, the expression of Arg-1was much more elevated in aged 4-NQO mice than young 4-NQO mice. Thus, earlier epithelial dysplasia coincided with an increased $T_{regs:}$ CD8 ratio, $CD11b^+Ly6G^+$ Arg-1^{high} MDSC in aged tongues. Taken together, these results show that aging causes premature development of oral carcinogenesis, which is associated with increased infiltration of immune-suppressive cells.

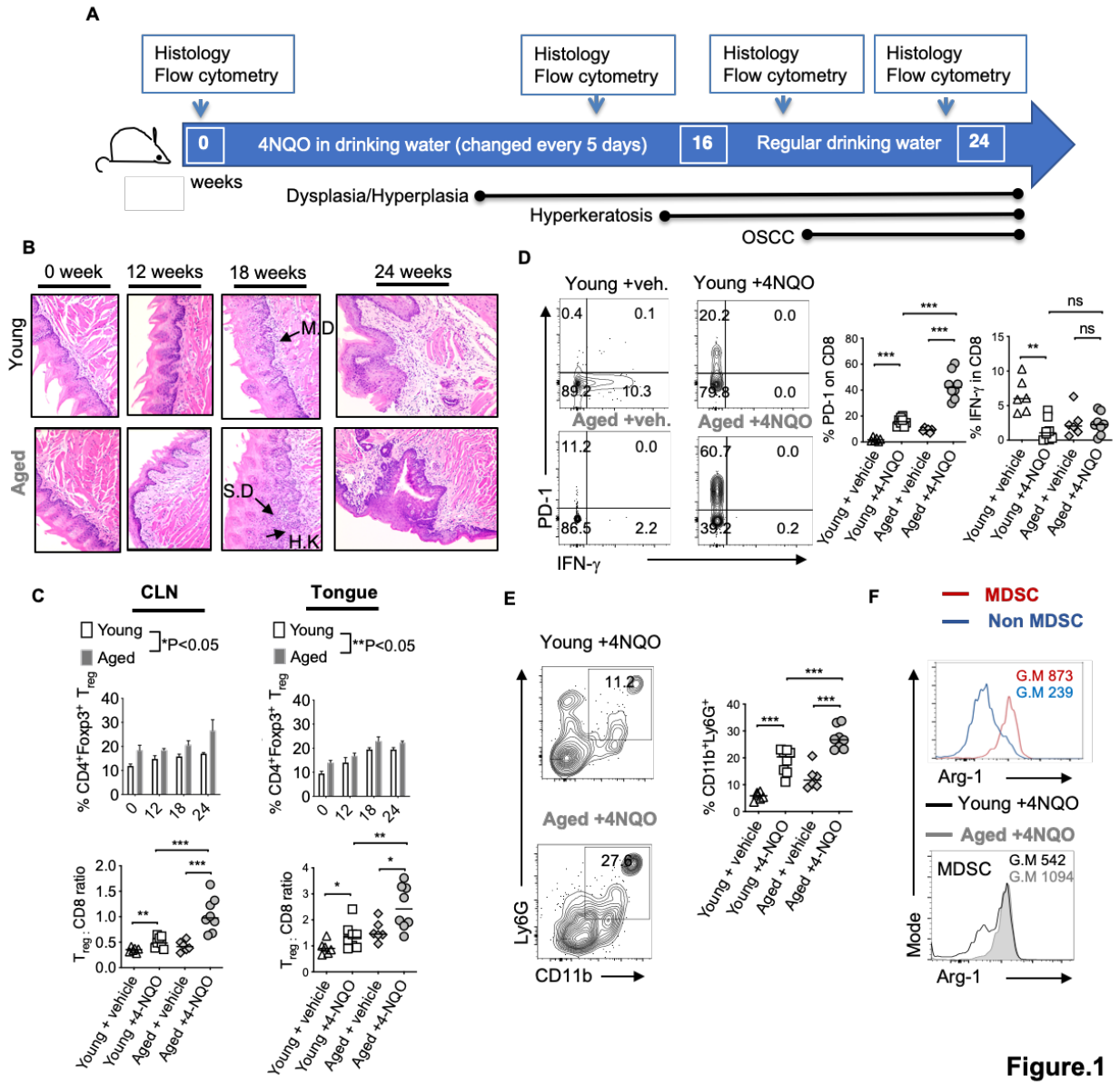

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Fig.1 Aged mice display accelerated development of OSCC and heightened levels of immunosuppressive cells in the tongue. A) 4-NQO was (50 µg/ml) administered in drinking water. Time-points for readouts and various stages of OSCC development in this model are shown. **B)** H&E immunohistochemistry images of the tongue at 200X magnification at indicated times [IF: Infiltration, MD: mild dysplasia, SD: severe dysplasia/hyperplasia, HK: Hyperkeratosis]. Samples were processed for flow cytometry. (**C**) Statistics showing %T_{regs} (top) and T_{reg}:CD8 ratio (bottom) in draining cervical lymph nodes (CLN) (left) or tongue (right) at 16 weeks (**D**) Contour plots (left) and statistics (right) showing % of PD-1 and IFN-γ expressing $CD8^+$ cells. **E**) Representative contour plots (left) and

statistics (right) showing % of CD11b and Ly6G expressing $CD45^+$ cells. **F**) Arg-1 expression in MDSC ($CD45^+CD11b^+Ly6G^+$) *vs* non MDSC ($CD45^+CD11b^+Ly6G^{-neg}$ cells (top) and in MDSC in young and aged 4-NQO treated mice (bottom) (Geometric Mean = G.M)(Mann-Whitney test * $P<0.05$).

Heightened levels of intra-tumoral T_{regs}, MDSC, and IL-1β in OSCC patients. To validate the findings in developed tumors in humans, we examined the surgically removed OSCC tumors, tumor lesion cytobrushes along with control biopsies that were taken 2-3 cm from the tumor margin or contralateral control tissue cytobrush samples from patients (**Fig. 2B, S3**). Similar to 4-NQO treated mice, human OSCC tumors showed very high proportions of $CD25^+Foxp3^+$ T_{reg} cells, sometimes (up to 42%, Mean 22%)(**Fig. 2B**). This was significantly higher than what is found in gingival mucosa of healthy individuals (*$P<0.05$)[26]. Among $CD45^+$ cells, there were also higher proportions of $CD14^{neg}CD11B^+CD33^+$ MDSC cells (**Fig. 2C**), which had higher ARG-1 expression, compared to non-MDSC cells(**Fig. 2D**). Because IL-1β signaling is critical for induction of immunomodulatory ROR-γt^+FOXP3^+ cells in oral mucosal tissues and is implicated in oral tumor progression[26; 28; 29], we examined the expression of IL-1β. IL-1β was significantly increased in the supernatants derived from human tumors and resected tumor supernatants (**Fig. 2E**), as well as in immune cells (**Fig. 2F**). However, the IL-1 receptor antagonist (IL-1RA) levels were significantly lower in tumor samples (**Fig.S4**) suggesting that IL-1 antagonism is associated with health while IL-1β was associated with tumor development. Notably, MDSC cells showed heightened levels of IL-1β than other cells, implying that IL-1β is likely linked to immune-suppressive milieu in tumors (**Fig. 2F**).

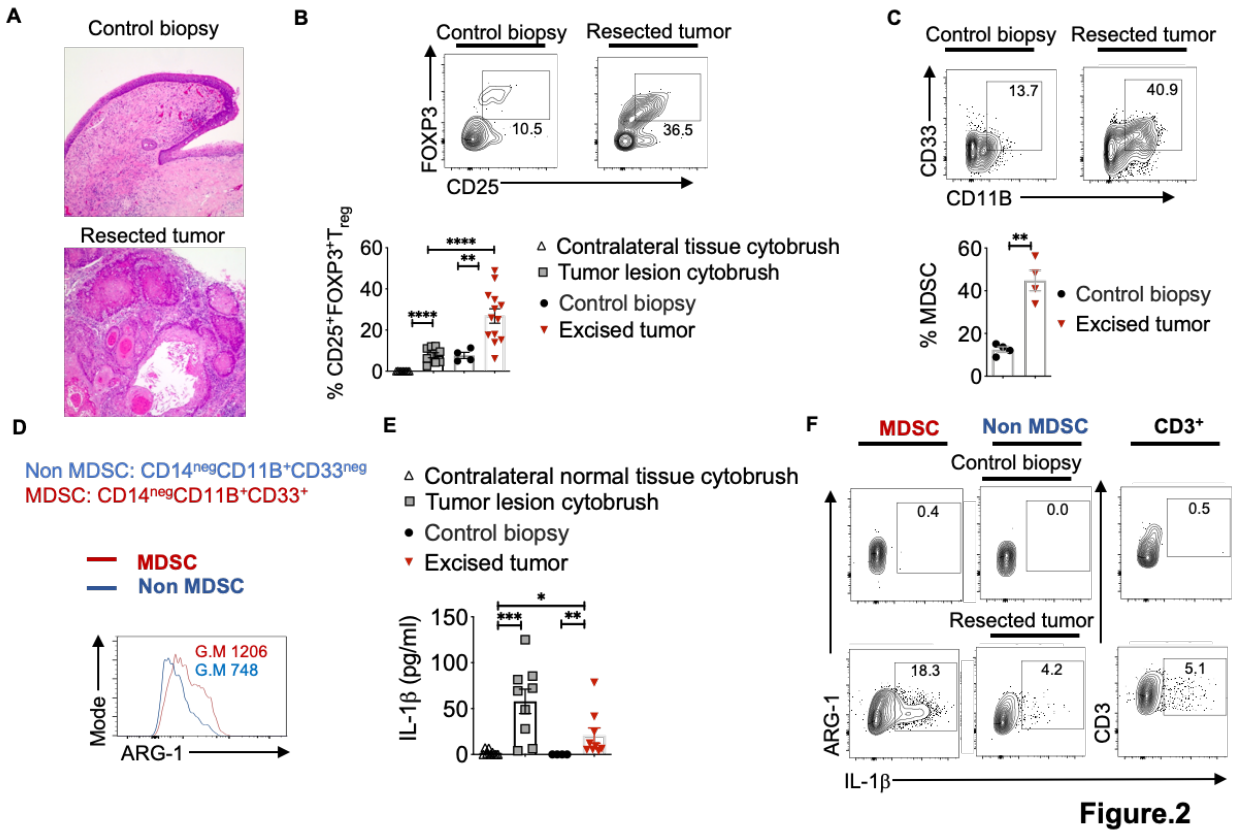

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Fig.2. Heightened levels of intra-tumoral T_{regs}, MDSC, and IL-1β in OSCC patients. Human oral tissue samples were obtained by excision (tumor resection), cytobrushing (mostly epithelial cells), or by biopsy (control tissue 2-3 cm from the tumor margin) under an approved IRB protocol. Samples were digested by collagenase-1A and restimulated with PMA/Ionomycin for 4 hours. (**A**) Contour plots gated on $CD45^+CD3^+CD4^+$ lymphocytes (top) and statistical analysis (bottom) showing % of T_{regs}. (**B**) Contour plots gated on $CD45^+$ $CD3^-$cells and statistical analyses (bottom) showing % of MDSC. (**C**) ARG-1 expression in MDSC *vs* non-MDSC cells. (**D**) Supernatants were collected from these cultures and assessed for IL-1β using ELISA (**E**) Contour plots showing IL-1β expressing MDSC(left), non-MDSC (middle), and T cells (right). Mean +/-SEM are shown. (Mann-Whitney test * P<0.05).

Aged mice have distinct T_{reg} phenotype and elevated levels of IL-1β compared to young mice.

The degree of phenotypic and functional heterogeneity of tissue/intra-tumoral T_{regs} can be high [30], and evaluating them solely with FOXP3 and CD25 markers without examining anti-tumor $CD8^+$ T cells can sometimes be misleading. Besides recruiting thymic T_{regs} in to tumors via chemotaxis, T_{regs} can be induced *in situ* in tumors due to the abundance of mediators such as nitric oxide synthase, indoleamine 2, 3-dioxygenase 1, Transforming growth factor- β1, and adenosine released from tumor cells, TAM, and MDSC [31]. Some of these $FOXP3^+$ cells may also be critical for tissue repair or become dysfunctional depending on the inflammatory cytokine milieu[32]. Indeed we[26; 33-37] and others have shown distinct functions of T-bet^+Foxp3^+cells, ROR-γt^+Foxp3^+ cells, and PD-1^+Foxp3^+ cells, whose functions are significantly altered by cytokines including IL-6 and IL-1β in an mTOR dependent manner in oral mucosa[26]. Because CD25, PD-1, T-bet, ROR-γt, Suppression of Tumorigenicity 2 (ST-2) expression were all implicated in determining intra-tumoral T_{reg} functions positively and negatively in other tissues[7; 28; 38; 39], we examined the expression of the molecules, comparing aged and young T_{regs}, 16 weeks after 4-NQO administration. While the expression of CD25 (ability to consume IL-2) [10; 40], Ki-67 (proliferation), and ST-2 (tissue T_{reg} and tumorigenicity marker) [7] did not change between young and aged T_{regs}, CD103 (TGF-β1 dependent, tolerogenic, and tumor-specific T cell marker)[41], PD-1, ROR-γt, and T-bet were significantly up-regulated in aged T_{regs} (**Fig.3A)**. Consistent with the higher expression of ROR-γt in T_{regs} and earlier dysplasia in aging mice, increased numbers of IL-1β expressing cells were found among infiltrating immune cells in aged mice as early as 5 and 12 weeks after 4-NQO administration (**Fig. 3B, C**). IL-6 levels were also elevated in aged tongue supernatants(**Fig. S5**). Moreover, at later times, IL-1β expression was found in epithelial tumor cells and immune cell infiltrates in both the groups, but was dramatically elevated in aged 4-NQO treated mice when

compared to their young counterparts (**Fig 3D**). These results showed that T_{regs} found in tongues during early tumor development in 4-NQO treated mice are phenotypically distinct, appearing to be functionally more immunosuppressive in aged mice, and may be linked with higher expression of IL-1β in the milieu.

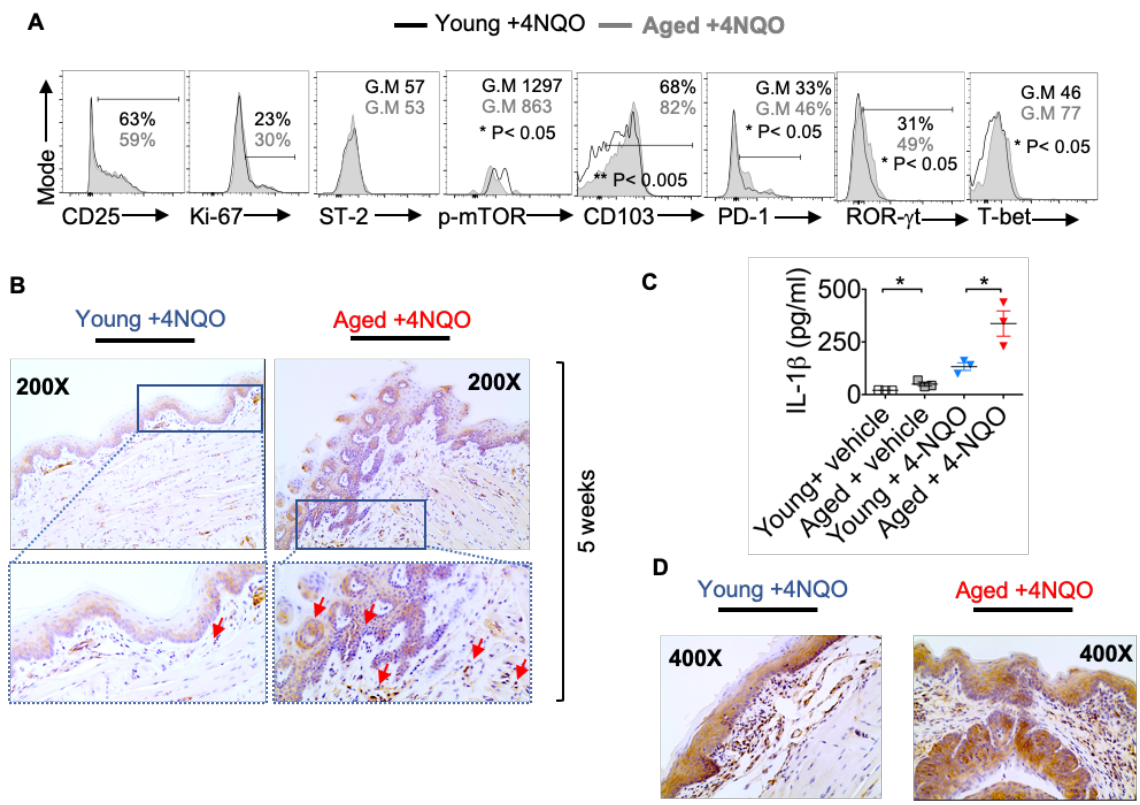

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Fig.3. Aged mice have distinct T_{reg} phenotype and elevated levels of IL-1β compared to young mice. 16 weeks after 4-NQO administration, single-cell suspension from tongue was re-stimulated with PMA/Ionomycin for 4 hours. **A)** Flow cytometry histograms showing indicated protein expression in $CD45^+CD3^+CD4^+Foxp3^+$cells. Tongue tissues were processed for IL-1β immuno-histochemical staining at 5 (**B**), or 24 (**D**), weeks. **C**) Supernatants

collected at 12 weeks were processed for IL-1β ELISA. Mean +/- SEM in 3 mice/group (Mann-Whitney test * $P< 0.05$).

Partial T_{reg} depletion reduces tumor progression.

Although tumorigenesis coincides with increases in $Foxp3^+$ T_{regs}, the function of these cells has not been validated in the 4-NQO model. Therefore, we employed $Foxp3^{DTR}$-eGFP reporter (FDTR) mice that express the diphtheria toxin receptor on $Foxp3^+$ cells and allow targeted T_{reg} depletion by intraperitoneal diphtheria toxin (DT) injection (20 μg/10 gm body weight; n=4/group)[36; 40]. We took two approaches; 1) Early and long-term T_{reg} depletion where DT was injected every 5 days, in the first 16 weeks of 4-NQO treatment 2) Late and short-term T_{reg} depletion where DT was injected every 5 days, from the 16th -21st weeks of 4-NQO treatment n=6/group)(**Fig.4A**). Early T_{reg} depletion caused systemic autoimmunity characterized by splenomegaly, lymphadenopathy, CD4 hyperactivation ($CD44^{high}$, IFN-γ^{high}), and mortality in 30% of mice which confounded the effects on early tumorigenesis (*not shown*). However, late and short-term T_{reg} depletion caused only partial T_{reg} reduction, but the mice had significantly reduced dysplasia and OSCC at 24 weeks (**Fig.4B, C, S6A**). The frequencies of $CD11b^+Ly6G^+$ and IL-$1\beta^+$ cells were unchanged with T_{reg} depletion, showing that these components are likely upstream to T_{reg} induction and infiltration during tumorigenesis (**Fig. S6B, C**). However, the PD-1 expression was diminished and IFN-γ expression was partially restored in $CD8^+$ T cells(**Fig.4D**). Collectively, these results show that infiltrating T_{regs} control anti-tumorigenic functions of $CD8^+$ T cells and contribute to OSCC tumorigenesis and progression.

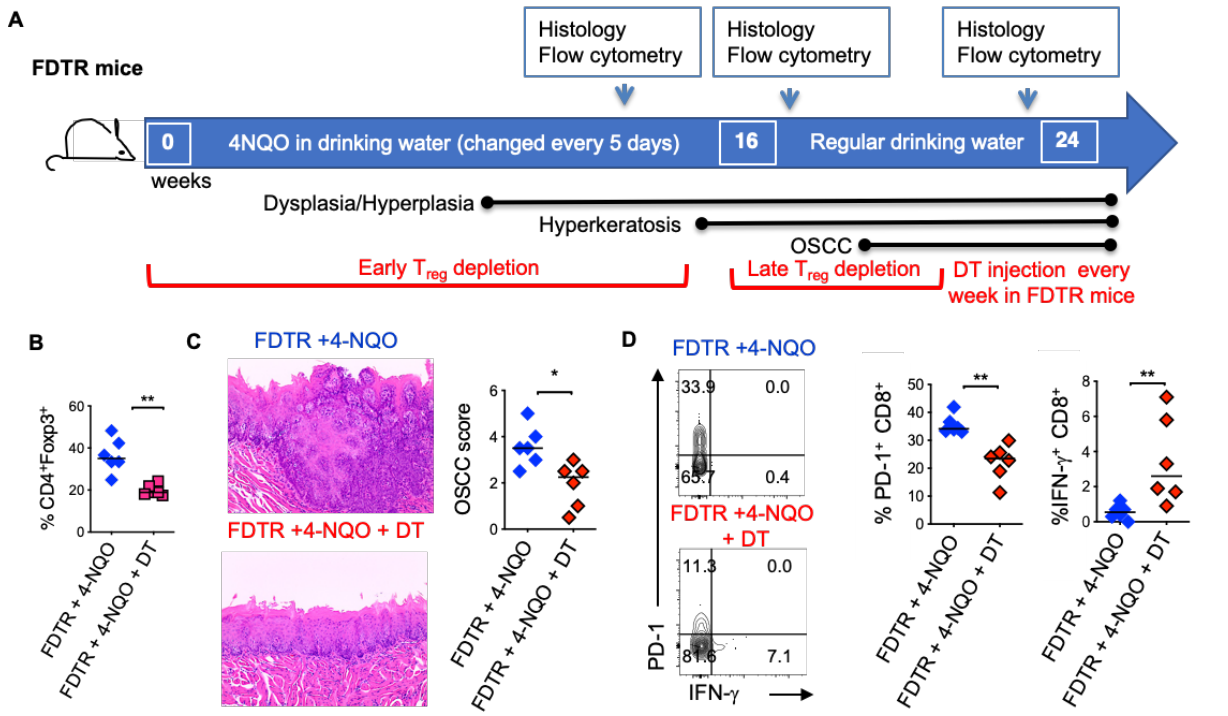

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Fig.4. Partial T_{reg} depletion reduces tumor progression. A) T_{regs} were depleted in FDTR mice by injecting diphtheria toxin (DT) at late time points (every 5 days between 16th -21st weeks of 4-NQO treatment). **B)** Statistics showing $CD4^+Foxp3^+$cells based on flow cytometry analyses at 20 and 24 weeks. **C)** Immunohistochemistry H&E staining (left) and OSCC score based on invasive hyperkeratosis, and nesting (0-5) (right) **D**) Contour plots (left) and statistics (right) showing PD-1 and IFN-γ expressing $CD8^+$ T cells. Mean +/- SEM in 6 mice/group (Mann-Whitney test * P< 0.05).

Candida and Zymosan exacerbate and accelerate dysplasia and hyperplasia.

Previous evidence has also shown that oral carriage of *Candida* is higher in elderly individuals and patients with OSCC[42-46]. We and others have previously shown that *Candida* infection can also induce IL-1β expression and T_{reg} infiltration in oral mucosa[26; 47]. Because aforementioned results showed IL-1β was positively linked with tumorigenesis, we hypothesized

that *Candida* infection and consequent IL-1β induction might worsen OSCC outcome. Therefore, we examined the effect of *Candida* infection and Zymosan during carcinogenesis in 4-NQO treated mice. We sublingually applied *Candida* (10^7 blastospores) or D-Zymosan (TLR-2 depleted Zymosan; 1 mg/100ul) under anesthesia WT or TLR-2 knockout (TLR-2 KO) C57BL/6 mice every week between 4th and 8th weeks of 4-NQO administration. These treatments significantly accelerated and worsened dysplasia/hyperplasia (**5A, B**), increased IL-1β$^+$ cells and T_{regs} in the tongue at 12 weeks (**Fig. 5C, D, S7)**, and induced OSCC around 21 weeks (**Fig. 5E)**. Importantly, premature OSCC development was also observed in TLR-2 KO mice, revealing that Zymosan and *Candida* mediated exacerbation of OSCC development was independent of TLR-2 signaling. In fact, in the absence of TLR-2, the numbers of IL-1β$^+$ cells were increased implying the involvement of anti-fungal Dectin-1 signaling in OSCC.

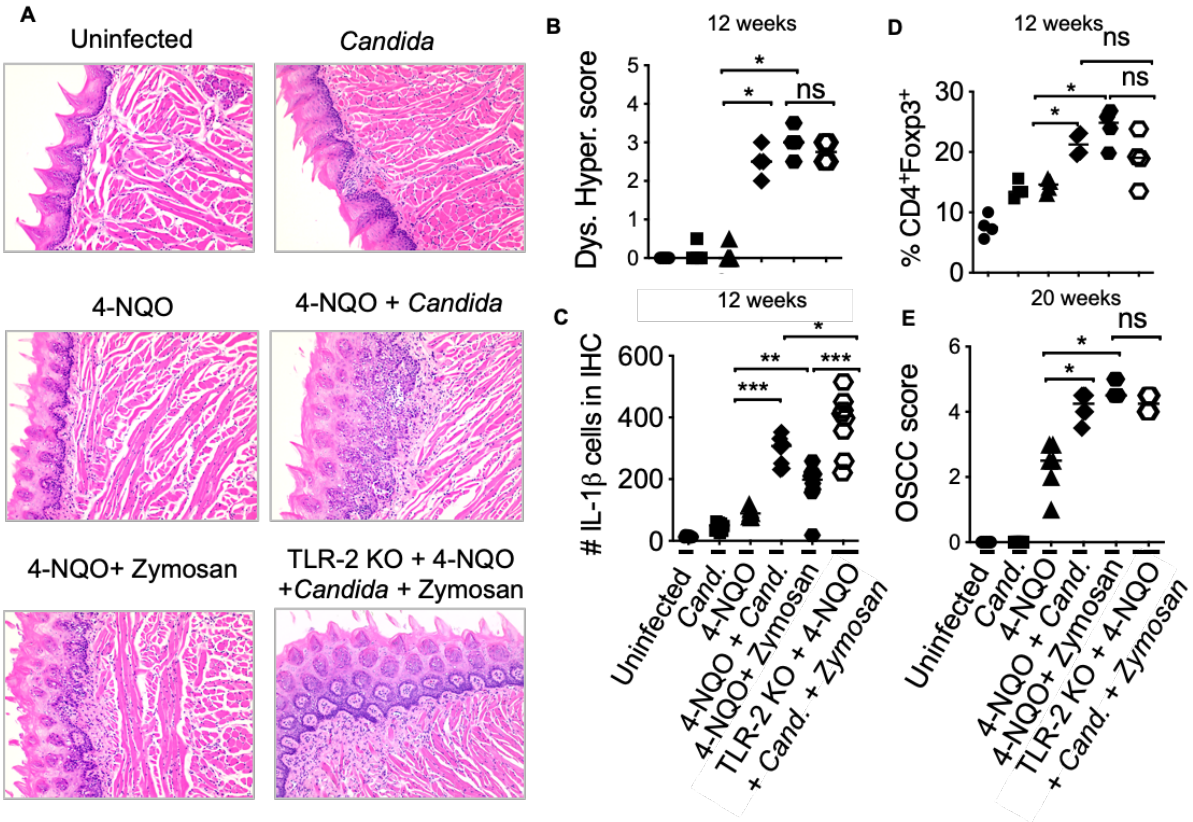

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Fig.5. *Candida* and Zymosan exacerbate and accelerate dysplasia and hyperplasia. A) 4-NQO was administered in WT or TLR-2 KO C57BL/6 mice as in **Fig.3A** (6 mice/group). Zymosan (1mg), *Candida* (10^7 blastospores), or both were applied sublingually under anesthesia every week between the 4th and 8th weeks of 4-NQO administration. H&E staining to assess dysplasia and hyperplasia (**B**), IL-1β immunohistochemistry (**C**), and flow cytometry staining for T_{reg} assessment (**D**) were performed at 12 weeks. OSCC scores were assigned based on hyperplasia, invasive hyperkeratosis, and nests after 20 weeks after 4-NQO administration. Statistics show Mean +/- SEM (Mann-Whitney test * $P< 0.05$).

Aged mice show elevated Dectin-1 signaling in immune cells in the tongue and increased fungal abundance in saliva. Dectin-1 is expressed by myeloid phagocytes[48] and binds specifically to fungal β-1,3 glucans, endogenous galectins, and annexins on apoptotic cells[49]. Dectin1 signaling activation induces the phosphorylation of Syk and consequently the expression of IL-1β. We and others have shown that Dectin-1 signaling can regulate T_{reg} alterations and macrophage metabolism[50-53]. Fungal dysbiosis and Dectin-1 have also been implicated in inflammatory diseases and cancers[54-56]. Thus, previous reports and our above-mentioned results led us to hypothesize that Dectin-1 might be involved in OSCC development and earlier predisposition of aged mice to OSCC. Corroborating to this tenet, we found that 4-NQO induced tumorigenesis involves a significant elevation of Dectin-1 expression and Syk activation in immune infiltrates ($CD45^+$ cells) (**Fig.6A**). Dectin-1 expression and Syk activation were further increased in aged 4-NQO treated mice suggesting the involvement of Dectin-1 signaling in accelerated tumorigenesis in aging mice(**Fig.6B**). Although non-significant there was also an inclination towards increased fungal abundance in the saliva of 4-NQO treated mice, as measured by fungal abundance qPCR[57; 58]. Interestingly, aged 4-NQO treated mice that displayed accelerated dysplasia and OSCC (**Fig. 1A**), showed a significantly higher fungal

abundance than control mice (**Fig. 6C**). Together, these results revealed that increased fungal abundance and resultant aberrant Dectin-1 signaling might play a role in accelerated tumorigenesis in aged mice.

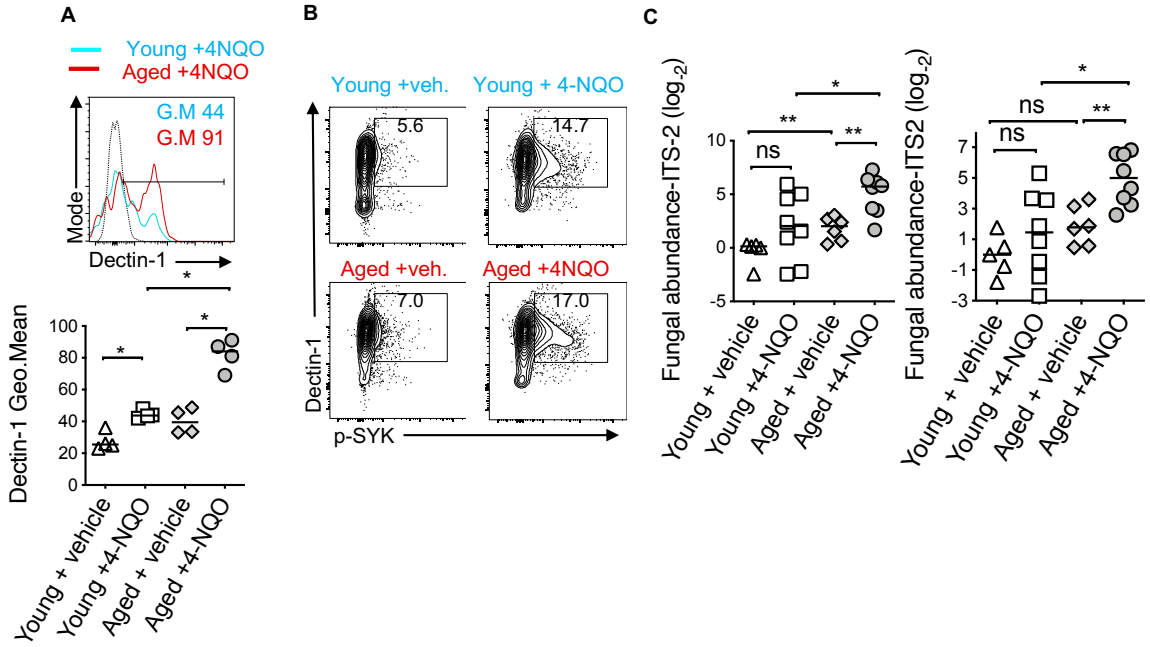

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Fig.6. Aged mice show elevated Dectin-1 signaling in immune cells in the tongue and increased fungal abundance in saliva. A) Tongue samples were processed for flow cytometry. Contour plots (left) and statistics (right) showing Dectin-1 expressing CD45$^+$ cells. **B**) Contour plots showing % of Dectin-1$^+$ and phosphorylated SYK (p-SYK) expressing CD45$^+$ cells. **C**) Saliva swabs collected at 12, 15, and 23 weeks were pooled. qPCR was performed to quantify fungal DNA using ITS-2 and fungi-quant PCR primers [57; 58]. Relative abundance was standardized to input DNA using β-actin as reference. (Mann-Whitney test * $P<0.05$).

Dectin-1 deficiency reduces immunosuppressive cells and OSCC tumor burden. Next, we investigated the role of Dectin-1 in tumorigenesis using the mice deficient in Dectin-1 signaling (Dectin KO mice). Monitoring the body-weight we found that weight loss was much less

pronounced in Dectin-1 KO than WT mice **(Fig.7A)**. Dectin-1 KO mice also had significant reduction in IL-1β^+ cells **(Fig.7B, S8)**, T_{reg} induction (**Fig.7C, S9**), T_{reg}:CD8 ratio(**Fig.7D**), PD-1 induction in CD8$^+$ T cells(**Fig.7E, G**), and MDSC cells(**Fig.7F**). However, coinciding with IFN-γ restoration in CD8 T cells(**Fig.7E, G**), mice showed significantly slower progression of dysplasia and lower numbers and sizes of OSCC tumors(**Fig.7H, I**). Taken together, these results show Dectin-1 signaling is critical for establishing an immunosuppressive milieu and the development of oral tumorigenesis.

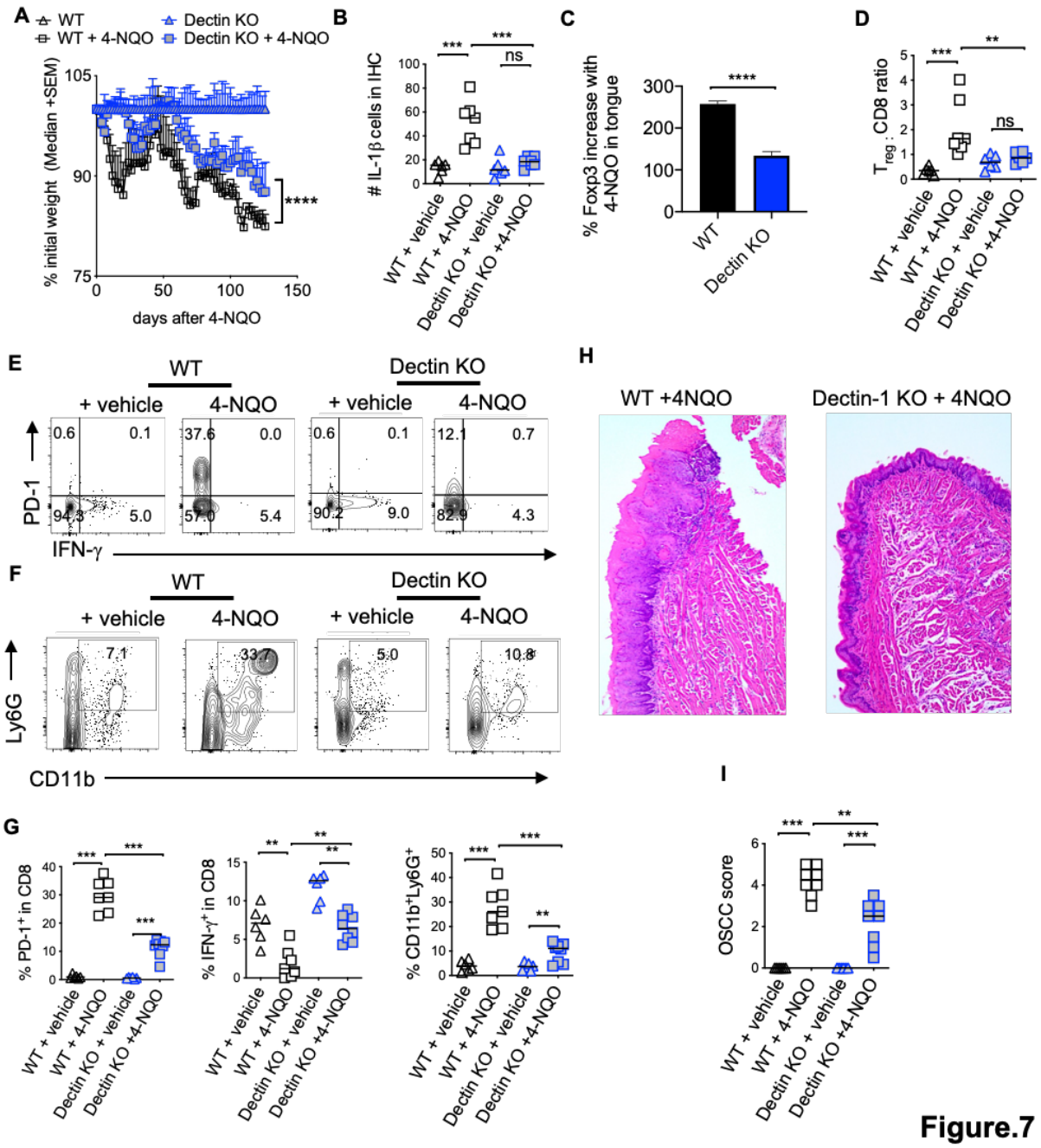

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Fig.7. Dectin-1 deficiency reduces immunosuppressive cells and OSCC tumor burden. Tongue tissues were processed for immunohistochemistry (IHC) and flow cytometry at 23 weeks after 4-NQO administration. (**A**) Statistics showing the number of IL-1β+ cells in the entire field of 200X IHC image. Flow cytometry-based statistics showing % increase in T_{regs} in 4-NQO treated mice compared to Veh. controls (**B**), T_{reg}:CD8 ratio (**C**), the

% of PD-1 (**D**), and IFN-γ expressing CD8 cells (**E**), the % of CD11b and Ly6G expressing $CD45^+$ cells (**F**). (**G**) H&E immunohistochemistry of tongue (left) and blinded scoring (right). (Mann-Whitney test except for **B** where t-test with Welch's correction was performed, * $P<0.05$).

Discussion

Aging skews host $CD4^+$ T cells and T_{regs} towards an inflammatory T_{reg} type, which fails to control immunopathology during an infection. Instead, there is an increased accumulation of $Foxp3^+$ cells, a proportion of which was associated with increased $CD4^+$ T cell hyperactivation and altered levels of IL-6 and IL-1β in mice and humans in oral mucosa *in vivo* [26]. To our knowledge, no study has examined T_{reg} interactions in TIME with relevance to aging and OSCC carcinogenesis. While there are contentious data on age-related changes in T_{regs} in periphery/blood [59; 60], there is no report to date on intra-tumoral T_{regs} and their interactions with other immune suppressive cells in the context of aging. In this study, we found that mouse and human OSCC tissues showed not only an increased accumulation of T_{regs} but also of MDSC. Moreover, aged mice showed increased and early filtration of these cells and were also more susceptible to early dysplasia and tumor development compared to young mice. The 4-NQO mouse model mimicked the multistage carcinogenesis of human OSCC, so that we could investigate the immunological events in tumors and tumor-draining lymph nodes in early stages [61; 62]. In this model, we found that infiltration of immunosuppressive cells was one of the early immunological events of OSCC initiation. We also validated these findings in developed human OSCC tumors, comparing them to adjacent normal control tissues. These data provided a compelling link among aging, immunosuppressive milieu, and oral cancer. While many previous studies have shown that high levels of intra-tumoral T_{regs} associate with poor prognosis, a few studies show that intra-tumoral T_{regs} in some cancers correlate with improved disease outcomes[63-66]. However, multivariate analysis shows that T_{reg}

levels are not independently prognostic of OSCC but cytolytic T cells that co-infiltrate along with T_{regs} appear to drive the favorable prognosis [15]. Therefore we examined $FOXP3^+$ cells in relation to $CD8^+$T cell frequency and MDSC. Increased intra-tumoral T_{reg}: CD8 ratio rather than the mere increases in T_{reg} frequencies is more informative about pro-tumorigenic functions of T_{regs} during OSCC. Indeed, examining T_{reg}: CD8 ratio has allowed previous investigators to obtain consistent data that have clearly shown that a higher ratio is associated with poor prognosis and survival in human studies [67; 68]. While we also characterized the T_{regs} examining their inflammatory and tissue markers during early carcinogenesis in aging mice, more studies are required to determine the mechanisms underlying their altered phenotype and their contribution to OSCC development.

IL-1β is a cancer-inflammation associated cytokine that is abundant in breast, colon, lung, esophageal, and OSCC cancers[69]. It is linked to a poor prognosis for patients with esophageal cancer[70]. Targeting IL-1β has been shown to hinder oral carcinogenesis in the 4-NQO model, and is considered in the clinical settings[29; 71]. IL-1β is also involved in inducing immunomodulatory $Foxp3^+$ROR-γt^+ cells in microbiome dependent manner[26; 29; 36; 37; 51; 72-79]. $Foxp3^+$ROR-γt^+ cells have been shown to be present in tumors and contribute to tumor immune evasion and autoimmunity control[28; 80]. But the events preceding excessive IL-1β expression and at the IL-1β/T_{reg} interface are largely unknown. Our results also show an excessive IL-1β production in tongues of aged 4-NQO treated mice compared to young mice, which demonstrates that IL-1β is clearly associated with the establishment of a worse immunosuppressive milieu. These results are distinct from an acute infection milieu and steady state-conditions where aging oral mucosa shows IL-1β impairment [26]. Although it is conceivable that early carcinogenesis milieu triggers elevated levels of IL-1β in aged mice, the sequence of events that

promote elevated levels of IL-1β, T_{reg} accrual, and MDSC increase during aging need to be addressed. Furthermore, we found increased Dectin-1 expression in immune infiltrates in aging mice. Also, signaling through Dectin-1 promoted T_{reg} and MDSC cell infiltration. While it is clear that immune cells show activation of Syk signaling, the precise subset of cells that responds through Dectin-1 signaling hyperactivation in the context of aging remains to be addressed. The microbiome has been shown to be a significant contributor to OSCC in the 4-NQO mediated carcinogenesis[81; 82]. Aging is also associated with changes in the resident microbiome, reduced microbiota diversity, and an increase in certain types of bacteria and fungi, resulting in local immunological changes, chronic inflammation, and other several geriatric conditions[83-85]. *Candida* carriage and related co-morbidities are significantly more frequent in elderly individuals[86]. The role of microbiome dysbiosis in enhancing Dectin-1 signaling, immunosuppressive milieu, and OSCC development, and how these are dysregulated during aging are critical questions to be addressed and will be studied in future investigations. Taken together, our study sheds new light on the immune-oncological events in TIME providing some key insights into mechanisms of immune evasion. By linking MDSC and T_{regs} with a higher predisposition of aged oral mucosa to tumor development, our study also provides important insights in to mechanisms of higher susceptibility of the aging population to oral cancer. By revealing the pro-tumorigenic role of Dectin-1 signaling and T_{regs}, our study may also provide a way to control intra-tumoral T_{regs} without affecting the peripheral T_{reg} cell repertoire.

Author contributions

PP supervised the project, designed the study, performed experiments, analyzed data, and wrote the manuscript. QP and AT provided tumor resections from human participants under an approved

protocol and contributed to discussions. NB and SJ performed the experiments and analyzed ELISA and qPCR data. PM weighed the mice and performed validation qPCRs. CQ and SJ performed microscopy of the histology slides, scored the histology data in masked fashion, performed animal breeding, and processed tissues for flow cytometry. MG and AW read the manuscript and contributed to discussions.

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Declaration of interests

The authors declare no competing interests.

Materials and methods

Mice and human samples

Mouse experiments were performed following all guidelines and regulations under an approval from the CWRU Institutional Animal Care and Use Committee. Young (3-4 months of age), aged (20-24 months of age) C57BL/6 mice, TLR-2$^{-/-}$ and Dectin-1$^{-/-}$ mice were purchased from Jackson laboratories. Human oral tissue samples were obtained by excision (tumor resection), cytobrushing (mostly epithelial cells), or by biopsy (control tissue 2-3 cm from the tumor margin) under an approved protocol approved by the University Hospitals Cleveland Medical Center Institutional Review Board.

4-NQO administration in mice

4-NQO was dissolved in 1:1 solution of propylene glycol and DMSO and administrated in drinking water (50 -100 μg/ml) for 16 weeks, followed by the regular drinking water for another 6 weeks. Mice were sacrificed at the end of 24-25 week 4-NQO regimen. Young mice developed OSCC tumors after 24- 25 weeks and aged mice around 21-22 weeks after the start of 4-NQO administration (**Fig.1A**). Control mice received the propylene glycol/DMSO vehicle. Mouse body weight was measured every other day until sacrifice. 5-, 12 and 15- week time-points were considered early timepoints for the evaluation of immune cell changes in the tongue. Scores were assigned using tongue immuno-histochemistry using established OSCC criteria[61]. Epithelial hyperplasia and dysplasia scores were designated at 12 weeks after the 4-NQO regimen as follows: 0 = No immune infiltrates and the presence of cohesive epithelium;1 =Sparse immune infiltrates with mild epithelial hyperplasia; 2= Sparse immune infiltrates and moderate epithelial hyperplasia and dysplasia; 3 = Severe epithelial hyperplasia and dysplasia and moderate hyperkeratosis; 4 = Severe epithelial hyperplasia and dysplasia and hyperkeratosis; 5= Presence of invasive hyperkeratosis. OSCC scores were given at 20 or 24-25 weeks after the 4-NQO regimen as follows: 0 = No immune infiltrates and the presence of cohesive epithelium;1 =Sparse immune infiltrates with mild epithelial hyperplasia; 2= Moderate epithelial hyperplasia and dysplasia; 3 = Severe epithelial hyperplasia and dysplasia and moderate hyperkeratosis; 4 = Severe epithelial hyperplasia and dysplasia and hyperkeratosis; 5= Presence of invasive hyperkeratosis, cell nests and OSCC.

Antibodies

Purified or fluorochrome conjugated mouse and human α-CD25 (3C7 and 7D4), CD4, IFN-γ, Foxp3, CD45, CD8, CD11C, CD38, HLADR, Phospho-Syk (Ser473), IL-1β, IL-6, Arg-1, PerCP-eFluor 710 conjugated Ly-6G Monoclonal Antibody (1A8-Ly6g), APC conjugated CD11b Monoclonal Antibody (M1/70) and Dectin-1 antibodies were all purchased from Life Technologies/Thermofisher and BD-Biosciences.

Quantitative-PCR

DNA was isolated from mouse saliva swabs using the PureLink™ Microbiome DNA Purification Kit (Invitrogen). ITS and FungiQuant qPCR primers [57; 58] (Invitrogen), and SYBR Green PCR Kit (BioBasic) were used for performing qPCR, employing the real time PCR machine (Applied Biosystems). The relative amount of fungal DNA of interest was estimated from its Ct values, which were normalized to the host β-actin DNA levels.

Histochemistry and immunohistochemistry of proteins

For immunocytochemical hematoxylin and eosin (H&E), and immuno-histochemistry staining, tongue tissues were rinsed with PBS, fixed with 10% formalin overnight, and rehydrated in 70% ethanol overnight for paraffin embedding. This was followed by paraffin-sectioning and staining by the commercial facility (Histoserv, Inc, MD). Antibodies were used at 1-5 μg/ml concentrations.

Flow cytometry and ELISA

Cells were re-stimulated with PMA (50 ng/ml) and Ionomycin (500 ng/ml) for 4 hours in complete RPMI-1640 (Hyclone) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml

streptomycin, 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate and 50 μM β-mercaptoethanol [40]. Brefeldin-A (10 μg/ml) added in last 2 hours before harvesting the cells and supernatants. For single-cell flow cytometry staining, cells washed in PBS or PBS/BSA before surface and intracellular staining. Live-Dead viability staining was used to remove dead cells in the flow cytometry analyses. Appropriate un-stain, isotype, secondary antibody, single stain and FMO controls were used. For p-Syk staining, the cells were washed, fixed and were stained with Phosflow staining kit from BD Biosciences using manufacturer's protocol. For examining T cells, leukocyte singlets, Live-dead^{neg}, CD45^{+}CD3^{+}CD4^{+} or CD8^{+} gates were used. For examining MDSC, leukocyte singlets, Live-dead^{neg}, CD45^{+}CD3^{neg} gates were used. Data was acquired using BD Fortessa cytometers and were analyzed using FlowJo 9.8 or 10.5.3 software. Mouse IL-1β and IL-6 ELISA kits from Boster Bio (Pleasanton, CA) were used to assess the protein levels in the supernatants.

Oral *Candida* infection and Zymosan application

Mice were infected with *Candida* as previously described [36; 87]. Briefly, they were sublingually applied under anesthesia by placing a 3 mm diameter cotton ball saturated with 1 x 10^7 *Candida albicans* (SC5314) blastospores for 90 min. Mice were re-infected as indicated. We applied Zymosan sublingually in the same manner[88].

Statistical analysis

P values were calculated by Mann-Whitney test in Prism 6.1 (GraphPad Software, Inc.) assuming random distribution. Welch's correction and student t tests were used where indicated. One or two way ANOVA analyses were also used for grouped analyses. $P < 0.05$* was considered significant.

Supplemental information

Supplementary figures are attached separately.

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