Calnexin Impairs the Antitumor Immunity of CD4\(^+\) and CD8\(^+\) T Cells

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Abstract

Elucidation of the mechanisms of T-cell–mediated antitumor responses will provide information for the rational design and development of cancer immunotherapies. Here, we found that calnexin, an endoplasmic reticulum (ER) chaperone protein, is significantly upregulated in oral squamous cell carcinoma (OSCC). Upregulation of its membraneous expression on OSCC cells is associated with inhibited T-cell infiltration in tumor tissues and correlates with poor survival of patients with OSCC. We found that calnexin inhibits the proliferation of CD4\(^+\) and CD8\(^+\) T cells isolated from the whole blood of healthy donors and patients with OSCC and inhibits the secretion of IFN\(\gamma\), TNF\(\alpha\), and IL2 from these cells. Furthermore, in a melanoma model, knockdown of calnexin enhanced the infiltration and effector functions of T cells in the tumor microenvironment and conferred better control of tumor growth, whereas treatment with a recombinant calnexin protein impaired the infiltration and effector functions of T cells and promoted tumor growth. We also found that calnexin enhanced the expression of PD-1 on CD4\(^+\) and CD8\(^+\) T cells by restraining the DNA methylation status of a CpG island in the PD-1 promotor. Thus, this work uncovers a mechanism by which T-cell antitumor responses are regulated by calnexin in tumor cells and suggests that calnexin might serve as a potential target for the improvement of antitumor immunotherapy.

Introduction

Although T-cell immunity plays a critical role in mediating antitumor immunity, the molecular mechanisms underlying impaired T-cell T-cell immunity are not fully understood. Immune checkpoint blockade with mAbs directed against the inhibitory immune receptors CTLA-4, PD-1, and PD-L1 has emerged as a successful treatment approach that has shown durable response rates observed with cancer immunotherapies. Here, we found that calnexin, an endoplasmic reticulum (ER) chaperone protein, is significantly upregulated in oral squamous cell carcinoma (OSCC). Upregulation of its membraneous expression on OSCC cells is associated with inhibited T-cell infiltration in tumor tissues and correlates with poor survival of patients with OSCC. We found that calnexin inhibits the proliferation of CD4\(^+\) and CD8\(^+\) T cells isolated from the whole blood of healthy donors and patients with OSCC and inhibits the secretion of IFN\(\gamma\), TNF\(\alpha\), and IL2 from these cells. Furthermore, in a melanoma model, knockdown of calnexin enhanced the infiltration and effector functions of T cells in the tumor microenvironment and conferred better control of tumor growth, whereas treatment with a recombinant calnexin protein impaired the infiltration and effector functions of T cells and promoted tumor growth. We also found that calnexin enhanced the expression of PD-1 on CD4\(^+\) and CD8\(^+\) T cells by restraining the DNA methylation status of a CpG island in the PD-1 promotor. Thus, this work uncovers a mechanism by which T-cell antitumor responses are regulated by calnexin in tumor cells and suggests that calnexin might serve as a potential target for the improvement of antitumor immunotherapy.

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cell carcinoma (OSCC). Our work thus uncovers a mechanism by which antitumor responses are regulated by calnexin expressed in tumor cells and suggests that calnexin may serve as a target for antitumor immunotherapy. Elucidation of these mechanisms will reveal clues as to the next steps that need to be taken to potentially overcome resistance to immunotherapy.

Materials and Methods

Patients and tissue samples

For Western blot analyses, 8 pairs of primary OSCC samples and corresponding normal oral epithelial tissues were obtained during surgeries at the Hospital of Stomatology, Sun Yat-sen University (Guangzhou, Guangdong, P.R. China). For real-time PCR analyses, 33 pairs of primary OSCC samples and corresponding normal oral epithelial tissues were obtained during surgeries at the Hospital of Stomatology, Sun Yat-sen University (Guangzhou, Guangdong, P.R. China). For IHC and immunofluorescence analyses, the expression of calnexin was investigated using a tissue microarray (TMA) containing samples from 357 patients with primary OSCC who were treated at the Hospital of Stomatology, Sun Yat-sen University (Guangzhou, Guangdong, P.R. China), between January 2007 and January 2009. All specimens were confirmed histologically by hematoxylin and eosin staining, and tumor tissue was present in more than 80% of the specimens. The follow-up interval was calculated from the date of surgery to the date of death or last clinical evaluation. The detailed information of these patients is described in Supplementary Table S2. This study protocol was approved by the Institutional Review Board of the Hospital of Stomatology, Sun Yat-sen University (Guangzhou, Guangdong, P.R. China) and was conducted in agreement with the Helsinki Declaration, and written informed consent was obtained from all study participants.

Cell lines and reagents

The HSC-3 cell line was purchased from the cell bank of the Japanese Collection of Research Bioresource (JCRB). SCC15, SCC25, CAL27, B16F10, and M849 cells were purchased from the ATCC. The normal keratinocyte cell line (NOK-SI) was kindly provided by J. Silvio Gutkind (UCSD, San Diego, CA). Cell lines used in these experiments were passaged a maximum of four times before the experiments. Cells were tested for Mycoplasma contamination and identified by short tandem repeat.

Mice

C57BL/6 mice were purchased from the experimental animal center of Sun Yat-sen University (Guangzhou, Guangdong, P.R. China). NOO-Pckdcm6Cd52II2genc6Cd22/Nju (NCG) mice were purchased from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). All experiments were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University (Guangzhou, Guangdong, P.R. China) and performed following local rules.

Flow cytometry

Single-cell preparations were stained with antibodies purchased from eBioscience, BD Biosciences, and BioLegend. Isotype-matched control mAbs were used. Intracellular staining was done using a Foxp3/Transcription factor staining kit or Intracellular Fix & Perm Set according to the manufacturer's instructions (BD Biosciences). Briefly, cells were stimulated with 50 ng/mL PMA (Sigma-Aldrich) and 5 μg/mL ionomycin (Sigma-Aldrich) in the presence of GolgiPlug (BD Biosciences). After 4 hours, cells were stained for dead cells using a SYTO dye (eBioscience) and surface markers then fixed prior to intracellular staining for cytokines. Data were analyzed using a FACSort flow cytometer (BD Biosciences) and using FlowJo software (Tree Star). The following antibodies were used for flow cytometry: anti-mouse CD4 (clone GK1.5); anti-mouse CD8α (clone 53-6.7); anti-mouse CD3 (clone 17A2); anti-mouse/rat Foxp3 (clone FJK-16s); anti-mouse CD45.2 (clone 104); anti-mouse ki-67 (clone 16A8); anti-mouse TNFα (clone MP6-XT22); and anti-mouse IFNγ (clone MOB-47).

Xenograft assays in immunodeficient mice

CNX-knockdown (sh-CNX) cells or control cells (6 × 10^6) were injected subcutaneously into right flank of NCG mice and 1 × 10^6 human peripheral blood mononuclear cells (PBMCs) were injected via the tail vein after tumor implantation. The animals were monitored for tumor formation every 2 days and euthanized 3 weeks later. Tumor length (L) and width (W) were measured at the end of the experiment, and tumor volume was calculated by the formula (L × W^2)/2. Serum cytokines were analyzed at the indicated time points, and human CD3 T cells were counted after tumor dissociation.

Tumor experiment

B16F10 tumor cells were retrovirally transduced with sh-CNX or a control and selected with puromycin (3 μg/mL). For tumor vaccination, naïve C57BL/6 mice were immunized with 1 × 10^6 irradiated B16F10 (1 × 10^6 rad) cells that were inoculated subcutaneously into the left flank. On day 14, the vaccinated mice were challenged with live transduced tumor cells that were inoculated subcutaneously into the right flank. A CNX-Ig fusion protein or Flag-Ig (200 μg) was injected intraperitoneally (i.p.) into each mouse once a week. Tumor growth was monitored every 2 days. The mice were euthanized when the tumor size reached 15 mm diameter.

Isolation of tumor-infiltrating leukocytes from tissues

Tumor-infiltrating leukocytes (TILs) from the xenograft tumors were prepared according to the protocol described previously (22, 23). Briefly, tumors were dissected and homogenized using a GentleMACS dissociator (Miltenyi Biotech), digested with 0.05% collagenase IV (Sigma-Aldrich), 0.02% DNase I (Roche) at 37°C for 1 hour prior to centrifugation on Percoll density gradient (40%-80%), and the TILs were washed and resuspended in RPMI.

Retroviral constructs and transduction of OSCC cell lines

HSC3 cells were transfected with CNX shRNA or empty vector (Genechem) using Polybrene. The cells were trypsinized and replated in 0.5 μg/mL puromycin 48 hours after transfection. Two months later, the puromycin-resistant stable line was established and maintained in medium with 1 μg/mL puromycin. The transfected cells were incubated for 24 hours and harvested for real-time PCR and Western blot analysis.

In vitro antigen-specific T-cell response assay

OSCC tumor cells were isolated from fresh specimen; single-cell suspensions were obtained as described above. Human PBMCs from 6 healthy donors and 8 patients with OSCC were
density-enriched by Ficoll (TBD). Tumor antigens were prepared as described previously (24). Briefly, 2 \times 10^5 tumor cells were subjected to four freeze (liquid nitrogen) and thaw (37°C water bath) cycles to obtain a crude lysate as tumor antigen. After removal of large particles by centrifugation and sterilization by filtering (0.22 \mu m), the protein concentration in the supernatant was measured (Coomassie blue protein assay kit, Thermo Scientific) and aliquots stored at -80°C until use. A total of 1 \times 10^5 PBMCs were stimulated for 48 hours in the presence or absence of tumor lysate from the same patient and 1 \mu g/mL PHA-M (Sigma Aldrich). GolgiPlug was added for 5 hours to the cells in culture. After 5 hours, cells were stained for viability using a FVD dye (Sigma Aldrich). Horseradish peroxidase staining was visualized with 3,3-diaminobenzidine (Gene Company), and antibody (Thermo Scientific) and sequentially immunoblotted with calnexin mAb (C5C9, Cell Signaling Technology). Anti-CD3 (17A2, eBioscience), anti-CD4 (OKT4, eBioscience), anti-CD8 (HIT8A, eBioscience), anti-IL5, anti-IL10, anti-TNF\alpha, and IFN\gamma (C5C9, Cell Signaling Technology) as loading controls. The proteins were using GAPDH antibody (Takara). Quantitative real-time PCR was done using SYBR Green I Dye (Roche) and according to the protocol of Light Cycle 480 kits (Roche). The primer sequences of CNX were 5'-CATGATGGA-CAATGATGATGACAC-3' (forward) and 5'-CTAGGGCTTTG GTATAC-3'. Results were normalized to the expression of GAPDH (forward, 5'-AATTTGCGATG TGAAGG-3', reverse, 5'-ACA- CATTGGCGGTAGGACA-3').

**Western blot analysis**

Cells and tissues were lysed with radioimmunoprecipitation assay (RIPA) buffer containing proteases inhibitor cocktail (Sigma Aldrich) and ultrasonication. Protein quantification was performed using BCA Protein Assay Reagent (Thermo Fisher Scientific), and 45 mg protein per sample was loaded into SDS-PAGE and sequentially immunoblotted with calnexin mAb (C5C9, Cell Signaling Technology). The proteins were using GAPDH antibodies (D16H11, Cell Signaling Technology) as loading controls.

**CTL killing assay**

Tumor antigen–specific CD8+ human T-cell clones were generated from PBMCs from a healthy donor by in vitro stimulation using dendritic cells loaded with corresponding peptide epitopes (irradiated HSC3 cells were used as tumor antigens). CNX-overexpressing HSC3 cells or control cells were labeled with CFSE and cocultured with CTLs at an effector-to-target ratio (E/T) of 5:1 and 10:1 for 4 hours. Then, 0.1 \mu g of DAPI was added to each sample, and the samples were immediately analyzed by flow cytometry. CTL killing (%) = CFSE+ DAPI+ cells/total CFSE+ cells \times 100%.

**Statistical analysis**

Baseline characteristics were described by mean and SD for continuous variables or described by numbers and percentages for categorical variables. To compare the baseline characteristics between different groups, Student t test was used for continuous variables, whereas \chi^2 tests were used for categorical variables. Overall survival was calculated and described by Kaplan–Meier method. The difference of survival curves was tested by log-rank test. Univariate and multivariate Cox proportional models were used to analyze the associations between baseline characteristics and overall survival, and the HRs with 95% confidence interval (CI).
were calculated. All statistical analyses were performed by GraphPad Prism 7.0 and Stata/MP 14.0. All tests were two-sided, and a $P$ value of less than 0.05 was considered significant.

**Results**

Upregulation of membranous calnexin is correlated with reduced T-cell infiltration

ITRAQ-coupled 2D LC-MS/MS technique was used to study the protein expression patterns of OSCC tumor tissue and control normal tissue. In total, 6 pairs of tissue lysates were analyzed. When the protein patterns of the primary tumor and its corresponding normal tissue were compared, multiple proteins were found to be differentially expressed. Supplementary Table S1 shows 43 proteins that are significantly upregulated (>2-fold). Calnexin exhibited a higher expression in cancer tissues (2.7-fold elevation) when compared with the corresponding normal tissues. We further confirmed its expression by qRT-PCR (Fig. 1A), Western blotting (Fig. 1B), and IHC staining (Fig. 1C) in OSCC cells; paired cancer and adjacent

![Graph](image_url)

**Figure 1.** Calnexin expression is upregulated in OSCC, and its membranous expression is correlated with reduced infiltration of T cells in OSCC tissues. A, qRT-PCR analyses of calnexin (CNX) expression in tumor and adjacent normal tissues derived from the same OSCC patient ($n \equiv 33$; left); qRT-PCR analyses of CNX expression in OSCC cell lines (right). NOK cells served as control cells. B, Western blotting analysis of CNX expression in the cell lines (top) and paired tissues (bottom). N, normal tissue; Ca, cancer ($n \equiv 8$). C, IHC analysis of CNX expression in tumor and adjacent normal tissues derived from a typical patient with progressive OSCC. D, Representative immunofluorescence microscopic images of CNX in OSCC tissue and melanoma tissue. In addition to be primarily expressed in the cytosol of OSCC cells, a significant fraction of calnexin colocalized with Wheatgerm agglutinin (WGA) in the cell membrane of tumor cells (scale bar, 50 μm). E, The gating strategy of CNX$^+$ epithelial cell and T cells in single-cell suspension of fresh surgical specimen. F, The number of CNX$^+$ epithelial cells among every gram of OSCC tissue, was correlated with CD4$^+$ and CD8$^+$ T cells in corresponding single-cell suspensions, $n \equiv 20$. Pearson correlation coefficient was used. Bar graph, mean ± SEM. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$. One representative experiment of three is depicted.
noncancerous tissues were derived from the same patients with OSCC. We found that calnexin was significantly upregulated in OSCC tissues.

In addition, immunofluorescence analysis suggested that although most of the calnexin protein expression was observed in the cytosol of OSCC cells, a considerable fraction of calnexin colocalized with WGA in the cell membrane of tumor cells. Membrane localization of calnexin has been found in several tumors, including OSCC and melanoma (Fig. 1D), suggesting that calnexin may exert its biological or immunologic regulation functions as either a secreted or membrane-bound form. Because T cells play a critical role in mediating antitumor immunity, and impaired T-cell infiltration is positively correlated with poorer prognosis of tumor patients (29), we first determined whether there was any correlation between membranous expression of calnexin and T-cell infiltration in tumor tissues. Cell surface calnexin expression was determined by flow cytometry. We found that higher calnexin membranous expression was negatively correlated with the numbers of infiltrated CD4+ and CD8+ T cells in OSCC tumor tissues (Fig. 1E and F).

Upregulation of membranous calnexin correlated with poor clinical prognosis

Given that calnexin membranous expression was upregulated in OSCC tissues, we next determined whether calnexin expression in the cytosol and cell membrane was correlated with tumor prognosis. To address this, IHC staining of calnexin was performed to assess the expression of calnexin in samples from 357 patients with primary OSCC. Representative images of the intensity stages are shown in Fig. 2A. Overall, cytoplastic calnexin expression was categorized into low (178 of the 357 tumor samples, 49.86%) and high (179 of the 357 tumor samples, 50.14%) expression groups using the cutoff point 5.04 based upon the median of SID scores of the total patients. In addition, 71 of the 357 tumor samples (19.89%) showed apparent calnexin expression at the plasma membrane, whereas 286 of the 357 tumor samples (80.11%) showed negative staining of calnexin at the plasma membrane (Supplementary Table S2). Representative images are shown in Fig. 2A. The expression of calnexin was assessed for association with a number of clinicopathologic variables (Supplementary Table S2).

Kaplan-Meier survival curves show, overall patient survival was not significantly different when compared between low and high cytoplastic expression of calnexin (P = 0.405), whereas patients with positive calnexin membranous expression had a significantly reduced overall survival than patients with negative expression [3-year OS: 47.89% (35.93–58.88%) vs. 66.43% (60.64–71.58%), P = 0.016; Fig. 2B]. In the univariate analysis, calnexin membranous expression (P = 0.018) along with nodal stage (P < 0.001), clinical TNM stage (P = 0.030), and radiotherapy (P = 0.042) were significantly associated with overall survival. Adjusted for nodal stage and radiotherapy, patients with positive membranous expression of calnexin was significantly associated with reduced overall survival, compared with patients with negative expression (HR, 1.59; 95% CI, 1.10–2.30; P = 0.013; Fig. 2C). There was no significant association between cytoplastic expression of calnexin and overall survival among patients with OSCC (Supplementary Tables S3 and S4).

Calnexin inhibits T-cell proliferation and antitumor effector functions

Because upregulation of calnexin in OSCC tissue was correlated with reduced infiltration of CD4+ and CD8+ T cells, we hypothesized that calnexin impairs the antitumor immunity of effecter T cells. A calnexin-Ig fusion protein (CNX-Ig) was generated to examine the regulatory roles of calnexin in T-cell responses. Indeed, we found that when immobilized on a microplate, calnexin-Ig, but not control-Ig, suppressed the proliferation of bulk purified CD4+ and CD8+ T cells in response to anti-CD3 stimulation (Fig. 3A) and inhibited the production of effector molecules such as IFNγ, TNFα, and IL2 (Fig. 3B). Furthermore, calnexin inhibited the antitumor cytolytic functions of CD8+ T cells against HSC3 tumor cells (Fig. 3C). These data collectively suggested that calnexin inhibited the proliferation and antitumor effecter functions of CD4+ and CD8+ T cells. Although the receptor for calnexin is unknown, we speculated that the engagement of calnexin-R on T cells suppresses T-cell receptor (TCR) signaling. To test this hypothesis, proximal TCR signaling events were examined using calnexin-Ig. LAT is a proximal signaling adaptor that is phosphorylated upon TCR stimulation and forms a complex with multiple signaling molecules, including SH2 domain containing a leukocyte protein of 76 kDa (SLP76) and phospholipase C (PLC)-γ1 (30). Immobilized calnexin-Ig substantially reduced the amount of SLP76 recruited to the CD3 complex, as well as its phosphorylation. When total cell lysates were examined, the phosphorylation of several downstream signaling molecules, such as Akt and Erk1/2 was also impaired (Fig. 3D).

In addiction to demonstrating the inhibitory effect of calnexin on peripheral blood T cells from healthy donors, we also evaluated its role in circulating blood T cells from patients with OSCC. Tumor lysates from the same patients were used as tumor antigens. We found that PBMCs from OSCC patients cocultured with calnexin-Ig showed inhibitory effects on the proliferation of CD8+ T cells (Fig. 4A) and reduced the number of functional CD8+ T cells producing IFNγ by nearly half (Fig. 4B). These findings were confirmed by our cytometric bead assay (CBA) results, which showed decreased production of IFNγ and TNFα but increased production of IL10 by T cells (Fig. 4C). These changes were more significant in the tumor antigen–experienced cells. Because an increase in IL10 production by T cells was observed, the induction of Treg was examined. However, calnexin could not promote Treg production in an antigen-specific manner (Supplementary Fig. S1). These data collectively suggested that calnexin inhibited the proliferation and antitumor effecter functions of CD8+ T cells in patients with OSCC in an antigen-dependent manner.

Calnexin promotes OSCC tumor growth in a humanized mouse model

Because calnexin inhibits the proliferation and effecter functions of CD4+ and CD8+ T cells, we next determined whether calnexin-mediated impairment of antitumor T-cell responses contributes to tumor growth. Because OSCC tumor cells do not grow well in wild-type mice, a humanized mouse model was used (31, 32). HSC3 tumor cells expressing shRNA-CNX (sh-CNX) or shRNA-control (sh-NEG) were inoculated into NCG mice, and the mice were engrafted with human PBMCs after tumor implantation. The mice were euthanized before experiencing weight loss, a symptom of graft-versus-host disease (GVHD) that occurs in this.

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humanized mouse model (Fig. 5A). In this model, the tumor growth in the sh-CNX group was lower than that in the sh-NEG group (P = 0.047; Fig. 5B). We also detected increased frequencies of multifunctional CD3^+ T cells producing IFNγ in the sh-CNX group compared with the sh-NEG group upon PBMC engraftment (Fig. 5C and D). We also examined control mice without PBMC injection in HSC3 tumor model and found that in contrast to the results from humanized mice, calnexin silencing promoted tumor growth in the immunodeficient mice (Fig. 5E and F), indicating that calnexin might have another tumor-intrinsic role that is independent of its function on T cells. These data indicated that calnexin suppressed antitumor immunity and promoted OSCC.

Figure 2.
Upregulation of membranous calnexin is correlated with poorer overall survival rates of patients with OSCC. A, The calnexin (CNX) cytoplasmic expression was determined on the basis of the staining intensity and proportion, and then divided into high and low expression groups using the cutoff point 5.04 based upon the median of SID scores of the total patients. The calnexin plasma membrane expression was determined, and the samples were divided into positive and negative expression groups. Representative images showing strong-, moderate- and weak-intensity cytoplasmic staining and positive/negative membranous staining in tumor tissue samples derived from patients with OSCC. B, Analysis of the associations between the cytoplasmic and membranous expression of calnexin and overall survival among 357 patients with OSCC. Kaplan-Meier survival curves showed that patients with positive membranous expression of calnexin had a significantly reduced overall survival than patients with negative expression (P = 0.016). Calnexin cytoplasmic expression was not significantly associated with overall survival (P = 0.405). The P value was determined by log-rank test. C, Adjusted multivariable risk factor cohort of overall survival. Calnexin membranous expression was significantly associated with overall survival (P = 0.013).
Calnexin Impairs T-cell Antitumor Immunity

Calnexin inhibited the proliferation of CD4<sup>+</sup>/CD8<sup>+</sup> T cells and the cytotoxicity of CD8<sup>+</sup> T cells. Fresh PBMCs were isolated from 6 healthy donors. A, CFSE-labeled, bulk-purified pan T cells were stimulated by plate-bound anti-CD3 together with coabsorbed calnexin-Ig (CNX-Ig) or control-Ig (Flag-Ig) protein. Top, representative CFSE dilution profiles. Bottom, the percentage of CFSE-low cells was quantified. B, Culture supernatants in A were collected at the indicated times. The concentrations of IL2, IFNγ, and TNFα were analyzed by ELISA. C, Tumor antigen-specific CD8<sup>+</sup> human T-cell clones (CTL) were generated from PBMCs of a healthy donor by in vitro stimulation using dendritic cells loaded with irradiated HSC3 cells. Calnexin-overexpressing HSC3 cells (CNX) or control cells (Vector) were labeled with CFSE and cocultured with CTLs at an effector-to-target ratio (E/T) of 5:1 and 10:1 for 4 hours. D, Engagement of calnexin during TCR activation maximally suppresses proximal adaptor signaling. Naive pan-T cells were stimulated by plate-bound anti-CD3 together with coabsorbed calnexin-Ig (CNX-Ig) or control-Ig (Flag-Ig) protein. Then, cell lysates were prepared, and the phosphorylation status of SLP76, Erk1/2, and AKT were examined by immunoblotting. Bar graph is shown as the mean ± SEM (n = 6); N.S., not significant. * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001. One representative experiment of three is depicted.

tumor growth via inhibiting the proliferation and effector functions of CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

Calnexin deficiency promotes antitumor immunity and controls tumor growth
We next developed a mouse melanoma model to determine whether calnexin-mediated impairment of T cells contributes to tumor growth. We injected mice subcutaneously with B16F10 cells expressing shRNA targeting calnexin (sh-CN) or control shRNA (sh-NEG) and monitored tumor growth. To generate protective immunity, naïve mice were vaccinated with irradiated B16F10 tumor cells in advance (Fig. 6A). We found that knockdown of calnexin in melanoma tumor cells significantly inhibited melanoma growth in mice, whereas administration of calnexin-Ig enhanced melanoma growth (Fig. 6B). Furthermore, knockdown of calnexin in melanoma tumor cells increased the infiltration of
CD3⁺, CD4⁺, and CD8⁺ T cells in melanoma tumors (Fig. 6C and D) and enhanced the expression of Ki67 in CD4⁺ and CD8⁺ T cells (Fig. 6E). In addition, treatment with calnexin-Ig inhibited this infiltration in melanoma tumors (Fig. 6C and D) and the expression of Ki67 in these T cells (Fig. 6E). Moreover, knockdown of calnexin in melanoma tumor cells enhanced the expression of the antitumor effector molecules IFNγ and TNFα by CD4⁺ and CD8⁺ T cells in melanoma tumors, and this effect was significantly reversed by treatment with calnexin-Ig (Fig. 6F). No differences in Tregs and MDSC frequencies among TILs were found (Supplementary Fig. S2). There were no significant differences in the proliferation and effector functions of CD4⁺ and CD8⁺ T cells located in the spleen, lymph nodes and PBMCs between the groups (Supplementary Fig. S3). To confirm that there is no intrinsic enhancement of tumor growth in the absence of T-cell–mediated antitumor immunity, tumors were inoculated in T-cell–deficient nude mice. As shown in Supplementary Fig. S4, administration of calnexin-Ig no longer
Calnexin promotes OSCC tumor growth in humanized NCG mice. A, Schematic diagram showed the experiment protocol used to determine the role of calnexin (CNX) in OSCC tumor growth in immune-integrity environment. On day 1, mouse was transplanted with HSC3 cells transduced with sh-CNX or sh-NEG, mice were sacrificed, and tumors and tumor volume kinetics were measured and calculated using the following formula: $V = \frac{L \times W^2}{2}$. B and C, The bar graph shows the increased frequencies of CD3+ T cells and functional T cells producing IFNγ in the sh-calnexin group compared with the sh-NEG group after PBMC engraftment. D, Schematic diagram shows the experiment protocol used to determine the role of calnexin in OSCC tumor growth in immune-deficient environment. HSC3 cells transduced with shRNA targeting calnexin (sh-CNX) or control shRNA (sh-NEG) were injected subcutaneously at indicated time. E, Representative in situ images of OSCC tumors in NCG mice and tumor volume kinetics were measured and calculated using the formula described in D. F, Bar graph, mean ± SEM ($n = 5$). *, $P < 0.05$. One representative experiment of two is depicted.

Calnexin enhances the expression of PD-1 by repressing PD-1 promoter methylation

Given that T-cell surface receptors such as TIGIT, CTLA-4, PD-1, and LAG-3 play critical roles in inhibiting T-cell responses, we next determined whether upregulation of calnexin might enhance the expression of these molecules and therefore induce impairment of the proliferation and effector functions of CD4+ and CD8+ T cells in tumors. To address this, we analyzed the expression of TIGIT, CTLA-4, PD-1, and LAG-3 on CD4+ and CD8+ T cells derived from melanoma tumor samples. We found that knockdown of calnexin in melanoma tumor cells significantly decreased the expression of PD-1, but not TIGIT, CTLA-4, PD-1, or LAG-3, in CD4+ and CD8+ T cells derived from melanoma tumor samples (Fig. 7A). In contrast, calnexin-Ig treatment partly reversed the decrease in PD-1 expression on CD4+ and CD8+ T cells conferred by knockdown of calnexin in melanoma tumors (Fig. 7A). Similar results were found in an MB49 tumor model with calnexin-Ig treatment (Fig. 7B). In addition, calnexin-Ig enhanced the expression of PD-1 on CD8+ T cells in PBMCs derived from patients with progressive OSCC, and this enhancement was more significant in tumor antigen–experienced T cells, as shown in Fig. 7C. Thus, these data suggested that calnexin enhanced the expression of PD-1 on CD4+ and CD8+ T cells in OSCC in an antigen-dependent manner.

We then determined the mechanism by which calnexin enhanced the expression of PD-1 on T cells in tumors. Because PD-1 promoter CpG island methylation status plays a central role in mediating PD-1 expression (33, 34), we analyzed the methylation of this region using bisulfite sequencing in T cells from OSCC patients’ PBMCs (Fig. 7D). In contrast to control-Ig, T cells

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Calnexin expressed in tumor cells inhibits the antitumor protective immunity of CD4⁺ and CD8⁺ T cells in a mouse melanoma model. A, Schematic diagram shows the protocol used to determine the effect of calnexin (CNX) on melanoma tumor growth in mice. B, Kinetics of tumor volumes in mice as indicated (n = 4–5). C, Flow cytometry analysis of the number of CD4⁺ and CD8⁺ T cells infiltrated in tumors derived from mice with the indicated treatments. Note that knockdown of calnexin (CNX) significantly increased the number of infiltrated CD4⁺ or CD8⁺ T cells in tumors. However, treatment with calnexin-Ig decreased the number of tumor-infiltrated CD4⁺ or CD8⁺ T cells (n = 4–5). D, IHC analysis of tumors derived from mice with indicated treatments suggested that significantly larger numbers of infiltrated CD3⁺ T cells in tumors were observed in the calnexin-deficient group. E, Flow cytometry analysis of the Ki67 expression on tumor infiltrated CD4⁺ and CD8⁺ T cells. F, Representative flow cytometric analysis of expression of IFNγ and TNFα in CD4⁺ or CD8⁺ T cells isolated from tumors derived from mice with the indicated treatments; note that knockdown of calnexin in melanoma tumor cells enhanced expression of the antitumor effector molecules IFNγ and TNFα produced by CD4⁺ and CD8⁺ T cells, and this enhancement of effector functions was significantly reversed by treatment with the calnexin-Ig. Bar graph, mean ± SEM (n = 4–5); *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; N.S., not significant. One representative experiment of two is depicted.
Figure 7.
Calnexin promotes the expression of PD-1 on CD4⁺/CD8⁺ T cells in tumor by restraining the DNA methylation status of a CpG region in the PD-1 (PDCD1) promoter. A, Representative flow cytometric analysis and dot plot data show the expression of PD-1, TIGIT, CTLA-4, PD-1H, and LAG-3 on CD4⁺ or CD8⁺ T cells isolated from mice in Fig. 6. The data showed that knockdown of calnexin (CNX) in B16F10 tumor cells significantly reduced the expression of PD-1, but not TIGIT, CTLA-4, PD-1H, or LAG-3, on CD4⁺ or CD8⁺ T cells (n = 4–5). B, Representative flow cytometric analysis and dot plot data from an MB49 tumor model showed that treatment with calnexin-Ig, but not Flag-Ig protein, significantly enhanced the expression of PD-1 on CD4⁺ and CD8⁺ T cells (n = 4–5). One representative experiment of two is depicted. C, Representative flow cytometric data and bar graph data showed that calnexin-Ig increased the expression of PD-1 among CD8⁺ T cells in the presence of OSCC tumor antigen (n = 8). D, Schematic of the CpG island and bisulfite pyrosequencing region in the PDCD1 promoter. TSS, transcription start site; red letters, CG sites for bisulfite pyrosequencing. Bisulfite pyrosequencing was used to detect the methylation of PD-1 promoter CpG island. E, The average methylation in the calnexin-Ig and Flag-Ig group was calculated. Note that recombinant calnexin-Ig significantly suppressed PD-1 promoter CpG island methylation in T cells. The data are representative of three independent experiments. Bar graph is shown as mean ± SEM; *, P < 0.05; **, P < 0.01; ***, P < 0.001; N.S., not significant.
Calnexin has been reported to play a role in the folding and quality control of newly synthesized glycoproteins (14, 35). A wide variety of important cellular and viral glycoproteins are known substrates of calnexin, including HIV gp120 and gp160, class I MHC heavy chain, and TCR subunits (36, 37). Although several reports have shown that calnexin expression may be associated with the progression of breast cancer, lung cancer, and colorectal cancer, most previous studies of calnexin focused on the relationship between the expression of calnexin and clinical outcomes (38–40). Whether calnexin regulates the T-cell response during tumor development is unknown. Here, we first identified that upregulation of calnexin in tumor cells could inhibit the infiltration of T cells in tumors and the proliferation and effector functions of CD4+ and CD8+ T cells. As increasing evidence has suggested that the infiltration and effector functions of T cells in tumors are critical for antitumor immunity, this finding therefore reveals a mechanism responsible for poor survival of tumor patients.

A finding of this study is the establishment of an immunologic link between calnexin and PD-1 on T cells. We found that knockdown of calnexin in melanoma tumor cells significantly decreased the expression of PD-1. In addition, calnexin-Ig treatment partly reversed the decrease of PD-1 expression on T cells. Calnexin-Ig also enhanced the expression of PD-1 on T cells in PBMCs derived from patients with progressive OSCC by inhibiting the PD-1 promoter CpG island (Fig. 7E). Whether calnexin regulates the T-cell response during tumor development is unknown. Here, we first identified that upregulation of calnexin in tumor cells could inhibit the infiltration of T cells in tumors and the proliferation and effector functions of CD4+ and CD8+ T cells. As increasing evidence has suggested that the infiltration and effector functions of T cells in tumors are critical for antitumor immunity, this finding therefore reveals a mechanism responsible for poor survival of tumor patients.

Discussion

In this study, we discovered that the ER chaperone protein calnexin was highly upregulated in OSCC tumor tissues and multiple tumors. Upregulation of membranous calnexin was positively correlated with poor prognosis of patients with OSCC. We found that calnexin played a central role in inhibiting the infiltration and effector functions of T cells and promoting the expression of PD-1 on CD4+ and CD8+ T cells in tumors, which therefore enhanced tumor growth, demonstrating the potential of calnexin as a new antitumor immunotherapy target.

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