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Amphiregulin promotes activated regulatory T cell-suppressive function via the AREG/EGFR pathway in laryngeal squamous cell carcinoma

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Abstract

Background Activated regulatory T cells (aTregs) play a vital role in promoting a tumor immunosuppressive microenvironment in laryngeal squamous cell carcinoma (LSCC). However, the regulatory factors that induce the generation of aTregs are not clear. Herein, we investigated the effect of amphiregulin (AREG) on the production of aTregs in the tumor microenvironment of LSCC.

Methods Immunohistochemical (IHC) analysis was conducted to examine the expression of AREG and FOXP3, and their association with clinical parameters and patient outcomes was demonstrated. The expression level of EGFRs in three functional subsets of Tregs was assessed, and the induction of $CD4^+$ T cells into aTregs in the presence or absence of AREG or Gefitinib was analyzed using flow cytometry.

Results Our results showed a higher expression level of AREG was significantly related to advanced clinical stage and worse survival, particularly with increased infiltration of Tregs in LSCC tumor tissue. The in vitro study showed that AREG significantly promoted the differentiation of aTregs, and enhanced the inhibitory effect of Tregs on T cell proliferation, which could be reversed by epidermal growth factor receptor (EGFR) inhibitors. In addition, we found that EGFR was highly expressed in aTregs, but not in other subsets of Tregs. It is suggested that AREG might induce aTregs, and enhance the immunosuppressive function of Tregs via the AREG/EGFR signal pathway.

Conclusions Collectively, this study revealed the role and mechanism of AREG in negative immune regulation, and targeting AREG might be a novel immunotherapy for LSCC.

Keywords Amphiregulin, Epidermal growth factor receptor, Laryngeal squamous cell carcinoma, Immunosuppressive microenvironment, Regulatory T cells

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Background

Laryngeal squamous cell carcinoma (LSCC), is the most common malignant tumor of the upper respiratory tract, accounting for 25-30% of head and neck squamous cell carcinoma (HNSCC) [1]. And the LSCC is the most common pathological type of laryngeal carcinoma, which is mainly male [2]. From 1990 to 2017, the number of cases and deaths from LSCC increased by 58.7% and 33.9%, respectively, globally [3]. There are about 185,000 patients with LSCC in the world in 2020, of which 99,000 patients with LSCC died [4]. Despite the continuous development of surgery, radiotherapy and chemotherapy, laryngeal cancer still has a high recurrence rate and metastasis rate, and the five-year survival rate of patients has not been significantly improved [5]. Immunotherapy activates the patient's own immune system to combat tumor cells, and its development has improved the prognosis for many different malignancies [6, 7]. However, despite the effectiveness of immunotherapy in certain patients, there is a limited overall response rate of LSCC to immunotherapy that might be in part attributed to immunosuppressive factors present in the tumor microenvironment (TME) of LSCC [8, 9]. Therefore, understanding the impact of the immune suppressive microenvironment in LSCC may help to develop novel treatments and improve treatment outcomes.

The tumor immune suppressive microenvironment refers to a number of immune inhibitory cells and molecules surrounding the tumor that can suppress the function of immune cells, thus reducing the effectiveness of immune therapy. It has been reported that the strong immunosuppressive properties in the TME of LSCC has a marked influence on the progression, metastasis, and immunotherapeutic resistance [10]. Regulatory T cells (Tregs) can be divided into 3 functionally distinct subsets, of which activated Tregs (aTregs; $FOXP3^{hi}CD45RA^{-}CD4^{+}$) is a subset of Tregs with immunosuppressive functions [11]. Our previous studies demonstrated that aTregs infiltrate into tumor margins and stroma, and the infiltration is closely related to a poor prognosis of LSCC [12, 13]. However, it is unclear what factors promote the infiltration of aTregs in LSCC, thus promoting an immunosuppressive TME.

Amphiregulin (AREG), which is secreted by tumor cells, T cells, mast cells, and type 2 innate lymphoid cells (ILC2) [10, 14], plays a role as a growth factor in the promotion of epithelial cell growth, inflammation resolution, and tissue regeneration through its interaction with epidermal growth factor receptors (EGFRs) [9]. Recent studies have provided evidence that AREG plays an important role in Treg-mediated immune regulation, and its overexpression is strongly associated with decreased survival [15]. Moreover, it has been shown that tumor cells can secret a large amount of AREG, which subsequently enhances the immunosuppressive function of Tregs via EGFRs [16]. Study has also indicated that AREG may play a role in promoting the differentiation of Tregs in the TME [17]. To the best of our knowledge, the regulatory role of AREG on aTregs has not been reported and remains unclear.

In the present study, we first evaluated the role of AREG in the clinical staging and prognosis of patients with LSCC. Then, we compared the expression levels of EGFRs in functionally distinct subsets of Tregs, and EGFRs were found to be significantly up-regulated in aTregs, but not in resting Tregs (rTregs) or non-Tregs (nTregs). Finally, we evaluated the effect of AREG up-regulation or EGFR kinase activity inhibition on the differentiation and function of aTregs in vitro. Our results revealed the role and mechanism of AREG in negative immune regulation, and targeting AREG may be a novel method for altering the immune suppressive microenvironment of LSCC.

Table 1 Clinicopathological characteristics of LSCC patients

Characteristic		No. cases	%
Total		68	100
Gender	Male	64	94.1
	Female	4	5.9
Age(year)	< 60	32	47.1
Tumor Differentiation	≥ 60	36	52.9
	High	26	38.2
	Moderate	22	32.4
	Low	20	29.4
Stage (AJCC)	I-II	47	69.1
	III- IV	21	30.9
Tumor status	T_{1-2}	49	72.1
	T_{3-4}	19	27.9
Lymph node metastases	N_0	57	83.8
	N_1	11	16.2
Distant metastases	M_0	68	100.0
	M_1	0	0.0

LSCC, laryngeal squamous cell carcinoma; AJCC, American joint cancer staging committee.

Materials and methods

Patients sample and clinical information

Formalin-fixed, paraffin-embedded tumor samples were collected from 68 laryngeal squamous cell carcinoma (LSCC) patients who were diagnosed at the First Affiliated Hospital of Sun Yat-sen University. The associated clinical and pathological parameters including age, sex, tumor stage (AJCC), and tumor node metastasis (TNM) stage are shown in Table 1.

Immunohistochemical analysis

Detection of AREG (1:50 dilution, R&D Systems, Minneapolis, MN, USA), FOXP3 (1:50 dilution, Abcam, Cambridge, MA, USA) was performed on 4 μm-thick,

paraffin-embedded sections of tissue by immunohistochemical (IHC) analysis. The densities of AREG and FOXP3 positive cells were scored by 2 independent pathologists who were blind to patient clinical data. The IHC score of AREG was graded as [18]: negative (score 0), weakly positive (score 1), moderately positive (score 2), and strongly positive (score 3). Patients were then divided into low AREG expression (score 0 or 1) and high AREG expression (score 2 or 3) groups.

Expression and prognostic analysis of AREG in HNSCC

The processed RNA-Seq FPKM data and corresponding patient survival information for 502 HNSCC tissues and 44 adjacent normal tissues were acquired from The Cancer Genome Atlas (TCGA) data portal (https://portal.gdc.cancer.gov/cart). The expression of AREG in HNSCC tissues and normal tissues was compared, and the overall survival (OS) and disease-free survival (DFS) in patients with high and low AREG expression were determined using the survival and glmnet R packages.

Cell culture

The LSCC cell line SNU46 was purchased from Professor Ja-Lok Ku (Seoul National University College of Medicine), and cells were cultured under standard conditions. The tumor culture supernatants (TSNs) were prepared as described previously [19]. Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated by Ficoll density gradient centrifugation (MP Biomedicals, Santa Ana, USA). $CD4^+$ T cells were isolated from PBMCs using a MACS CD4 T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), and were cultured in 96-well plates with RPMI medium in the presence of 10% fetal calf serum (Gibco) and 1% penicillin/streptomycin (Gibco).

Induction of aTregs by AREG in vitro.

To clarify the induction effect of AREG on aTregs, $CD4^+$ T cells were seeded at 2.5×10^5 cells per well in round-bottomed 96-well plates and they were co-cultured the in the presence or absence of 100 ng/ml recombinant amphiregulin (R&D Systems) or 200 ng/ml gefitinib (AstraZeneca, London, UK) with 20% TSNs for 48 h. Then proportions of aTregs induced were analyzed.

The $CD4^+CD45RA^-FOXP3^{hi}$ cells were used to label the aTregs; $CD4^+CD45RA^+FOXP3^{low}$ cells were used to label rTregs; and $CD4^+D45RA^-FOXP3^{lo}$ cells were used to label nTregs [20]. The antibodies used were anti-hCD4-FITC, anti-hCD45RA-eFluor 450, and anti-hFOXP3-PE (eBioscience, San Diego, CA, USA), and were analyzed using CytoFLEX S (Beckman Coulter, California, USA).

T-cell proliferation by CFSE

The Tregs were sorted from PBMCs by $CD4^+CD25^{hi}CD127^{lo}$ T cell loaded magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). For suppression assays, sorted Tregs were co-cultured with autologous $CD4^+$ T cells labeled with 2 μM carboxyfluorescein succinimidyl amino ester (CFSE) at a ratio of 1:2 in the presence of anti-CD3 (OKT3, 5 μg/ml, eBioscience) and anti-CD28(CD28.2, 2 μg/ml, eBioscience) in 96-well round-bottom plates. Subsequently, amphiregulin (100 ng/ml, R&D Systems) with or without gefitinib (200 ng/ml, AstraZeneca, London, UK) was added to the co-cultured systems. After 3 days, flow cytometry was used to measure the proliferation of $CD4^+$ T cells. Proliferation was defined as the percentage of cells which had undergone at least one division.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6 software (San Diego, USA), and R software (version 3.6.2). The means of 2 groups were compared with the 2-tailed Mann-Whitney test and Student's *t*-test. Multiple comparisons were performed with the Kruskal-Wallis test. To determine the role of AREG in Treg subset proportions, the Friedman test followed by Dunn's multiple comparisons test was used to avoid individual differences. The OS was defined as the period from surgery to death. The Kaplan-Meier method and log-rank test were used for analyzing survival data. Data were represented as means ± standard error of the mean (SEM). Statistical significance was accepted with $p<0.05$.

Results

Upregulation of AREG is associated with worse prognosis and more Treg production

To determine the correlation between AREG expression and clinical outcomes, we analyzed the AREG expression in LSCC tumor tissue ($n=68$) using the IHC. The results showed that higher expression of AREG was associated with a more advanced clinical stage (Fig. 1A-D, $p<0.05$), but no significant association with lymph node metastasis ($p=0.1862$) (Fig. 1E). The Kaplan–Meier analysis revealed that patients with higher AREG expression had a significantly shorter OS compared to those with lower AREG expression (Fig. 1F, $p=0.0046$). To further explore the role of AREG in HNSCC, we conducted an additional Kaplan–Meier analysis using TCGA data. The results were consistent, showing a similar conclusion (Supplementary Fig. 1), which underscores the significant role of AREG in HNSCC .

We further analyzed the expression of AREG and FOXP3 (the marker gene of Tregs) in LSCC, and the results showed that there were more Treg cells infiltration in high AREG expression specimens than those in low AREG expression (Fig. 1G, $p=0.0483$). These results suggested that the up-regulation of AREG in LSCC might contribute to the immunosuppressive microenvironment

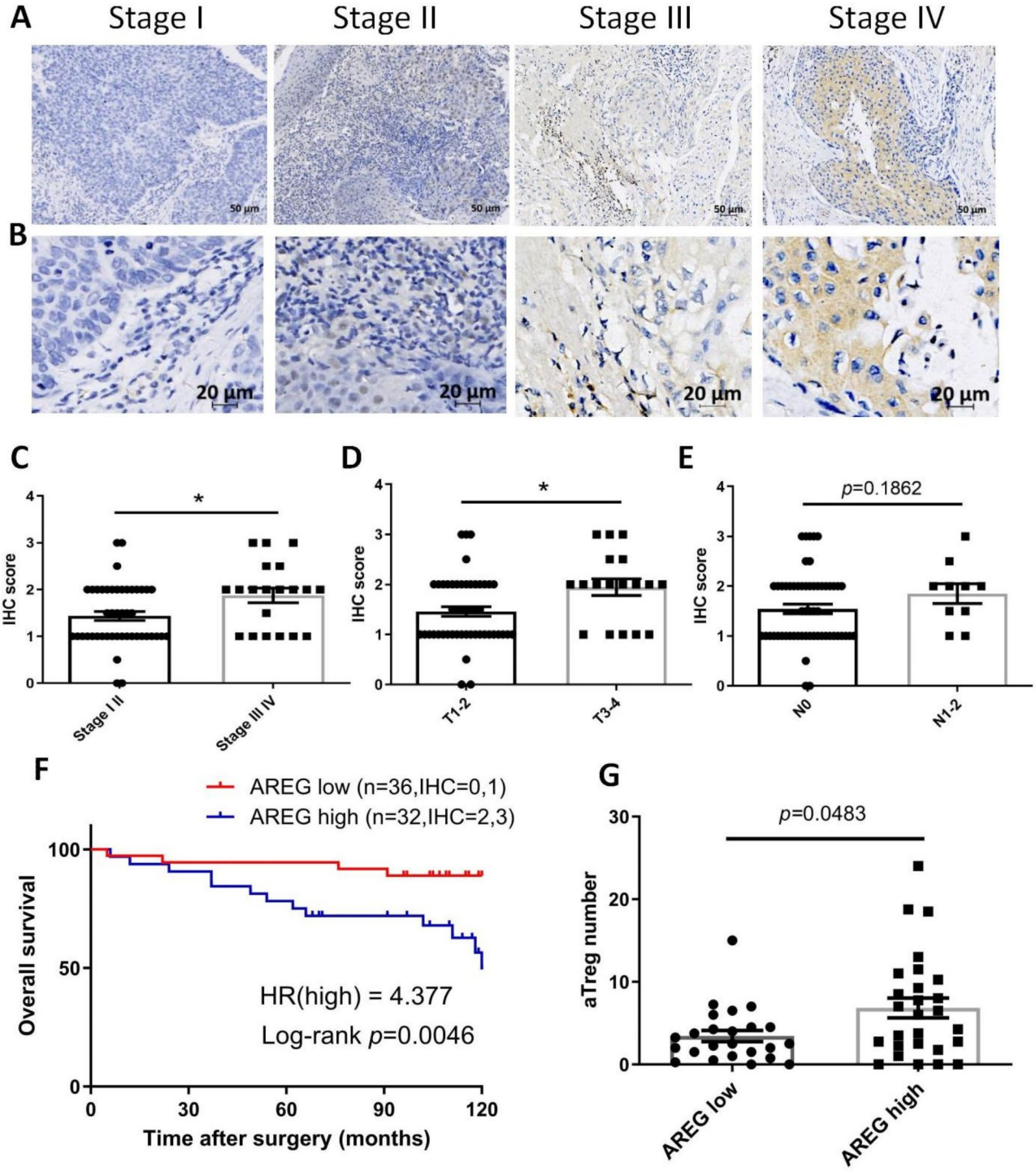

Fig. 1 The expression of AREG in LSCC specimens is correlated with clinical staging and patient overall survival. (**A, B**) Representative IHC images of AREG expression in paraffin-embedded LSCC tumor sections ($n=68$). Scale bar: 50 μm in Fig. 1A, 20 μm in Fig. 1B. (**C-E**) IHC score was higher in advanced T stages (T3 and T4) and advanced clinical stages (III and IV) than in early T stages (T1 and T2) and early clinical stages (I and II). (**F**) Accumulation of AREG in the tumor microenvironment predicted poor survival of patients with LSCC (Kaplan-Meier method and log-rank test). Low AREG expression ($n=36$) and high AREG expression ($n=32$) groups are represented by red and blue lines, respectively. **G**) Higher Treg infiltration was founded in high AREG expression specimens than those in low AREG expression ($n=51$). Data presented as mean ± SEM. *$p<0.05$

by regulation of Tregs, and lead to a poor prognosis of patients with LSCC.

AREG up-regulates the proportion of aTregs through EGFRs

To determine effect of AREG on Treg differentiation, we co-cultured $CD4^+$ T cells in the presence or absence of AREG or gefitinib. The results showed that AREG significantly up-regulated the proportion of aTregs in $CD4^+$ T cells, and this effect was reversed by gefitinib (an EGFR specific blocker) (Fig. 2, $p<0.01$).

We further examined the expression of EGFRs in different functional subsets of Tregs by flow cytometry analysis. The analysis showed that EGFRs were significantly overexpressed in aTregs, comparing to rTreg or nTreg subsets (91.32% vs. 5.08% vs. 4.58%, Fig. 3).

AREG enhances the immunosuppressive effect of Tregs

To further investigate the effect of AREG on the immunosuppressive function of aTregs, we developed a co-cultured system of Tregs cultured with AREG and/or gefitinib and autologous CFSE-labeled $CD4^+$ T cells. The gate strategy of Flow Cytometry was showed in Supplementary Fig. 2. It was indicated that the $CD4^+$ T cell proliferation was significantly inhibited in the AREG treatment group, compared to the control group or the group treated with both the AREG and gefitinib (Fig. 4, $p<0.05$).

In conclusion, our study indicates that AREG might promote the production of aTregs and enhance the immunosuppressive function of Tregs by up-regulating EGFR expression, which contributes to tumor immune escape.

Discussion

The TME consists of mesenchymal cells and recruited immune cells, which exert immunosuppressive and tumor-promoting effects [21]. An increasing body of research has revealed that Tregs play an important role as immunosuppressive cells that accumulate within the TME, and contribute to the evasion of tumors by inducing T-cell anergy and immunosuppression during tumor development [22, 23]. Furthermore, studies have reported that selectively eliminating or reducing the infiltration of Tregs can activate tumor-specific effector T cells and enhance the effectiveness of immunotherapy [24, 25]. In our previous study, aTregs, a functionally heterogeneous subset of Tregs, were found to be the dominant immunosuppressive functional subset of Tregs in the TME of LSCC, and had a role in tumor immune escape in LSCC [12]. Although the pivotal role of Tregs in the TME has been widely discussed [26, 27], the initial factors regulating the generation of aTregs are still unclear and have become an increasing focus of research.

Cetuximab, an EGFR monoclonal antibody, is recommended for the treatment of patients with advanced stage HNSCC and those with distant metastases [28–30]. Kogashiwa et al. showed that patients with recurrent/metastatic HNSCC and higher expression of AREG appeared to have longer survival from treatment with cetuximab [31]. However, the mechanism has not been clarified. AREG is a member of the epidermal growth factor (EGF) family, while EGFR is a receptor protein related to the EGF family. AREG can bind to EGFRs as a ligand, and activate the EGFR signaling pathway during tumor growth and progression [32]. In this study, we found that up-regulation of AREG in LSCC tissue was closely related to an advanced stage and poor prognosis of LSCC, which

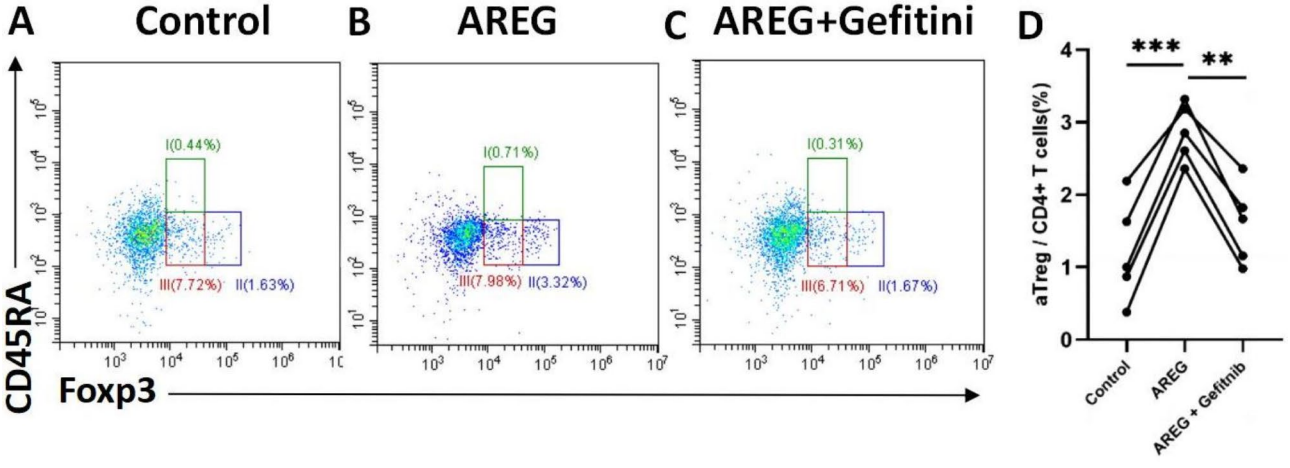

Fig. 2 AREG upregulates the proportion of aTregs through EGFR. $CD4^+$ T cells were isolated from PBMCs and cultured in the presence or absence of 100 ng/ml recombinant AREG or 200 ng/ml Gefitinib for 48 h and flow cytometry analysis were used to identify the subsets of Tregs. Generation proportion of Tregs subsets in different treatment groups control (**A**), AREG (**B**), AREG + Gefitinib (**C**) and the results were analyzed statistically (D). rTregs (I Tregs), aTregs (II Tregs), and nTregs (III Tregs); $N=5$, $^{**}p<0.01$, $^{***}p<0.001$

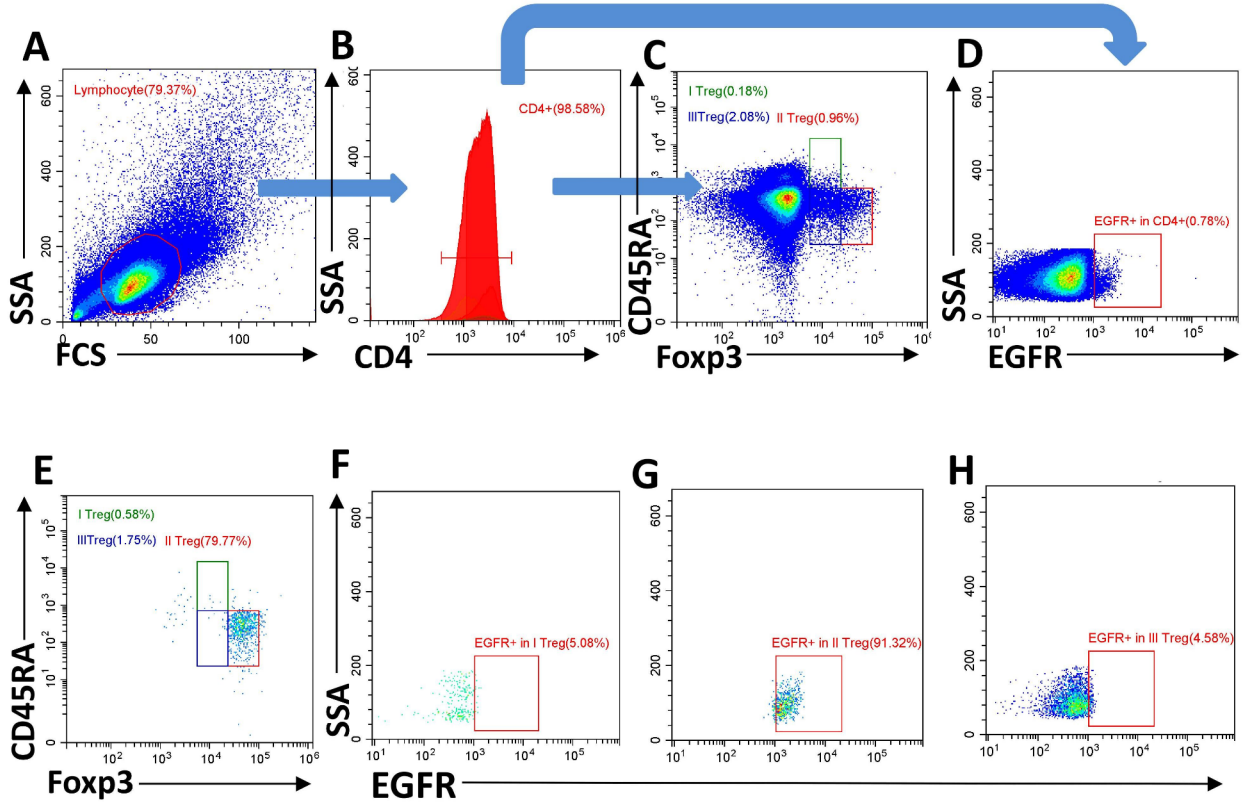

Fig. 3 EGFR was highly expressed in aTregs. The expression level of EGFR in three functional subsets of Tregs was detected by using the flow cytometry analysis. (**A-D**) Gating Strategy was shown. (**E**) The proportion of three functional subsets of Tregs in $EGFR^{+}CD4^{+}$T cells. (**F-H**) The expression level of EGFR was detected on rTregs (I Tregs), aTregs (II Tregs) an nTregs (III Tregs) respectively. All experiments were repeated three times

indicates that AREG could be involved in the occurrence and development of LSCC. Furthermore, we also found that higher expression of AREG was associated greater infiltration of Tregs in the TME of LSCC. This suggests that AREG might be involved in the regulation of Tregs.

Studies have demonstrated that the expression of AREG in the TME an increase the expression of surface molecules and inhibitory cytokines of Tregs, such as EGFR and interleukin (IL)-10 [33]. Moreover, stimulation with AREG markedly enhances Treg function in vitro, which suggests that AREG is necessary for efficient Treg cell function [16]. Miyara et al. [11] reported that rTregs could swiftly convert to aTregs with specific stimulations in vivo, but the mechanism is not known. Our results showed that AREG cand induce the production of more aTregs, but the effect can be reversed by the EGFR specific inhibitor gefitinib.

Recent studies have revealed more than 90% of LSCC overexpress EGFRs [34]. In order to further explore the association between Tregs and EGFRs, we analyzed the expression level of EGFRs on different functional heterogeneous subsets of Tregs, and the results showed that EGFRs were mainly expressed on aTregs. This suggests that AREG might promote an increase of aTreg production by up-regulating EGFR expression in LSCC.

Studies have also revealed that the up-regulated AREG protein enhances the suppressive function of Tregs via the EGFR/GSK-3β/FOXP3 axis in vitro and in vivo [15]. Our results suggest that Tregs have a stronger immunosuppressive ability for T cell proliferation when treated with AREG in vitro. However, the addition of gefitinib significantly reduced the Treg-mediated immunosuppression which AREG enhanced. This indicates that inhibition of EGFR expression in Tregs can significantly reduce the effect of AREG on the immunosuppressive function of Tregs.

Conclusion

This study revealed that AREG has an important role in the differentiation of aTregs by activating AREG/EGFR signaling pathway, which enhances the immunosuppressive microenvironment in LSCC. This may be a newly identified mechanism of tumor immune escape. Targeting AREG may be a novel strategy to reverse the aTreg-mediated immunosuppressive effect, and thus increase the effectiveness of immunotherapy.

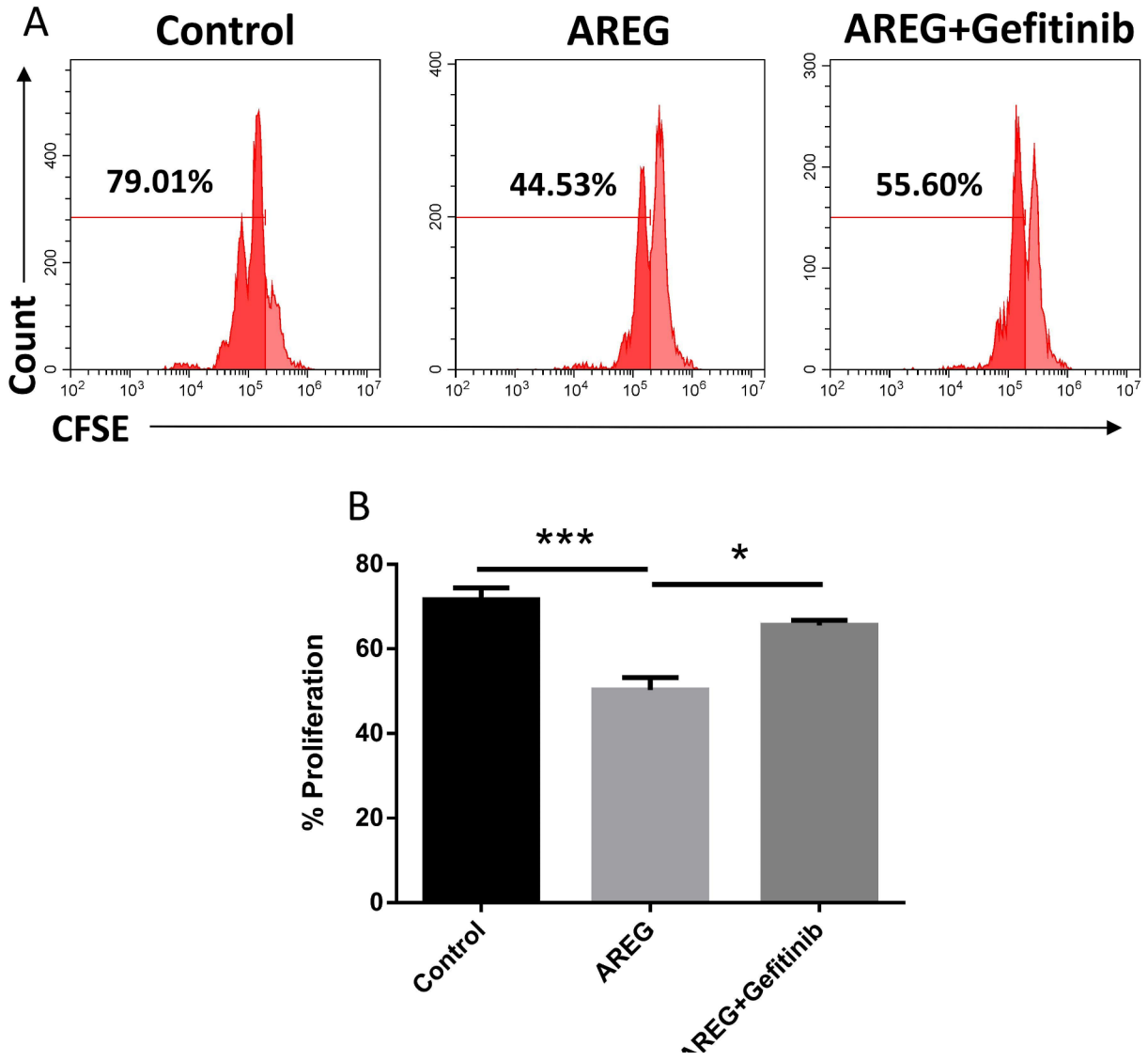

Fig. 4 AREG enhances the immunosuppressive effect of Tregs. $CD4^{+}CD25^{hi}CD127^{lo}$ T cells were sorted by magnetic cell separation from PBMCs and were cocultured with $CD4^{+}$ T cells labeled with CFSE for 3 days in the presence or absence of 100 ng/ml recombinant AREG or 200 ng/ml Gefitinib. Proliferation of $CD4^{+}$ T cells was determined in different treatment groups control by CFSE analysis (**A**) and the results were analyzed statistically (**B**). *$p < 0.05$, ***$p < 0.001$

Abbreviations

aTregs	Activated regulatory T cells
LSCC	Laryngeal squamous cell carcinoma
HNSCC	Head and neck squamous cell carcinoma
AREG	Amphiregulin
EGFR	Epidermal growth factor receptor
TME	Tumor microenvironment
Tregs	Regulatory T cells
ILC2	Type 2 innate lymphoid cells
rTregs	Resting Tregs
nTregs	Non-Tregs
TNM	Tumor node metastasis
IHC	Immunohistochemical
TCGA	The Cancer Genome Atlas
OS	Overall survival
DFS	Disease-free survival
PBMCs	Peripheral blood mononuclear cells
CFSE	Carboxyfluorescein succinimidyl amino ester
SEM	Standard error of the mean
EGF	Epidermal growth factor

Supplementary Information

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Supplementary Material 1

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Author contributions
Hang Li: Conception and design of the study, and drafting the article. Ruihua Fang: Analysis and interpretation of data. Yihui Wen: Conceptualization, review & editing, Funding acquisition and final approval of the version to be submitted. Renqiang Ma: Review & editing, Supervision, Project administrationa and Funding acquisition. Yudong Long: Methodology, Formal analysis. Rui He: Methodology, Validation. Huanhuan Lyu: Methodology, Formal analysis. Lin Chen: Immunohistochemistry and CFSE analysis.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participants
The study was conducted in accordance with ethical guidelines and was approved by the ethics committee of the First Affiliated Hospital of Sun Yat-sen University (Approval Number: SYSU-IACUC-[2020]165). All patients were exempt from informed consent. This study was performed in accordance with the Declaration of Helsinki.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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