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

Natarajan Bhaskaran[#1], Sangeetha Jayaraman[#1], Cheriese Quigley[1], Prerna Mamileti[1], Mahmoud Ghannoum[2,3], Aaron Weinberg[1,4], Jason Thuener[5], Quintin Pan[2,5], Pushpa Pandiyan*[1,2,4].

[#] equal contribution

[1]Department of Biological Sciences, School of Dental Medicine, [2]Department of Pathology, [3]Dermatology, School of Medicine, [4]Case Comprehensive Cancer Center, Case Western Reserve University, [5]Otolaryngology-Head and Neck surgery, University Hospitals Cleveland Medical Center, Cleveland, Ohio, 44106.

* To whom correspondence should be addressed.

E-mail: pxp226@case.edu

Running title:

Aging and oral carcinogenesis

Key words: T_{reg}, Foxp3, Treg17, IL-1β, Candida, oral cancer, OSCC, tumorigenesis, aging

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 the mouse 4-Nitroquinoline 1-oxide(4-NQO) oral carcinogenesis model, we found that tongues of aged mice displayed increased propensity for epithelial cell dysplasia, hyperplasia, and accelerated OSCC development, which coincided with 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. Confirming this tenet, dectin-1 deficient mice showed diminished IL-1β, reduced infiltration of T_{regs} and MDSC in the tongues, as well as slower progression and reduced severity of tumor burden. Taken together, these data identify an important role of 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 in the elderly are not completely understood. 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 responses against tumors resulting in cancer immune evasion [5-14]. Depending on the tumor type, T_{regs} may constitute between 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]. Thus there is considerable interest in understanding the involvement of these cells in many cancers[21-25]. However, molecular mechanisms that instruct intra-tumoral T_{reg} recruitment, proliferation, stability, or functions in the context of their interactions with anti-tumor CD8 cells

70 and other immune-suppressive cells remain elusive. Understanding these mechanisms will lead to
71 new combinatorial strategies in the face of PD-1/PD-L1 blockade resistance, leading to improved
72 patient outcomes. Having previously linked some of the immunosenescent cytokines in T_{reg}
73 accrual and aging previously [26], we now show that dectin-1 signaling is required for increased
74 IL-1β, MDSC, and T_{regs} during carcinogenesis, and that this enhancement in aged mice contributes
75 to the acceleration in tumor development in them.
76

77 **Results**

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

80 To obtain mechanistic insights into immunological changes driving OSCC and to define aging-
81 dependent alterations in early immunological events during carcinogenesis, we used the 4-NQO
82 oral carcinogenesis mouse model. This constitutes 16-weeks of 4-NQO administration in drinking
83 water followed by another 6-weeks with regular drinking water (**Fig. 1A**). We compared young
84 (3-4 months of age) and aged (20-24 months of age) C57BL/6 mice treated with 4-NQO. Control
85 mice received the propylene glycol vehicle in drinking water. At indicated times, we examined
86 the tongue pathology by immuno-histochemistry using established OSCC criteria (**Fig. 1B**).
87 Thickened keratinized layer (Hyperkeratosis; HK), lesions showing enlarged nuclei with increased
88 nuclear to cytoplasmic ratio, loss of typical epithelial cell organization (dysplasia), intrusion into
89 the connective tissue, and presence of cell nests were used for scoring on a 0-5 scale. Aged mice
90 demonstrated earlier and higher incidence of accelerated progression of hyperkeratosis,
91 hyperplasia, and dysplasia during early time points (**12- 22 weeks**) (**Fig. 1B**). Also, more mice in
92 the aged group showed these changes at early time-points (**Fig. S1**). These data are consistent

93 with a previous study that elegantly showed similar results in aged mice [27]. Since aging-related
94 differences in OSCC were not significant after 23-24 weeks, we focused on early events of
95 carcinogenesis in defining altered and accelerated dysplasia in aging mice. Therefore, we
96 examined the rest of the parameters at this early time (12- 16 weeks) window. We isolated the
97 tongue/gingival tissues and the oral draining cervical lymph nodes (CLN) and determined the
98 frequency of $CD3^+CD4^+Foxp3^+$ T_{regs} by flow cytometry (n=6/ Veh. group; n=8/ 4-NQO group).
99 4-NQO carcinogenesis process also increased the frequency of $Foxp3^+$ T_{regs} in oral tissues and
100 draining lymph nodes, which increased with time(**Fig. 1C, S2**). Aged mice displayed significantly
101 higher proportions of T_{regs} when compared to younger mice (**Fig. 1C**). Correlating with the
102 increase in T_{regs}, the percentage of $CD3^+CD8^+$ cells decreased, and the aged mice had a
103 significantly higher T_{reg}: CD8 ratio than young mice (**Fig. 1D, left**). $CD8^+$ T cells in the tongue
104 also upregulated PD-1 but showed a decreased expression of IFN-γ in 4-NQO treated mice in both
105 groups (**Fig. 1D, right**). Also, tongues from 4-NQO treated mice showed increased accumulation
106 of $CD11b^+Ly6G^+$MDSC cells that were at higher levels in aged mice than young mice (**Fig. 1E**).
107 Consistent with their MDSC phenotype, $CD11b^+Ly6G^+$ cells were Arginase-1^{high} (Arg-1), when
108 compared to $CD11b^+Ly6G^-$ cells (**Fig. 1F, top**). Moreover, the expression of Arg-1 was much
109 more elevated in aged 4-NQO mice than young 4-NQO mice (**Fig. 1F, bottom**). Thus, earlier
110 epithelial dysplasia coincided with an increased $T_{regs:}$ CD8 ratio and $CD11b^+Ly6G^+$ Arg-1^{high}
111 MDSC in aged tongues. Taken together, these results show that aging in the context of the
112 carcinogen 4NQO, causes premature development of oral carcinogenesis, which is associated with
113 increased infiltration of immune-suppressive cells.
114

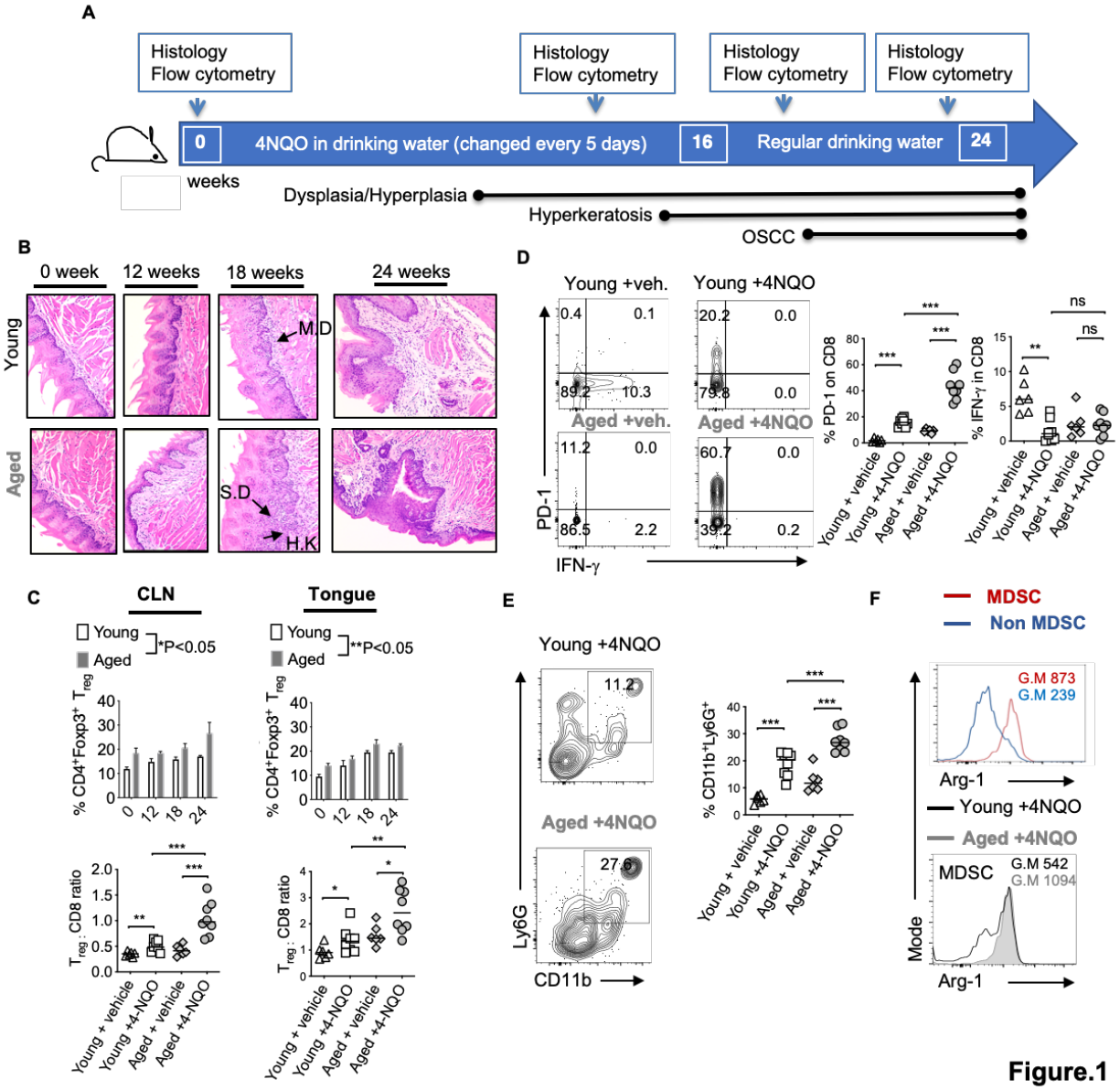

Figure.1

Figure legend

Fig. 1. Aged mice display accelerated development of OSCC and heightened levels of immunosuppressive cells in the tongue. 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 **(A)**. 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 **(B)**. Statistics showing %T_{regs} (**C**, top) and T_{reg}:CD8 ratio (**C**, bottom) in the 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)

124 and statistics (right) showing % of CD11b and Ly6G expressing $CD45^+$ cells. Arg-1 expression in MDSC
125 ($CD45^+CD11b^+Ly6G^+$) *vs* non MDSC ($CD45^+CD11b^+Ly6G^{-neg}$ cells (**F**, top) and in MDSC in young and aged 4-NQO
126 treated mice (**F**, bottom) (Geometric Mean = G.M)(Mann-Whitney test * P<0.05).
127

128 **Heightened levels of intra-tumoral T_{regs}, MDSC, and IL-1β in OSCC patients.** To validate 4-
129 NQO findings, we examined surgically excised human OSCC tumors, control biopsies that
130 included tissue derived from a site 2-3 cm from the tumor margin, as well as tumor lesion
131 cytobrushings and contralateral control tissue cytobrushings from patients (**Fig. 2A, S3**). Tumor
132 tissues were obtained from 16 patients that included eight former smokers, four current smokers,
133 and four non-smokers. Similar to 4-NQO treated mice, human OSCC tumors showed very high
134 proportions of $CD25^+Foxp3^+$ T_{reg} cells, (sometimes up to 42%; Mean 22%) (**Fig. 2B**). This was
135 significantly higher than what is found in gingival mucosa of healthy individuals (*P<0.05)[26].
136 Among $CD45^+$ cells, there were also higher proportions of $CD14^{neg}CD11B^+CD33^+$ MDSC cells
137 (**Fig. 2C**), which had higher ARG-1 expression, compared to non-MDSC cells(**Fig. 2D**). Because
138 IL-1β signaling is critical for induction of immunomodulatory ROR-γt^+FOXP3^+ cells in oral
139 mucosal tissues and is implicated in oral tumor progression[26; 28; 29], we examined the
140 expression of IL-1β. IL-1β was significantly increased in the supernatants derived from human
141 tumors and resected tumor supernatants (**Fig. 2E**), as well as in immune cells (**Fig. 2F**). However,
142 the IL-1 receptor antagonist (IL-1RA) levels were significantly lower in tumor samples (**Fig. S4**)
143 suggesting that IL-1 antagonism is associated with health while IL-1β was associated with tumor
144 development. Notably, MDSC cells showed heightened levels of IL-1β than other cells, implying
145 that IL-1β is likely linked to immune-suppressive milieu in tumors (**Fig. 2F**).

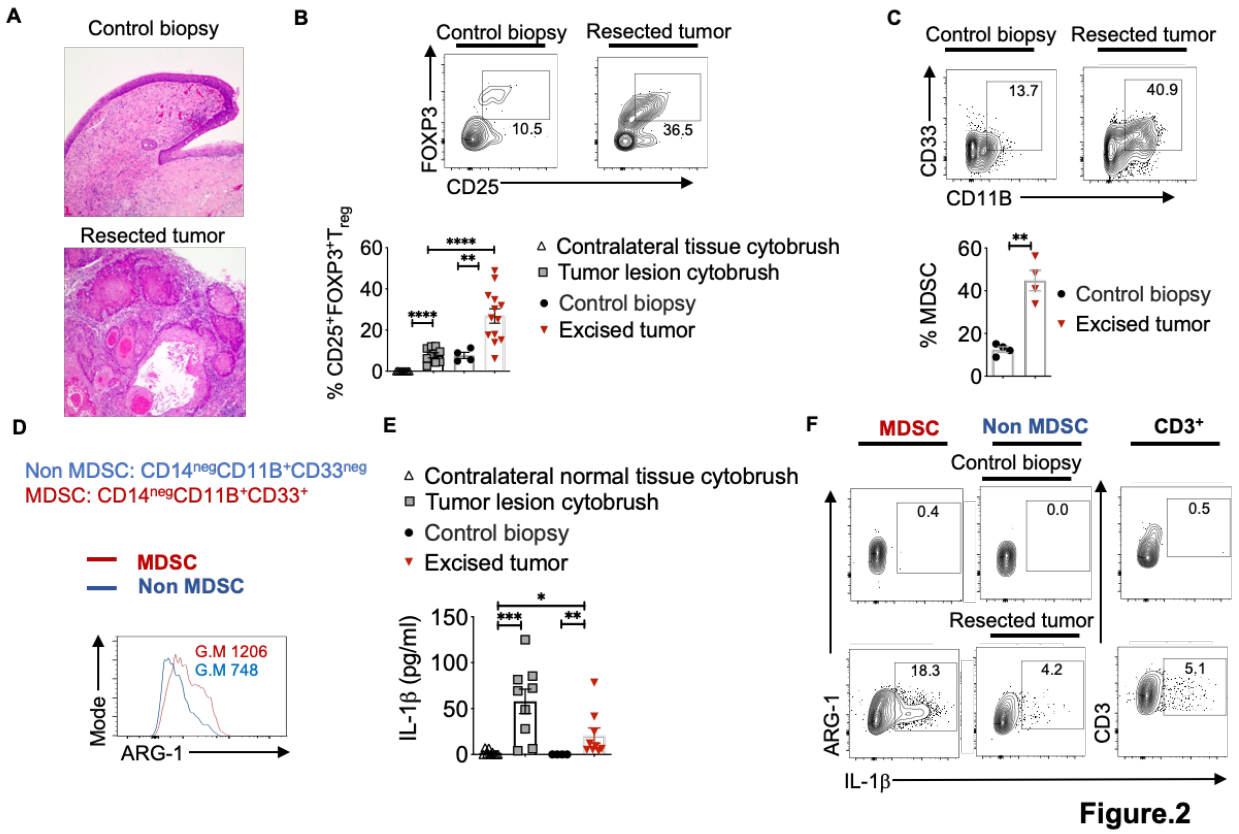

Figure.2

Figure legend

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. H&E immunohistochemistry images of the tongue at 200X magnification (**A**). Samples were digested by collagenase-1A and restimulated with PMA/Ionomycin for 4 hours. Contour plots gated on $CD45^+CD3^+CD4^+$ lymphocytes (**B**, top) and statistical analysis (**B**, bottom) showing % of T_{regs} and 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) (**F**). Mean +/-SEM are shown. (Mann-Whitney test * $P<0.05$).

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

159 The degree of phenotypic and functional heterogeneity of tissue/intra-tumoral T_{regs} can be high
160 [30], and evaluating them solely with FOXP3 and CD25 markers without examining anti-tumor
161 $CD8^+$ T cells can sometimes be misleading. Besides recruiting thymic T_{regs} into tumors via
162 chemotaxis, T_{regs} can be induced *in situ* in tumors due to the abundance of mediators such as nitric
163 oxide synthase, indoleamine 2, 3-dioxygenase 1, transforming growth factor- β1, and adenosine
164 released from tumor cells, TAM, and MDSC [31]. Some of these $FOXP3^+$ cells may also be
165 critical for tissue repair or become dysfunctional depending on the inflammatory cytokine
166 milieu[32]. Indeed we[26; 33-37] and others have shown distinct functions of T-bet^+Foxp3^+cells,
167 ROR-γt^+Foxp3^+ cells, and PD-1^+Foxp3^+ cells, whose functions are significantly altered by
168 cytokines including IL-6 and IL-1β in an mTOR dependent manner in oral mucosa[26]. Since
169 CD25, PD-1, T-bet, ROR-γt, Suppression of Tumorigenicity 2 (ST-2) expression were all
170 implicated in determining intra-tumoral T_{reg} functions positively and negatively in other tissues[7;
171 28; 38; 39], we examined the expression of the molecules, in aged vs. young T_{regs}, 16 weeks after
172 4-NQO administration. While the expression of CD25 (ability to consume IL-2) [10; 40], Ki-67
173 (proliferation), and ST-2 (tissue T_{reg} and tumorigenicity marker) [7] did not change between young
174 and aged T_{regs}, CD103 (TGF-β1 dependent, tolerogenic, and tumor-specific T cell marker)[41],
175 PD-1, ROR-γt, and T-bet were significantly up-regulated in aged T_{regs} (**Fig. 3A)**. Consistent with
176 the higher expression of ROR-γt in T_{regs} and earlier dysplasia in aging mice, increased numbers of
177 IL-1β expressing cells were found among infiltrating immune cells in aged mice as early as 5 and
178 12 weeks after 4-NQO administration (**Fig. 3B, C**). IL-6 levels were also elevated in aged tongue
179 supernatants(**Fig. S5**). Moreover, at later times, IL-1β expression was found in epithelial tumor
180 cells and immune cell infiltrates in both the groups, but was dramatically elevated in aged 4-NQO
181 treated mice when compared to their young counterparts (**Fig. 3D**). These results showed that T_{regs}

182 found in tongues during early tumor development in 4-NQO treated mice are phenotypically
183 distinct, appearing to be functionally more immunosuppressive in aged mice, and may be linked
184 to higher expression of IL-1β in the milieu.
185

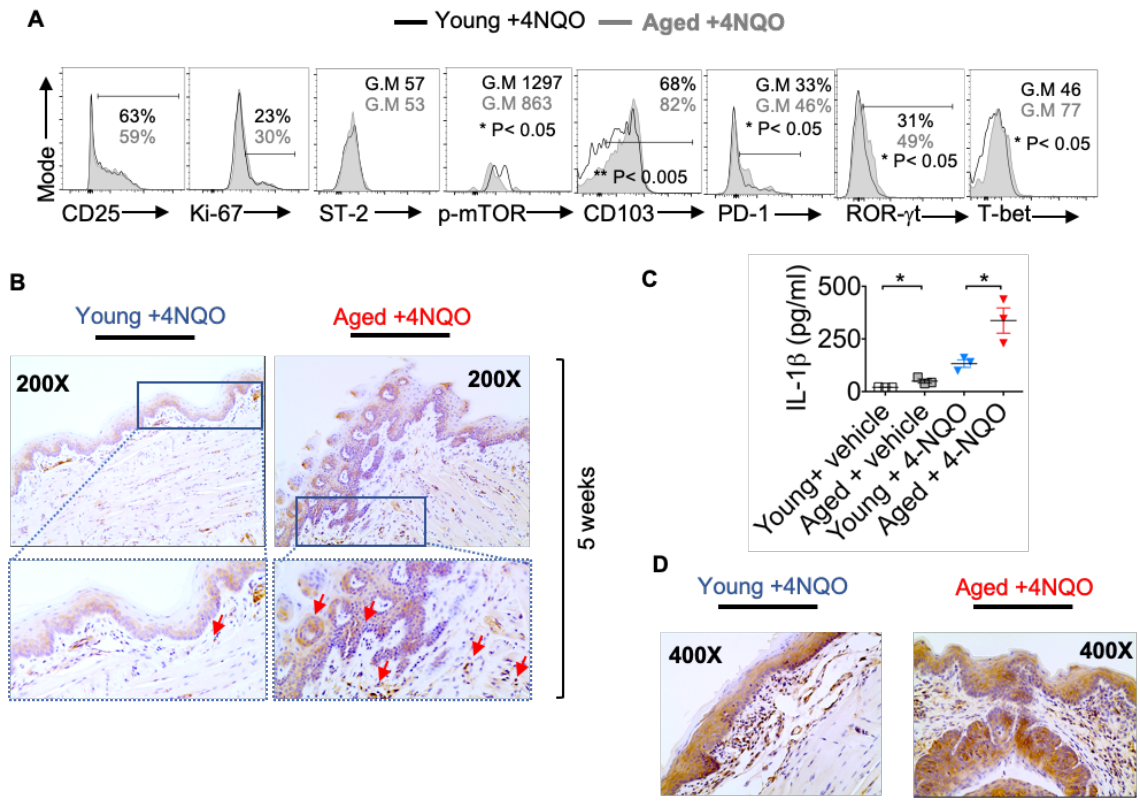

Figure.3

186

187 **Figure legend**

188 **Fig. 3. Aged mice have distinct T_{reg} phenotype and elevated levels of IL-1β compared to young mice.** 16 weeks
189 after 4-NQO administration, single-cell suspension from tongue was re-stimulated with PMA/Ionomycin for 4 hours.
190 Flow cytometry histograms showing indicated protein expression in $CD45^+$ $CD3^+$ $CD4^+Foxp3^+$cells **(A)**. Tongue
191 tissues were processed for IL-1β immuno-histochemical staining at 5 weeks(**B**). Supernatants collected at 12 weeks
192 were processed for IL-1β ELISA. Mean +/- SEM in 3 mice/group (Mann-Whitney test * P< 0.05) **(C)**. Tongue tissues
193 were processed for IL-1β immuno-histochemical staining at 24 weeks(**D**).
194

195 **Partial T_{reg} depletion reduces tumor progression.**

196 Although tumorigenesis coincides with increases in Foxp3$^+$ T_{regs}, the function of these cells has
197 not been validated in the 4-NQO model. Therefore, we employed Foxp3^{DTR}-eGFP reporter (Foxp3
198 diphtheria toxin receptor; FDTR) mice that express the diphtheria toxin receptor on Foxp3$^+$ cells
199 and allow targeted T_{reg} depletion by intraperitoneal diphtheria toxin (DT) injection (20 μg/10 gm
200 body weight; n=4/group)[36; 40]. We took two approaches; 1) Early and long-term T_{reg} depletion
201 where DT was injected every 5 days, in the first 16 weeks of 4-NQO treatment 2) Late and short-
202 term T_{reg} depletion where DT was injected every 5 days, from the 16th -21st weeks of 4-NQO
203 treatment n=6/group)(**Fig. 4A)**. Early T_{reg} depletion caused systemic autoimmunity characterized
204 by splenomegaly, lymphadenopathy, CD4 hyperactivation (CD44^{high}, IFN-γ^{high}), and mortality in
205 30% of mice which confounded the effects on early tumorigenesis (*not shown*). However, late and
206 short-term T_{reg} depletion caused only partial T_{reg} reduction, but the mice had significantly reduced
207 dysplasia and OSCC at 24 weeks (**Fig. 4B, C, S6A**). The frequencies of CD11b$^+$Ly6G$^+$ and IL-
208 1β$^+$ cells were unchanged with T_{reg} depletion, showing that these components are likely upstream
209 to T_{reg} induction and infiltration during tumorigenesis (**Fig. S6B, C**). However, the PD-1
210 expression was diminished and IFN-γ expression was partially restored in CD8$^+$ T cells(**Fig. 4D**).
211 Collectively, these results show, for the first time in a 4-NQO OSCC model, that infiltrating T_{regs}
212 control anti-tumorigenic functions of CD8$^+$ T cells and contribute to OSCC tumorigenesis and
213 progression.

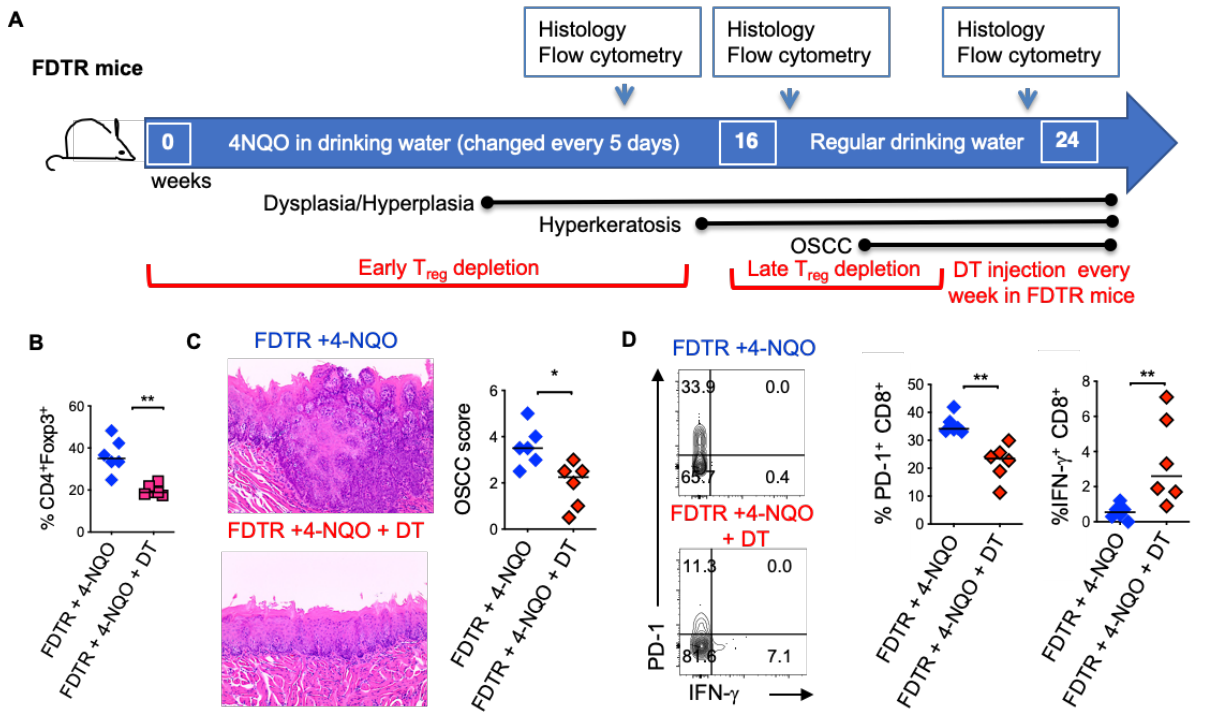

Figure.4

214

215 **Figure legend**

216 **Fig. 4. Partial T_{reg} depletion reduces tumor progression.** T_{regs} were depleted in FDTR mice by injecting diphtheria
217 toxin (DT) at late time points (every 5 days between 16th -21st weeks of 4-NQO treatment) **(A)**. Statistics showing
218 $CD4^{+}Foxp3^{+}$cells based on flow cytometry analyses at 20 and 24 weeks (**B**). Immunohistochemistry H&E staining
219 (left) and OSCC score based on invasive hyperkeratosis, and nesting (0-5) (right) **(C)**. Contour plots (left) and
220 statistics (right) showing PD-1 and IFN-γ expressing $CD8^{+}$ T cells (**D**). Mean +/- SEM in 6 mice/group (Mann-
221 Whitney test * P< 0.05).

222

223 ***Candida* and zymosan exacerbate and accelerate dysplasia and hyperplasia.**

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

228 infection and consequent IL-1β induction might worsen OSCC outcome. Therefore, we examined
229 the effect of *Candida* infection and zymosan during carcinogenesis in 4-NQO treated mice. We
230 sublingually applied *Candida* (10^7 blastospores) or D-zymosan (TLR-2 depleted zymosan; 1
231 mg/100ul) under anesthesia WT or TLR-2 knockout (TLR-2 KO) C57BL/6 mice every week
232 between 4th and 8th weeks of 4-NQO administration. These treatments significantly accelerated
233 and worsened dysplasia/hyperplasia (**Fig. 5A, B**), increased IL-1β^+ cells and T_{regs} in the tongue at
234 12 weeks (**Fig. 5C, D, S7)**, and induced OSCC around 21 weeks (**Fig. 5E)**. Importantly, premature
235 OSCC development was also observed in TLR-2 KO mice, revealing that zymosan and *Candida*
236 mediated exacerbation of OSCC development was independent of TLR-2 signaling. In fact, in the
237 absence of TLR-2, the numbers of IL-1β^+ cells were increased implying the involvement of anti-
238 fungal dectin-1 signaling in OSCC.

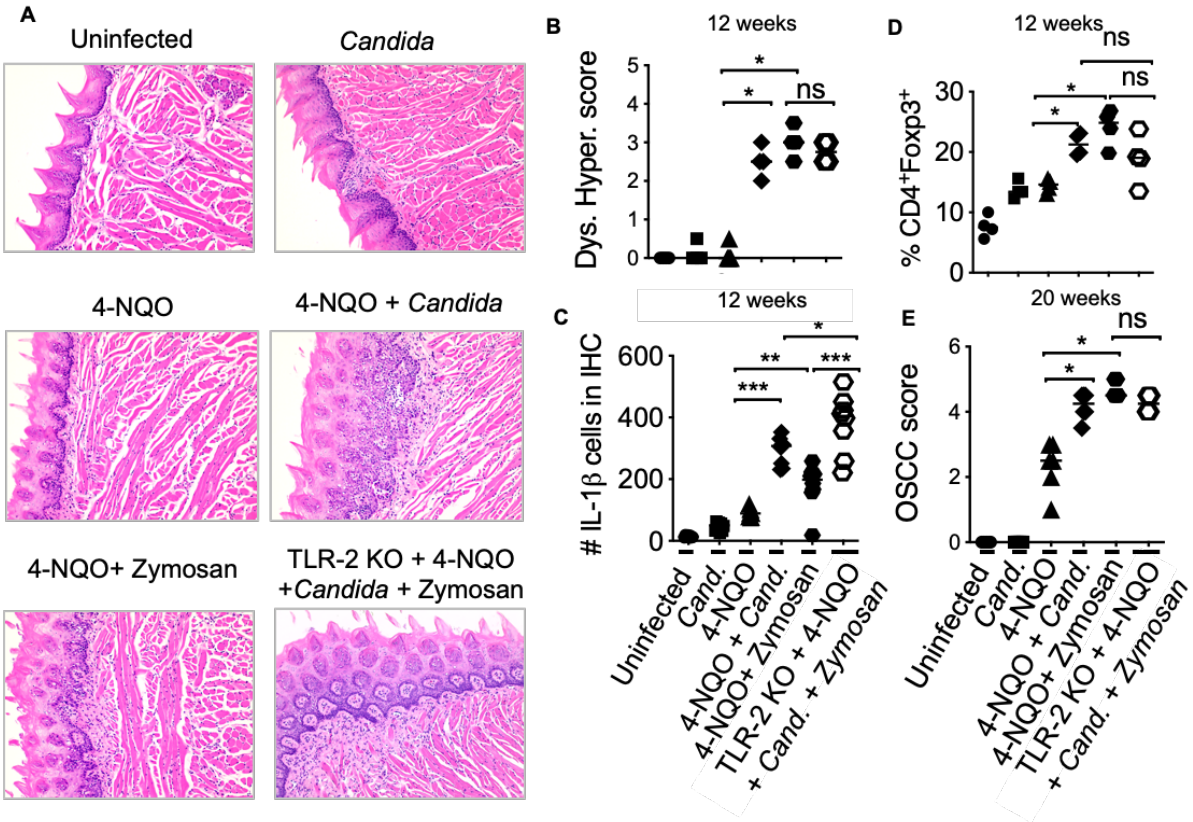

Figure.5

239

240 **Figure legend**

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

249

250 **Aged mice show elevated Dectin-1 signaling in immune cells in the tongue and increased**
251 **fungal abundance in saliva.** Dectin-1 is expressed by myeloid phagocytes[48] and binds
252 specifically to fungal β-1,3 glucans, endogenous galectins, and annexins on apoptotic cells[49].
253 Dectin1 signaling activation induces the phosphorylation of Syk and consequently the expression
254 of IL-1β. We and others have shown that dectin-1 signaling can regulate T_{reg} alterations and
255 macrophage metabolism[50-53]. Fungal dysbiosis and dectin-1 have also been implicated in
256 inflammatory diseases and cancers[54-56]. Thus, previous reports and our above-mentioned
257 results led us to hypothesize that dectin-1 might be involved in OSCC development and earlier
258 predisposition of aged mice to OSCC. Confirming the hypothesis, 4-NQO induced tumorigenesis
259 promoted a significant elevation of dectin-1 expression and Syk activation in immune infiltrates
260 ($CD45^+$ cells) (**Fig. 6A**). Dectin-1 expression and Syk activation were further increased in aged 4-
261 NQO treated mice suggesting the involvement of dectin-1 signaling in accelerated tumorigenesis
262 in aging mice(**Fig. 6B**). Although not-significant, there was also a trend towards increased fungal
263 abundance in the saliva of 4-NQO treated mice, as measured by fungal abundance qPCR[57; 58].
264 Interestingly, aged 4-NQO treated mice that displayed accelerated dysplasia and OSCC (**Fig. 1A**),

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

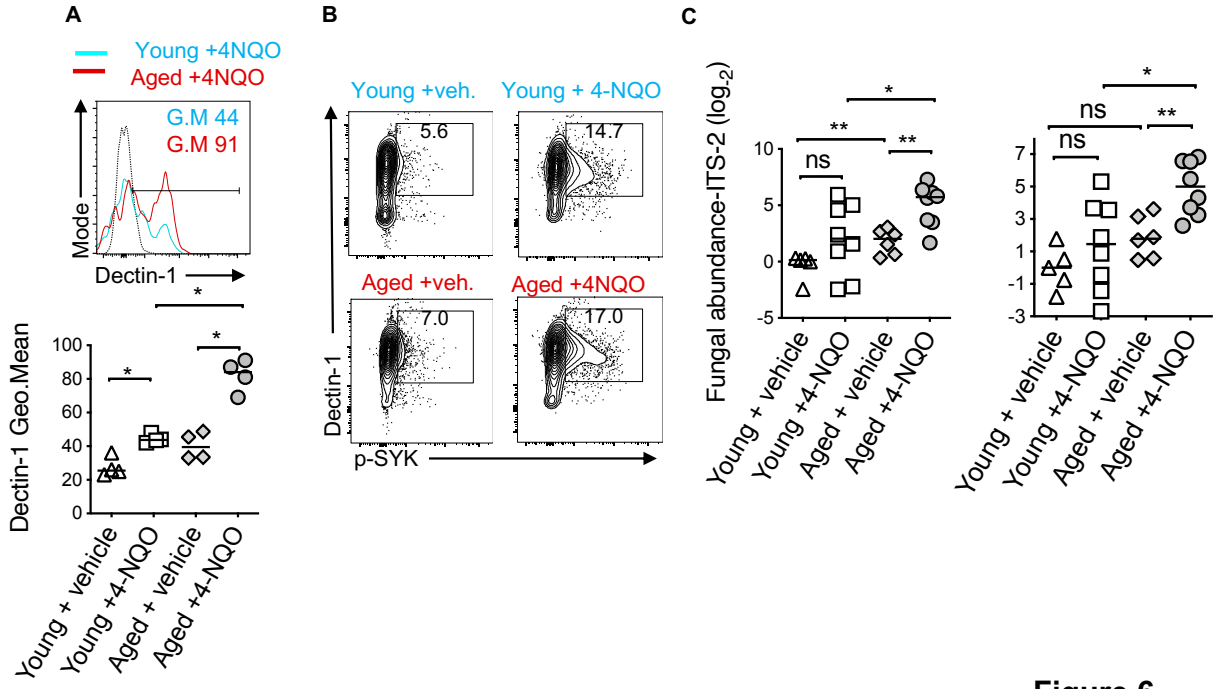

Figure.6

268

269 **Figure legend**

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

276

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

280 pronounced in Dectin-1 KO than WT mice **(Fig. 7A)**. Dectin-1 KO mice also had significantly
281 reduced IL-1β^+ cells **(Fig. 7B, S8)**, T_{reg} induction (**Fig. 7C, S9**), T_{reg}:CD8 ratio(**Fig. 7D**), PD-1
282 induction in $CD8^+$ T cells(**Fig. 7E, G**), and MDSC cells(**Fig.7F**). However, coinciding with IFN-
283 γ restoration in CD8 T cells(**Fig. 7E, G**), mice showed significantly slower progression of
284 dysplasia and lower numbers and sizes of OSCC tumors(**Fig. 7H, I**). Based on the phenotype of
285 Dectin-1 KO mice, dectin-1 signaling appears to play a clear role in OSCC development. Taken
286 together, these results show dectin-1 signaling is critical for establishing an immunosuppressive
287 milieu and the development of oral tumorigenesis.

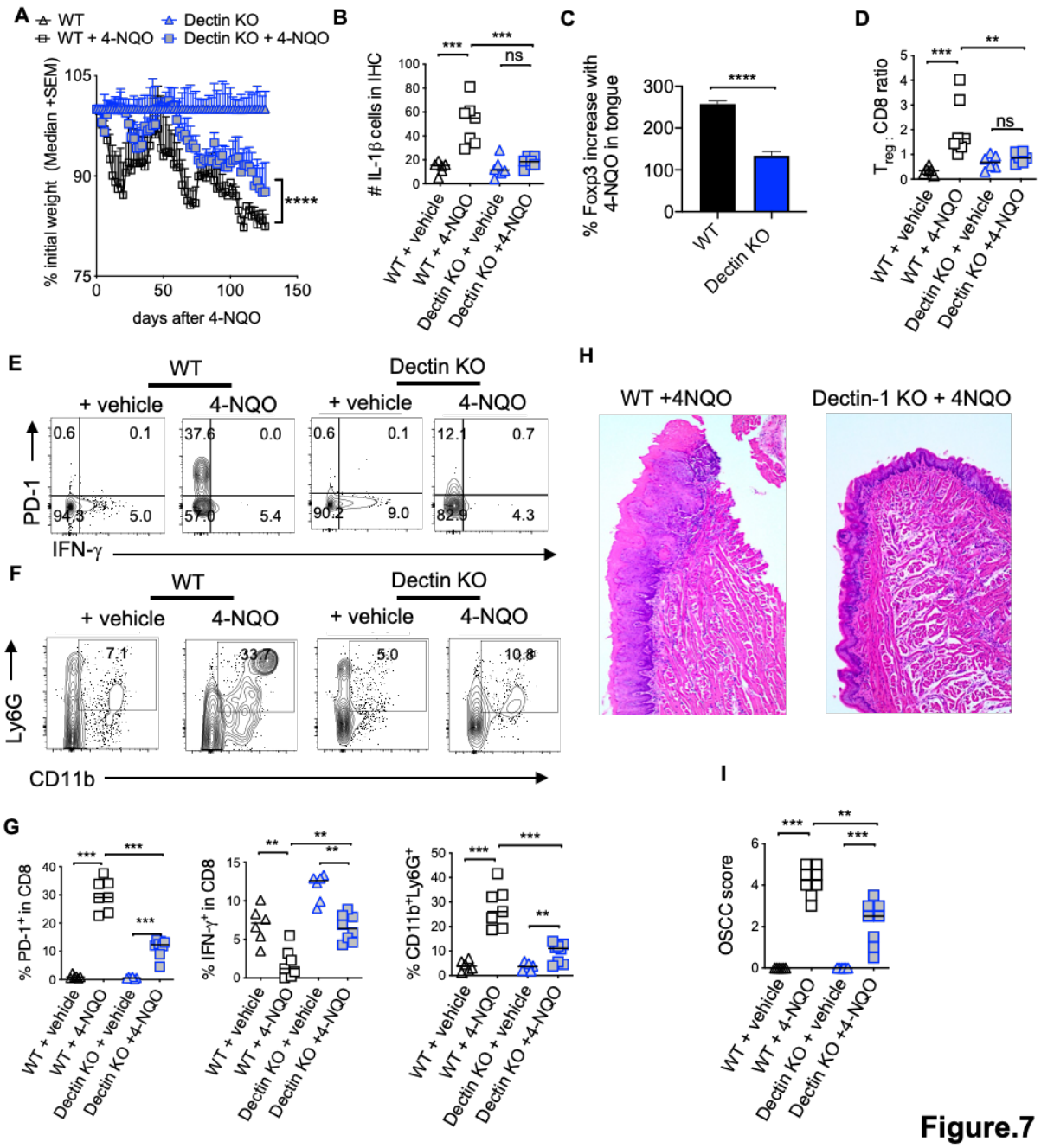

Figure.7

Figure legend

Fig. 7. Dectin-1 deficiency reduces immunosuppressive cells and OSCC tumor burden. Statistics showing the body weight loss, comparing the indicated groups (**A**). Tongue tissues were processed for immunohistochemistry (IHC) and flow cytometry at 23 weeks after 4-NQO administration. Statistics showing the number of IL-1β+ cells in the entire field of 200X IHC image (**B**). Flow cytometry-based statistics showing % increase in T_{regs} in 4-NQO treated

294 mice compared to Veh. controls (**C**), T_{reg}:CD8 ratio (**D**), the % of PD-1 and IFN-γ expressing CD8 cells (**E**), the % of
295 CD11b and Ly6G expressing $CD45^+$ cells (**F**), and the statistical analysis for all of the above (**G**). H&E
296 immunohistochemistry of tongue (**H**), and blinded OSCC scoring (**I**). (Mann-Whitney test except for (**B),** where t-
297 test with Welch's correction was performed, * $P<0.05$).
298

299 **Discussion**

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

318 T_{regs} in some cancers correlate with improved disease outcomes[63-66]. However, multivariate
319 analysis shows that T_{reg} levels are not independently prognostic of OSCC but cytolytic T cells that
320 co-infiltrate along with T_{regs} appear to drive the favorable prognosis [15]. Therefore we examined
321 $FOXP3^+$ cells in relation to $CD8^+$T cell frequency and MDSC. Increased intra-tumoral T_{reg}: CD8
322 ratio rather than increases in T_{reg} frequencies is more informative about pro-tumorigenic functions
323 of T_{regs} during OSCC. Indeed, examining T_{reg}: CD8 ratio has allowed investigators to obtain
324 consistent data showing clearly that a higher ratio is associated with poor prognosis and survival
325 in human studies [67; 68]. While we also characterized the T_{regs} by examining their inflammatory
326 and tissue markers during early carcinogenesis in aging mice, more studies are required to
327 determine the mechanisms underlying their altered phenotype and their contribution to OSCC
328 development.
329
330 IL-1β is a cancer-associated pro-inflammatory cytokine that is abundant in breast, colon,
331 lung, esophageal, as well as OSCC cancers[69]. It is linked to poor prognosis for patients with
332 esophageal cancer[70]. Targeting IL-1β has been shown to hinder oral carcinogenesis in the 4-
333 NQO model, and is considered in the clinical settings[29; 71]. IL-1β is also involved in inducing
334 immunomodulatory $Foxp3^+$ROR-γt^+ cells in microbiome dependent manner[26; 29; 36; 37; 51;
335 72-79]. $Foxp3^+$ROR-γt^+ cells have been shown to be present in tumors and contribute to tumor
336 immune evasion and autoimmunity control[28; 80]. But the events preceding excessive IL-1β
337 expression and at the IL-1β/T_{reg} interface are largely unknown. Our data showed that there was
338 excessive IL-1β production in tongues of aged 4-NQO treated mice compared to young mice,
339 demonstrating that IL-1β is clearly associated with the establishment of a worse
340 immunosuppressive milieu. These results are distinct from an acute infection milieu and steady

341 state-conditions where aging oral mucosa shows IL-1β suppression [26]. Although it is
342 conceivable that early carcinogenesis environment triggers elevated levels of IL-1β in aged mice,
343 the sequence of events that promote elevated levels of IL-1β, T_{reg} accrual, and MDSC increase
344 during aging need to be addressed. Furthermore, we found increased dectin-1 expression in
345 immune infiltrates in aging mice. Also, signaling through dectin-1 promoted T_{reg} and MDSC
346 accrual. While it is clear that immune cells show activation of Syk signaling, the precise subset of
347 cells that responds through dectin-1 signaling hyperactivation in the context of aging remains to
348 be addressed. While we hypothesize that changes in fungal stimulation emanating from the
349 commensal fungal microbome could lead to dectin-1 activation, this possibility remains to be
350 further confirmed in the future. The microbiome is a significant contributor to OSCC in the 4-
351 NQO mediated carcinogenesis model [81; 82]. Aging is also associated with changes in the
352 resident microbiome, reduced microbiota diversity, and an increase in certain types of bacteria and
353 fungi, resulting in local immunological changes, chronic inflammation, and other geriatric
354 sequelae [83-85]. *Candida* carriage and related co-morbidities are significantly more frequent in
355 elderly individuals[86]. The role of microbiome dysbiosis in enhancing dectin-1 signaling,
356 immunosuppressive settings, and OSCC development, and how these are dysregulated during
357 aging are critical questions to be addressed and will be studied in future investigations. Taken
358 together, our study sheds new light on the immune-oncological events in TIME providing some
359 key insights into mechanisms of immune evasion. By linking MDSC and T_{regs} with a higher
360 predisposition of aged oral mucosa to tumor development, our study also provides important
361 insights into mechanisms of higher susceptibility of the aging population to oral cancer. By
362 revealing the pro-tumorigenic role of dectin-1 signaling and T_{regs}, our study may also provide a
363 way to control intra-tumoral T_{regs} without affecting the peripheral T_{reg} cell repertoire.

364

365 **Author contributions**

366 PP supervised the project, designed the study, performed experiments, analyzed data, and wrote
367 the manuscript. QP and AT provided tumor resections from human participants under an approved
368 protocol and contributed to discussions. NB and SJ performed the experiments and analyzed
369 ELISA and qPCR data. PM weighed the mice and performed validation qPCRs. CQ and SJ
370 performed microscopy of the histology slides, scored the histology data in a masked fashion,
371 performed animal breeding, and processed tissues for flow cytometry. MG and AW read the
372 manuscript and contributed to discussions.

373

374 **Acknowledgments**

375 PP was supported by the CWRU School of Dental Medicine departmental startup funding and
376 NIH Head and Neck Cancer SPORE pilot grant (P30 CA043703).

377

378 **Declaration of interests**

379 The authors declare no competing interests.

380

381

382

383

384

385

386

387 **Materials and methods**

388

389 **Mice and human samples**

390 Mouse experiments were performed following all guidelines and regulations under an approval
391 from the CWRU Institutional Animal Care and Use Committee. Young (3-4 months of age), aged
392 (20-24 months of age) C57BL/6 mice, TLR-2$^{-/-}$ and Dectin-1$^{-/-}$ mice were purchased from Jackson
393 laboratories. Human oral tissue samples were obtained by excision (tumor resection), cytobrushing
394 (mostly epithelial cells), or by biopsy (control tissue 2-3 cm from the tumor margin) under an
395 approved protocol approved by the University Hospitals Cleveland Medical Center Institutional
396 Review Board.

397

398 **4-NQO administration in mice**

399 4-NQO was dissolved in 1:1 solution of propylene glycol and DMSO and administrated in drinking
400 water (50 -100 μg/ml) for 16 weeks, followed by the regular drinking water for another 6 weeks.
401 Mice were sacrificed at the end of 24-25 week 4-NQO regimen. Young mice developed OSCC
402 tumors after 24- 25 weeks and aged mice around 21-22 weeks after the start of 4-NQO
403 administration (**Fig. 1A**). Control mice received the propylene glycol/DMSO vehicle. Mouse body
404 weight was measured every other day until sacrifice. 5-, 12 and 15- week time-points were
405 considered early timepoints for the evaluation of immune cell changes in the tongue. Scores were
406 assigned using tongue immuno-histochemistry using established OSCC criteria[61]. Epithelial
407 hyperplasia and dysplasia scores were designated at 12 weeks after the 4-NQO regimen as follows:
408 0 = No immune infiltrates and the presence of cohesive epithelium;1 =Sparse immune infiltrates
409 with mild epithelial hyperplasia; 2= Sparse immune infiltrates and moderate epithelial hyperplasia

410 and dysplasia; 3 = Severe epithelial hyperplasia and dysplasia and moderate hyperkeratosis; 4 =
411 Severe epithelial hyperplasia and dysplasia and hyperkeratosis; 5= Presence of invasive
412 hyperkeratosis. OSCC scores were given at 20 or 24-25 weeks after the 4-NQO regimen as
413 follows: 0 = No immune infiltrates and the presence of cohesive epithelium;1 =Sparse immune
414 infiltrates with mild epithelial hyperplasia; 2= Moderate epithelial hyperplasia and dysplasia; 3 =
415 Severe epithelial hyperplasia and dysplasia and moderate hyperkeratosis; 4 = Severe epithelial
416 hyperplasia and dysplasia and hyperkeratosis; 5= Presence of invasive hyperkeratosis, cell nests
417 and OSCC.
418
419 **Antibodies**
420 Purified or fluorochrome conjugated mouse and human α-CD25 (3C7 and 7D4), CD4, IFN-γ,
421 Foxp3, CD45, CD8, CD11C, CD38, HLADR, Phospho-Syk (Ser473), IL-1β, IL-6, Arg-1, PerCP-
422 eFluor 710 conjugated Ly-6G Monoclonal Antibody (1A8-Ly6g), APC conjugated CD11b
423 Monoclonal Antibody (M1/70) and dectin-1 antibodies were all purchased from Life
424 Technologies/Thermofisher and BD-Biosciences.
425
426 **Quantitative-PCR**
427 DNA was isolated from mouse saliva swabs using the PureLink™ Microbiome DNA Purification
428 Kit (Invitrogen). ITS and FungiQuant qPCR primers [57; 58] (Invitrogen), and SYBR Green PCR
429 Kit (BioBasic) were used for performing qPCR, employing the real time PCR machine (Applied
430 Biosystems). The relative amount of fungal DNA of interest was estimated from its Ct values,
431 which were normalized to the host β-actin DNA levels.
432

433 **Histochemistry and immunohistochemistry of proteins**

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

439

440 **Flow cytometry and ELISA**

441 Cells were re-stimulated with PMA (50 ng/ml) and Ionomycin (500 ng/ml) for 4 hours in complete
442 RPMI-1640 (Hyclone) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml
443 streptomycin, 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate and 50 μM β-
444 mercaptoethanol [40]. Brefeldin-A (10 μg/ml) added in last 2 hours before harvesting the cells and
445 supernatants. For single-cell flow cytometry staining, cells washed in PBS or PBS/BSA before
446 surface and intracellular staining. Live-Dead viability staining was used to remove dead cells in
447 the flow cytometry analyses. Appropriate un-stain, isotype, secondary antibody, single stain and
448 FMO controls were used. For p-Syk staining, the cells were washed, fixed and were stained with
449 Phosflow staining kit from BD Biosciences using manufacturer's protocol. For examining T cells,
450 leukocyte singlets, Live-dead^{neg}, $CD45^{+}CD3^{+}CD4^{+}$ or $CD8^{+}$ gates were used. For examining
451 MDSC, leukocyte singlets, Live-dead^{neg}, $CD45^{+}CD3^{neg}$ gates were used. Data was acquired using
452 BD Fortessa cytometers and were analyzed using FlowJo 9.8 or 10.5.3 software. Mouse IL-1β and
453 IL-6 ELISA kits from Boster Bio (Pleasanton, CA) were used to assess the protein levels in the
454 supernatants.

455

456 **Oral *Candida* infection and Zymosan application**

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

461

462 **Statistical analysis**

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

467

468 **Supplemental information**

469 Supplementary figures are attached separately.

470

471 **References**

472

473 [1] M.C. White, D.M. Holman, R.A. Goodman, and L.C. Richardson, Cancer Risk Among Older
474 Adults: Time for Cancer Prevention to Go Silver. Gerontologist 59 (2019) S1-S6.
475 [2] Y. Harari, and M. Kupiec, Do long telomeres affect cellular fitness? Curr Genet 64 (2018) 173-
476 176.
477 [3] S.S. Jose, K. Bendickova, T. Kepak, Z. Krenova, and J. Fric, Chronic Inflammation in Immune
478 Aging: Role of Pattern Recognition Receptor Crosstalk with the Telomere Complex? Front
479 Immunol 8 (2017) 1078.
480 [4] P. Scaffidi, and T. Misteli, Lamin A-dependent nuclear defects in human aging. Science 312
481 (2006) 1059-63.
482 [5] V. Bronte, S. Brandau, S.H. Chen, M.P. Colombo, A.B. Frey, T.F. Greten, S. Mandruzzato,
483 P.J. Murray, A. Ochoa, S. Ostrand-Rosenberg, P.C. Rodriguez, A. Sica, V. Umansky, R.H.
484 Vonderheide, and D.I. Gabrilovich, Recommendations for myeloid-derived suppressor cell
485 nomenclature and characterization standards. Nat Commun 7 (2016) 12150.

[6] T.M. Grzywa, A. Sosnowska, P. Matryba, Z. Rydzynska, M. Jasinski, D. Nowis, and J. Golab, Myeloid Cell-Derived Arginase in Cancer Immune Response. Front Immunol 11 (2020) 938.
[7] A. Hatzioannou, A. Banos, T. Sakelaropoulos, C. Fedonidis, M.S. Vidali, M. Kohne, K. Handler, L. Boon, A. Henriques, V. Koliaraki, P. Georgiadis, J. Zoidakis, A. Termentzi, M. Beyer, T. Chavakis, D. Boumpas, A. Tsirigos, and P. Verginis, An intrinsic role of IL-33 in Treg cell-mediated tumor immunoevasion. Nat Immunol 21 (2020) 75-85.
[8] C. O'Higgins, F.J. Ward, and R. Abu Eid, Deciphering the Role of Regulatory CD4 T Cells in Oral and Oropharyngeal Cancer: A Systematic Review. Front Oncol 8 (2018) 442.
[9] M. Onda, K. Kobayashi, and I. Pastan, Depletion of regulatory T cells in tumors with an anti-CD25 immunotoxin induces CD8 T cell-mediated systemic antitumor immunity. Proc Natl Acad Sci U S A 116 (2019) 4575-4582.
[10] P. Pandiyan, L. Zheng, and M.J. Lenardo, The molecular mechanisms of regulatory T cell immunosuppression. Frontiers in immunology 2 (2011) 60.
[11] S. Sakaguchi, T. Yamaguchi, T. Nomura, and M. Ono, Regulatory T cells and immune tolerance. Cell 133 (2008) 775-87.
[12] E.M. Shevach, Mechanisms of foxp3+ T regulatory cell-mediated suppression. Immunity 30 (2009) 636-45.
[13] A. Chaudhry, and A.Y. Rudensky, Control of inflammation by integration of environmental cues by regulatory T cells. J Clin Invest 123 (2013) 939-44.
[14] I.K. Gratz, M.D. Rosenblum, and A.K. Abbas, The life of regulatory T cells. Ann N Y Acad Sci 1283 (2013) 8-12.
[15] R. Mandal, Y. Senbabaoglu, A. Desrichard, J.J. Havel, M.G. Dalin, N. Riaz, K.W. Lee, I. Ganly, A.A. Hakimi, T.A. Chan, and L.G. Morris, The head and neck cancer immune landscape and its immunotherapeutic implications. JCI Insight 1 (2016) e89829.
[16] C. Tanchot, M. Terme, H. Pere, T. Tran, N. Benhamouda, M. Strioga, C. Banissi, L. Galluzzi, G. Kroemer, and E. Tartour, Tumor-infiltrating regulatory T cells: phenotype, role, mechanism of expansion in situ and clinical significance. Cancer Microenviron 6 (2013) 147-57.
[17] T.S. Nowicki, S. Hu-Lieskovan, and A. Ribas, Mechanisms of Resistance to PD-1 and PD-L1 Blockade. Cancer J 24 (2018) 47-53.
[18] L. Wen, H. Lu, Q. Li, Q. Li, S. Wen, D. Wang, X. Wang, J. Fang, J. Cui, B. Cheng, and Z. Wang, Contributions of T cell dysfunction to the resistance against anti-PD-1 therapy in oral carcinogenesis. J Exp Clin Cancer Res 38 (2019) 299.
[19] C. Tay, Y. Qian, and S. Sakaguchi, Hyper-Progressive Disease: The Potential Role and Consequences of T-Regulatory Cells Foiling Anti-PD-1 Cancer Immunotherapy. Cancers (Basel) 13 (2020).
[20] B. Pang, Y. Zhen, C. Hu, Z. Ma, S. Lin, and H. Yi, Myeloid-derived suppressor cells shift Th17/Treg ratio and promote systemic lupus erythematosus progression through arginase-1/miR-322-5p/TGF-beta pathway. Clin Sci (Lond) 134 (2020) 2209-2222.
[21] P. Pandiyan, and M.J. Lenardo, The control of CD4+CD25+Foxp3+ regulatory T cell survival. Biology direct 3 (2008) 6.
[22] W. Pierson, B. Cauwe, A. Policheni, S.M. Schlenner, D. Franckaert, J. Berges, S. Humblet-Baron, S. Schonefeldt, M.J. Herold, D. Hildeman, A. Strasser, P. Bouillet, L.F. Lu, P. Matthys, A.A. Freitas, R.J. Luther, C.T. Weaver, J. Dooley, D.H. Gray, and A. Liston,

531 Antiapoptotic Mcl-1 is critical for the survival and niche-filling capacity of Foxp3(+)
532 regulatory T cells. Nat Immunol 14 (2013) 959-65.
533 [23] L. Barron, H. Dooms, K.K. Hoyer, W. Kuswanto, J. Hofmann, W.E. O'Gorman, and A.K.
534 Abbas, Cutting edge: mechanisms of IL-2-dependent maintenance of functional regulatory
535 T cells. J Immunol 185 (2010) 6426-30.
536 [24] X. Tai, B. Erman, A. Alag, J. Mu, M. Kimura, G. Katz, T. Guinter, T. McCaughtry, R.
537 Etzensperger, L. Feigenbaum, D.S. Singer, and A. Singer, Foxp3 transcription factor is
538 proapoptotic and lethal to developing regulatory T cells unless counterbalanced by
539 cytokine survival signals. Immunity 38 (2013) 1116-28.
540 [25] X. Wang, A.L. Szymczak-Workman, D.M. Gravano, C.J. Workman, D.R. Green, and D.A.
541 Vignali, Preferential control of induced regulatory T cell homeostasis via a Bim/Bcl-2 axis.
542 Cell death & disease 3 (2012) e270.
543 [26] N. Bhaskaran, F. Faddoul, A. Paes da Silva, S. Jayaraman, E. Schneider, P. Mamileti, A.
544 Weinberg, and P. Pandiyan, IL-1beta-MyD88-mTOR Axis Promotes Immune-Protective
545 IL-17A(+)Foxp3(+) Cells During Mucosal Infection and Is Dysregulated With Aging.
546 Front Immunol 11 (2020) 595936.
547 [27] V.K. Vincent-Chong, H. DeJong, L.J. Rich, A. Patti, M. Merzianu, P.A. Hershberger, and M.
548 Seshadri, Impact of Age on Disease Progression and Microenvironment in Oral Cancer. J
549 Dent Res 97 (2018) 1268-1276.
550 [28] S. Downs-Canner, S. Berkey, G.M. Delgoffe, R.P. Edwards, T. Curiel, K. Odunsi, D.L.
551 Bartlett, and N. Obermajer, Suppressive IL-17A(+)Foxp3(+) and ex-Th17 IL-
552 17A(neg)Foxp3(+) Treg cells are a source of tumour-associated Treg cells. Nat Commun
553 8 (2017) 14649.
554 [29] T. Wu, Y. Hong, L. Jia, J. Wu, J. Xia, J. Wang, Q. Hu, and B. Cheng, Modulation of IL-1beta
555 reprogrammes the tumor microenvironment to interrupt oral carcinogenesis. Sci Rep 6
556 (2016) 20208.
557 [30] T. Saito, H. Nishikawa, H. Wada, Y. Nagano, D. Sugiyama, K. Atarashi, Y. Maeda, M.
558 Hamaguchi, N. Ohkura, E. Sato, H. Nagase, J. Nishimura, H. Yamamoto, S. Takiguchi, T.
559 Tanoue, W. Suda, H. Morita, M. Hattori, K. Honda, M. Mori, Y. Doki, and S. Sakaguchi,
560 Two FOXP3(+)CD4(+) T cell subpopulations distinctly control the prognosis of colorectal
561 cancers. Nat Med 22 (2016) 679-84.
562 [31] M. Chu, Y.X. Su, L. Wang, T.H. Zhang, Y.J. Liang, L.Z. Liang, and G.Q. Liao, Myeloid-
563 derived suppressor cells contribute to oral cancer progression in 4NQO-treated mice. Oral
564 Dis 18 (2012) 67-73.
565 [32] N. Arpaia, J.A. Green, B. Moltedo, A. Arvey, S. Hemmers, S. Yuan, P.M. Treuting, and A.Y.
566 Rudensky, A Distinct Function of Regulatory T Cells in Tissue Protection. Cell 162 (2015)
567 1078-89.
568 [33] P. Pandiyan, S. Younes, S. Ribeiro, A. Talla, N. Bhaskaran, D. McDonald, A. Weinberg, and
569 R. Sekaly, Mucosal regulatory T cells and T helper 17 cells in HIV associated immune
570 activation. . Frontiers in immunology 7 (2016) 228.
571 [34] A. Weinberg, S. Tugizov, P. Pandiyan, G. Jin, S. Rakshit, A. Vyakarnam, and J.R. Naglik,
572 Innate immune mechanisms to oral pathogens in oral mucosa of HIV-infected individuals.
573 Oral diseases 26 Suppl 1 (2020) 69-79.
574 [35] S.A. Younes, A. Talla, S. Pereira Ribeiro, E.V. Saidakova, L.B. Korolevskaya, K.V. Shmagel,
575 C.L. Shive, M.L. Freeman, S. Panigrahi, S. Zweig, R. Balderas, L. Margolis, D.C. Douek,
576 D.D. Anthony, P. Pandiyan, M. Cameron, S.F. Sieg, L.H. Calabrese, B. Rodriguez, and

577 M.M. Lederman, Cycling CD4+ T cells in HIV-infected immune nonresponders have
578 mitochondrial dysfunction. The Journal of clinical investigation 128 (2018) 5083-5094.
579 [36] N. Bhaskaran, C. Quigley, C. Paw, S. Butala, E. Schneider, and P. Pandiyan, Role of Short
580 Chain Fatty Acids in Controlling Tregs and Immunopathology During Mucosal Infection.
581 Front Microbiol 9 (2018) 1995.
582 [37] P. Pandiyan, N. Bhaskaran, M. Zou, E. Schneider, S. Jayaraman, and J. Huehn, Microbiome
583 dependent regulation of Tregs and Th17 cells in mucosa. Front Immunol 3 (2019).
584 [38] D.E. Lowther, B.A. Goods, L.E. Lucca, B.A. Lerner, K. Raddassi, D. van Dijk, A.L.
585 Hernandez, X. Duan, M. Gunel, V. Coric, S. Krishnaswamy, J.C. Love, and D.A. Hafler,
586 PD-1 marks dysfunctional regulatory T cells in malignant gliomas. JCI Insight 1 (2016).
587 [39] A.G. Levine, A. Mendoza, S. Hemmers, B. Moltedo, R.E. Niec, M. Schizas, B.E. Hoyos, E.V.
588 Putintseva, A. Chaudhry, S. Dikiy, S. Fujisawa, D.M. Chudakov, P.M. Treuting, and A.Y.
589 Rudensky, Stability and function of regulatory T cells expressing the transcription factor
590 T-bet. Nature 546 (2017) 421-425.
591 [40] P. Pandiyan, L. Zheng, S. Ishihara, J. Reed, and M.J. Lenardo, CD4(+)CD25(+)Foxp3(+)
592 regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4(+) T
593 cells. Nat Immunol 8 (2007) 1353-62.
594 [41] T. Duhen, R. Duhen, R. Montler, J. Moses, T. Moudgil, N.F. de Miranda, C.P. Goodall, T.C.
595 Blair, B.A. Fox, J.E. McDermott, S.C. Chang, G. Grunkemeier, R. Leidner, R.B. Bell, and
596 A.D. Weinberg, Co-expression of CD39 and CD103 identifies tumor-reactive CD8 T cells
597 in human solid tumors. Nat Commun 9 (2018) 2724.
598 [42] M. Perera, N.N. Al-Hebshi, I. Perera, D. Ipe, G.C. Ulett, D.J. Speicher, T. Chen, and N.W.
599 Johnson, A dysbiotic mycobiome dominated by Candida albicans is identified within oral
600 squamous-cell carcinomas. J Oral Microbiol 9 (2017) 1385369.
601 [43] R.J. Bastiaan, and P.C. Reade, The prevalence of Candida albicans in the mouths of tobacco
602 smokers with and without oral mucous membrane keratoses. Oral Surg Oral Med Oral
603 Pathol 53 (1982) 148-51.
604 [44] R.A. Cawson, Leukoplakia and oral cancer. Proc R Soc Med 62 (1969) 610-5.
605 [45] P.P. Dwivedi, S. Mallya, and A. Dongari-Bagtzoglou, A novel immunocompetent murine
606 model for Candida albicans-promoted oral epithelial dysplasia. Med Mycol 47 (2009) 157-
607 67.
608 [46] J.F. O'Grady, and P.C. Reade, Candida albicans as a promoter of oral mucosal neoplasia.
609 Carcinogenesis 13 (1992) 783-6.
610 [47] S.C. Cheng, F.L. van de Veerdonk, M. Lenardon, M. Stoffels, T. Plantinga, S. Smeekens, L.
611 Rizzetto, L. Mukaremera, K. Preechasuth, D. Cavalieri, T.D. Kanneganti, J.W. van der
612 Meer, B.J. Kullberg, L.A. Joosten, N.A. Gow, and M.G. Netea, The dectin-
613 1/inflammasome pathway is responsible for the induction of protective T-helper 17
614 responses that discriminate between yeasts and hyphae of Candida albicans. J Leukoc Biol
615 90 (2011) 357-66.
616 [48] M. Inoue, and M.L. Shinohara, Clustering of pattern recognition receptors for fungal
617 detection. PLoS Pathog 10 (2014) e1003873.
618 [49] K. Bode, F. Bujupi, C. Link, T. Hein, S. Zimmermann, D. Peiris, V. Jaquet, B. Lepenies, H.
619 Weyd, and P.H. Krammer, Dectin-1 Binding to Annexins on Apoptotic Cells Induces
620 Peripheral Immune Tolerance via NADPH Oxidase-2. Cell Rep 29 (2019) 4435-4446 e9.

621 [50] N. Bhaskaran, S. Cohen, Y. Zhang, A. Weinberg, and P. Pandiyan, TLR-2 Signaling Promotes
622 IL-17A Production in CD4+CD25+Foxp3+ Regulatory Cells during Oropharyngeal
623 Candidiasis. Pathogens 4 (2015) 90-110.
624 [51] P. Pandiyan, and J. Zhu, Origin and functions of pro-inflammatory cytokine producing
625 Foxp3(+) regulatory T cells. Cytokine 76 (2015) 13-24.
626 [52] F. Osorio, S. LeibundGut-Landmann, M. Lochner, K. Lahl, T. Sparwasser, G. Eberl, and C.
627 Reis e Sousa, DC activated via dectin-1 convert Treg into IL-17 producers. Eur J Immunol
628 38 (2008) 3274-81.
629 [53] S.C. Cheng, J. Quintin, R.A. Cramer, K.M. Shepardson, S. Saeed, V. Kumar, E.J.
630 Giamarellos-Bourboulis, J.H. Martens, N.A. Rao, A. Aghajanirefah, G.R. Manjeri, Y. Li,
631 D.C. Ifrim, R.J. Arts, B.M. van der Veer, P.M. Deen, C. Logie, L.A. O'Neill, P. Willems,
632 F.L. van de Veerdonk, J.W. van der Meer, A. Ng, L.A. Joosten, C. Wijmenga, H.G.
633 Stunnenberg, R.J. Xavier, and M.G. Netea, mTOR- and HIF-1alpha-mediated aerobic
634 glycolysis as metabolic basis for trained immunity. Science 345 (2014) 1250684.
635 [54] D. Daley, V.R. Mani, N. Mohan, N. Akkad, A. Ochi, D.W. Heindel, K.B. Lee, C.P.
636 Zambirinis, G.S.B. Pandian, S. Savadkar, A. Torres-Hernandez, S. Nayak, D. Wang, M.
637 Hundeyin, B. Diskin, B. Aykut, G. Werba, R.M. Barilla, R. Rodriguez, S. Chang, L.
638 Gardner, L.K. Mahal, B. Ueberheide, and G. Miller, Dectin 1 activation on macrophages
639 by galectin 9 promotes pancreatic carcinoma and peritumoral immune tolerance. Nat Med
640 23 (2017) 556-567.
641 [55] I.D. Iliev, and I. Leonardi, Fungal dysbiosis: immunity and interactions at mucosal barriers.
642 Nat Rev Immunol 17 (2017) 635-646.
643 [56] T. Wang, C. Fan, A. Yao, X. Xu, G. Zheng, Y. You, C. Jiang, X. Zhao, Y. Hou, M.C. Hung,
644 and X. Lin, The Adaptor Protein CARD9 Protects against Colon Cancer by Restricting
645 Mycobiota-Mediated Expansion of Myeloid-Derived Suppressor Cells. Immunity 49
646 (2018) 504-514 e4.
647 [57] I.D. Iliev, V.A. Funari, K.D. Taylor, Q. Nguyen, C.N. Reyes, S.P. Strom, J. Brown, C.A.
648 Becker, P.R. Fleshner, M. Dubinsky, J.I. Rotter, H.L. Wang, D.P. McGovern, G.D. Brown,
649 and D.M. Underhill, Interactions between commensal fungi and the C-type lectin receptor
650 Dectin-1 influence colitis. Science 336 (2012) 1314-7.
651 [58] C.M. Liu, S. Kachur, M.G. Dwan, A.G. Abraham, M. Aziz, P.R. Hsueh, Y.T. Huang, J.D.
652 Busch, L.J. Lamit, C.A. Gehring, P. Keim, and L.B. Price, FungiQuant: a broad-coverage
653 fungal quantitative real-time PCR assay. BMC Microbiol 12 (2012) 255.
654 [59] I. Harpaz, U. Bhattacharya, Y. Elyahu, I. Strominger, and A. Monsonego, Old Mice
655 Accumulate Activated Effector CD4 T Cells Refractory to Regulatory T Cell-Induced
656 Immunosuppression. Front Immunol 8 (2017) 283.
657 [60] K.A. Hwang, H.R. Kim, and I. Kang, Aging and human CD4(+) regulatory T cells. Mech
658 Ageing Dev 130 (2009) 509-17.
659 [61] R. Hasina, L.E. Martin, K. Kasza, C.L. Jones, A. Jalil, and M.W. Lingen, ABT-510 is an
660 effective chemopreventive agent in the mouse 4-nitroquinoline 1-oxide model of oral
661 carcinogenesis. Cancer Prev Res (Phila) 2 (2009) 385-93.
662 [62] C.H. Lee, J.S. Chang, S.H. Syu, T.S. Wong, J.Y. Chan, Y.C. Tang, Z.P. Yang, W.C. Yang,
663 C.T. Chen, S.C. Lu, P.H. Tang, T.C. Yang, P.Y. Chu, J.R. Hsiao, and K.J. Liu, IL-1beta
664 promotes malignant transformation and tumor aggressiveness in oral cancer. J Cell Physiol
665 230 (2015) 875-84.

666 [63] E.J. de Ruiter, M.L. Ooft, L.A. Devriese, and S.M. Willems, The prognostic role of tumor
667 infiltrating T-lymphocytes in squamous cell carcinoma of the head and neck: A systematic
668 review and meta-analysis. Oncoimmunology 6 (2017) e1356148.
669 [64] S.J. Santegoets, C.L. Duurland, E.S. Jordanova, J.J. van Ham, I. Ehsan, S.L. van Egmond,
670 M.J.P. Welters, and S.H. van der Burg, Tbet-positive regulatory T cells accumulate in
671 oropharyngeal cancers with ongoing tumor-specific type 1 T cell responses. J Immunother
672 Cancer 7 (2019) 14.
673 [65] Y. Takeuchi, and H. Nishikawa, Roles of regulatory T cells in cancer immunity. Int Immunol
674 28 (2016) 401-9.
675 [66] O. Stasikowska-Kanicka, M. Wagrowska-Danilewicz, and M. Danilewicz,
676 Immunohistochemical Analysis of Foxp3(+), CD4(+), CD8(+) Cell Infiltrates and PD-L1
677 in Oral Squamous Cell Carcinoma. Pathol Oncol Res 24 (2018) 497-505.
678 [67] E. Sato, S.H. Olson, J. Ahn, B. Bundy, H. Nishikawa, F. Qian, A.A. Jungbluth, D. Frosina, S.
679 Gnjatic, C. Ambrosone, J. Kepner, T. Odunsi, G. Ritter, S. Lele, Y.T. Chen, H. Ohtani, L.J.
680 Old, and K. Odunsi, Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high
681 CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer.
682 Proc Natl Acad Sci U S A 102 (2005) 18538-43.
683 [68] Y. Watanabe, F. Katou, H. Ohtani, T. Nakayama, O. Yoshie, and K. Hashimoto, Tumor-
684 infiltrating lymphocytes, particularly the balance between CD8(+) T cells and CCR4(+)
685 regulatory T cells, affect the survival of patients with oral squamous cell carcinoma. Oral
686 Surg Oral Med Oral Pathol Oral Radiol Endod 109 (2010) 744-52.
687 [69] M. Shiiba, K. Saito, H. Yamagami, D. Nakashima, M. Higo, A. Kasamatsu, Y. Sakamoto, K.
688 Ogawara, K. Uzawa, Y. Takiguchi, and H. Tanzawa, Interleukin-1 receptor antagonist
689 (IL1RN) is associated with suppression of early carcinogenic events in human oral
690 malignancies. Int J Oncol 46 (2015) 1978-84.
691 [70] M.F. Chen, M.S. Lu, P.T. Chen, W.C. Chen, P.Y. Lin, and K.D. Lee, Role of interleukin 1
692 beta in esophageal squamous cell carcinoma. J Mol Med (Berl) 90 (2012) 89-100.
693 [71] C.A. Dinarello, Why not treat human cancer with interleukin-1 blockade? Cancer Metastasis
694 Rev 29 (2010) 317-29.
695 [72] G. Landskron, M. De la Fuente, P. Thuwajit, C. Thuwajit, and M.A. Hermoso, Chronic
696 inflammation and cytokines in the tumor microenvironment. J Immunol Res 2014 (2014)
697 149185.
698 [73] E.M. El-Omar, M. Carrington, W.H. Chow, K.E. McColl, J.H. Bream, H.A. Young, J.
699 Herrera, J. Lissowska, C.C. Yuan, N. Rothman, G. Lanyon, M. Martin, J.F. Fraumeni, Jr.,
700 and C.S. Rabkin, Interleukin-1 polymorphisms associated with increased risk of gastric
701 cancer. Nature 404 (2000) 398-402.
702 [74] J. Hulkkonen, J. Vilpo, L. Vilpo, T. Koski, and M. Hurme, Interleukin-1 beta, interleukin-1
703 receptor antagonist and interleukin-6 plasma levels and cytokine gene polymorphisms in
704 chronic lymphocytic leukemia: correlation with prognostic parameters. Haematologica 85
705 (2000) 600-6.
706 [75] L. Li, and V.A. Boussiotis, The role of IL-17-producing Foxp3+ CD4+ T cells in
707 inflammatory bowel disease and colon cancer. Clin Immunol 148 (2013) 246-53.
708 [76] L. Li, J. Kim, and V.A. Boussiotis, IL-1beta-mediated signals preferentially drive conversion
709 of regulatory T cells but not conventional T cells into IL-17-producing cells. J Immunol
710 185 (2010) 4148-53.

711 [77] L. Li, N. Patsoukis, V. Petkova, and V.A. Boussiotis, Runx1 and Runx3 are involved in the
712 generation and function of highly suppressive IL-17-producing T regulatory cells. PloS
713 one 7 (2012) e45115.
714 [78] T. Miyao, S. Floess, R. Setoguchi, H. Luche, H.J. Fehling, H. Waldmann, J. Huehn, and S.
715 Hori, Plasticity of Foxp3(+) T cells reflects promiscuous Foxp3 expression in conventional
716 T cells but not reprogramming of regulatory T cells. Immunity 36 (2012) 262-75.
717 [79] E. Sefik, N. Geva-Zatorsky, S. Oh, L. Konnikova, D. Zemmour, A.M. McGuire, D. Burzyn,
718 A. Ortiz-Lopez, M. Lobera, J. Yang, S. Ghosh, A. Earl, S.B. Snapper, R. Jupp, D. Kasper,
719 D. Mathis, and C. Benoist, MUCOSAL IMMUNOLOGY. Individual intestinal symbionts
720 induce a distinct population of RORgamma(+) regulatory T cells. Science 349 (2015) 993-
721 7.
722 [80] B.S. Kim, H. Lu, K. Ichiyama, X. Chen, Y.B. Zhang, N.A. Mistry, K. Tanaka, Y.H. Lee, R.
723 Nurieva, L. Zhang, X. Yang, Y. Chung, W. Jin, S.H. Chang, and C. Dong, Generation of
724 RORgammat(+) Antigen-Specific T Regulatory 17 Cells from Foxp3(+) Precursors in
725 Autoimmunity. Cell Rep 21 (2017) 195-207.
726 [81] P. Stashenko, S. Yost, Y. Choi, T. Danciu, T. Chen, S. Yoganathan, C. Kressirer, M. Ruiz-
727 Tourrella, B. Das, A. Kokaras, and J. Frias-Lopez, The Oral Mouse Microbiome Promotes
728 Tumorigenesis in Oral Squamous Cell Carcinoma. mSystems 4 (2019).
729 [82] J.S. Wu, M. Zheng, M. Zhang, X. Pang, L. Li, S.S. Wang, X. Yang, J.B. Wu, Y.J. Tang, Y.L.
730 Tang, and X.H. Liang, Porphyromonas gingivalis Promotes 4-Nitroquinoline-1-Oxide-
731 Induced Oral Carcinogenesis With an Alteration of Fatty Acid Metabolism. Front
732 Microbiol 9 (2018) 2081.
733 [83] T.W. Buford, (Dis)Trust your gut: the gut microbiome in age-related inflammation, health,
734 and disease. Microbiome 5 (2017) 80.
735 [84] R.I. Clark, and D.W. Walker, Role of gut microbiota in aging-related health decline: insights
736 from invertebrate models. Cell Mol Life Sci 75 (2018) 93-101.
737 [85] H.J. Zapata, and V.J. Quagliarello, The microbiota and microbiome in aging: potential
738 implications in health and age-related diseases. J Am Geriatr Soc 63 (2015) 776-81.
739 [86] F. Barchiesi, E. Orsetti, S. Mazzanti, F. Trave, A. Salvi, C. Nitti, and E. Manso, Candidemia
740 in the elderly: What does it change? PLoS One 12 (2017) e0176576.
741 [87] P. Pandiyan, H.R. Conti, L. Zheng, A.C. Peterson, D.R. Mathern, N. Hernandez-Santos, M.
742 Edgerton, S.L. Gaffen, and M.J. Lenardo, CD4(+)CD25(+)Foxp3(+) regulatory T cells
743 promote Th17 cells in vitro and enhance host resistance in mouse Candida albicans Th17
744 cell infection model. Immunity 34 (2011) 422-34.
745 [88] N. Bhaskaran, A. Weinberg, and P. Pandiyan, Th17 inflammation model of oropharyngeal
746 candidiasis in immunodeficient mice. Journal of visualized experiments : JoVE (2015).
747

748