Anti-CD47 treatment enhances anti-tumor T-cell immunity and improves immunosuppressive environment in head and neck squamous cell carcinoma

Lei Wu, Guang-Tao Yu, Wei-Wei Deng, Liang Mao, Lei-Lei Yang, Si-Rui Ma, Lin-Lin Bu, Ashok B. Kulkarni, Wen-Feng Zhang, Lu Zhang, and Zhi-Jun Sun

The State Key Laboratory Breeding Base of Basic Science of Stomatology & Key Laboratory of Oral Biomedicine, Ministry of Education, Wuhan, Hubei, China; Department of Oral Maxillofacial-Head Neck Oncology, School and Hospital of Stomatology, Wuhan University, Wuhan, Hubei, China; Functional Genomics Section, Laboratory of Cell and Developmental Biology, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD USA

ABSTRACT
Head and neck squamous cell carcinoma (HNSCC) is considered as an immunosuppressive disease, with impaired tumor-infiltrating T lymphocytes and increased suppressive immune cells. The efficacy of CD47 antibodies in immune checkpoint therapy is not clearly understood in HNSCC. In this study, human tissue microarrays and immunocompetent transgenic mouse models were used to explore the expression of CD47 and the use of CD47 antibodies in HNSCC. We identified overexpression of CD47 in HNSCC as compared with the control normal human tissue and also in HNSCC mouse models. The expression of CD47 also correlated with clinicopathological parameters as well as outcome. Furthermore, inhibition of CD47 delayed tumor growth and improved tumor microenvironment by stimulating effector T cells and decreasing suppressive immune cells and regulating the function of CD11b+Ly6G+ MDSC. Our data suggest that CD47 blockade may be a potential immunotherapeutic target in human HNSCC.

Introduction
The hallmarks of cancer including sustaining proliferative signaling, evasion of growth suppressors, and resistance to apoptosis, replicative immortality, and escaping the immune destruction lead to initiation and progression of cancer.1 Cancer cells have various strategies to avoid recognition and destruction by the immune system, thereby evading immune surveillance.1 Many immune cells play a key role in evading immune surveillance in the tumor microenvironment of head and neck squamous cell carcinoma (HNSCC) including myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs) and tumor-associated macrophages (TAMs).2 MDSCs represent a diverse immunosuppressive cell population in various cancer types, inhibiting activated T cells, impairing natural killer (NK) cells, and facilitating stem cell-like cancer cells.3,4 MDSCs identified by expression of CD11b and Gr-1 (Ly6C+Ly6G+) in mice, and the subpopulations of MDSCs have also been defined as polymorph nuclear (CD11b+Ly6G+Ly6C0) (PMN) and monocytic (CD11b+Ly6G−Ly6C0) (M)-MDSCs.5 The CD4+CD25+Foxp3+ regulatory T cells (Tregs) promote tumor progression by regulating the immune system with the inhibition of actions of activated T cells in peripheral lymphoid tissues.8

CD47, a “don’t eat me” signal for phagocytic cells, is expressed on the surface of multiple human tumor types including ovarian carcinomas, renal cell carcinoma (RCC), human hepatocellular carcinoma (HCC), acute myeloid leukemia (AML) and other human solid tumor.9–12 CD47 expressed on tumor cells evade immune surveillance through binding to signal regulatory protein-α (SIRPα), a protein expressed on macrophages and dendritic cells, thereby resulting in the inhibition of phagocytosis.13 CD47 also binds to thrombospondin-1 (TSP1), which is a ligand in T cells of CD47,14 to inhibit T-cell activation in the immune system.15,16 Recent reports indicated that CD47 is involved in the process of adaptive immunity response by impairing T cell immune response.17,18 Blockade of CD47 led to an efficient and rapid elimination of multiple tumor cell types and achieved a good effect in the malignant tumor.19,20

Immune checkpoint molecules such as programmed cell death protein 1 (PD1) are the major mechanism of immune resistance, particularly dampening T cell activation to avoid tumor cell elimination.21 Immune checkpoint blockade, enhancing T cell anti-tumor activity and revitalize suppressed immune environment, have produced encouraging results in the treatment of multiple cancers including head neck cancer.22 Recent studies reported that antibody blockade of CD47 enhances NK cell-mediated killing of HNSCC cells in vitro and CD47 suppression increased tumor delay in vivo using the SCC VII HNSCC model in immune competent mice.22,23 Using the same mouse model, David et al. reported that anti-CD47 treatment induced tumor delay depends on T cell–mediated antitumor immunity.27 In order to further explore the efficiency of the anti-CD47 treatment as another immune checkpoint therapy, we utilized our HNSCC mouse model for in vivo experiments.
The present study was designed to investigate the precise effect of CD47 inhibition in the modulation of tumor infiltrating T cells, MDSCs, Tregs and immune checkpoint molecules in HNSCC.

**Results**

**Overexpression of CD47 in HNSCC tissue and dysplasia is associated with clinical outcome of primary HNSCC**

Many studies suggested that CD47 was overexpressed in multiple types of cancers.\(^9,10,12,24\) This prompted us to gain insight into the expression of CD47 in HNSCC and normal mucosa. We identified mRNA and DNA expression of CD47 in human HNSCC by Oncomine database.\(^25\) CD47 mRNA or DNA copy number were significantly increased in 13 out of 17 HNSCC datasets (Fig. S1). Moreover, analysis of Cromer and TCGA dataset revealed significant increase in the mRNA level and DNA copy number of CD47 in HNSCC as compared with their control counterpart (Fig. S1). Furthermore, immunohistochemical staining of specimen from 48 cases of oral mucosa, 43 cases of dysplasia (Dys) and 165 cases of primary HNSCC showed higher CD47 expression in human HNSCC tissue as

![Figure 1](https://example.com/Figure1.png)

**Figure 1.** Overexpression of CD47 in human HNSCC confers poor prognosis of HNSCC patients. Representative hematoxylin-eosin, immunohistochemical staining (A) and quantification (B) of CD47 in human head neck squamous cell carcinoma (HNSCC, \(n=165\)) as compared with dysplasia (Dys, \(n=48\)) and oral mucosa (\(n=43\)). Each dot is presented as an independent core. CD47 staining was significantly alternated in different pathological grades (C, Grade II–III vs Grade I, \(P<0.05\)), the primary tumor with positive or negative lymph node metastasis status (D, N1+N2 vs N0, \(P<0.05\)). (E) Kaplan–Meier survival analysis indicated that high CD47 expression confers poor overall survival in the patient with HNSCC (\(P\text{-value}=0.0495\)) with the median of CD47 protein expression histoscore (the histoscore = 58.37) as a cut-off.
compared with normal oral mucosa. Interestingly, positive immunostaining of CD47 was mainly localized in cancer cells, especially in the invasive layer of cancer cells (Fig. 1A). Quantification of CD47 expression also showed that CD47 was significantly increased in human HNSCC tissue and dysplasia as compared with in oral mucosa (Fig. 1B). Further detailed analysis of HNSCC tissue revealed that CD47 staining was significantly increased in advanced pathology grade HNSCC (II+III vs. I, \( P < 0.05 \), Fig. 1C) and primary HNSCC with lymph node metastasis (N1+N2 vs. N0, \( P < 0.05 \), Fig. 1D). Most importantly, in 165 primary HNSCC with follow-up data, Kaplan–Meier survival analysis indicated that CD47 high expression confers poor overall survival in the patient with HNSCC (\( n = 165 \), \( P \)-value = 0.0459, Fig. 1E) with the median of CD47 protein expression histoscore (the histoscore = 58.37) as a cut-off value.

**Overexpression of CD47 in human HNSCC is associated with PD-1/PD-L1, Foxp3 and myeloid-derived suppressor cell markers**

Cumulative studies demonstrated that overexpression of CD47 may correlate with immunosuppressive status, including elevated expression inhibitory marker PD-1 and PD-L1, accumulation of Treg and MDSCs. This finding prompted us to determine the correlation between the CD47 and suppressive cells. We analyzed the PD-1, PD-L1, Foxp3 and MDSC markers (CD11b and CD33) by immunohistochemical staining in human HNSCC as shown in Fig. 2A. The expression of CD47 was positively correlated with PD-1 (\( P < 0.001 \)) and PD-L1 (\( P < 0.01 \), Fig. 2B). Similarly, the expression of Treg marker Foxp3 (\( P < 0.001 \)) and MDSC markers CD11b (\( P < 0.01 \)) and CD33 (\( P < 0.01 \)) was positively correlated with the CD47 (Fig. 2C). Hierarchical clustering indicated that CD47

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Overexpression of CD47 in human HNSCC is associated with inhibitory checkpoints and suppressive immune cell. (A) Representative immunohistochemical staining of CD47, PD-1, PD-L1, Foxp3, CD11b and CD33 in human HNSCC specimens. Scale bar, 25 \( \mu m \). (B) The expression of CD47 was positively correlated with PD-1 (\( P < 0.001 \), \( r = 0.2715 \)) and PD-L1 (\( P < 0.01 \), \( r = 0.2527 \)). (C) The expression of CD47 were positively correlated with Foxp3 (\( P < 0.001 \), \( r = 0.2857 \)), CD11b (\( P < 0.01 \), \( r = 0.2424 \)) and CD33 (\( P < 0.01 \), \( r = 0.2154 \)) in human HNSCC. (D) Hierarchical clustering indicated a close relation of CD47 with PD-1 in primary HNSCC. (statistic including 165 primary HNSCC).
expression may mostly correlate with PD-1 expression in the human HNSCC sample (Fig. 2D). Together, these results suggest that the activation of CD47 is correlated with PD-1/PD-L1 expression, Tregs, and MDSCs in human HNSCC.

**Anti-CD47 treatment delays tumor growth in Tgfbr1/Pten 2 cKO HNSCC mouse model**

Loss of PTEN has been described as a frequent molecular event in HNSCC.28 Conditional deletion of the tumor suppressor Tgfbr1 in epithelial cells, which abrogates TGF-\(\beta\) signaling, leads to accumulation of TGF-\(\beta\)1 ligands in stromal cells.29,30 A combined deletion of important tumor suppressors Pten and Tgfbr1 (2 cKO) leads to a fast and full penetration of HNSCC tumorigenesis in these mice.31 Pathologically, the HNSCC mice were similar to human HNSCC with abundant infiltration of inflammatory cells.31 Considering the negative role and rather high expression of CD47 in human HNSCC, we subsequently examined the expression of CD47 in this mice model. As shown in Fig. 3A, CD47 was highly expressed in mouse HNSCC compared with wild-type mouse. The results of Western blot and immunofluorescence were consistent with

![Figure 3](image-url)

Figure 3. Anti-CD47 treatment by monoclonal antibody delays tumorigenesis in immunocompetent transgenic HNSCC mouse models. Representative immunohistochemical staining (A), western blot with GAPDH as a loading control of CD47 (B) and immunofluorescence staining for CD47 (red immunofluorescence), CK14 (green) and nucleus (blue) (C) in Tgfbr1/Pten 2cKO mice HNSCC compared with wild-type tongue. (D) Tgfbr1/Pten 2cKO mice bearing carcinoma by tamoxifen were treated with the CD47 antibody (aCD47) intraperitoneally (i.p) every other day for 28 days or isotype control treated (n = 5 mice respectively). (E) Tumor burden and representative photos of mice with head and neck tumor (right panel) after treatment with aCD47 or isotype IgG in day 18 and day 32 after tamoxifen gavage. Data presented as mean ± SEM.
that of immunohistochemical staining (Fig. 3B and C). Based on this finding, specific mouse CD47 monoclonal antibody was used to explore the role of CD47 in this mouse model. To test the effect of CD47 on the progression of HNSCC in vivo, a chemopreventive investigation on the Tgfbr1/Pten 2 cKO mouse model was performed. The mice received the intraperitoneal injection of CD47 blocking antibody (MIAP301), known to functionally blockade CD47-SIRPα interactions, rat IgG2aa, 10 mg/kg per mouse; n = 5 mice respectively) or isotype control rat IgG2ac (2A3, 10 mg/kg per mouse; n = 5 mice respectively) every other day (Fig. 3D). Tumor growth was assessed every third day by direct measurements of tumor size. We observed that the tumor burden of head and neck sufficiently reduced by systemic anti-CD47 treatment (Fig. 3E). Meanwhile, the results of body weight changes in two groups indicated that treatment with an anti-mouse-CD47 mAb did not cause significant weight changes between CD47 blockade and control group (P > 0.05, Fig. S2A). There were no significant differences between the anti-CD47 treatment group and control group in major organs using H&E staining (Fig. S2B), indicating that we did not identify readily detectable cytotoxicity in our mouse model upon anti-CD47 treatment. Together, these results show that inhibition of CD47 suppresses HNSCC progression. In addition, anti-CD47 treatment effectively rescued immunosuppressive status as indicated by the decrease of spleen index and size of lymph node (Fig. S2 C and D).

**Anti-CD47 treatment reduced PD-1 expression and increased IFN-γ secretion in the effector T cells of HNSCC mouse model**

Based on the significant effect on peripheral immune organs with anti-CD47 treatment, we hypothesized that the tumor-bearing Tgfbr1/Pten 2cKO mouse was in an immunosuppressive state in the tumor progression. Considering that elevated immunosuppressive cells, the population of CD4+ CD25hi Treg cells was evaluated by ow cytometry 3 days later. Results indicated that CD47 treated mice as compared with isotype control counterparts (Fig. 3D). These results demonstrated that anti-CD47 treatment significantly reduced the population of CD4+ CD25+ Tregs in aCD47 treated 2cKO mice as compared with isotype control (Fig. 5F). Quantification of immunofluorescence of CD11b+ Gr1+ MDSCs was shown in supplementary figure 3D.

**Anti-CD47 treatment regulates CD11b+ Ly6G+ MDSC function of HNSCC mouse model**

To further explore the effect of anti-CD47 treatment on the immunosuppressive function of CD11b+Ly6G+Ly6C−, CD11b+Ly6G−Ly6C+, and CD4+CD25+ Tregs cells, these subsets were sorted from the spleens of anti-CD47 treated 2cKO mice and control group and cultured with CFSE-labeled effector T cells. Their capacity to suppress proliferation of effector T cells was evaluated by flow cytometry 3 days later. Results indicated that anti-CD47 treatment resulted in a decrease in suppressive function of CD11b+Ly6G+Ly6C− MDSCs compare with the control group (Fig. S3E, Fig. 6A and B). However, when cultured with effector T cells, anti-CD47 treated CD11b+Ly6G−Ly6C+ MDSC suppressed as well as control group CD11b+Ly6G+Ly6C− MDSC (Fig. 6C and D). And anti-CD47 treatment did not regulate the suppressive function of CD4−CD25+ Treg cells between the groups (Fig. 6E and F).

**Anti-CD47 treatment inhibited tumor proliferation and may influence Akt-dependent pathway**

The previous study reported that treatment with the TSP-1-derived peptide 4N1, a widely used CD47 agonist, facilitated the tumor proliferation via an Akt-dependent pathway. Recent reports have demonstrated that PI3K/AKT/mTOR pathway was broadly activated in tumorigenesis of HNSCC. To further explore the influence to proliferation through the anti-CD47 treatment, western blots of AKT, the phosphorylation status of AKT, S6 and phosphorylation status of S6 and immunohistochemistry of Ki-67 and p-AKT were performed. The anti-CD47 treatment significantly decreased...
phospho-S6S235/236 and phospho-AKT5473 levels and Ki-67 level compared with isotype group (Fig. 7A and B). Meanwhile, the level of SIRPa was also significantly decreased in anti-CD47 treated group compared with control group (Fig. 7A). Immunofluorescence staining also confirmed the above results (Fig. 7C). Together, these results demonstrate that anti-CD47 treatment affected tumor proliferation and meanwhile, may influence Akt-dependent pathway in our HNSCC mouse model.

**Discussion**

Multiple lines of evidence have shown that the immune system is a powerful tool for preventing tumor initiation as well as for eliminating tumor cells. Immunotherapy with targeted checkpoint agents has led to remarkable advances in the treatment of cancer. We found here the expression of CD47 was significantly increased in HNSCC, which was true both in human HNSCC samples and mouse model HNSCC tissues compared with their control counterparts. Remarkably, elevated CD47 expression was correlated with positive lymph nodes and advanced HNSCC and confers poor prognosis in HNSCC patients. Interestingly, the anti-CD47 treatment could delay tumor progression and improve immunosuppressive state in immunocompetent HNSCC mouse model. Together, these results provide the rationale for using the anti-CD47 monoclonal antibody as a promising treatment for human HNSCC.

Previous study reported that anti-CD47 treatment with an anti-mouse-CD47 mAb (anti-CD47: MIAP301) could slowed the growth of tumor and reduce tumor burden in syngeneic
tumor models.\textsuperscript{18} And another report demonstrated that anti-CD47 treatment enables phagocytosis of mouse AML.\textsuperscript{10} Inconsistent with these reports, Willingham et al. do not show any therapeutic effects of MIAP301 in a syngeneic breast cancer model.\textsuperscript{40} In our study, anti-CD47 treatment with MIAP301 mAb delays tumor growth in Tgfbr1/Pten 2cKO HNSCC mouse model. The reason for this difference may be due to the different tumor categories as well as the mouse model.

Previous reports described that monoclonal antibodies directed against CD47 therapy inhibited tumor progression via the enabling of phagocytosis in xenograft models.\textsuperscript{10,41} Recent studies demonstrated inhibition of CD47 effectively enhanced antitumor the immune effect by directly triggering cytotoxic T cells in immune-competent mice.\textsuperscript{17,18} In our study, anti-CD47 treatment effectively enhanced antitumor effects by augmenting the amount and function of effector T cells and decreasing immunosuppressive cells, regulatory T cells and MDSCs, in immunocompetent transgenic HNSCC mouse models. And anti-CD47 treatment regulates CD11b\textsuperscript{+} Ly6G\textsuperscript{+} MDSC function in our mouse models, which may provide an additional benefit for improving tumor immune suppressive environment. While anti-CD47 treatment does not regulate CD11b\textsuperscript{+} Ly6C\textsuperscript{+} MDSC. The reason for this difference is unclear and we will explore in future experiments. Van et al. reported that CD47\textsuperscript{−/−} mice lead to selective augmentation of activated Foxp3\textsuperscript{+} CD4\textsuperscript{+} Treg cells that express CD103 and CD47 expression on Treg does not regulate their function.\textsuperscript{42} Another study reported that anti-CD47-mediated phagocytosis of cancer cells lead to a reduction in Foxp3\textsuperscript{+} regulatory T cells.\textsuperscript{43} Our study indicated that anti-CD47 treatment decreased regulatory T cells and did not

Figure 5. Anti-CD47 treatment significantly attenuated Tregs and MDSCs in Tgfbr1/Pten2 cKO mouse. (A) On the 41st day, the mice were euthanized for flow analysis. (n = 5 mice respectively) Representative flow cytometry plots of gating strategy of CD4\textsuperscript{+}CD25\textsuperscript{+} Foxp3\textsuperscript{+} Tregs in the spleen. (B) Quantification of Tregs from spleen, lymph nodes (LN), peripheral blood and tumor (Mean ± SEM. Unpaired t test, *P < 0.05). (C) Immunohistochemical staining of Foxp3 in aCD47 treated mouse HNSCC as compared with isotype IgG treated counterpart. (D) Representative flow cytometry plots of gating strategy of CD11b\textsuperscript{+} Ly6G\textsuperscript{+} and CD11b\textsuperscript{+} Ly6C\textsuperscript{+} MDSCs in lymph nodes. (E) Quantification of MDSCs (Ly6G and Ly6 C) from spleen, lymph nodes (LN), peripheral blood and tumor (Mean ± SEM. Unpaired t-test, *P < 0.05, **P < 0.01). (F) Representative immunofluorescence of CD11b\textsuperscript{+}Gr1\textsuperscript{+}MDSCs in Tgfbr1/Pten2 cKO mouse HNSCC in aCD47 group and isotype control group.
regulate the suppressive function of CD4^+CD25^hi Treg cells. Anti-CD47 cancer therapy may represent an additional approach for stimulating an effective cytotoxic T-cell response, which may contribute to antitumor effects.

Based on previous studies, these results offered the rationale for anti-CD47 alone as monotherapy for tumor therapy.\(^{10,11}\) However, the combination of anti-CD47 therapy with additional therapies may induce the synergistic stimulation to eradicate tumor cells.\(^{17,41,44,45}\) Chao et al. reveal data suggesting that the combination of anti-CD47 with anti-CD20 therapy eradicate non-Hodgkin lymphoma in vitro and in vivo.\(^{41}\) Yu et al. reported that blockade of PD-1 down-regulated the expression of CD47 in our HNSCC mouse model,\(^{46}\) which may provide a potential for the combination of anti-PD-1 and anti-CD47 treatment. Combining anti-CD47 with anti-CD40, anti-CTLA-4, anti-PD-1, or anti-PD-L1 may also lead to an increase in tumor cell elimination by the initiation of both innate and adaptive immune response. In the present study, we discovered that the anti-CD47 treatment alternate the anergic effector T cell as indicated by decrease expression PD-1 using syngeneic mouse models of cancer. Taken together, combining the therapy with anti-CD47 and alternate therapies may provide a rational candidate approach to induce tumor reduction.

The foremost important factors in cancer therapy are safety in clinical application. Immune checkpoint therapy has been reported to produce immune-driven dysimmune toxicities, defined as immune-related adverse events (irAEs), such as the anti-CTLA-4 monoclonal antibody, ipilimumab, which may be caused by unbalancing immune system.\(^{47,48}\) The blockade of CD47 as another immune checkpoint therapy is also expected to take toxicity into consideration. Our results demonstrated that we did not identify readily detectable cytotoxicity in our mouse model upon anti-CD47 treatment. Although there are no reports on the toxicity of anti-CD47 treatment in the clinical study, anti-CD47 is predicted to have minimal drug toxicity due to the lack of adequate ‘eat me’ signals in healthy cells.\(^{49}\)

In conclusion, we identified that CD47 was highly expressed in HNSCC and it is associated with clinical outcome in primary
HNSCC. Blocking anti-CD47 with antibodies effectively stimulated cytotoxic T cells and improved immunosuppressive environment by decreasing immunosuppressive cells in immunocompetent HNSCC mouse model. Thus, our findings present a potential rationale for targeting CD47 for effective therapy against HNSCC in the future clinical application.

Materials and methods

**Immunocompetent transgenic HNSCC mouse models**

All animals and experiments were bred and performed in accordance with guidelines approved by Institutional Animal Care and Use Committee of the Wuhan University, Wuhan, China and the NIH guidelines for the use of laboratory animals in pathogen-free ASBL3 animal center of the Wuhan University. The time inducible tissue-specific Tgfbr1/Pten double knockout mice (K14-CreERtam+/--; Tgfbr1^floox/fox^; Pten^floox/fox^; Tgfbr1/Pten 2cKO) were maintained and genotyped based on previous protocols. Tgfbr1^floox/fox^/Pten^floox/fox^ mice (K14-CreERtam+/--; Tgfbr1^floox/fox^; Pten^floox/fox^; Tgfbr1/Pten 2cKO) from same cage were chosen as wild type (WT) control. Tamoxifen was administered in the oral cavity of Tgfbr1/Pten 2cKO mice for 5 consecutive days to induce homozygous deletion of Tgfbr1 and Pten in head and neck epithelia. All the mice had the same mixed background of FVBN/CD1/129/C57.

**Drugs and MIAP301 treatment**

The anti-mouse CD47-blocking *in vivo* monoclonal antibody (MIAP301, rat IgG2α) was purchased from BioXcell (West Lebanon, NH, USA) and was stored at 4°C in the dark at a concentration of 9.87 mg/ml. The anti-CD47 antibody is a rat-derived antibody and can act by: i) CD47-SIRPa checkpoint blockade, ii) opsonisation and Fc-receptor-mediated immune effector function, or iii) both. And it is known to functionally inhibit CD47-SIRPa interactions. For the preparation of working solution, this antibody was diluted to 2 mg/ml just before use. The *in vivo* isotype control (Clone: 2A3, rat IgG2α) was used as an isotype control for prophylactic tumorigenesis experiments. After oral gavage with tamoxifen (2 mg/kg) for 5 consecutive days (Day 1 to Day 5), the Tgfbr1/Pten 2cKO mice were then treated with intraperitoneal injections of 0.2 mg of the anti-CD47 antibody or IgG control every other day from Day 12 (n = 5 mice). All animals were routinely monitored.
and tumor size measurements were performed every other day. Tumor volume was calculated using the formula: tumor volume = length × width × height using a micrometer caliper. The endpoint was determined based on a systematic evaluation by the veterinary doctor. Then, animals were euthanized and immune organs and tumors were harvested as soon as possible. Subsequently, the tumor tissues were fixed in formalin or frozen at −80°C for immunostaining or Western blot analysis.

**Flow cytometry**

Spleen, draining lymph nodes (DLN), blood and tumors of HNSCC mouse model were processed for flow cytometric analysis as previously described. Especially, the tumor tissues and immune organs were dissociated and processed by the gentleMACS Dissointator and mouse tumor dissociation kit (Miltenyi Biotec). The following anti-mouse antibodies were used for staining: FITC-conjugated anti-CD4 (Clone GK1.5), CD11b (Clone M1/70), PE-conjugated anti-Ly6G (Clone 1A8), PD1 (Clone 29F.1), IFN-γ (Clone, XMG1.2) PE-Cy7-conjugated anti-Ly6C (Clone HK1.4), Percp-Cy5.5-conjugated anti-CD8 (Clone 53–6.7) and mouse regulatory T cell staining kit #3; and isotype-matched IgG controls (eBioscience, San Diego, CA). All the antibodies were used as per the manufacturer’s recommended protocols. The cells were analyzed with a FACS caliber flow cytometer equipped with CellQuest software (Becton Dickinson, Mountain View, CA), and data analyzed using FlowJo (Treestar, Ashland, OR) and gated by the side scatter and forward scatter. Dead cells were excluded based on 7AAD staining (Invitrogen).

**Suppression assay**

The CD11b<sup>+</sup>Ly6<sup>C<sub>D</sub></sup>/Ly6G<sup>−</sup>Ly6<sup>C<sub>hi</sub></sup> MDSCs and CD11b<sup>+</sup>Ly6G<sup>+</sup>Ly6<sup>C<sub>hi</sub></sup> MDSCs were sorted from spleens of anti-CD47 treated 2cKO mice and control group by mouse Myeloid-Derived Suppressor Cell Isolation Kit (Miltenyi Biotec, Germany). The naive CD8<sup>+</sup> T cells were isolated from lymph nodes of WT mice by Naïve CD8α<sup>+</sup> T Cell Isolation Kit (Miltenyi Biotec, Germany). CD4<sup>+</sup>CD25<sup>hi</sup> cells from spleens of anti-CD47 treated 2cKO mice and control group were sorted as Tregs cells by flow cytometry. The naive CD8<sup>+</sup> T cells were labeled with CFSE, and then were stimulated with 25 μg/ml αCD3, 2.5 μg/ml αCD28 and 30 IU/ml rIL-2 (BD PharmingenTM, USA). The activated T cells were respectively cocultured with CD11b<sup>+</sup>Ly6G<sup>−</sup>Ly6<sup>C<sub>lo</sub></sup> MDSCs and CD11b<sup>+</sup>Ly6G<sup>−</sup>Ly6<sup>C<sub>hi</sub></sup> MDSCs, and Tregs at ration of 1:1 in 96-well round-bottom plate. 72 h later, cells were assessed for CFSE analysis by flow cytometry.

**Western blot**

The Western blot analysis was performed according to our previously described procedures. Briefly, tumor tissues were carefully dissected and lysed with a T-PER buffer, which contains 1% phosphatase inhibitors and complete mini cocktail (Roche). Lysates of each sample were denatured and then loaded in 8–12% SDS-polyacrylamide gel electrophoresis. Subsequently, proteins were transferred to polyvinylidene fluoride membranes and blocked with milk for 1 hour, then incubated with primary antibodies: CD47 (Abcam ab175388), SIRPα (D6I3M), AKT (11E7), p-AKT (D9E), S6 (54D2), and p-S6 (D57.2.2E) (Cell signaling Technology) overnight. On day two, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL). The blots were developed by an enhanced chemiluminescence kit (West Pico, Thermo). GAPDH (Cell Signaling Technology) was used as a loading control. Each experiment was repeated at least three times.

**Human HNSCC samples**

Human HNSCC samples were obtained from the patients in the Hospital of Stomatology of Wuhan University with informed consent. All samples were analyzed by two independent pathologists of the Department of Oral Pathology, the Wuhan University. The custom made human HNSCC tissue microarrays used in this study were described as previously. The study of human tissue samples was approved by the Institutional Medical Ethics Committee of School and Hospital of Stomatology, the Wuhan University.

**Immunochemistry, images analyses, scoring system and immunofluorescence**

Tumors and control counterpart tissues such as tongue mucosa were dissected from the mice and fixed in 10% formalin overnight. The tissue sections were paraffin-embedded and sectioned as previously described. The sections were incubated with primary antibodies for CD47(ab175388), PD-1(D4W2) and D7D5W), PD-L1(E1L3N), Foxp3(D2W8E® and D6O8R), CD11b(EPR1344), CD33 (WM53), IFN-γ (GB11107), Ki-67 (ab15580), p-AKT (D9E) or isotype-matched IgG controls overnight. The staining was performed by ABC kits (Vector). All immunohistochemically stained slides were scanned using an Aperio ScanScope CS scanner (Vista, CA, USA). Histologic quantification of slides was operated using Aperio ScanScope quantification software (Version 9.1). The histoscore values were performed as previously described. Briefly, histoscore of membrane and nuclear staining quantification was performed according to the formula (3+ percent cells) × 3+(2+ percent cells) × 2+(1+ percent cells) × 1 and the formula total intensity/total cell number was used to evaluate the histoscore of pixel quantification. In this case, the normalized score is 0–300. The hierarchical analysis was conducted by Cluster 3.0 with Pearson’s correlation coefficient and the results were visualized by Java Treeview. Closely related biomarkers were tightly put together.

For immunofluorescence staining, the slides were blocked with 10% goat serum for 1 hr and then incubated overnight at 4°C with primary antibodies:CD47(ab175388), CK14 (PROGEN German), CD11b (NB110-89474), Gr-1(RB6-8C5), SIRPα (D6I3M) On the second day, the sections were incubated with fluorochrome labeled secondary antibodies for 1 hr (Alexa 594 anti-rabbit Invitrogen and Alexa 488 Yeasen 34506ES60). Then, the slides were stained with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). The images were detected with a laser scanning confocal microscope (Leica).
Statistical analysis

Statistical data analysis was performed with GraphPad Prism 6.03 (GraphPad Software, Inc., La Jolla, CA) statistical packages. The data among each group were analyzed by One-way ANOVA, followed by the post-Tukey multiple comparison tests or unpaired t test. Two-tailed Pearson statistical analyses were used for correlated expression of these markers. Mean values ± SEM with a difference of P < 0.05 were considered statistically significant (ns, p > 0.05; *P < 0.05; **P < 0.01; ***P < 0.001).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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References

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646–74. doi:10.1016/j.cell.2011.02.013. PMID:21376230
2. Azoury SC, Gilmore RC, Shukla V. Molecularly targeted agents and immunotherapy for the treatment of head and neck squamous cell cancer (HNSCC). Discov Med. 2016;21:507–16. PMID:27448787.
3. Murdoch C, Muthana M, Coffelt SB, Lewis CE. The role of myeloid cells in the promotion of tumour angiogenesis. Nat Rev Cancer. 2008;8:618–31. doi:10.1038/nrc2444. PMID:18633355.
4. Gabrilovich DI, Nagarah S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol. 2009;9:162–74. doi:10.1038/nri2506. PMID:19197294.
5. Peng D, Tanikawa T, Li W, Zhao L, Vatan L, Szluga W, Wan S, Wei S, Wang Y, Liu Y, et al. Myeloid-Derived Suppressor Cells Endow Stem-like Qualities to Breast Cancer Cells through IL6/STAT3 and therapeutic antibody target on human acute myeloid leukemia stem cells. Cell. 2011;144:646–74. doi:10.1111/j.1365-2457.2011.07071.x. PMID:22039616.
6. Nagaraj S, Gupta K, Pisarev V, Kinarsky L, Sherman S, Kang L, Herber DL, Schneck J, Gabrilovich DI. Altered recognition of stem cells. Cell. 2009;138:286–99. doi:10.1016/j.cell.2009.05.045. PMID:19632179.
7. Xiao Z, Chung H, Banan B, Manning PT, Ott KC, Lin S, Capoccia BJ, Subramanian V, Hiebsch RR, Upadhyia GA, et al. Antibody mediated therapy targeting CD47 inhibits tumor progression of hepatocellular carcinoma. Cancer Lett. 2015;360:302–9. doi:10.1016/j.canlet.2015.02.036. PMID:25721088.
8. Campbell IG, Freemont PS, Foulkes W, Trowsdale J. An ovarian tumor marker with homology to vaccinia virus contains an IgV-like region and multiple transmembrane domains. Cancer Res. 1992;52:5416–20. PMID:1394148.
9. Chao MP, Weissman IL, Majeti R. The CD47-SIRPα pathway in cancer immune evasion and potential therapeutic implications. Curr Opin Immunol. 2012;24:225–32. doi:10.1016/j.coi.2012.01.010. PMID:22321013.
10. Brooke G, Holbrook JD, Brown MH, Barclay AN. Human lymphocytes interact directly with CD47 through a novel member of the signal regulatory protein (SIRP) family. J Immunol. 2004;173:2562–70. doi:10.4049/jimmunol.173.4.2562. PMID:15294972.
11. Li Z, He L, Wilson K, Roberts D. Thrombospondin-1 inhibits TCR-mediated T lymphocyte early activation. J Immunol. 2001;166:2427–36. doi:10.4049/jimmunol.166.4.2427. PMID:11160302.
12. Kaur S, Kuznetsova SA, Pendrak ML, Sipes JM, Romeo MJ, Li Z, Zhang L, Roberts DD. Heparan sulfate modification of the transmembrane receptor CD47 is necessary for inhibition of T cell receptor signaling by thrombospondin-1. J Biol Chem. 2011;286:14991–5002. doi:10.1074/jbc.M110.179663. PMID:21334308.
13. Soto-Pantoja DR, Terabe M, Ghosh A, Ridnour LA, DeGraff WG, Wink DA, Berzofsky JA, Roberts DD. CD47 in the tumor microenvironment limits cooperation between antitumor T-cell immunity and radiotherapy. Cancer Res. 2014;74:6771–83. doi:10.1158/0008-5472.CAN-14-0037-T. PMID:25297630.
14. Liu X, Pu Y, Cron K, Deng L, Kline J, Frazier WA, Xu H, Peng H, Fu YX, Xu MM. CD47 blockade triggers T cell-mediated destruction of immunogenic tumors. Nat Med. 2015;21:1209–15. doi:10.1038/nm.3931. PMID:26322579.
15. Chan KS, Espinosa I, Chao M, Wong D, Ailles L, Diehn M, Gill H, Presti Jr., Chang HY, van de Rijn M, et al. Identification, molecular characterization, clinical prognosis, and therapeutic targeting of human bladder tumor-initiating cells. Proc Natl Acad Sci U S A. 2009;106:14016–21. doi:10.1073/pnas.0906513106. PMID:19666525.
16. Manna PP, Frazier WA. CD47 mediates killing of breast tumor cells via Gi-dependent inhibition of protein kinase A. Cancer Res. 2004;64:1026–36. doi:10.1158/0008-5472.CAN-03-1708. PMID:14871834.
17. Pardoll DM. The blockade of immune checkpoints in cancer immuno-therapy. Nat Rev Cancer. 2012;12:252–64. doi:10.1038/nrc3239. PMID:22437870.
18. Kim MJ, Lee JC, Lee JJ, Kim S, Lee SG, Park SW, Sung MW, Heo DS. Association of CD47 with natural killer cell-mediated cytotoxicity of head-and-neck squamous cell carcinoma lines. Tumour Biol. 2008;29:28–34. doi:10.1159/000132568.
19. Mashimer JB, Soto-Pantoja DR, Ridnour LA, Shih HB, Degraf WG, Tsokos M, Wink DA, Isenberg JS, Roberts DD. Radioprotection in normal tissue and delayed tumor growth by blockade of CD47 signaling. Sci Transl Med. 2009;1:3ra7. doi:10.1126/scitranslmed.3000139. PMID:20161613.
20. Yang C, Gao S, Zhang H, Xu L, Liu J, Wang M, Zhang S. CD47 is a Potential Target for the Treatment of Laryngeal Squamous Cell Carci- noma. Cell Physiol Biochem. 2016;40:126–36. doi:10.1159/000452530. PMID:27853370.
21. Rhodes DR, Kalyana-Sundaram S, Mahavisno V, Varambally R, Yu J, Briggs BB, Barrette TR, Anseth MJ, Kincead-Beal C, Kulkarni P, et al. Oncomine 3.0: genes, pathways, and networks in a collection of 18,000 cancer gene expression profiles. Neoplasia. 2007;9:166–80. doi:10.1593/neoplasia.1072. PMID:17356713.
22. Vonderheide RH. CD47 blockade as another immune checkpoint therapy for cancer. Nat Med. 2015;21:1122–3. doi:10.1038/nm.3965. PMID:26444633.
23. Ferris RL. Immunology and Immunotherapy of Head and Neck Cancer. J Clin Oncol. 2015;33:3293–304. doi:10.1200/JCO.2015.61.1509. PMID:26351330.
24. Leemans CR, Braakhuis BJ, Braekenhoff RH. The molecular biology of head and neck cancer. Nat Rev Cancer. 2011;11:9–22. https://dx.doi. org/10.1038/nrc2982. PMID:21160525.
30. Bian Y, Hall B, Sun ZJ, Molinolo A, Chen W, Gutkind JS, Wakefield LM, et al. Progressive tumor formation in mice with conditional deletion of TGF-beta signaling in head and neck epithelia is associated with activation of the PI3K/Akt pathway. Cancer Res. 2009;69:5918–26. doi:10.1158/0008-5472.CAN-08-4623. PMID:19584284.

31. Bian Y, Terse A, Du J, Hall B, Molinolo A, Zhang P, Chen W, Flanders KC, Gutkind JS, Waes CV, Larsson J, Karlsson S, Gies JP, et al. Loss of TGF-beta signaling and PTEN promotes head and neck squamous cell carcinoma through cellular senescence and cancer-related inflammation. Oncogene. 2012;31:3322–32. doi:10.1038/onc.2011.494. PMID:22037217.

32. Wherry EJ. T cell exhaustion. Nat Immunol. 2011;12:492–9. PMID:21739672.

33. Yu GT, Bu LL, Zhao YY, Mao L, Deng WW, Wu TF, Zhang WF, Sun ZJ. CTLA4 blockade reduces immature myeloid cells in head and neck squamous cell carcinoma. Oncology. 2016;35:115594. doi:10.1158/2162402X.2016.115594. PMID:27471622.

34. Freiser ME, Serafini P, Weed DT. The immune system and head and neck squamous cell carcinoma: from carcinogenesis to new therapeutic opportunities. Immunol Res. 2013;57:52–69. doi:10.1007/s12026-013-8462-3. PMID:23421836.

35. Stagg J, Smyth MJ. Extracellular adenosine triphosphate and adenosine in cancer. Oncogene. 2010;29:5346–58. doi:10.1038/onc.2010.292. PMID:20661219.

36. Sick E, Boukharai A, Deramaudt T, Ronde P, Bucher B, Andre P, Gies JP, et al. Anti-CD47 antibody-mediated phagocytosis of cancer by macrophages primes an effective antitumor T-cell response. Proc Natl Acad Sci U S A. 2013;110:11103–8. doi:10.1073/pnas.1305691110. PMID:23690610.

37. Lo J, Lau EY, So FT, Lu P, Chan VS, Cheung VC, Ching RH, Cheng BY, Ma MK, Ng IO, et al. Anti-CD47 antibody suppresses tumour growth and augments the effect of chemotherapy treatment in hepatocellular carcinoma. Liver Int. 2016;36:737–45. doi:10.1111/liv.12963. PMID:26351778.

38. Ngo M, Han A, Lakatos A, Sahoo D, Hachej SY, Weiskopf K, Beck AH, Weissman IL, Boiko AD. Antibody Therapy Targeting CD47 and CD271 Effectively Suppresses Melanoma Metastasis in Patient-Derived Xenografts. Cell Rep. 2016;16:1701–16. doi:10.1016/j.celrep.2016.07.004. PMID:27477289.

39. Yu GT, Bu LL, Huang CF, Zhang WF, Chen WJ, Gutkind JS, Kulkarni AB, Sun ZJ. PD-1 blockade attenuates immune-suppressive myeloid cells due to inhibition of CD47/SIRPalpha axis in HPV negative head and neck squamous cell carcinoma. Oncotarget. 2015;6:42067–80. doi:10.18632/oncotarget.9595. PMID:26573233.

40. Gao J, He Q, Subudhi S, Aparicio A, Zurita-Saavedra A, Lee DH, James C, Suarez-Almazor M, Sharma P. Review of immune-related adverse events in prostate cancer patients treated with ipilimumab. MD Anderson experience. Oncogene. 2015;34:5411–7. doi:10.1038/ons.2015.5. PMID:25659583.

41. Boutros C, Tarhini A, Routier E, Lambotte O, Ladurie FL, Carbonnel F, Izzeddine H, Marabelle A, Champiat S, Berdelou A, et al. Safety profiles of anti-CTLA-4 and anti-PD-1 antibodies alone and in combination. Nat Rev Clin Oncol. 2016;13:473–86. doi:10.1038/nrclinonc.2016.58. PMID:27141885.

42. McCracken MN, Cha AC, Weissman IL. Molecular Pathways: Activating T Cells after Cancer Cell Phagocytosis from Blockade of CD47 "Don’t Eat Me" Signals. Clin Cancer Res. 2015;21:3597–601. doi:10.1158/1078-0432.CCR-14-2520. PMID:26116271.

43. Zhang L, Sun ZJ, Bian Y, Kulkarni AB. MicroRNA-135b acts as a tumor promoter by targeting the hypoxia-inducible factor pathway in genetically defined mouse model of head and neck squamous cell carcinoma. Cancer Lett. 2013;331:230–8. doi:10.1016/j.canlet.2013.01.003. PMID:23340180.

44. Oldenborg PA, Gresham HD, Lindberg FP. CD47-signal regulatory protein alpha (SIRPalpha) regulates Fcgamma and complement receptor-mediated phagocytosis. J Exp Med. 2001;193:855–62. doi:10.1084/jem.193.7.855. PMID:11283158.

45. Deng WW, Mao L, Yu GT, Bu LL, Ma SR, Liu B, Gutkind JS, Kulkarni AB, Zhang WF, Sun ZJ.LAG-3 confers poor prognosis and its blockade reshapes antitumor response in head and neck squamous cell carcinoma. Oncology. 2016;35:1239905. doi:10.1080/00085472.2016.1239905. PMID:27999760.

46. Woodman N, Pinder SE, Tajadura V, Le BX, Gillet C, Delannoy P, Burchell JM, Julien S. Two E-selectin ligands, BST-2 and LGALS3BP, predict metastasis and poor survival of ER-negative breast cancer. Int J Oncol. 2016;49:265. PMID:27176937.

47. Wu L, Deng WW, Yu GT, Mao L, Bu LL, Ma SR, Liu B, Zhang WF, Sun ZJ. B7-H4 expression indicates poor prognosis of oral squamous cell carcinoma. Cancer Immunol Immunother. 2016;65:1035–45. doi:10.1007/s00262-016-1867-9. PMID:27383830.

48. Wu L, Deng WW, Huang CF, Bu LL, Yu GT, Mao L, Zhang WF, Liu B, Sun ZJ. Expression of VISTA correlated with immunosuppression and synergized with CD8 to predict survival in human oral squamous cell carcinoma. Cancer Immunol Immunother. 2017;66:627–36. doi:10.1007/s00262-017-1968-0. PMID:28236118.

49. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A. 1998;95:14863–8. doi:10.1073/pnas.95.25.14863. PMID:9843981.