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Specific blockade CD73 alters the 'exhausted' phenotype of T cells in head and neck squamous cell carcinoma

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Abstract

The adenosine-induced immunosuppression hampers the immune response toward tumor cells and facilitates the tumor cells to evade immunosurveillance. CD73, an ecto-5-nucleotidase, is the ectoenzyme dephosphorylating extracellular AMP to adenosine. Here, using immunocompetent transgenic head and neck squamous cell carcinoma (HNSCC) mouse model, immune profiling showed high expression of CD73 on $CD4^+$ and $CD8^+$ T cells was associated with an 'exhausted' phenotype. Further, treatment with anti-CD73 monoclonal antibody (mAb) significantly blunted the tumor growth in the mouse model, and the blockade of CD73 reversed the 'exhausted' phenotype of $CD4^+$ and $CD8^+$ T cells through downregulation of total expression of PD-1 and CTLA-4 on T cells. Whereas the population of $CD4^+CD73^{hi}$/$CD8^+CD73^{hi}$ T cells expressed higher CTLA-4 and PD-1 as compared to untreated controls. In addition, the human tissue microarrays showed the expression of CD73 is upregulated on tumor infiltrating immune cells in patients with primary HNSCC. Moreover, CD73 expression is an independent prognostic factor for poor outcome in our cohort of HNSCC patients. Altogether, these findings highlight the immunoregulatory role of CD73 in the development of HNSCC and we propose that CD73 may prove to be a promising immunotherapeutic target for the treatment of HNSCC.

Keywords

CD73; T cell; head and neck squamous cell carcinoma; programmed cell death protein 1; cytotoxic T-lymphocyte-associated protein 4

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is a highly immunosuppressive disease.[1] The immunosuppression, which is induced by the exhaustion of effector cells, production of immunosuppressive mediators and recruitment of immunosuppressive cells, facilitates HNSCC cells to escape from immunosurveillance and prevents tumor elimination via multiple mechanisms.[2–4] The immunosuppressive status of HNSCC raises the possibility that immunotherapy could be an effective approach to HNSCC treatment.[5] Recently, the immunotherapy has revolutionized the treatment of cancers. Therapeutics that target inhibitory immune checkpoints, such as programmed cell death protein 1(PD-1), have generated durable and effective anti-tumor responses in cancer patients, including HNSCC.[6] However, some patients are non-responsive to this kind of immunotherapy, indicating that alternative immunosuppressive mechanisms must be studied.[7] Thus, a better understanding of the various aspects of tumor-induced immunosuppression would benefit the exploration and development of novel immunotherapeutic strategies.

Adenosine is one of the immunosuppressive mediators involved in tumor progression.[8, 9] Adenosine plays a crucial role in the hypoxia-driven immunosuppression and, thereby hampers the immune response towards tumor cells in various cancer.[10] CD73, a GPI-anchored enzyme, is a crucial enzyme in conversion of immune-activating ATP into immunosuppressive adenosine.[11, 12] Current studies demonstrate that CD73 is widely expressed on tumor cells in several types of cancers, including HNSCC cells, and the expression of CD73 confers poor prognosis.[13, 14] Notably, CD73 on regulatory T cells (Tregs) impairs anti-tumor T cell responses.[15, 16] Therapeutics that target CD73 alleviates suppression of the adaptive immune system, thereby enhancing anti-tumor responses.[17, 18] Nevertheless, whether CD73 blockade reverses the immunosuppression and constitutes an effective therapeutic target in HNSCC remains to be shown.

Inhibitory checkpoints contribute to exhaustion of T cells and serve as the markers for dysfunctional lymphocytes.[19] Recent studies indicated anti-CD73 treatment may synergize with inhibitory checkpoint blockade in cancer immunotherapy.[20] However, the underlying mechanism is not investigated. A recent report pointed out CD73 expression on $CD8^+$ T cells is inversely associated with cell activation in HIV patients.[21] Therefore, detailed studies on the relationship between CD73 and inhibitory checkpoint on $CD8^+$ T cells will be beneficial to the understanding of the immunosuppression in HNSCC.

In the present study, we utilized immunocompetent transgenic mice model to determine the expression of CD73 on immune cells. We also carried out *in vivo* studies to investigate the immunomodulatory effects on T cells and therapeutic potentials of the blockade of CD73 with monoclonal antibody (mAb). At last, the human tissue microarrays were used to analyze the expression of CD73 and its relationship with clinic-pathological parameters.

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Materials and Methods

Mice

The *Tgfbr1*/*Pten* 2cKO mice was described as previously.[22, 23] In briefly, the mice (*Tgfbr1*$^{flox/flox}$; *Pten*$^{flox/flox}$; *K14-CreER*$^{tam+/-}$) was generated from crosses between *Tgfbr1* cKO mice (*K14-CreER*^{tam}; *Tgfbr1*$^{flox/flox}$) and *Pten*$^{flox/flox}$ mice. Deletion of *Tgfbr1* and *Pten* in head and neck epithelia induces tumor lesion, after tamoxifen was applied in the oral cavity for 5 consecutive days. The mice were genotyped as previously reported.[22] The mice were housed and maintained under SPF conditions in the School and Hospital of Stomatology at Wuhan University. All animal experiments were approved and supervised by the Institutional Animal Care and Use Committee of Wuhan University. Mice were handled under the NIH guidelines for the Care and Use of Laboratory Animals.

In vivo CD73 antibody treatment

The *Tgfbr1/Pten* 2cKO mice were randomized into two groups (6 in each group) after tamoxifen induction. Then, the mice received treatment with isotype control (control group, rat IgG2a, Clone:2A3, 100μg/mouse; BioXcell) or anti-CD73 mAb (aCD73 group, *In Vivo* CD73 monoclonal antibody, Clone:TY/23, BioXcell) by intraperitoneally injection (twice per week). Throughout the study, the tumor size was measured with a caliper every 5 day. The photos were taken at day 7 and day 25. The treatment was stopped at 4 weeks after the initial treatment. Two days later, the mice were euthanized and the tissues were harvested for the flow cytometry analysis.

Flow cytometry

Single-cell suspensions from spleen, draining lymph nodes and peripheral blood were prepared as previously described.[24] After non-specific Fc-receptor blocking, the cells were stained with the antibodies against: CD4 (RM4–5), CD8 (53–6.7), CD73 (TY/11.8), PD-1 (J43), IFN-γ (XMG1.2)(eBioscience) and CTLA-4 (UC10–4B9, BioLegend). The staining of IFN-γ and Foxp3 was performed according to the Staining Intracellular Antigens for Flow Cytometry Protocol (eBioscience). Respective isotype was set. Dead cells were excluded by 7-AAD staining. Samples were analyzed on a CytoFLEX flow Cytometer (Beckman Coulter) and analyzed by CytExpert (Beckman Coulter).

Human HNSCC tissue samples

Human HNSCC specimens were obtained from patients at Department of Oral and Maxillofacial Surgery (School and Hospital of Stomatology, Wuhan University). In the tissue microarrays, the tissue samples contain 22 normal mucosae and 134 primary HNSCC. Informed consents were obtained from all patients. The study was approved by the School and Hospital of Stomatology of Wuhan University Medical Ethics Committee. The clinical stages were assessed according to the International Union Against Cancer (UICC 2010), and histological grading was confirmed by two pathologists independently.

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Immunohistochemistry

The immunohistochemistry protocol was described as previously.[25] In brief, the slides were deparaffinized and rehydrated. The antigens were retrieved by boiling in sodium citrate. Sections were blocked with goat serum, then stained with CD73 (Cell Signaling Technology). The procedure was done with the DAB kit (Mxb Bio). Negative control was set in parallel.

Scoring System

Pannoramic MIDI (3DHISTECH Ltd.) scanner was exploited for scanning all slices and quantifying the histoscore of each sample with background subtraction as previously described.[22] Immunohistochemical staining of the membrane, nuclei and cytoplasm in the interested region was quantified respectively by the Quant Center module of Pannoramic Viewer software (3DHISTECH Ltd.). Histoscore was calculated as previously described.[22] Briefly, scanned high power field of each sample, membranous and nuclear immunostaining was quantified using the algorithm: (1 × the percentage of weakly positive staining) + (2 × the percentage of moderately positive staining) + (3 × the percentage of strongly positive staining).[26, 27] Quantification of cytoplasmic intensity was calculated as total intensity/total cell number. The threshold for scanning of different positive cells was set by a pathologist. To evaluate the expression of CD73 on tumor infiltrating lymphocytes, the histoscore of CD73 was determined by the calculated method of membranous staining.

Data bases

We downloaded mRNA expression data of head and neck squamous carcinomas (HNSC) from the Cancer Genome Atlas (TCGA).[28] The data on TCGA were stored in four levels and we adopted level 3. The HNSC dataset contains mRNA expression levels of 520 samples from tumor patients and 44 from normal controls. R package TCGAbiolinks was used to download and parse data from TCGA database and to create expression matrices as previously described.[29] TCGAbiolinks was also used to perform univariate Kaplan-Meier survival analysis on the downloaded data.

Gene Set Enrichment Analysis

Gene set enrichment analysis was performed on the GSEA software (Broad Institute, Massachusetts Institute of Technology) as described in the previous article.[30] Gene sets were downloaded from Molecular Signatures Database (MSigDB; Broad Institute, Massachusetts Institute of Technology). All gene sets that met the false discovery rate (FDR) q-value ≤ 0.25 were considered significant as indicated in the previous article.[30]

Statistical Analysis

The statistical significance of the differences was determined by Student t test for comparison between groups and One-way ANOVO followed Tukey test for three groups. GraphPad Prism 6.0 (Graph Pad Software Inc) was used for the statistical analysis mentioned above. For univariate analysis of overall survival, Kaplan-Meier estimate and the log-rank test was used with GraphPad Prism 6.0. And for multivariate analyses of overall survival, Cox proportional hazards regression model was used with SPSS 22.0 (IBM). The

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data was displayed as mean ± SEM. For all tests, statistical differences were considered significant if $p < 0.05$.

Results

CD73 is upregulated on CD4$^+$ and CD8$^+$ T cells and associated with 'exhausted' phenotype in tumor-bearing mice

The immunocompetent mouse model is a powerful tool for cancer immunology. According to previous report, a *Tgfbr1/Pten* double conditional knockout (2cKO) HNSCC mouse model was generated (Fig. 1a). In this model, *Pten* knockout results in the activation of PI3K/Akt pathway, and the *Tgfbr1* deletion in the epithelia of head and neck enhanced paracrine effect of TGF-β and the recruitment of immunosuppressive cells in the tumor stroma.[23] This model was selected to study tumor immunity owing not only to the similarity of pathology with human HNSCC but also to the immunocompetence of the mouse model.[23, 24] Firstly, we utilized immunohistochemistry to test the expression of CD73, the result showed the CD73 expression was upregulated in tumor bearing mice compared with normal oral mucosa (Fig. 1b). Further, by comparing the expression of CD73 between wild type mice and 2cKO tumor bearing mice, we observed significantly increased CD73 expression on CD4$^+$ T cells (Supporting information 1a, Fig. 1c and 1d) and CD8$^+$ T cells (Fig. 1e) isolated from spleen, draining lymph node and peripheral blood. To characterize different subsets of CD4$^+$ and CD8$^+$ T cells based on CD73 expression, we examined the expression of CTLA-4 and PD-1 on CD4$^+$ and CD8$^+$ T cells (Fig. 2a and 2b). Results revealed that there was a relatively larger fraction of CTLA-4$^+$ population among CD4$^+$CD73^{hi} subset than CD4$^+$CD73^{lo} subset, whereas the CD4$^+$CD73^{neg} subset had the smallest fraction of CTLA-4-expressing cells (Fig. 2c). Likewise, similar results were obtained in CTLA-4$^+$ cells enriched in CD8$^+$CD73^{hi} subsets (Fig. 2d). PD-1 expressing cells followed a similar pattern with the CD4$^+$CD73^{hi}/CD8$^+$CD73^{hi} having the highest proportion of PD-1 and CD4$^+$CD73^{neg}/CD8$^+$CD73^{neg} having the lowest (Fig. 2e and 2f). Collectively, these results indicate that CD73 is relevant to the 'exhausted' phonotype of T cells in tumor-bearing mice.

Anti-CD73 mAb treatment blunted tumor growth in HNSCC mouse model

Given the high expression of CD73 in HNSCC mouse model, we speculated CD73 may be potential target for HSNCC treatment. In an effort to explore the effect of CD73 blockade on tumor progression, the *In Vivo* CD73 mAb (Clone:TY/23, BioXcell) was used. A schematic drug delivery strategy is shown in Fig. 3a. Intraperitoneally infusion of anti-CD73 mAb (TY/23,100μg/mouse) or rat IgG2a (Clone:2A3, 100μg/mouse) twice per week significantly delayed tumor growth ($p<0.05$ at day 20 and $p<0.001$ at day 25, Fig. 3b and 3c). These results suggest that the blockade of CD73 could delay the tumor growth in HNSCC mouse model and verify the feasibility of target CD73.

Targeting CD73 in tumor-bearing mice reversed 'exhausted' phenotype of CD4$^+$ and CD8$^+$ T cells

Based on the closely relationship between CD73 and 'exhausted' phenotype of T cells, we tested whether the blockade of CD73 has an effect on the effector function and

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phenotype of $CD4^+$ and $CD8^+$ T cells. Firstly, we validated the on-target effect of the CD73 mAb. As shown in Fig. 4a, a significant reduction of $CD73^+$ population on $CD4^+$ and $CD8^+$ T cells was observed after anti-CD73 mAb treatment. Then we analyzed the percentage of $CD4^+$ and $CD8^+$ T cells based on lymphocyte gate. There was a trend toward increased $CD4^+$ population and $CD8^+$ population, but the difference did not reach statistical significance except that $CD8^+$ population was significantly increased in lymph nodes (Fig. 4b and Supporting Information Fig.1b). To further test whether anti-CD73 mAb treatment restored cytokine production, we examined the production of IFN-γ in $CD4^+$/$CD8^+$ T cells after PMA stimulation *in vitro*. There is a significantly increased IFN-γ production in $CD4^+$/$CD8^+$ T cells after anti-CD73 mAb treatment (Fig. 4c). Remarkably, the expression of CTLA-4 and PD-1 in CD73 mAb treatment group exhibited a significantly decrease on $CD4^+$ and $CD8^+$ T cells as compared with isotype control group except that $CD4^+$ T cells did not express significantly less PD-1 in peripheral blood group (Fig. 4d and Supporting Information Fig.1c). We also demonstrated that CD73 is highly expressed on $CD4^+CD25^{hi}Foxp3^+$ Tregs in the tumor bearing mice (Supporting information Fig.2a). To investigate the impact of anti-CD73 mAb on Tregs, we measured the proportion of $CD25^{hi}Foxp3^+$ Tregs on $CD4^+$ T cells. As shown, unaltered Tregs proportion was observed (Supporting information Fig.2b). In addition, we also measured the expression of CD73 and CTLA-4 on Tregs. Indeed, we observed that both CD73 and CTLA-4 expression were significantly downregulated after anti-CD73 mAb treatment (Supporting information Fig.2c). All these data demonstrate that the blockade of CD73 in HNSCC in part alleviates suppression of T cells.

Anti-CD73 mAb treatment increased the frequency of $CD4^+CD73^{hi}$/$CD8^+CD73^{hi}$ T cells expressing CTLA-4 and PD-1

Because tumors use nonoverlapping mechanisms to escape immunosurveillance, single blockade of inhibitory checkpoint often fails to reach anticipated effects.[20] In this study, specific blockade of CD73 reduced the population of $CTLA\text{-}4^+CD4^+$, $CTLA\text{-}4^+CD8^+$, $PD\text{-}1^+CD4^+$ and $PD\text{-}1^+CD8^+$ as compared with isotype treated counterpart (Fig. 4d). Further analysis revealed the decrease in CTLA-4 and PD-1 in the subpopulation of $CD73^{neg}$ $CD4^+$ and $CD8^+$ T cells, but, surprisingly, the remaining $CD73^+$ ($CD73^{hi}$ and $CD73^{lo}$) $CD4^+$ and $CD8^+$ T cells expressed more CTLA-4 (Fig. 5a) and PD-1 (Fig. 5b) than those in isotype control group. This trend was even more obvious in $CD4^+CD73^{hi}$ and $CD8^+CD73^{hi}$ exhausted T cells characterized by high expression of PD-1 or CTLA-4 (Fig. 5a–5c). These results may indicate a CD73 resistant phenotype in $CD4^+$ or $CD8^+$ exhausted T cell during the blockade of CD73 and provide a rationale for the treatment with CD73 blockade in combination with PD-1 or CTLA-4.

CD73 is upregulated on the tumor infiltrating immune cells in primary human HNSCC

Due to the anti-tumor effect of CD73 blockade in HNSCC mouse model, we want to preliminarily explore the expression of CD73 in human HNSCC samples. To determine whether CD73 expression is associated with human HNSCC, we took advantage of publicly available datasets through Oncomine.[31] *NT5E* (encoding CD73) mRNA or DNA copy number were found to be significantly increased in 8 out of 17 datasets at a certain cut-off ($p<0.05$, fold change>1.5, Supporting Information Fig. 3a). The mRNA level of

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NT5E was significantly higher in HNSCC samples as compared with oral mucosa (All $p < 0.05$, Supporting Information Fig. 3b and 3c). Since elevated CD73 gene expression has been observed, we wished to investigate the protein expression of CD73 in human HNSCC specimen. Immunohistochemical study for CD73 were performed, and automatic membrane staining quantification software was used to assess CD73 expression in the tumor infiltrating immune cells of human HNSCC. As indicated in Fig.6a and 6b, the results showed expression level of CD73 was significantly higher in HNSCC (n=134) as compared with normal mucosa (n=22, $p<0.05$). Then, we assessed the relationships between CD73 expression and various clinicopathological features. However, there were no significant differences in CD73 histoscores in different pathological grades, tumor sizes and lymph node status (Fig. 6c). In addition, no significant correlation was observed between the expression of CD73 and gender, age, cigarette smoking or alcohol consumption in primary HNSCC (Supporting Information Fig. 4). Our data thus suggest that primary human HNSCC exhibited elevated CD73 expression.

CD73 is correlated with poor prognosis and negative regulation of immunity

A total of 69.4% (93/134) of HNSCC specimens were positive for CD73 in the tumor infiltrating immune cells. To investigate whether the positive expression of CD73 in the tumor infiltrating immune cells influenced overall survival rate, we divided the specimens into two groups ($CD73^+$ group and $CD73^-$ group) and evaluated the difference of overall survival rate. Univariate analysis with Kaplan-Meier estimates revealed the specimens with CD73 positive staining were more likely to have an unfavorable survival ($p = 0.0027$, Fig. 6d). Consistent with this result, CD73 positive expression was found to be an independent prognostic factor in multivariate analysis with Cox hazard regression model ($p = 0.018$, Supporting Information Table 1). Taking advantage of the cancer genome atlas (TCGA) HNSC dataset, we performed survival analysis and Gene Set Enrichment Analysis (GSEA) enrichment between two groups based on *NT5E* mRNA expression level (Supporting Information Fig. 5 and supporting information Table 2. *NT5E* top 25% vs bottom 25%). Survival analysis showed $NT5E^{hi}$ group had significantly poorer prognosis than $NT5E^{lo}$ group ($p = 1.2\times10^{-3}$, Fig. 6e). And the cutoff we selected in Kaplan Meier survival plots (*NT5E* top 25% vs bottom 25%) was based on the best separation of survival curves of the high/low expression groups. Overall Survival of HNSCC patients, dichotomized according to the mean and median of NT5E mRNA level, still separated, with significance values of 0.0099 and 0.0043 respectively (Supporting Information Fig. 6). GSEA revealed that processes which negatively regulated immune effector were more active in $NT5E^{hi}$ group (q-value = 0.05, NES = 1.55, Fig. 6f). Thus, the CD73 expression has a clinical significance as a poor prognostic factor and these results raised the possibility that CD73 may have negative immunoregulatory role in HNSCC. Taken together, the results above prompted CD73 may be a potential target in human HNSCC and the efficiency of anti-CD73 in human HNSCC should be investigated in the future.

Discussion

Exhausted T cells express multiple inhibitory receptors, such as PD-1 and CTLA-4, and lose effector functions.[19] Using flow cytometry, we demonstrated the expression of

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PD-1 and CTLA-4 was significantly increased $CD73^+CD4^+$ and $CD73^+CD8^+$ T cells in the tumor-bearing mice, which indicated these $CD73^+$ population cell may represent an immunosuppressive status of HNSCC. In agreement with our observations, a previous research reported that $CD4^+CD73^+$ population downregulated T-cell activation.[32] As the upstream component, CD73 works with A2AR to exert immunosuppressive function. A2AR activation suppress IFN-γ production but not T cell proliferation and this effect would last even after the removal of A2AR agonist.[33] In consistent with that report, our results show CD73 blockade increase IFN-γ and decrease CTLA-4 as well as PD-1 expression in $CD4^+$ and $CD8^+$ T cells, which indicated that the T cell effector function was reinvigorated. Although the expression of CD73 is highly expressed on Tregs, Tregs is only a small part of $CD4^+$ T cells. Thus, the increased frequency of $CD73^+$ T cells may largely represent an upregulation of CD73 on $CD4^+$ and $CD8^+$ T cells in the tumor bearing mice. And the decrease of CTLA-4 and PD-1 is in large part determined by T cells, especially for $CD8^+$ T cells. We have observed the co-occurrence of CD73 and immune-inhibitory molecules in $CD4^+$ and $CD8^+$ T cells and that CD73 inhibition lead to some degree of decrease of CTLA4 and PD-1. Therefore, we propose that high CD73 expression is a marker of exhausted T cells. Similarity, CD39 was defined as a marker of $CD8^+$ T cell exhaustion in a recent research.[34]

Nevertheless, the populations of $CD4^+$ and $CD8^+$ T cells remain unchanged. Interestingly, the remaining $CD73^+$ population express even higher levels of PD-1 and CTLA-4. This result, to some extent, supports that targeting CD73 enhances the therapeutic efficiency of PD-1 or CTLA-4 blockade.[20] Thus, we hypothesize that anti-CD73 mAb treatment will reverse a large part of 'exhausted' $CD4^+$ and $CD8^+$ T cells to immune-competent, however, the remaining $CD73^+CD4^+$ and $CD73^+CD8^+$ T cells, especially the $CD73^{hi}$ subset, gains more inhibitory markers and becomes even more dysfunctional. Thus, this subset of $CD73^{hi}$ T cells may represent a resistance to anti-CD73 mAb treatment.

Ectopic over-expression of CD73 involved in tumor progression is frequently observed.[16, 36] In the present study, using immunohistochemistry we identify that CD73 is upregulated in the tumor infiltrating immune cells of HNSCC samples compared with normal mucosa. Increased expression of CD73 on tumor cells has been observed, and is associated with poor prognosis in human HNSCC.[13] In our study, we found CD73 is also highly expressed on the tumor infiltrating immune cells in human HNSCC samples, and confers poor prognosis in our cohort. Tumor cells expressed CD73 could convert AMP to adenosine, which exerts potent immunosuppressive effect.[16] However, CD73 highly expressed on T cell may be identified as an exhausted marker. Although there have been multiple reports relating CD73 to anti-tumor immunity in other tumors,[37–41] only few of them are focused on HNSCC.[42] Harnessing TCGA transcriptome sequencing data and R package TCGAbiolinks,[28, 29] we find out that *NT5E* mRNA level is also associated with negative immune regulation in HNSCC patients. Among the enriched genes, TGFB1 and TGFB2 were key factors in immunosuppression and TGFB1 was shown to induce immunosuppressive microenvironment in tumors.[43, 44] NDFIP1 negatively regulates T cell activation partly by limiting IL-2 expression.[45] although MKP-5 (DUSP10) is required for naïve $CD4^+$ T cell activation, it negatively regulates $CD4^+$ and $CD8^+$ T cells in adaptive immunity.[46] IL-10 is known as an immunosuppressive cytokine which is secreted by a range

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of cells, IL-10 suppress the activation and proliferation of antigen-specific CD4 T cells that are vital to anti-tumor immunity.[47] IL-33 induces long-term immunosuppression by expanding Tregs population.[48]

The contribution of adenosine in the modulation of immune response depends on CD39 and CD73, which converting ATP into AMP and adenosine.[12] CD39 is found on both human and mouse Tregs, whereas the expression of CD73 on human and mouse Tregs is ambiguous.[49] Some study proved CD73 is dramatically expressed on murine Tregs, but low levels of CD73 are detectable in human Tregs.[49] In contrast to this result, most of the Tregs expressed CD73 in human HNSCC patients.[42] The ultimate goal of current study is to develop a potential therapeutic strategy for targeted CD73 in human HNSCC. And the current study is based on the HNSCC mouse model. Therefore, it is necessary to evaluate the expression and function of CD73 on T cells in human HNSCC. Indeed, we demonstrated that CD73 was overexpressed in tumor infiltrating immune cells of human HNSCC. However, lack of experimental research using human T cells is limitation of this study. Thus, detailed exploration of CD73 function on human T cells modulating tumor immunity in the future may facilitate to understand the immunosuppressive mechanisms in human HNSCC. Currently, The MEDI9447 (monoclonal antibody specific for CD73) alone and in combination with MEDI4736 (anti-PD-L1 monoclonal antibody) in select advanced solid tumor is recruiting participants (NCT02503774).

In summary, we elucidate that the expression of CD73 on T cells represents an ‘exhausted’ phenotype in competent HSNCC mouse model. Furthermore, *in vivo* assay showed the blockade of CD73 restrains tumor progression and reverses a large part of ‘exhausted’ $CD4^+$ and $CD8^+$ T cells to immune-competent. Moreover, we showed a potential resistant population remaining CD73 positive during the anti-CD73 treatment. The human tumor samples reveal that CD73 is overexpressed in tumor-infiltrating immune cells, and the positive expression of CD73 is correlated with the poor prognosis in human HNSCC. Taken together, these data suggest that CD73 has a negative immunoregulatory function and it may prove to be a potential target for immunotherapy in HNSCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations:

CTLA-4	cytotoxic T-lymphocyte-associated protein 4
GSEA	gene set enrichment analysis
HNSCC	head and neck squamous cell carcinoma

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LN	lymph node
mAb	monoclonal antibody
NT5E	ecto-5'-nucleotidase
OS	overall survival
PB	peripheral blood
PD-1	programmed cell death protein 1
SP	spleen
TB	tumor bearing mice
TCGA	the Cancer Genome Atlas
Tregs	regulatory T cells
WT	wild type mice

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Novelty and Impact?

Cancer immunotherapy brings tremendous promise for the cancer patients. In this article, we disclose the relationship between CD73 and ‘exhausted’ phenotype of T cells, and reveal CD73 blockade blunts the tumor growth and reverses the ‘exhausted’ phenotype of T cells. More interestingly, a CD73 resistant phenotype in exhausted T cell during the blockade of CD73 was indicated. Further, based on human tissue microarrays, the highly expression and prognostic role of CD73 was proved.

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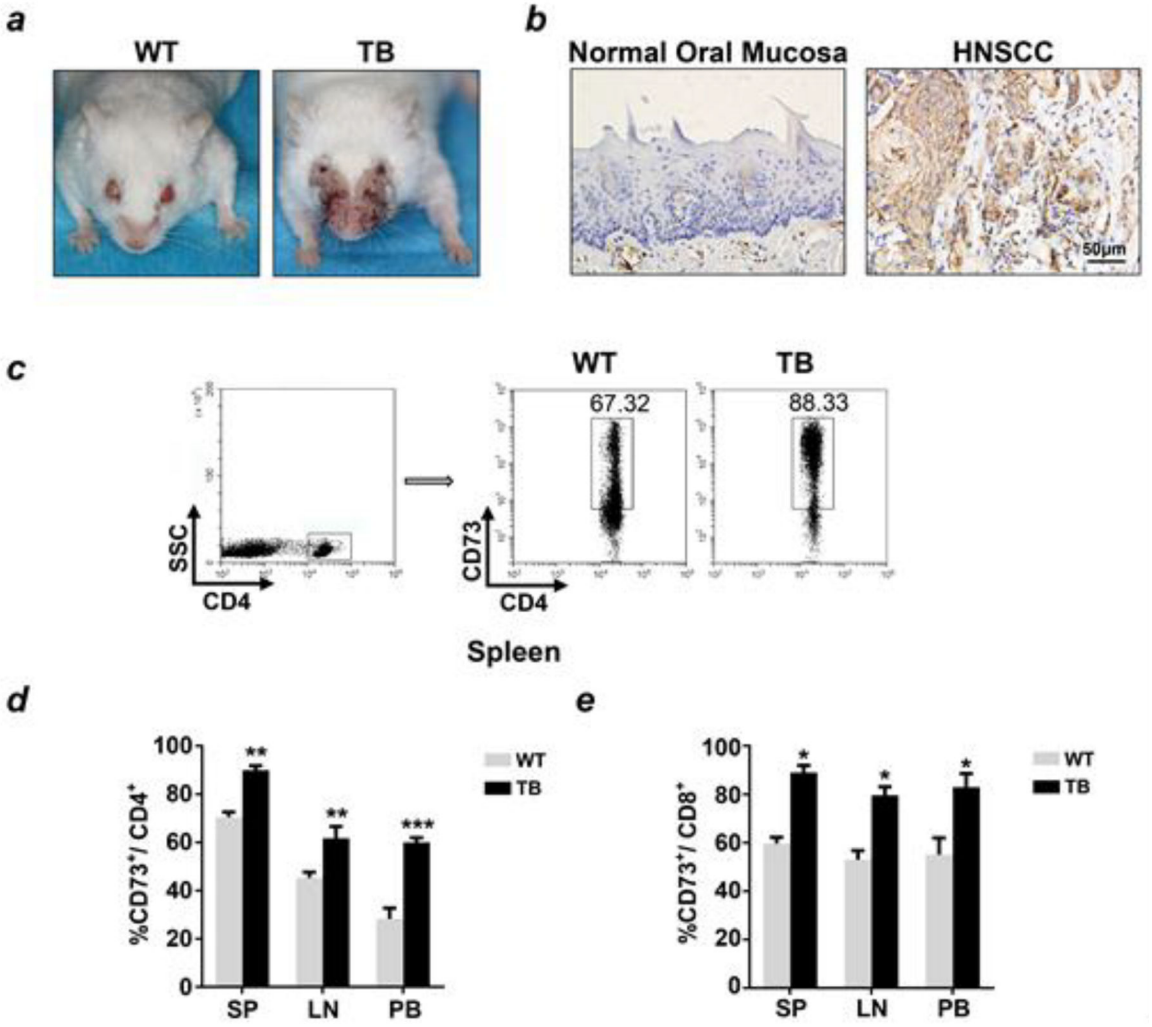

Figure 1. CD73 is upregulated on $CD4^+$ and $CD8^+$ T cells.

(*a*) The typical photos of wild type (WT) mice and *Tgfbr1/Pten* double conditional knockout tumor bearing (TB) mice. (*b*) The representative immunohistochemical staining of CD73 in normal mucosa from WT mice and HNSCC from TB mice. Scale bar = 50μm (*c*) Representative gating strategy showed the expression of CD73 in the $CD4^+$ gated population in the spleen of WT mice and TB mice. The percentage of CD73 in the $CD4^+$ gated population (*d*) and $CD8^+$ gated population (*e*) compared between WT mice and TB mice were shown in bar graph. Cells were harvested from spleen (SP), draining lymph nodes (LN), and peripheral blood (PB). Data are shown as mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

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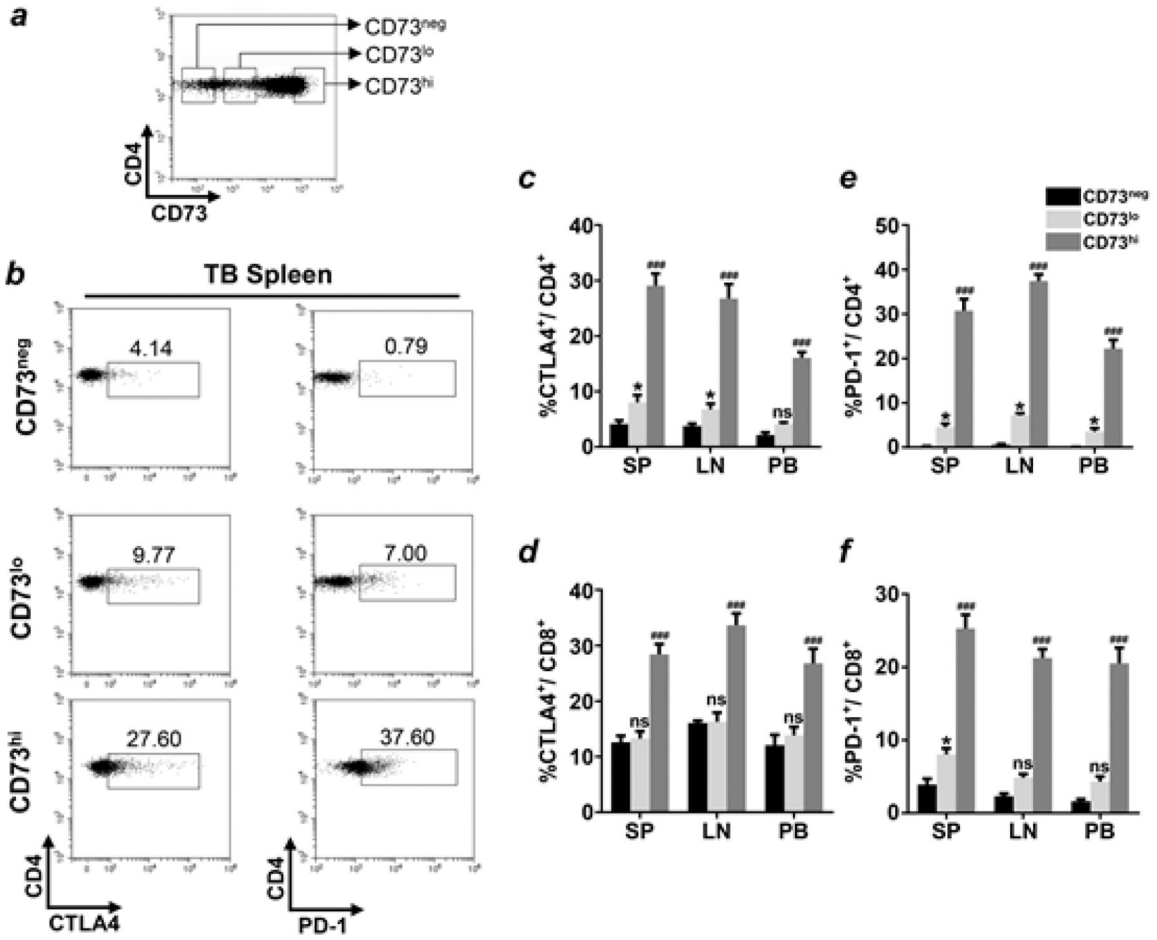

Figure 2. CD73 expression is associated with 'exhausted' phenotype in tumor-bearing mice.

(*a*) The gating strategy for the subgroup of CD73 (CD73 negative:$CD73^{neg}$; CD73 low: $CD73^{lo}$ and CD73 high: $CD73^{hi}$) on $CD4^+$ T cells. (*b*) The gating strategy for CTLA-4 (left panel) and PD-1(right panel) on different $CD4^+$ CD73 population. Quantifications of (*c*) CTLA-4^+CD4^+, (*e*) PD-1^+CD4^+, (*d*) CTLA-4^+CD8^+ and (*f*) PD-1^+CD4^+ T cells in $CD73^{neg}$, $CD73^{lo}$ and $CD73^{hi}$ cells from 2cKO tumor-bearing mice were shown in bar graph. Cells were harvested from spleen, draining lymph node, and peripheral blood. Data are shown as mean ± SEM. one-way ANOVA post Tukey test, ns: no significance, * $p < 0.05$, $CD73^{lo}$ versus $CD73^{neg}$; ### $p < 0.001$, $CD73^{hi}$ versus $CD73^{neg}$.

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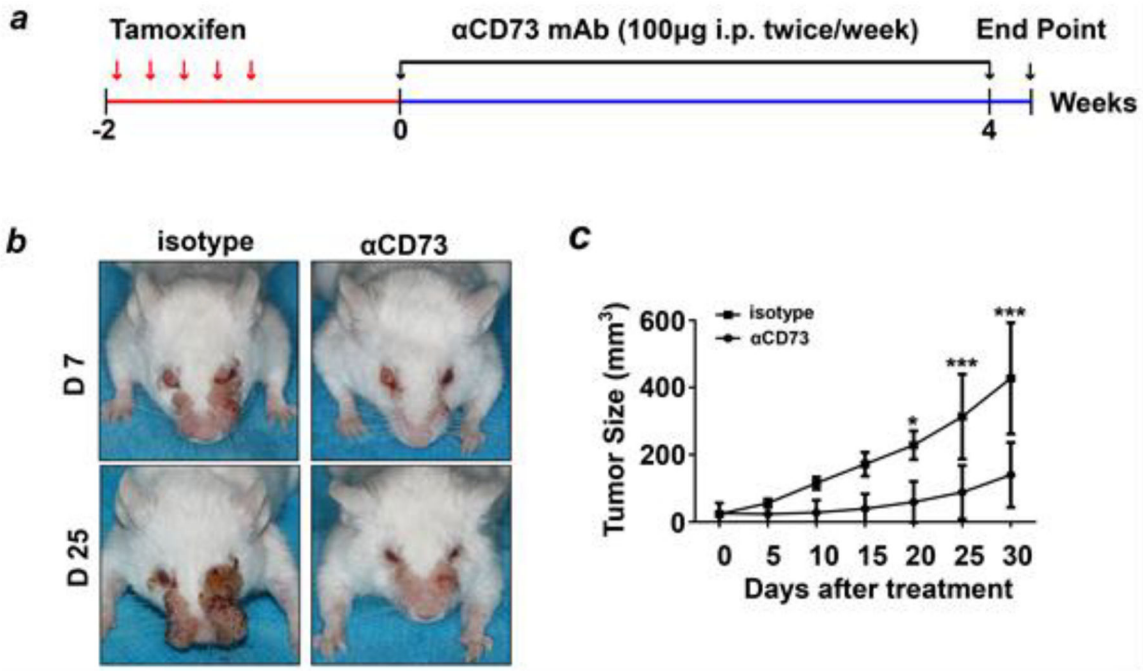

Figure 3. Anti-CD73 treatment blunts tumor growth in *Tgfbr1/Pten* 2cKO mouse model.

(*a*) Schematic drug delivery strategy. *Pten/Tgfbr1* 2cKO mice were randomized into two groups and treated with isotype control (control group, rat IgG2a, Clone:2A3, 100μg/mouse; BioXcell; n=6 mice; 100μg/mouse) and anti-mouse CD73 group (αCD73 group, *In vivo* CD73 monoclonal antibody, Clone:TY/23, BioXcell; n=6 mice; 100μg/mouse). (*b*) Representative photos of HNSCC in control group and αCD73 group at days 7 and days 25. (*c*) Tumor growth in control group and αCD73 group. Tumor sizes are presented as mean ± SEM and statistical significance was determined on day 20. *p< 0.05, ***p<0.001.

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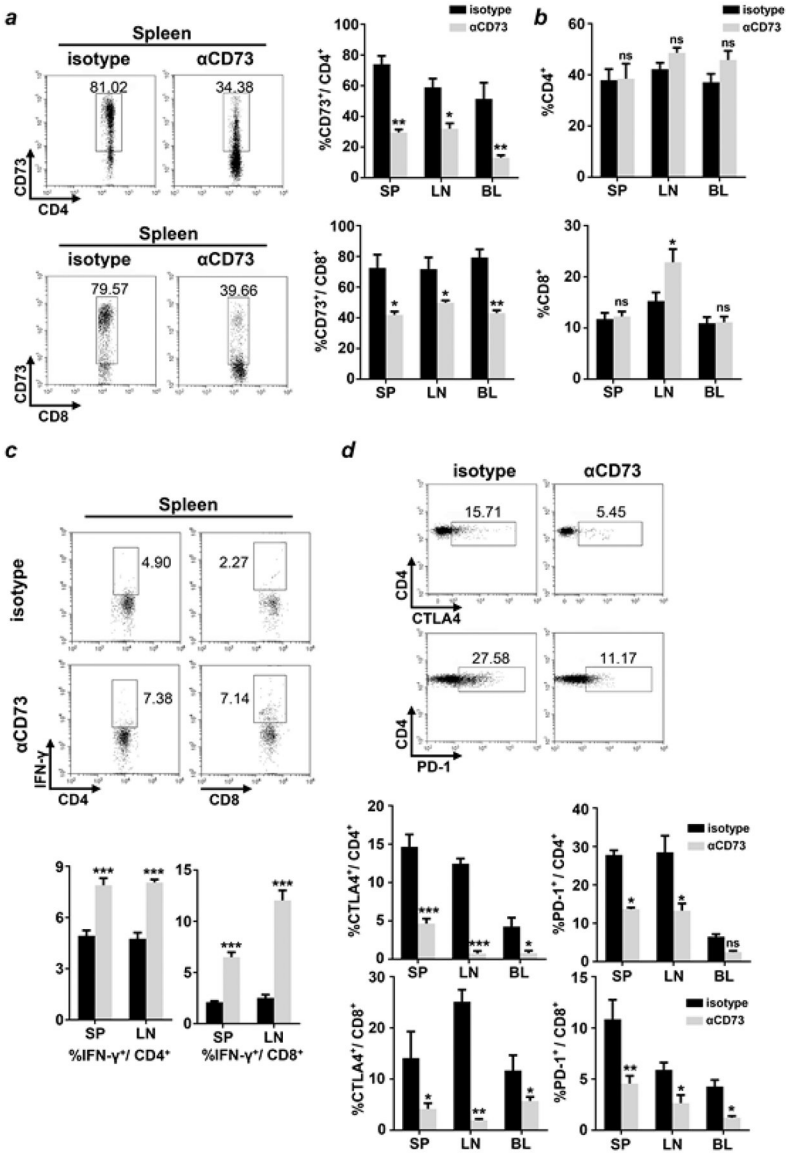

Figure 4. Targeting CD73 in tumor-bearing mice reverses 'exhausted' phenotype of CD4$^+$ and CD8$^+$ T cells.

(*a*) Representative flow cytometric dot plot and quantification of CD73$^+$CD4$^+$ and CD73$^+$CD8$^+$ T cells population in isotype or αCD73 treated *Tgfbr1/ Pten* 2cKO mice. Cells were harvested from spleen (SP), draining lymph nodes (LN), and peripheral blood (PB). Data are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$. (*b*) Quantification of CD4$^+$ and CD8$^+$ T cells population in isotype or aCD73 treated *Tgfbr1/Pten* 2cKO mice. (*c*) Representative flow cytometric dot plot (upper panel) and the percentage (lower panel) of IFN-γ$^+$CD4$^+$ and IFN-γ$^+$CD8$^+$T cells population in spleen and lymph nodes from control group and αCD73 group. Data presented as mean ± SEM. ***$p < 0.001$. (*d*) The representative dot plot for CTLA-4 and PD-1 on CD4$^+$ population. And quantification of CTLA-4$^+$CD4$^+$, PD-1$^+$CD4$^+$, CTLA-4$^+$CD8$^+$ and PD-1$^+$CD4$^+$ T cells in control group and αCD73 group were shown in bar graph. Data are shown as mean ± SEM. one-way ANOVA post Tukey test, *$p < 0.05$, **$p < 0.01$.

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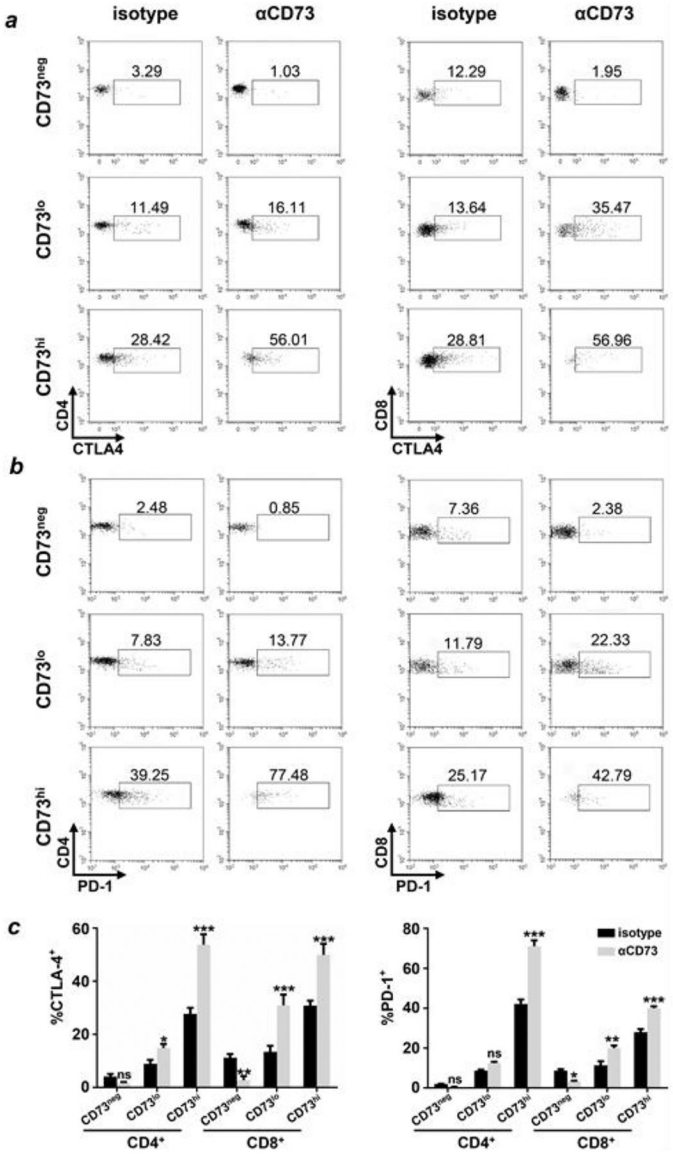

Figure 5. Anti-CD73 mAb treatment enriches 'exhausted' phenotype in $CD4^+CD73^{hi}$ and $CD8^+CD73^{hi}$ T cells.

(*a*) Representative dot plots of CTLA-4 and PD-1 on different CD73 subsets ($CD73^{neg}$, $CD73^{lo}$ and $CD73^{hi}$) of $CD4^+$ or $CD8^+$ T cells in isotype or αCD73 treated *Tgfbr1/ Pten* 2cKO mice. Cells were harvested from spleen. (*b*) Quantification of CTLA-4 and PD-1 on different CD73 subsets ($CD73^{neg}$, $CD73^{lo}$ and $CD73^{hi}$) of $CD4^+$ or $CD8^+$ T cells in isotype or αCD73 treated *Tgfbr1/ Pten* 2cKO mice. Data are shown as mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

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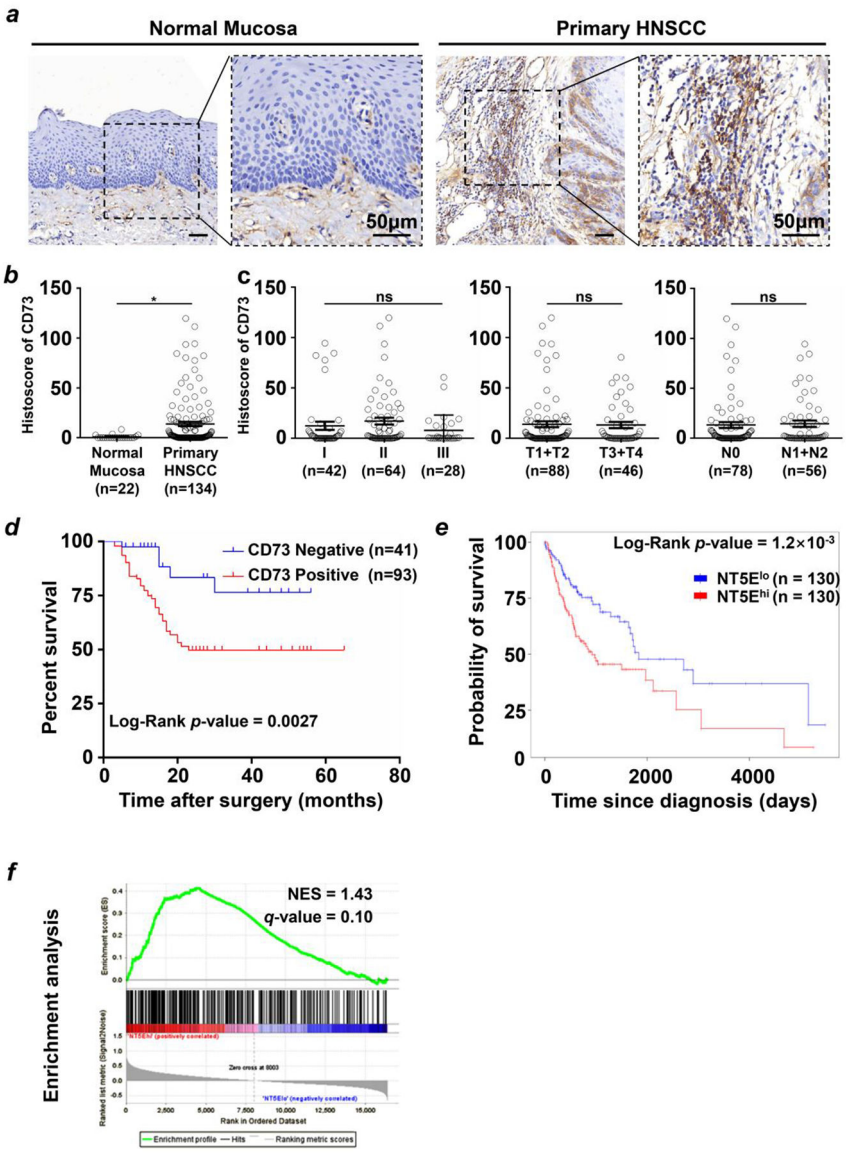

Figure 6. CD73 is elevated on tumor infiltrating immune cells and correlated with poor prognosis in human HNSCC.

(*a*) Immunohistochemical staining of CD73 in human primary HNSCC and normal mucosa. Scale bar, 50μm. (*b*) Quantitative analysis of histoscore of CD73 expression in normal mucosa and primary HNSCC. Each dot presents an individual sample. unpaired *t* test **p*<0.05. (*c*) The quantitative analysis of CD73 histoscore is performed in pathological grades (I–III), tumor size (T1+T2, T3+T4) and lymph node status (negative, N0; positive, N1+N2). ns: no significance. (*d*)Kaplan-Meier survival analysis and Log-rank test displayed overall survival of primary HNSCC patients with CD73 positive (n=94) vs. CD73 negative (n=41). (*e*) Kaplan-Meier survival analysis and Log-rank test displayed OS of *NT5E* mRNA level of HNSCC patients in TCGA database. *p* (*NT5E*^{hi}, n=130) vs. low CD73 expression (*NT5E*^{lo}, n = 130) = 0.0012. (*f*) Based on the TCGA dataset, GSEA showed genes

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associated with negatively regulated immune effector were significantly enriched in *NT5E*^{hi} group compared with *NT5E*^{lo} group.