The aryl hydrocarbon receptor suppresses immunity to oral squamous cell carcinoma through immune checkpoint regulation

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Immune checkpoint inhibitors represent some of the most important cancer treatments developed in the last 20 y. However, existing immunotherapy approaches benefit only a minority of patients. Here, we provide evidence that the aryl hydrocarbon receptor (AhR) is a central player in the regulation of multiple immune checkpoints in oral squamous cell carcinoma (OSCC). Orthotopic transplant of mouse OSCC cells from which the AhR has been deleted (MOC1AhR-KO) results, within 1 wk, in the growth of small tumors that are then completely rejected within 2 wk, concomitant with an increase in activated T cells in tumor-draining lymph nodes (tdLNs) and T cell signaling within the tumor. By 2 wk, AhR+ control cells (MOC1Cas9), but not MOC1AhR-KO cells up-regulate exhaustion pathways in the tumor-infiltrating T cells and expression of checkpoint molecules on CD4+ T cells (PD-1, CTLA4, Lag3, and CD39) and macrophages, dendritic cells, and Ly6g+ myeloid cells (PD-L1 and CD39) in tdLNs. Notably, MOC1AhR-KO cell transplant renders mice 100% immune to later challenge with wild-type tumors. Analysis of altered signaling pathways within MOC1AhR-KO cells shows that the AhR controls baseline and IFNγ-induced Ido and PD-L1 expression, the latter of which occurs through direct transcriptional control. These observations 1) confirm the importance of malignant cell AhR in suppression of tumor immunity, 2) demonstrate the involvement of the AhR in IFNγ control of PD-L1 and Ido expression in the cancer context, and 3) suggest that the AhR is a viable target for modulation of multiple immune checkpoints.

Significance

Immune checkpoint inhibitors have emerged as critical therapeutics for several cancer types, including head and neck squamous cell carcinoma. However, enthusiasm remains constrained by the fact that only a minority of patients benefit. Therefore, there is a need to identify new immunotherapy targets. Here, we provide evidence supporting our hypothesis that the aryl hydrocarbon receptor (AhR) influences multiple immune checkpoints in a model of oral squamous cell carcinoma (OSCC). Remarkably, transplant of AhR-deleted OSCC cells generates completely protective tumor immunity characterized by a decrease in multiple immune checkpoints (PD-L1, CD39, CTLA4, PD1, and Lag3) on malignant and/or immune cells. These results have important implications for understanding the biology of cancer immunosuppression and for targeting the AhR for cancer immunotherapy.

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growth and tumor-specific immunity in vivo. Since studies with environmental AhR ligands (e.g., tobacco smoke) suggest interactions between the AhR and PD-L1 (35), and previous studies demonstrate AhR transcriptional control of CD39 expression (13), we also considered the possibility that malignant cell AhR contributes to immunosuppression via these or other important immune checkpoints.

**Results**

**Validation of AhR Deletion from Oral Squamous Cell Carcinoma (OSCC).** Our working hypothesis is that the AhR within malignant cells drives immunosuppression in the TME and that AhR deletion may break the amplification cycle, reverse immunosuppression, and result in tumor rejection. To test this hypothesis, we used a murine orthotopic (tongue) oral cancer (MOC) model, characterized by high MHC I expression, multiple neoantigens, and susceptibility to anti-PD-L1 checkpoint therapy (32, 33, 36).

AhR knockout through targeting of exon 1 (MOC1AhR-KO) cells was confirmed by Western blotting and by lack of response to the potent AhR ligand 6-formylindolo[3,2-b]carbazole (FICZ), as measured by an AhR-dependent reporter gene assay (SI Appendix, Fig. S1 A and B). Similarly, little or no Cyp1a1 or Cyp1b1, prototypical AhR target genes, was detected in MOC1Cas9 or MOC1AhR-KO cells by qPCR in the presence or absence of FICZ (SI Appendix, Fig. S1C). As shown with human OSCC (6), and breast cancer (11, 18), MOC1AhR-KO cells exhibited impaired migration as compared with MOC1 wild-type (MOC1WT) cells or MOC1Cas9 control cells, which express Cas9 but no guide RNA (SI Appendix, Fig. S1 D and E). In agreement with our previous observations (6, 11, 18, 37), AhR deletion did not affect MOC1 cell proliferation in vitro (SI Appendix, Fig. S1F). These studies show that the AhR controls OSCC migration, but not growth or viability in vitro.

**AhR Expression in MOC1 Cells Is Required for Sustained In Vivo Orthotopic Tumor Growth.** To evaluate the effect that AhR expression in tumor cells has on the establishment of an immunosuppressive TME, we injected $3 \times 10^5$ MOC1AhR-KO or control MOC1Cas9 cells into the center of tongues from C57BL/6J mice. Both MOC1AhR-KO and MOC1Cas9 cell cultures generated small tumors within 7 d (Fig. 1 A, Left and Fig. 1B). However, the tumors generated with MOC1AhR-KO cells completely disappeared by week 2 and no further growth was observed over a 7-wk period (Fig. 1 A, Right and Fig. 1B).

We postulated that the rapid clearance of the tumor cells in the MOC1AhR-KO-injected mice reflects a robust immune response to MOC1AhR-KO cells. To test this hypothesis, we injected MOC1Cas9 or MOC1AhR-KO cells into the tongues of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (Fig. 1C). MOC1Cas9 tumors grew faster in NOD/SCID than in C57BL/6J mice (compare Fig. 1 A and C), a result consistent with a significant contribution of the immune system to slowing OSCC growth. MOC1AhR-KO cells grew at the same rate as MOC1Cas9 cells in NOD/SCID mice (Fig. 1C), indicating that MOC1AhR-KO cells are not inherently growth defective and that the immune compartment is important for MOC1AhR-KO tumor growth suppression.

To determine the mechanism(s) responsible for tumor growth inhibition, we investigated the transcriptional profile of the tumor environment using the 770-gene set NanoString PanCancer Immune Profiling Panel. We analyzed RNA isolated from the right lateral half of tongues of mice 1, 2, 4, and 7 wk after transplant of MOC1Cas9 or MOC1AhR-KO cells and identified 82, 206, 377, and 516 significantly differentially expressed genes at those timepoints, respectively ($P < 0.05$) (Fig. 1D).

Consistent with the rapid clearance of MOC1AhR-KO but not MOC1Cas9 tumors, NanoString data revealed significantly higher expression of gene modules defining cytotoxic T lymphocytes (CTLs) in MOC1AhR-KO tumors as compared with tissue from naive mice or from the control tumor at the 3-wk timepoint (Fig. 1 E, Top). By 2 wk, when MOC1AhR-KO tumors were cleared, the CTL signal in MOC1AhR-KO-injected tumors was no longer significantly different from either the naive or the Cas9 controls (Fig. 1 E, Bottom). While expression of the CTL module tended to be higher in control MOC1Cas9 tumors than in naive tissue at both timepoints, it did not reach statistical significance ($P = 0.051$).

Ingenuity Pathway Analysis (IPA) of the differentially expressed genes revealed that, 1 wk after injection, transcriptional modules associated with increased T cell responsiveness, including Th1 and Th2 pathway activation, CD28, iCOS-iCOSL, NF-κB activation, interferon, TREM1, and IL-6 signaling pathways, were significantly higher in MOC1AhR-KO-transplanted tongues than MOC1Cas9-transplanted tongues (Fig. 1F and SI Appendix, Table S1). Notably, IL-6 is a biomarker of OSCC progression (38) and NF-κB and IL-6 have both been reported to be regulated by the AhR in various contexts (8, 28, 39). The inverse pattern was seen by 2 wk, when tumors became undetectable in the MOC1AhR-KO-injected tongues. Notably, concomitant with clearance of the MOC1AhR-KO tumor by 2 wk and through 7 wk, transcriptional modules associated with T cell exhaustion were significantly lower in tongues from MOC1AhR-KO-injected mice than in controls.

These data indicate the successful clearance of the MOC1AhR-KO tumor by the immune system by 2 wk and an ongoing but insufficient/exhausted immune response to the MOC1Cas9 tumor throughout the experimental period.

**Control MOC1Cas9 but Not MOC1AhR-KO Cells Induce Multiple Immune Checkpoint Markers.** We next hypothesized that the transcriptional changes associated with immune modulation in the tongues of MOC1AhR-KO-implemented mice (Fig. 1) would be reflected in the distribution of immune cells expressing markers of T cell exhaustion and immune checkpoint mediators in the submandibular and cervical tumor-draining lymph nodes (tdLNs). Analysis of the submandibular tdLNs in humans is prognostic of HNSCC outcomes and a surrogate for tumor-mediated immunosuppression (40, 41). Note that this flow cytometry analysis could not be performed within the tumor itself, since an insufficient number of leukocytes can be recovered from this highly muscular organ for flow cytometric analysis of multiple cell subsets.

While the percentage of CD4+ and CD8+ cells was similarly reduced in tdLNs of both the MOC1Cas9 and MOC1AhR-KO, implanted mice at 1 wk compared to naive mice (Fig. 2A), the absolute number of CD4+ and CD8+ cells was significantly higher ($P < 0.05$) in the tdLNs from the MOC1AhR-KO-implemented mice compared to MOC1Cas9-implemented mice and naive mice (Fig. 2B), a result consistent with a rapid and effective immune response to the MOC1AhR-KO cells. The number and percentage of CD4+ and CD8+ T cells in tdLNs from MOC1AhR-KO-injected mice returned to baseline levels at 2 wk, when tumors had been cleared, while the number of CD4+ and CD8+ T cell subsets in the tdLNs from MOC1Cas9-injected mice increased over time (Fig. 2A and B).

Further, the percentage and absolute number of CD4+ IFNγ+ T cells spiked at 1 wk ($P < 0.0001$) and then returned to baseline at 2 wk in the tdLNs from MOC1AhR-KO-transplanted mice while the percentage and absolute number of CD4+ IFNγ+ T cells from the tdLNs of MOC1Cas9-transplanted mice slowly rose over the first 4 wk of the experiment (Fig. 2 C and E).

With regard to immune checkpoint/exhaustion markers on T cells, there was a significant increase in the percentage of Lag3+, CTLA4+, and CD39+CD4+ T cells at 1 to 2 wk in the tdLNs from MOC1AhR-KO-injected mice (Fig. 2D). These increases were reflected in significant increases in the absolute number of these cells at 1 to 2 wk (Fig. 2F). The absolute number of PD1+CD4+ T cells also spiked at 1 wk in MOC1AhR-KO-implemented mice while their
Fig. 1. AhR knockout in MOC1 cells prevents orthotopic tumor growth. Control MOC1Cas9 or MOC1AhR-KO ($3 \times 10^5$) cells were injected into the right lateral side of the tongue $\sim 1.5$ mm from the tip of the tongue of C57BL/6 or NOD/SCID mice using a 27 1/2 gauge needle so that a bulbous mass formed in the center of the tongue. Tumor size was determined with a caliper. For histological analyses or mRNA extraction, entire tongues were removed and bisected down the middle from tip to back. In instances where histology was performed, the right half of the tongue was used for RNA isolation and the left half for histology; otherwise the entire tongue was used for RNA isolation. (A) Tongue volumes were measured once/week for 28 d (Left) or twice/week for 7 wk (Right) after injection. Small white tumors were visible 1 wk after injection of MOC1Cas9 or MOC1AhR-KO cells. Data are means ± SEM, representative of one experiment (Left) or more than three independent experiments (Right), n = 4 to 8 mice per group. P values are derived using two-way ANOVA, **P < 0.01 and ****P < 0.0001. (B) Representative H&E staining of tongue tissue from mice injected 1, 2, 3, 4, or 7 wk prior with MOC1Cas9 cells or MOC1AhR-KO cells. (C) Tongue volume in immunodeficient NOD/SCID mice injected orthotopically with $3 \times 10^5$ MOC1AhR-KO cells or MOC1Cas9 control cells. Data are means ± SEM, n = 6 mice per group. (D–F) Gene expression analysis using the NanoString PanCancer Immune Profiling Panel on mRNA isolated from the right half of tongues from MOC1Cas9 cell-injected mice compared to MOC1AhR-KO-injected mice at various timepoints after tumor injection, n = 3 to 6 mice per group. (D) Heat map of differentially regulated genes at 1, 2, 4, or 7 wk postinjection with a P < 0.05. Data are log2 transformed, row centered, and saturated at −2 and +2 for visualization. (E) Cytotoxic T cell profiling scores based on the gene expression profiles of naïve mice, MOC1AhR-KO-injected mice, or MOC1Cas9-injected mice. P values were derived using a one-way ANOVA and Tukey's multiple comparisons test. (F) IPA of the differentially regulated genes in MOC1AhR-KO-injected tongues compared to MOC1Cas9-injected tongues at 1, 2, 4, or 7 wk postinjection. Pathways associated with positive z-scores are in orange; pathways associated with negative z-scores are in blue; the relative strength of the z-score is represented by the intensity of the color.

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percentage and numbers returned to baseline after an initial spike. In contrast, the percentage and absolute number of CD4+ T cells expressing PD1, Lag3, CTLA4, and CD39 steadily increased after injection of MOC1Cas9 control cells (Fig. 2D and F).

With regard to non-T cells, injection of either MOC1Cas9 or MOC1AhR-KO cells tended to increase the percentage and number of PD-L1+ granulocytic-myeloid-derived suppressor cells (G-MDSC; defined as CD45+CD11b+Ly6c−Ly6g+high) and dendritic cells (DCs, defined as CD45+CD11c+), as well as CD39+ G-MDSC cells and macrophages (defined as CD45+CD11b+Ly6g−Ly6c+) in the tdLNs 1 wk after tumor cell transplantation (Fig. 3A, B, D, and E). The percentage and numbers of these cell subsets decreased essentially to baseline levels in the tdLNs of mice transplanted with MOC1AhR-KO cells by 2 wk, while they increased gradually in the tdLNs of mice transplanted with MOC1Cas9 cells (Fig. 4A, B, D, and E).

We also observed a significant increase in the percentage and number of CCR2+ macrophages in MOC1Cas9-transplanted mice at the 8-wk timepoint (Fig. 3C and F), suggesting an increased ability of macrophages to migrate to the tumor. We then returned to the NanoString data to determine if the same changes in immune checkpoint/exhaustion markers could be observed on the mRNA level in tongues as was seen by flow cytometry within tdLNs. Indeed, a similar profile of Pdcd1 (PD-1), Lag3, Ccl2, Cd274 (PD-L1), and Ifnγ mRNA levels was observed.

Thus, all five genes from MOC1AhR-KO-injected tongues tended to increase at 1 wk (although only Cd274 was statistically significant) and to return to baseline at 2 wk, while all five genes increased over time in the MOC1Cas9-injected tongues (Fig. 3G). Interestingly, we also observed a steady increase in mRNA expression of the Ccl2 chemokine in the tongues from MOC1Cas9-injected mice (Fig. 3H), a result that parallels the increase in CCR2+ macrophages in tdLNs of MOC1Cas9 but not MOC1AhR-KO-injected mice and is consistent with recruitment of potentially immunosuppressive CCR2+ macrophages by its ligand (CCL2) to the tumor, as in glioblastoma (13). CCL2 production and tumor infiltration of CCR2+ macrophages has been linked to poor outcomes in OSCC (42–44).

AhR Knockout Induces a Long-Lasting, Systemic, Antitumor Immune Response. Given the immune profile observed in recipients of MOC1AhR-KO cells, we hypothesized that mice previously exposed to MOC1AhR-KO tumors would have significant immunologic memory for the neoantigens expressed in MOC1WT cells. To test this hypothesis, we challenged C57BL/6J mice with 3×10⁵ MOC1WT cells 100 d after a previous orthotopic injection of 3×10⁵ MOC1AhR-KO cells. As expected, MOC1AhR-KO cells injected orthotopically failed to generate tumors after 2 wk (Fig. 4A, Left, red line). Remarkably, MOC1AhR-KO-injected mice challenged orthotopically 100 d later with MOC1WT cells also failed to generate wild-type tumors (Fig. 4A, Right, red line), suggesting a long-term memory response. Immunophenotyping of submandibular and cervical LNs 70 d after the secondary challenge with MOCWT cells in mice previously "immunized" with MOC1AhR-KO cells showed increased percentages of

Fig. 2. AhR knockout in MOC1 cells alters the phenotype of T cells in tdLNs. Mice were injected orthotopically with MOC1Cas9 or AhRKO MOC1 cells as in Fig. 1. tdLNs were harvested from mice injected 1, 2, 3, 4, or 8 wk prior with tumor or from naive mice (shown as 0-wk timepoint) and analyzed by flow cytometry for the percentage and absolute number of (A and B) CD4+ and CD8+ T cells; (C and E) IFNγ+ CD4+ T cells; and (D and F) PD1+, Lag3+, CTLA4+, and CD39+ CD4 T cells. Data are means ± SEM, n = 4 to 6 mice per group. P values are derived using two-way ANOVA and Sidak’s multiple comparisons test. Black hashtags represent a significant difference between MOC1Cas9-injected mice and naive mice; red hashtags represent a significant difference between MOC1AhR-KO-injected mice and naive mice; and black stars represent a significant difference between MOC1Cas9-injected mice and MOC1AhR-KO-injected mice. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.
CD4+ and CD8+ T cells and decreased representation of PD-1+, Lag3+, and CD39+ CD4+ T cells as compared with naïve mice injected with wild-type MOC1WT cells (Fig. 4B). While no tumors were detected at 70 d, tongues from MOC1AhR-KO-immunized mice were not “normal” in that NanoString analysis of tongue mRNA showed: 1) a different transcriptomic profile and an increase in T cells in general and cytotoxic cells in particular relative to tongues from naïve mice, and 2) an increase in multiple markers of T cell signaling activity, including Th1, Th2, OX40, Erk5, PKCθ, and NFκB signaling pathways (SI Appendix, Fig. S2).

Nearly the same results were seen with another immunogenic OSCC cell line, MOC22 (33, 34). That is, MOC22Cas9 control but not MOC22AhR-KO cells injected orthotopically generated a significant tumor burden (Fig. 4D) and a lower percentage of PD-L1+, Lag3+, CTLA4+, and CD39+ CD4+ T cells (Fig. 4E) in the LNs of mice initially injected subcutaneously with MOC1AhR-KO cells, then challenged with MOC1WT cells orthotopically 2 wk later and sacrificed 60 d after the MOC1WT challenge. The percentage of PD-L1+ G-MDSCs and DCs, and CD39+ G-MDSCs, was lower in LNs from MOC1AhR-KO-immunized, MOC1WT-challenged mice (Fig. 4F) and G). As with the primary challenge (Fig. 3C and F), the percentage of CCR2+ macrophages was significantly lower in LNs from MOC1AhR-KO-immunized and MOC1WT-challenged mice (Fig. 4H).

Finally, when MOC22WT cells were used as the secondary challenge, tumors grew approximately as well in naïve mice as in mice previously transplanted with MOC1AhR-KO cells (SI Appendix, Fig. S4), indicating that immunity induced in MOC1AhR-KO-injected mice is predominantly tumor specific. These results indicate that the potent immunity induced by MOC1AhR-KO cells is long lasting, systemic, tumor-specific, and takes no more than 2 wk to induce.

The AhR Modulates Immune Signaling in MOC1 Tumor Cells. To elucidate some of the AhR-dependent mechanisms that may be regulating tumor immunity, we compared the transcriptional profiles of control MOC1Cas9 and MOC1AhR-KO MOC1 cells. We identified 245 significantly differentially expressed genes (Fig. 5A and B; P < 0.05), including 180 genes that were down-regulated...
and 65 genes that were up-regulated in MOC1\textsuperscript{AhR-KO} cells as compared with MOC1\textsuperscript{Cas9} controls. Ingenuity Pathway Analysis of the differentially expressed genes revealed that AhR knockout down-regulated pro-tumorigenic inflammatory signaling pathways, including IL-1, IL-6, IL-8, STAT3, NF-κB, and iNOS (Fig. 5C). Conversely, several pathways associated with tumor suppressor function, including PPAR and PTEN (45, 46), were significantly higher. Correlating with our in vivo gene-profiling results obtained from the tumor microenvironment (Fig. 1D–F) and immune phenotyping data from tdLNs (Figs. 2–4), the T cell exhaustion signaling pathway was significantly lower in MOC1\textsuperscript{AhR-KO} cells than in MOC1\textsuperscript{Cas9} cells (Fig. 5C). With regard to specific genes, AhR knockout significantly down-regulated genes encoding PD-L1 (\textit{Cd274}) (Fig. 5D) and the chemokines CXCL2, CXCL3, and CXCL5 (Fig. 5B, red circles and Fig. 5D) known to promote the recruitment and generation of MDSCs (45, 47).
Finally, AhR knockout reduced expression of numerous components within the IFN\(\gamma\) signaling pathway (\(P = 2.65 \times 10^{-59}\), Fig. 5E), suggesting that AhR signaling may regulate the response of MOC1 cells to IFN\(\gamma\) produced by nonmalignant cells, e.g., IFN\(\gamma\)+ T cells that expand over time in the tdLN of MOC1Cas9-transplanted mice (Fig. 2D and F) and coincident with the accumulation of Ifn\(\gamma\) in the tongue TME (Fig. 3G). These results appear to reflect multiple mechanisms through which the AhR may moderate tumor immunity and suggest that chronic IFN\(\gamma\) signaling drives protumorigenic responses in these OSCC cells.

The AhR Transcriptionally Regulates Cd274/PD-L1. The NanoString data showing a significant decrease in MOC1 Cd274 after AhR deletion (Fig. 5D) suggest that the AhR may directly or indirectly regulate PD-L1 expression on malignant cells and thereby contribute to expression of T cell exhaustion markers in the tumor injection site (Fig. 1) and in the tdLN (Figs. 2 and 3). To confirm that the AhR regulates Cd274, Cxcl2, Cxcl3, and Cxcl5 in MOC1 cells and MOC1AhR-KO cells. Data are means ± SEM, \(n = 4\) biological replicates/group. \(* P < 0.05\) and \(**** P < 0.0001\) (Student’s t test). (E) IPA of the IFN\(\gamma\) signaling pathway in MOC1Cas9 or MOC1AhR-KO cells.

Fig. 5. The AhR modulates immune signaling pathways in MOC1 tumor cells. Gene expression analysis using the NanoString PanCancer Immune Profiling Panel on mRNA isolated from MOC1Cas9 compared with MOC1AhR-KO cells in vitro. \(n = 4\) biological replicates/group. (A) Heat map of differentially regulated genes with a \(P < 0.05\). Data are log, transformed, row centered, and saturated at −2 and +2 for visualization. (B) Volcano plot of differential gene expression. Yellow squares represent genes belonging to the inflammatory gene set identified by NanoString. Red circles highlight chemokines Cxcl2, 3, 5 involved in tumor inflammation. (C) IPA of the differentially regulated genes in MOC1Cas9 compared with MOC1AhR-KO cells. (D) mRNA expression of Cd274, Cxcl2, Cxcl3, and Cxcl5 in MOC1 cells and MOC1AhR-KO cells. Data are means ± SEM, \(n = 4\) biological replicates/group. \(* P < 0.05\) and \(**** P < 0.0001\) (Student’s t test). (E) IPA of the IFN\(\gamma\) signaling pathway in MOC1Cas9 or MOC1AhR-KO cells.

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in the percentage of PD-L1+ cells reflects a change in the amount of PD-L1 on a per cell basis, as indicated by lower mean fluorescence intensities (MFIs) in MOCI1AhR-KO cells or in control cells after TCDD treatment (Fig. 6 B, Right two graphs). That these AhR-dependent PD-L1 effects occurred at a transcriptional level was supported by a significant (P < 0.001) increase in PD-L1 reporter activity after treatment with the AhR agonist kyurenine or TCDD (Fig. 6C).

Analysis of the Cd274 promoter sequence revealed five potential consensus AhR response elements (AhREs) within a 1,943-bp region extending from -1,723 to +220 bp (beginning at nucleotides -1,555, -1,546, -374, +32, and +43). Deletion of a 680-bp region from -460 to +220 removing AhREs beginning at -374, +32, and +43, or a double deletion removing AhREs -1,555, -1,546, +32, and +43 but preserves AhRE -374 in a Cd274 reporter plasmid, completely ablated baseline reporter activity (Fig. 6D). Preservation of the -132 to +220 fragment containing AhREs +32 and +43 was sufficient for maximal reporter activity.

Site-specific mutations were then generated to determine if the critical sequences in the -132 to +220 fragment of the Cd274 promoter were functional AhREs. Mutation of either predicted AhRE +32 or +43 significantly suppressed promoter activity (Fig. 6E). These results are consistent with the hypothesis that the AhR regulates Cd274 expression through direct transcriptional activation of its promoter.

IFNγ Regulates Cd274 and Ido through the AhR. NanoString data (Fig. 5E) suggest that MOCI OSCC cells may be responsive to IFNγ signaling and data from Figs. 1, 2, and 3G indicate that IFNγ is likely to be present in the TME. As expected, qPCR analysis of mRNA from tongues injected 8 wk previously with MOCI1Cas9 or MOCI1AhR-KO cells indicated ~140-fold higher levels of Ifnγ in the former as compared with the latter (Fig. 7A). Since IFNγ induces PD-L1 expression on tumor and immune cells (46, 48), we evaluated the effect of exogenous IFNγ, in the context of AhR signaling, on PD-L1 expression by malignant cells in vitro.

Consistent with the NanoString (Fig. 5D) and previous qPCR (Fig. 6A) data, AhR knockout significantly reduced baseline Cd274 expression in MOCI1 cells as quantified by qPCR (Fig. 7B, first two bars). Consistent with previous studies (49), IFNγ increased Cd274 expression in MOCI1AhR-KO control cells (Fig. 7B). However, this Cd274 induction was significantly lower in MOCI1AhR-KO cells, indicating that IFNγ induction of Cd274 in MOCI1 cells is at least partially AhR dependent.

While these studies indicate that the AhR contributes to PD-L1 expression in malignant cells, they do not explain how AhR deletion in malignant cells decreases the percentage of PD-L1+ G-MDSCs and DCs in the tdLNs of MOCI1AhR-KO-injected mice. One possibility is through AhR control of Ido, the resulting production of AhR ligand(s) by malignant cells, and the activation of the AhR in immune cells. This AhR-mediated enhancement of Ido would not preclude involvement of IFNγ, which also induces Ido (49, 50). To assess these nonmutually exclusive possibilities, Ido1, Ido2, and Tdo2 were quantified in MOCI1AhR-KO and MOCI1AhR-KO MOCI1 cells in vitro by qPCR. Significant baseline levels of both Ido1 and Ido2 (Fig. 7 C, Left and Right first bars), but not Tdo2, were detected in control cells. In contrast, relatively little Ido1 or Ido2 was detected in MOCI1AhR-KO cells (Fig. 7C, second bars). IFNγ dramatically increased Ido1 and Ido2 levels. Remarkably, IFNγ-mediated induction of these genes was >70% lower in MOCI1AhR-KO cells than MOCI1Cas9 cells, demonstrating that, like IFNγ induction of Cd274 (Fig. 7B), IFNγ up-regulation of Ido1/2 is AhR regulated. Further, as could be predicted from these data, Ido1/2 levels in tongue tissue from MOCI1AhR-KO-injected mice were significantly lower than in MOCI1Cas9 tumors (Fig. 7D).

These data imply a role for the AhR in the direct transcriptional control of Cd274 and IFNγ induction of Cd274, Ido1, and Ido2. Further, chronic IFNγ production in the tumor microenvironment, as opposed to transient early IFNγ production as in the IFNγ spike seen 1 wk after MOCI1AhR-KO cell injection (Fig. 2C), may contribute to the stoking of a IFNγ→AhR→IDO→AhR ligand amplification loop in malignant cells and suppression of immune cells in the TME through AhR ligand production and AhR-dependent PD-L1 induction. Thus, this study directly links the AhR to control of IFNγ induction of critical immune checkpoints, i.e., PD-L1 and Ido.

Discussion

The AhR has been implicated in the pathogenesis of several cancer subtypes including HNSCC (6–16). While chronically active AhR within malignant cells has been linked to cancer aggressiveness (6, 7, 17, 18), AhR within immune cells has been linked to immunosuppression (21, 22, 24, 27, 28, 51, 52). Here, we tested the hypothesis that the AhR, chronically active within malignant cells, also has an impact on immune cells in the TME.

While MOCI1AhR-KO cells grew at the same rate as MOCI1Cas9 control cells in immunodeficient mice, they failed to grow for more than 1 wk in immunocompetent mice (Fig. 1 A–C). This complete rejection of MOCI1AhR-KO cells was characterized by a spike in T cell signaling in the tumor and CD4+ and CD8+ T cells in the tdLNs and was followed by a decrease in the number and percentage of CD4+ T cells expressing PD-1, Lag3, CTLA4, and CD39; G-MDSCs and DCs expressing PD-L1 and CD39; and M2s expressing CD39 and CCR2. This immune profile correlated with reduced PD-L1 and Ido expression in malignant cells and the induction of long-lasting, systemic, and tumor antigen-specific immunity leading to the conclusion that malignant cell AhR alters TME conditions to favor immunosuppression.

One mechanism through which malignant cell AhR may affect immunosuppression is through PD-L1 up-regulation (Fig. 6). AhR control of Cd274/PD-L1 expression in MOCI1 cells is reminiscent of a previous study demonstrating that tobacco carcinogen-induced AhR activation induces PD-L1 on normal lung epithelial cells (35). The significance of AhR control of PD-L1 is underscored by the finding that PD-1/PD-L1 blockade is most effective in lung cancer when malignant cells express high AhR levels (35). A second mechanism of immunosuppression may be through malignant cell production of excess kynurenine enforced by the AhR→IDO→AhR ligand loop (enhanced by IFNγ) and kynurenine’s effect on TME immune cells. As in malignant cells, kynurenine may enhance PD-L1 expression on G-MDSC, macrophages, and DCs, all of which express AhR (28, 53, 54). AhR activation, in some cases by kynurenine, also induces the immunosuppressive CD39 ectoenzyme on macrophages and T cells through direct transcriptional control (13, 23, 55, 56). Furthermore, PD-1 expression on CD8+ T cells in the TME has been linked to the AhR and “transcellular” Kyn produced by melanomas (31). Consistent with these possibilities is the increase in the T cell exhaustion module in the tongues of MOCI1Cas9- as compared with MOCI1AhR-KO-injected mice (Fig. 1F) and the enhanced expression of PD-L1 on G-MDSC and DCs, CD39 on G-MDSCs and macrophages (Fig. 3), and PD1 on T cells (Fig. 2) in MOCI1Cas9 as compared with MOCI1AhR-KO tdLNs.

All of these AhR-mediated effects seem likely to be exacerbated by chronic IFNγ production. Consistent with previous studies (32), we noted increasing IFNγ+ T cell numbers and IFNγ levels in MOCI1Cas9 tumors (Figs. 2C, 3G, and 7A). While IFNγ induces Cd274 transcription (46, 48), it was surprising to find that, in the MOC1 model, much of that effect is mediated by the AhR (Fig. 7B). Similarly, it was striking that the well-known induction of Ido by IFNγ (49, 50, 57) also was predominantly AhR controlled (Fig. 7C). While interactions between IFNγ and AhR signaling have been suggested (58), we report here that AhR control of IFNγ-driven outcomes, specifically PD-L1 and Ido induction, can be shown in the cancer context. The findings

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The AhR regulates Cd274/PD-L1 expression in malignant cells. (A) RT-qPCR expression of Cd274 mRNA in MOC1Cas9 or MOC1AhR-KO cells at baseline or after 24 h of treatment with TCDD (1 nM). mRNA levels in all graphs are normalized to Gapdh and to untreated MOC1WT cells. (B) Percent and MFI of PD-L1 expression on MOC1Cas9 or MOC1AhR-KO at baseline or MOC1WT cells 24 h after vehicle (0.1% DMSO) or TCDD (1 nM) treatment. (C) Normalized Cd274 promoter activity as measured with a pGL3-Luc luciferase reporter after 24 h of treatment with kynurenine (100 μM) or TCDD (1 nM). For A–C, data are means of at least three independent experiments with three wells per group + SEM. *P < 0.05, **P < 0.01, ***P < 0.001 (Student’s t test or one-way ANOVA). (D) The Cd274 promoter was mutated to delete whole sections of the sequence at each of three locations containing presumptive AhR binding sites. (E) Normalized wild-type or AHRE-mutated Cd274 promoter activity in MOC1 cells. The Cd274 promoter reporter was mutated at one or both of two presumptive AhR binding sites as indicated. For D and E, data are from each one of two identical experiments presented as means ± SEM, n = 4 wells/condition. ***P < 0.001, ****P < 0.0001 (one-way ANOVA and Tukey’s multiple comparisons test).
reflected dual roles of IFNγ in the tumor context, i.e., critical for activating acute antitumor responses yet contributing to immune evasion (59) when produced chronically. Chronic IFNγ production also may be important given the multiple other targets of IFNγ signaling in the MOC1 model (Fig. 5E) and in primary human OSCC (60).

Within the malignant cell itself, the AhR modulates expression of genes associated with self-renewal, invasion, metastasis, and inflammation (7, 11, 18). Here, we identified a number of AhR-regulated inflammatory signaling pathways similarly associated with cancer pathogenesis. While an IL-6 signaling module in MOC1AhR-KO–injected tongues was higher than in MOC1Cas9, indicating its association with cancer pathogenesis. While an IL-6 signaling module and inflammation (7, 11, 18). Here, we identified a number of signaling pathways in MOC1AhR-KO cells (Fig. 5F). Similarly, the IL-6 signaling pathway was significantly reduced after AhR knockout in vitro (Fig. 5C). These results support previous studies suggesting that the AhR regulates IL-6 expression to promote tumor growth (61–63). IL-6 is considered a biomarker of OSCC (38), especially in tobacco (i.e., environmental and chemical)-related cases (64). A role for AhR control of inflammation in the cancer context is further suggested by a decrease in IL-1 and IL-8 signaling pathways in MOC1AhR-KO cells (Fig. 5C).

AhR knockout significantly decreased chemokine (C-X-C motif) ligand mRNAs (Cxcl2, Cxcl3, and Cxcl5) all of which contribute to angiogenesis, migration, epithelial to mesenchymal transition (EMT), and immune evasion (65, 66). We also note that AhR knockout significantly decreased mRNAs encoding a number of genes that contribute to malignancy, including Tnf (P < 0.0003), Muc1 (P < 0.02), and Tigit (P < 0.00001). These data strongly suggest a role for the AhR amplification loop specifically in malignant cell aggression and potentially in protumorigenic inflammatory responses.

Finally, these results demonstrate outcomes induced with at least transferyptanoid‐derived endogenous ligands and, in some cases, environmental ligands like TCDD (e.g., Fig. 6). That said, other environmental or dietary AhR ligands may not generate similar outcomes, given that distinctly different effects have been seen with diverse AhR ligands in multiple contexts (37, 67).

In summary, we have identified mechanisms by which the AhR suppresses antitumor immune responses in OSCC through direct effects in malignant cells or indirect effects in the TME. The findings highlight the AhR as a critical suppressor of tumor immunity and strongly support the hypothesis that targeting the AhR is an effective approach for simultaneously inhibiting several complementary immune checkpoints especially in tumors that have been screened for AhR expression and activity, including through documentation of nuclear AhR localization or the expression of AhR biomarker genes (68, 69).

**Materials and Methods**

**Cell Culture.** MOC1 and MOC22 oral cancer cells (33) were kindly provided by R. Uppaluri, Dana Farber Cancer Institute, Boston, MA and cultured in Iscove's modified Dulbecco's media/DMEM/Ham's F-12 media containing 0.16 g/L transferyptan (Fisher Scientific). Cell culture details are described in SI Appendix.

**CRISPR-Cas-Mediated AhR Knockout.** Guide RNAs targeting the mouse Ahr gene (exon 1) were designed using the Zhang laboratory web resource (https://zlab.biologyguide-design-resources). sgRNA1, 5′-CCGGCTGGCCGCTGCCGCGG-3′; sgRNA2, 5′-AACATTGGACTACGGGAGGCCC-3′. Cell line selection and validation details are provided in SI Appendix.

**In Vivo Experiments.** Orthotopic transplants were performed as described (70). C57Bl/6 and NOD/SCID mice were anesthetized and tongues gently grasped and pulled out from the mouth using forceps. Using a 1-mL syringe attached to a 27 1/2 gauge needle, 3 × 10^6 MOC1 or MOC22 cells were slowly injected into the right lateral side of each tongue (∼1.5 mm from the tip of the tongue) so that a bulbous mass formed in the center of the tongue. Small white tumors were visible 1 wk after injection of either MOC1Cas9 or MOC1AhR-KO cells. For tumor samples taken thereafter, the entire tongue was removed by cutting the tongue at the back of the mouth and bisecting it down the middle from tip to back. Generally, the entire tongue was used for RNA isolation. In cases where histologies were done, the right half of the tongue was used for histology. For rechallenge experiments, 3 × 10^6 MOC1Cas9 or MOC22 cells were injected orthotopically 14, 100, or 150 d after or on the same day as orthotopic or subcutaneous (both flanks) injection of MOC1AhR-KO cells, as indicated in the figure legends. Tumor size was quantified using a caliper. Mouse sourcing and maintenance are detailed in SI Appendix.

**RT-qPCR.** TaqMan primer and probes sets were purchased from Applied Biosystems: Ahr (Mm00478930_m1), Ido1 (Mm00492590_m1), Ido2 (Mm00524210_m1), C2747 (Mm00452054_m1), Ifnγ (Mm01168134_m1), Cyp1A1 (Mm00487229_m1), Cyp1A2 (Mm00487218_m1), and Gapdh (Mm99999915_g1). See SI Appendix for details.

**NanoString nCounter Gene Expression.** Total RNA (100 ng) was isolated from MOC1AhR-KO, MOC1WT, and MOC1Cas9 cells from the right half (when

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**Fig. 7.** IFNγ regulates Cxcl2, Ido1, and Ido2 through the AhR. (A) RT-qPCR expression of Ifnγ mRNA isolated from the tongues of mice injected with MOC1Cas9 or MOC1AhR-KO cells 8 wk prior. mRNA levels were normalized to Gapdh and expressed relative to levels in tongues from naive mice. Data are means ± SEM, representative of three independent experiments, n = 8 mice/group. (B) RT-qPCR expression of Cxcl2 mRNA in MOC1Cas9 or MOC1AhR-KO cells at baseline or after treatment with IFNγ (100 μg/mL) for 24 h. Cxcl2 expression was normalized to Gapdh and to expression in untreated MOC1Cas9 cells. Data are means of three independent experiments with three wells/group ± SEM. (C) RT-qPCR expression of Ido1 and Ido2 mRNA in MOC1Cas9 or MOC1AhR-KO cells left untreated or treated with IFNγ (100 μg/mL) for 24 h. Expression is normalized to Gapdh and relative to expression in untreated MOC1Cas9 cells. Data are means of three independent experiments with three wells/group + SEM. (D) RT-qPCR expression of Ido1 and Ido2 in mRNA isolated from the tongues of mice injected 70 d previously with MOC1Cas9 or MOC1AhR-KO cells. mRNA levels are normalized to Gapdh and expressed relative to levels in tongues from naive mice. Data are means ± SEM and are representative of three independent experiments, n = 8 mice/group. Throughout, *P < 0.05, **P < 0.01, ***P < 0.001 (Student’s t test or one-way ANOVA).
histologies also were done) or the entire injected tongue and analyzed using the nCounter Pan Cancer Immune Profiling Panel (NanoString Technologies) according to the manufacturer’s instructions. Data were analyzed using the nSolver Analysis Software.

**Transient Transfection and AhR Activity Reporter Assay.** MOC1 cells were plated in a 24-well plate, allowed to adhere overnight, and cotransfected with the AhR response element-driven firefly luciferase reporter construct pGudluc (0.5 μg/mL) generously provided by M. Denison, University of California, Davis, CA) and cytomegalovirus (CMV)-green (0.25 μg/mL) using TransIT-2020 transfection reagent (Mirus). After 24 h, the cells were harvested in Glo lysis buffer (Promega). Luciferase activity was determined with the Bright-Glo luciferase system according to the manufacturer’s instructions (Promega). Luminescence and fluorescence were determined using a Synergy2 multiplate function plate reader (Bio-Tek). pGudluc luminescence was normalized to the CMV signal.

**Cd274 (PD-L1) Promoter Deletion and Mutagenesis.** The mouse Cd274 promoter was kindly provided by Xiaolong Yang, Queen’s University, Kingston, ON, Canada. See SI Appendix for details on the generation of deletion mutants.

**Flow Cytometry, Western Blotting, and Scratch-Wound Assay.** See SI Appendix for details.

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**In Vitro Stimulation of MOC1 Cells.** For some experiments, MOC1 cells were treated with vehicle (DMSO), TCCD (1 μM, Sigma-Aldrich), FICZ (0.5 μM, Sigma-Aldrich), or kynurenine (20 μM or 100 μM Sigma-Aldrich) for 24 h.

**Histology.** Tongue sections were fixed in 10% formalin, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin (H&E) for image by light microscopy. All images were captured using the same camera settings.

**Cell Counting Assay.** MOC1 cells (5 × 10^4) were plated in triplicate in 12-well plates. Cells were harvested with trypsin at indicated timepoints and counted by hemocytometer in a 0.4% Trypan Blue solution.

**Statistical Analyses.** Graphs were generated and statistical analyses were performed using Prism software (GraphPad) and using the statistical tests indicated in the figure legends. Details are provided in SI Appendix.

**Data Availability.** All study data are included in the article and/or supporting information.

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