Targeting STAT3 signaling reduces immunosuppressive myeloid cells in head and neck squamous cell carcinoma

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ABSTRACT
Cumulative evidence suggests that constitutively activated signal transducer and activator of transcription (STAT3) may contribute to sustaining immunosuppressive status, and that inhibiting STAT3 signaling represents a potential strategy to improve antitumor immunity. In the present study, we observed that high levels phosphorylated of STAT3 are significantly associated with the markers for both myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) in human head and neck squamous cell carcinoma (HNSCC). Additionally, we showed that targeting STAT3 signaling with a tolerable selective inhibitor S3I-201 significantly decreased immature myeloid cells such as MDSCs, TAMs and iDCs in genetically defined mice HNSCC model. These findings highlight that targeting STAT3 signaling may be effective to enhance antitumor immunity via myeloid suppressor cells in HNSCC.

Keywords: DCs, dendritic cells; HNSCC, head and neck squamous cell carcinoma; MDSCs, myeloid-derived suppressor cells; STAT3, signal transducer and activator of transcription 3; TAMs, tumor-associated macrophages

Introduction

HNSCC affects 600,000 new patients each year and accounts for over 90% of head and neck cancer cases. HNSCC arises in the oral cavity, larynx, hypo-pharynx and oropharynx, and it is the sixth most common malignancy worldwide. 4 Despite significant advances in the areas of reconstructive surgery, minimally invasive surgery, precisely targeted radiotherapy, chemotherapy, and monoclonal antibody therapy during the last three decades, the overall survival rates of HNSCC patients has been only modestly affected. 2,3 Moreover, HNSCC can occur in young patients without any known risk factors (tobacco use, alcohol consumption or infection with human papilloma virus). 3

In malignancy, the ability of tumors to evade immune surveillance plays a critical role in tumor initiation and progression. 4 Recent reports suggest cancer stromal cells, including TAMs, MDSCs, granulocytes, endothelial cells, fibroblasts, and T cells contribute to the initiation and progression of HNSCC. 5,7 Some studies have suggested that these tumor-associated myeloid cells (including TAMs, MDSCs and iDCs) were recruited in tumor environment and formed an organic whole to sustain an immunosuppressive state. 8 Recent advances in therapeutic antibodies, adoptive T-cell therapy (ACT) and cancer vaccines have provided relevant strategies to treat cancer patients. 9 A myriad of evidence suggests a critical role for the oncogenic transcriptional factor STAT3 (signal transducer and activator of transcription 3) in mediating tumor-induced immune suppression. 10-12 Nevertheless, the role of STAT3 in driving antitumor immunity against HNSCC remains poorly understood.

In the present study, the role of STAT3 in HNSCC immune invasion was explored by determining the correlation between the levels of p-STAT3 with the numbers of MDSCs and TAMs in human HNSCC. A chemopreventive experiment was performed using the selective p-STAT3 inhibitor S3I-201 in a HNSCC mice model, where populations of CD11b + Gr1 + MDSCs and CD11b + F4/80 + TAMs as well as effective T cells were directly analyzed in immune organs and in tumor tissues. This study demonstrates that therapy on targeting STAT3 signaling can lead to a remarkable decrease in MDSCs and TAMs. Inhibition of STAT3 through S3I-201 concurrently allowed enhanced immune surveillance through an increase in CD8 + and CD4 + T cells.

Results

Phosphorylation of STAT3 correlates with MDSC and TAM in human HNSCC

Recent studies have demonstrated that CD33 + CD11b + MDSCs and CD68 + CD163 + M2 type TAMs are major cancer immune suppressor cells in human HNSCC. 13 The
correlation of between p-STAT3 with markers of MDSCs and TAMs in HNSCC was initially investigated through a tissue microarray. The immunohistochemical staining of p-STAT3 at Tyrosine residue 705 (p-STAT3Tyr705) was localized in the nucleus of both the cancer cells and the immune cells while CD11b, CD33, CD68 and CD163 were only expressed in immune cells (Fig. 1A). In addition, p-STAT3Tyr705 was co-expressed with CD68 (Fig. S1A). The immunoreactivity of p-STAT3Tyr705, CD11b, CD33, CD68 and CD163 were consistently increased in HNSCCs (n = 86) compared with those in normal oral mucosa (n = 32) (Fig. 1A). Increased p-STAT3Tyr705 also was correlated with the expression of the TAM markers CD68 (p < 0.01, r = 0.2561) and CD163 (p < 0.05, r = 0.1903, Fig. 1B) as well as the MDSC markers CD11b (p < 0.01, r = 0.2500) and CD33 (p < 0.05, r = 0.1914, Fig. 1C). Hierarchal clustering analysis of the tissue microarray data linked CD11b/CD33 and CD68/CD163 as well as p-STAT3Tyr705 with HNSCC (Fig. 1D). The above results suggested that p-STAT3Tyr705 was strongly correlated with the appearance of TAMs and MDSCs in human HNSCC.

**STAT3 Phosphorylation in Tgfr1 cKO mice, Pten cKO mice and Tgfbr1/Pten 2cKO mice**

The immunohistochemical staining of p-STAT3Tyr705 was detected in the nucleus of the cancer cells of Tgfr1/Pten 2cKO mice (Fig. 2A). However, p-STAT3Tyr705 staining was negative in the wild-type mice tongue mucosa and partially positive in tongue of Tgfr1/Pten 2cKO (Fig. 2A). The p-STAT3Tyr705 positive staining gradually increases in intensity by examining wild-type mice tongue, Pten cKO mice
Figure 2. STAT3 Phosphorylation in Tgfr1 cKO mice, Pten cKO mice and Tgfr1/Pten 2cKO mice. (A) Representative immunohistochemical staining of p-STAT3Tyr705 in wide type (WT) tongue, Tgfr1/Pten 2cKO tongue and Tgfr1/Pten 2cKO tongue squamous cell carcinoma (TSCC). Scale bar, 50 μm. (B) Hematoxyline-eosin staining and immunohistochemically staining indicate increase p-STAT3Tyr705 expression in Pten conditional knock out (Pten cKO) mice HNSCC, Tgfr1 conditional knock out (Tgfr1 cKO) mice HNSCC and Tgfr1/Pten 2cKO mice HNSCC. Scale bars, 100 μm. (C) Western blot shows a significant increase in p-STAT3Tyr705 in Tgfr1/Pten 2cKO mice TSCC as compared with wide type and Tgfr1/Pten 2cKO tongue mucosa. (D) Western blot shows a significant increase in p-STAT3Tyr705 in Pten cKO mice HNSCC, Tgfr1 cKO mice HNSCC and Tgfr1/Pten 2cKO mice HNSCC. (E) Representative double immunofluorescence staining of CD11b/p-STAT3 in Tgfr1/Pten 2cKO mice HNSCC. Scale bar, 50 μm. (F) Representative double immunofluorescence staining of CD11c/p-STAT3 in Tgfr1/Pten 2cKO mice HNSCC. Scale bar, 50 μm.
Tgfbr1 cKO mice TSCC through to Tgfbr1/Pten 2cKO mice TSSC (Fig. 2B). Western blot results showed that increased p-STAT3 Tyr705 was an event associated with tumorigenesis of the Tgfbr1/Pten 2cKO mice compared with that in the wild type tongue mucosa (Fig. 2C). Western blot also suggested p-STAT3 gradually increased in wild-type mice, Pten cKO mice, Tgfbr1 cKO mice, through to Tgfbr1/Pten 2cKO mice TSCC (Fig. 2D). Representative double immunofluorescence staining photos showed CD11b (Fig. 2E) and CD11c (Fig. 2F) were both co-expressed with immature myeloid and DCs markers CD11b and CD11c in the Tgfbr1/Pten 2cKO mice HNSCC. These results indicate that loss of Tgfbr1 and Pten leads to the activation of the STAT3 signaling pathway and that p-STAT3 was also co-expressed with immature myeloid and DCs markers CD11b and CD11c in the Tgfbr1/Pten 2cKO HNSCC mouse model.

### S3I-201 induced STAT3 signaling inhibition delays tumorigenesis in Tgfbr1/Pten 2cKO mice

To investigate the correlation of STAT3 activation and immune evasion, we took advantage of our Tgfbr1/Pten 2cKO mice, through to Tgfbr1/Pten 2cKO mice TSCC (Fig. 2D). Treatment with S3I-201 induced STAT3 signaling inhibition delays CD11b and CD11c in the lymph nodes (Fig. S2B and Fig. 4B, p < 0.01). Double immunofluorescence staining also showed that S3I-201 treatment reduced CD11b+Gr1+ expression in the HNSCC mice tissues (Fig. 4E). Western blot showed that treatment with S3I-201 not only caused a significant decrease of p-STAT3, but also caused reductions in the myeloid cell chemokine CXCL1 in the HNSCC (Fig. 4F). Therefore, the inhibition of p-STAT3 effectively decreased the numbers of MDSCs in Tgfbr1/Pten 2cKO HNSCC.

Similar to MDSCs, CD11b+F4/80+ TAMs were also significantly increased in spleen (Figs. 5A and 5B, n = 6 respectively, p < 0.01), blood (Fig. S2A and Fig. 5B, *p < 0.05), and lymph nodes (Fig. S2A and Fig. 5B, **p < 0.01) in tumor-bearing mice vs. the wild type controls. Treatment with S3I-201 significantly reduced the number of TAMs in the spleen and lymph nodes of the Tgfbr1/Pten 2cKO mice while causing a modest non-significant decrease in the blood. Importantly, the number of TAMs in the tumors of Tgfbr1/Pten 2cKO mice were significantly reduced following treatment with S3I-201 (Figs. 5C and 5D, **p < 0.01). Recent reports indicate “don’t eat me” signal molecule CD47 and its phagocyte receptor SIRPα expressed in monocyte play an important role in both DC maturation and the recruitment of tumor-associated myeloid cells.17 By western blot and immunofluorescence, we demonstrated S3I-201 treatment remarkably decreased the expression of CD47 and SIRPα in Tgfbr1/Pten 2cKO mice with HNSCC (Figs. 5E and 5F). The above data suggests that inhibition of p-STAT3 may cause a decrease in TAMs by possibly inhibiting the CD47-SIRPα pathway.

### Blockade of p-STAT3 reverses the immune suppression by increasing effective CD4+ and CD8+ T cells as well as maturation of DCs in vivo

The balance between effector T cells (including CD4+ helper T cells and CD8+ cytotoxic T cells) and immunosuppressive cells (including TAMs, MDSCs, and immune cells expressing co-inhibitory immune checkpoint molecules) is a critical arbiter of effective anti-immunosuppressive activity.18 To further investigate the role of p-STAT3 inhibition on antitumor immunity, we analyzed the population of T cells in the mice. The inhibition of STAT3 activity significantly increased the percentage of CD4+ T cells in spleen, lymph nodes, blood and tumor in S3I-201 treated mice and compared to the untreated controls (Figs. 6A and 6B, n = 6, *p < 0.05). The number of CD8+ T cells was also significantly increased in mice lymph nodes (Figs. 6A and 6B, n = 6, **p < 0.01) and in tumor (Figs. 6A and 6B, n = 6, *p < 0.05) after S3I-201 treatment. This reversal in immune cell populations was also supported by the gross observation that p-STAT3 inhibition caused a significant decrease in spleen index vs. the PBS control, thereby leading to an overall spleen index comparable the wild-type mice (Fig. 6C). In summary, these results suggested that blockade of p-STAT3 could reverse tumor-induced immunosuppressive by re-establishing an adequate balance between the numbers of the effector T cells and immunosuppressive cells.

Both CD40 and MHC II cell populations were significantly decreased in the spleen, lymph nodes, and blood of the
tumor-bearing mice. In contrast, S3I-201 was able to cause a significant increase of CD40 and MHC-II cell populations in spleen of treated Tgfbr1/Pten 2cKO mice as compared to the untreated controls (Figs. S3A and S3B). MHC-II populations were also increased in the tumors of S3I-201 treated mice (Figs. S3A and S3B).

**Discussion**

Recent studies have demonstrated that suppression of the host immune system plays a major role in the progression of cancer development as well as resistance to cancer therapy. Although the blockade of STAT3 signaling achieved a significant success.
in many tumors through its effects on cancer and immune cells, the influence of this pathway in immune system should be further investigated. In the present study, our results demonstrate a significant correlation between levels of STAT3 activation and the numbers of immunosuppressive MDSCs and TAMs in human HNSCC. Furthermore, we demonstrate that activation of STAT3 is an important molecular event leading to HNSCC tumorigenesis upon the loss of both Tgfbr1 and Pten tumor suppressors. STAT3 signaling inhibition elicited antitumor effects at least by partially reversing the immunosuppressive status of the tumor bearing Tgfbr1/Pten mice. Inhibition of STAT3 activation through S3I-201 ultimately lead to decreased population of MDSCs, TAMs and increased maturation of DCs, CD4+ and CD8+ T cells.

Figure 4. The population of MDSCs was decreased in S3I-201 treatment Tgfbr1/Pten 2cKO mice. (A) Representative flow cytometry profiles showed increased CD11b+Gr1+ cells in spleen of HNSCC bearing Tgfbr1/Pten 2cKO mouse (PBS treatment, middle) as compared with wide type (WT) mice. CD11b+Gr1+ cell population was significantly decreased after S3I-201 treatment (right). (B) Quantification of the percent of CD11b+Gr1+ MDSCs in spleen, lymph nodes and blood of mice with or without S3I-201 treatment and wild type mice (Data presented as mean ± SEM, n = 6 mice respectively, ANOVA with post Tukey test. *p < 0.05; **p < 0.01; ***p < 0.001). (C) Representative flow cytometry profiles shows CD11b+Gr1+ cell population in tumor was significantly decreased after S3I-201 treatment. (D) Quantification the percent of CD11b+Gr1+ MDSCs in tumor of mice with or without S3I-201 treatment and wide type mice (Data presented as mean ± SEM, n = 6 mice respectively, ANOVA with post Tukey test. *p < 0.01). (E) Double immunofluorescence staining of CD11b+Gr1+ cell population was performed in mice HNSCC with or without S3I-201 treatment. Scale bar, 50 μm. (F) Western blot analysis revealed that the protein level of p-STAT3 and CXCL1 were reduced with selective STAT3 activity inhibition.
Figure 5. The population of TAMs was decreased in S3I-201 treatment Tgfbr1/Pten 2cKO mice. (A) Single cell suspension from spleen of HNSCC bearing mouse treated with S3I-201 or PBS and wide type mice were stained with anti-CD11b and anti-F4/80 antibody and the percentage of positive cells analyzed by flow cytometry, representative images are shown. (B) Quantification of CD11b^+ F4/80^+ TAMs in spleen, lymph nodes and blood of mice with or without S3I-201 treatment and wild type mice (Data presented as mean ± SEM, n = 6 mice respectively, ANOVA with post Tukey test. *p < 0.05; **p < 0.01). (C) Single cell suspension from tumor of HNSCC bearing mouse treated with S3I-201 or PBS and wide type mice were stained with anti-CD11b and anti-F4/80 antibody and percentage of positive cells analyzed by flow cytometry, representative images are shown. (D) Quantification of CD11b^+ F4/80^+ TAMs in tumor of mice with or without S3I-201 treatment and wide type mice (Data presented as mean ± SEM, n = 6 mice respectively, ANOVA with post Tukey test. **p < 0.01). (E) Double immunofluorescence images of CD47 and SIRPα in mice bearing tumor with or without S3I-201 treatment are shown. Scale bar, 50 μm. (F) Western blot analysis revealed that the protein level of CD47 and SIRPα were reduced with STAT3 activity inhibition.
inhibitor of STAT3 activity that blocks complex formation and its DNA-binding and transcriptional activities. More recently studies also suggested an association between STAT3 signaling with immature myeloid cells TAMs and MDSCs. Many studies have shown that STAT3 signaling is persistently activated in MDSCs which promotes expansion of MDSCs. Consistent with study by Kortylewski et al., blockade of STAT3 activity in our HNSCC model also caused marked decreases in MDSCs in the spleen, blood, lymph nodes, and tumors. The tumor microenvironment from the Tgfbr1/Pten 2cKO mice show high expression of CXCL1 marker known to recruit MDSCs. The present study demonstrated the reduction of CXCL1 through inhibition of STAT3 activation.

As a main component of tumor-infiltrating leukocytes, TAMs are known to play a significant role in HNSCC initiation and progression and resemble M2 macrophages, which promote tumor invasion and metastasis. Recent study have demonstrated that suppressing STAT3 activation can inhibit macrophage differentiation to M2 phenotype. Indeed, our study found that S3I-201 decreased M2 TAMs in our mice model. In many malignancies, CD47/SIRPα expression can act as a “do not eat me” signal and blockade of the CD47/SIRPα axis can cause an efficient and rapid phagocytosis of multiple tumor cell types. In the present study, the down-regulation of CD47/SIRPα axis enhanced the phagocytic ability of macrophages following p-STAT3 inhibition.

Many studies have shown that DCs, specialized antigen-presenting cells (APCs) recognize, process, and present antigens to T cells and play a critical role for the induction and maintenance of antitumor immune response. Abnormal dendritic cell (DC) differentiation is one of the major factors of tumor non-responsiveness. A recent report showed the role of hyperactivation of STAT3 in the accumulation of immature DCs, while mounting studies have proved that inhibition of the constitutive activation of STAT3 both in tumor cells and in diverse immune cells increases expression of CCL5, IL-12, TNF, IFNγ, IFNβ, CXCL10, CD40, CD80, CD86 and MHC class II molecules. In this current study, our results suggest that the co-stimulatory molecules of mature DCs, especially CD40 and MHC-II were remarkably increased following blockade of STAT3 activity. In addition, we demonstrated that inhibition of STAT3 signaling could reduce CD47-SIRPα, which is also known to restrain DC maturation.

In summary, our results prove direct clinical correlation between p-STAT3 and tumor-associated myeloid cells (MDSCs and TAMs) in human HNSCC. After blockade of p-STAT3, the host immunosuppressive status was reversed with decreases in the number of MDSCs, TAMs, and immature DCs and

Figure 6. Increase of effective T cells and reduction of exhausted T-cells in S3I-201 treatment Tgfbr1/Pten 2cKO mice. (A) Representative flow cytometry photos showed increase of CD4+ and CD8+ T cells in S3I-201 treatment group. (B) Quantification of CD4+ and CD8+ cell population in spleen, lymph node (LN), blood and tumor of wild type mice, Tgfbr1/Pten conditional knock out (Tgfbr1/Pten 2cKO) mice with or without S3I-201 treatment (Data presented as mean ± SEM, n = 6 mice respectively, ANOVA with post Tukey test. *p < 0.05; **p < 0.01). (C) Representative image and spleen index shows the comparison between S3I-201 treatment group and control group (Data presented as mean ± SEM, n = 3 mice respectively, ANOVA with post Tukey test. *p < 0.05).
increases in the number of CD8$^+$ T cells and CD4$^+$ T cells in our Tgfbr1/Pten 2cKO mice. Therefore, our study demonstrates that inhibiting the STAT3 pathway may be a promising target in HNSCC immunotherapy.

Materials and methods

Detailed methods and procedures are provided in the supplementary data

Tgfbr1/Pten 2cKO mice

The time inducible tissue-specific Tgfbr1/Pten 2cKO mice (Tgfbr1$^{fl/fl}$; Pten$^{fl/fl}$, K14-CreER$^{tam/+}$, FVB/N CD1/129/C57 mixed background) were maintained and genotyped as previously described.\textsuperscript{14,42} Tgfbr1 cKO mice HNSCC (K14-Cre$^{ER tam+-}$; Tgfbr1$^{fl/fl}$), Pten cKO mice HNSCC (K14-Cre$^{ER tam+-}$; Pten$^{fl/fl}$) tissue were gifted by Dr Ashok B. Kulkarni as previously described.\textsuperscript{14,42} All the mice were bred in the FVB/N CD1/129/C57 mixed background.

STAT3 signaling inhibitor S3I-201 treatment

S3I-201 (NCI74859) was purchased from Selleck Chemicals (S1155, Westlake Village, CA) and dissolved in dimethyl sulfoxide for use at indicated concentration. After oral gavage of tamoxifen for five consequent days, the Tgfbr1/Pten 2cKO mice were randomly divided into experiment group; 5 mg/kg S3I-201 was administered every other day (i.p. q.o.d, n = 6 mice) or a control group (PBS, i.p. q.o.d, n = 6 mice). S3I-201 and control treatment was performed at day 14 and maintained for 4 weeks. Syngeneic control mice (K14-Cre$^{ER tam+-}$; Tgfbr1$^{fl/fl}$; Pten$^{fl/fl}$) with same dose of tamoxifen were used as the wide type control (n = 6). All animals were inspected and monitored every other day. The tumor size was measured with a micrometer caliper and photographed every other day. The endpoint was determined according to a systematic evaluation by the veterinary doctor. The mice were euthanized at the end of the study, and the tumors were fixed in paraffin overnight or frozen at −80°C for immunostaining or western blot analysis.

Flow cytometry analysis

FACS was performed on single cell suspensions from tumor tissues, spleens, and lymph node as well as blood in Tgfbr1/Pten 2cKO mice with or without STAT3 blockade according to the detailed procedure in supplementary files.\textsuperscript{43} Wide-type controls with same dose tamoxifen were set for flow cytometry analysis.

Western blot

Spontaneous tumors that developed in Tgfbr1/Pten 2cKO mice were lysed in a T-PER buffer containing 1% phosphatase inhibitors and complete mini cocktail (Roche). Detailed procedures of immunoblotting were described previously.\textsuperscript{14}

Human HNSCC tissue array

Custom made human tissue microarray including 86 HNSCC and 32 normal oral mucosa were used for immunohistochemistry staining as previously described.\textsuperscript{14} These studies were carried out with the approval of School and Hospital of Stomatology of Wuhan University Medical Ethics Committee.

Immunohistochemistry and immunofluorescence

Immunohistochemically staining and double immunofluorescence staining slides were performed as previously described.\textsuperscript{44}

Hierarchical clustering, data visualization and statistical analysis

As we previously described,\textsuperscript{44} hierarchical clustering was done using Cluster program with average linkage based on Pearson’s correlation, visualized using the Tree View program. Data analyses were used Graph Pad Prism version 5.0 for Windows (Graph Pad Software Inc. La Jolla, CA). One-way ANOVA followed by the post-Tukey multiple comparison tests and unpaired t test were used to analyze the differences in positive cells and immunostaining among each group. Two-tailed Pearson’s statistics was used for correlation between p-STAT3, CD11b, CD33, CD68 and CD163. 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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