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Kv1.3 channels mark functionally competent CD8⁺ tumor infiltrating lymphocytes in head and neck cancer

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Abstract

Tumor infiltrating lymphocytes (TILs) are potent mediators of an anti-tumor response. However, their function is attenuated in solid tumors. CD8⁺ T cell effector functions such as cytokine and granzyme production depend on cytoplasmic Ca²⁺, which is controlled by ion channels. In particular, Kv1.3 channels regulate the membrane potential and Ca²⁺ influx in human effector memory T (T_{EM}) cells. In this study, we assessed the contribution of reduced Kv1.3 and Ca²⁺ flux on TIL effector function in head and neck cancer (HNC). We obtained tumor samples and matched peripheral blood from 14 patients with HNC. CD3⁺ TILs were comprised of 57% CD4⁺ (82% T_{EM} and 20% T_{reg}) and 36% CD8⁺ cells. Electrophysiology revealed a 70% reduction in functional Kv1.3 channels in TILs as compared to peripheral blood T cells from paired patients, which was accompanied by a decrease in Ca²⁺ influx. Immunofluorescence analysis showed that CD8⁺ TILs expressing high Kv1.3 preferentially localized in the stroma. Importantly, high expression of Kv1.3 correlated with high Ki67 and granzyme B expression. Overall, these data indicate that defective Kv1.3 channels and Ca²⁺ fluxes in TILs may contribute to reduced immune surveillance in HNC.

Keywords

Kv1.3 channels; Tumor infiltrating lymphocytes; Ca²⁺ flux; head and neck cancer; T cell function

Introduction

Head and neck cancers (HNC) represent a heterogeneous group of malignant tumors that originate primarily in the nasopharynx, nasal cavity, paranasal sinuses, oral cavity, oropharynx, salivary glands, larynx and hypopharynx and affect more than half a million individuals worldwide (1, 2). A majority of HNC cases present histologically as squamous

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cell carcinomas (SCC) (2). Head and neck squamous cell carcinoma (HNSCC) is the sixth most common type of cancer, with a 5-year survival of 50% (2–4). The heterogeneity of HNC tumors, the complex anatomy of the head and neck region and the proximity of these tumors to several vital organs and structures present a challenge in conventional treatment modalities of these cancers (2). The immune system plays an important role in the pathogenesis of HNC and immunotherapies aimed to boost the immune system are showing promising results in HNC patients (5, 6).

The response of the host immune system to the developing tumor is complex in nature and is poorly understood. Typically, tumors create a tumor microenvironment (TME) which enables them to survive and proliferate by evading recognition and initiation of an immune response from the host immune system (6, 7). The TME is infiltrated by a heterogeneous population of tumor infiltrating lymphocytes (TILs) (6–8). The extent of cytotoxic CD8⁺ (that are capable of killing the tumor cells) and CD4⁺ (helper T cells that facilitate the killing of tumor cells and/or regulatory T cells (T_{reg}) that have an immunosuppressive effect on other T cells) T cells' infiltration into the HNC tumor mass has repercussions on disease prognosis and responsiveness to therapy (5, 8, 9). Furthermore, the functional status of CD8⁺ and Th1 CD4⁺ TILs determines their ability to eradicate cancer cells. In various solid tumors, including HNC, TILs exhibit multiple functional defects that include reduced locomotion, proliferation, cytotoxicity and cytokine production (IL-2 and IFN γ) and increased cell death (3, 7, 10–15).

The function of T lymphocytes depends on Ca²⁺ signaling, which is controlled by multiple ion channels to regulate the Ca²⁺ influx into the T cell (16). Particularly, Kv1.3 channels regulate the membrane potential of human T lymphocytes and provide the electrochemical driving force for Ca²⁺ influx through Ca²⁺ release activated Ca²⁺ channels (CRAC), which is necessary for downstream effector functions (16–19). Unequivocal evidence exists of the importance of Kv1.3 and CRAC channels in T cell activation as their blockade suppresses cytokine production and proliferation (16, 18, 20). Although these ion channels play an important role in T cell function, their implications in cancer are poorly understood. Evidence for a role of ion channels in the failure of immune surveillance in cancer has been provided by conditional knockout mice lacking functional CRAC channels in T cells. These mice have increased susceptibility to tumor engraftment because their CD8⁺ cells are ineffective in killing tumor cells and producing IFN γ and TNF α (21). Currently, no information is available on either the ion channel expression or function in TILs in HNC. Understanding the ion channel dysregulation in TILs could be of value in developing new and effective cancer immunotherapies.

The current study was undertaken to unveil the Kv1.3 channel expression and function in TILs in HNC. Herein, we present evidence that Kv1.3 channels are downregulated in HNC TILs and this deficiency is associated with loss of effector functions such as decreased Ca²⁺ influx, cell proliferation and granzyme B (GrB) production.

Materials and Methods

Human subjects

Peripheral blood and tumor samples from 14 HNC patients were obtained from the University of Cincinnati Cancer Institute's (UCCI) Tumor Bank. Eligibility criteria for patient inclusion were: a diagnosis of SCC as confirmed by tissue biopsy, no preoperative administration of radiation or chemotherapy. Eligible patients underwent surgical removal of the solid tumors and a piece of the surgically resected tumor (> 0.1 g) was provided for TIL isolation. From the same patient, 2 ml of peripheral blood was obtained preoperatively. Blood samples were also drawn from 3 healthy volunteers (2 female, 1 male) between the ages of 35 and 67 and were used as healthy controls for electrophysiology and calcium measurement experiments. Informed written consent was obtained from all patients and healthy volunteers. The study and the informed consent forms were approved by the Institutional Review Board at the University of Cincinnati. Pathological and clinical data on the enrolled patients were provided by the UCCI tumor bank and are presented in Table 1 and Table 2 respectively. Time to median follow up for the HNC patients was 16 months (Range 3–40 months).

Cell isolation

Peripheral blood mononuclear cells (PBMCs) and CD3⁺ T cells were isolated from whole blood as previously described (22). Briefly, PBMCs were isolated from centrifugation of whole blood using Ficoll-Paque (GE Healthcare Bio-sciences) density gradient and CD3⁺ cells were further isolated from the PBMCs by negative selection using EasySep™ Human T-cell enrichment kit (StemCell Technologies). T cells were maintained in RPMI-1640 medium supplemented with 10% human serum, 200 u/ml penicillin, 200 mg/ml streptomycin, 1 mM L-glutamine, 25 mM HEPES (Sigma-Aldrich) and cells were used immediately for calcium measurement and electrophysiology.

TIL isolation

TILs were isolated by mechanical dissociation of surgically resected tumors within 2h of their removal. Briefly, the tumors were rinsed with PBS, dissected into ~2 mm³ fragments and transferred to a gentleMACS C-tube (Miltenyi Biotec) in sterile RPMI-1640 medium. The tumor fragments were dissociated in the gentleMACS dissociator (Miltenyi Biotec) using the A01 program for 25s. This dissociation was repeated three times, till a tumor cell suspension was obtained. This suspension was then filtered through a 100 µm sterile nylon mesh cell strainer, washed twice in PBS, resuspended in PBS + 2% FBS, layered over Ficoll-Paque density gradient and the TIL fraction was recovered after centrifugation. CD3⁺ TILs were isolated by negative selection from this fraction as mentioned above. These cells were assessed for viability and purity and were used immediately for phenotyping, electrophysiology and calcium measurements.

Cell surface antibody staining

Cell viability was assessed by live labeling 0.1×10^6 TILs with 7-AAD viability staining solution (Biolegend) (22). For purity, cells were stained with FITC anti-CD3 and PerCP

anti-CD19 antibodies. For phenotyping, approximately 0.5×10^6 TILs were stained for measuring the percentages of T_{reg} and T_{EM} cells using standard flow cytometry staining protocols (22). Briefly, for T_{reg} cells, TILs were labelled live with the following antibodies: FITC anti-CD3, PE anti-CD127, PerCP anti-CD8, APC anti-CD4, APC-Cy7 anti-CD25 and PB anti-CD45. For T_{EM} cells, TILs were labeled live with the following antibodies: FITC anti-CD45, PE anti-CD4, PerCP anti-CD8, PE-Cy7 anti-CCR7, APC anti-CD45RO, APC-Cy7 anti-CD3 and PB anti-CD45RA (all flow cytometry antibodies from BD Biosciences). Samples were fixed in 1% paraformaldehyde solution, read on a BD FACSCanto flow cytometer (BD Biosciences, San Jose, CA) and analyzed by FCS Express software (DeNovo Software.).

Calcium measurements

Ca^{2+} was measured using the Ca^{2+} add-back method (23). Briefly, $0.5-1 \times 10^6$ peripheral T cells and TILs were loaded with 1:1000 fold of 2 mg/ml Indo-1-AM ratiometric dye and 0.015% Pluronic F-127 (Life Technologies) in Hank's balanced salt solution (HBSS) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1% FCS for 30 min at 37 °C, then washed three times in HBSS supplemented with 10 mM HEPES (pH 7.0) and 1% FCS. Prior to measurements, cells were resuspended in a calcium-depleted solution prepared from the HBSS/HEPES solution mentioned above and supplemented with 0.5 mM EGTA (pH 7.4). The Indo-1 fluorescence ratio (indicative of the $[Ca^{2+}]_i$) in T cells was measured by flow cytometry on a LSRII flow cytometer (BD Biosciences). The following protocol was implemented: cells were exposed to thapsigargin (TG, 1 μ M) in 0 mM Ca^{2+} solution followed by 2 mM Ca^{2+} -containing solution (23). Analysis of the kinetics was performed using Flow-Jo software (Flow Jo LLC). Ca^{2+} fold change was measured as the ratio between the peak intensity ratio of Indo-1 upon addition of 2 mM Ca^{2+} and the mean baseline Indo-1 ratio at 0 mM Ca^{2+} .

Electrophysiology

Kv1.3 currents were measured in whole-cell voltage-clamp configuration using Axopatch 200B amplifier (Molecular Devices). The external solution contained (in mM): 145 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 5.5 glucose, 10 HEPES (pH 7.35). The pipette solution contained (in mM): 140 KF, 11 K_2EGTA , 1 $CaCl_2$, 2 $MgCl_2$, and 10 HEPES (pH 7.22) (24). Kv1.3 currents were elicited by 2-second-long voltage-step pulses of +50 mV from a holding potential of -120 mV. The amplitude of the peak current was read at +50 mV, and the current density was defined as the ratio of the peak current at +50 mV and the whole-cell cell capacitance (which is a measurement of cell surface area). The current density is proportional to the number of Kv1.3 channels per unit area. The inactivation kinetics was described with the inactivation time constant (τ_i), which was determined upon fitting single-exponential function ($I(t) = I_0 \times e^{-t/\tau_i} + C$, where I_0 : current amplitude, C: steady-state current at the end of depolarization) to the decay part of the current trace.

Immunohistochemistry and Immunofluorescence analysis

Formalin-fixed paraffin embedded (FFPE) and frozen blocks from tumor tissue resected from enrolled HNC patients (n=14) and surrounding normal tissue (n=9) were obtained from the UCCI tumor bank. Six 5 μ m sections per case were provided for immunohistochemical

studies. One slide per case prepared from the tumor and normal tissue FFPE blocks was stained with Hematoxylin-eosin (H&E), reviewed for histopathology by a pathologist affiliated with the UCCI tumor bank and the histopathology report is presented in Supplementary Table S1. Immunohistochemistry was performed on slides from FFPE tumor sections to assess for the presence of CD3⁺ cells in the tumor area and the slides were classified as “well- infiltrated” or “poorly-infiltrated” for each case (see Supplementary Methods).

For immunofluorescence experiments, 5 μ m thick sections prepared from frozen well-infiltrated tumor samples were fixed with pre-cooled acetone and blocked with PBS + 10% FBS. To measure cell infiltration, slides were stained with following primary antibodies: mouse anti-human CD4 (R&D systems) or mouse anti-human CD8 (R&D systems) and guinea pig polyclonal anti-human Kv1.3 (Alomone labs). The sections were washed and incubated with the following secondary antibodies: Alexa-647 anti-mouse and Alexa-555 anti-guinea pig (ThermoFisher Scientific). Slides were then washed and direct-labeled with Alexa 488- mouse anti-human pan cytokeratin (PCK, eBioscience), while the nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI, 1 μ g/ml, ThermoFisher Scientific). To measure proliferation and GrB production, frozen sections were fixed, blocked and stained with the following primary antibodies: mouse anti-human CD8, guinea pig polyclonal anti-human Kv1.3, rabbit monoclonal Ki-67 (Invitrogen). The sections were incubated with the following secondary antibodies: BV-480 anti-rabbit (BD Biosciences), Alexa-647 anti-mouse and Alexa-555 anti-guinea pig. Slides were then washed and direct labeled with Alexa-488 mouse anti-human PCK, and Alexa-405 mouse anti-human Granzyme B (Novus Biologicals). Confocal microscopy was performed on a Zeiss 710 laser scanning confocal microscope (Zeiss GmbH) using a 40 $\times$ water immersion lens at room temperature, the pinhole was set at 1 airy unit. Data were obtained using the “Multi Track” option of the microscope to exclude the cross-talk between the channels and images were acquired with the Zeiss Zen Image browser. The specificity of the polyclonal Kv1.3 antibody was determined by peptide adsorption studies (Supplementary Fig. S1). Staining was also performed with addition of secondary antibodies only and slides were imaged to assess the background fluorescence (Supplementary Fig. S2–S3).

Image analysis

Images were analyzed using ImageJ software (NIH, Bethesda). Briefly, in each field, a region of interest (ROI) was drawn around individual CD8⁺ or CD4⁺ cells. In the same ROI, the mean fluorescence intensities (MFI) were measured for Kv1.3 and either Ki-67 or GrB channels (at least 10 fields for each patient). For each individual, the Kv1.3 fluorescence intensities were sorted by values from low to high and the median value was determined. Values above the median were considered as “Kv1.3^{high}”, while those below the median were “Kv1.3^{low}” (Supplementary Fig. S4). Thus, for each individual, we classified the CD8⁺ cells as being either Kv1.3^{high} or Kv1.3^{low}. Similarly, “high” or “low” MFI values were determined for Ki-67 and GrB. We then calculated the numbers of Ki-67^{high} or GrB^{high} cells within the Kv1.3^{high} and Kv1.3^{low} populations for every case. For measuring the infiltration, for each microscopic field, areas of tumor and stroma were defined based on PCK fluorescence. CD8⁺ or CD4⁺ cells within the tumor and stroma were then manually counted

for a minimum of 10 fields. The percentages of Kv1.3^{high} cells were calculated within the tumor and stroma for each patient.

Statistical analysis

All data are presented as mean $\pm$ SEM, number of subjects or cells are indicated. Statistical analysis was performed using Student's t-test (paired or unpaired) with SigmaPlot (Systat Software Inc), p value of less than or equal to 0.05 was considered as statistically significant.

Results

Kv1.3 channels and Ca²⁺ fluxes are reduced in tumor infiltrating lymphocytes of HNC patients

The TME in solid tumors is characterized by the infiltration of T lymphocytes, and the composition of this T cell population along with its functional state can determine the outcome of the anti-tumor immune response of the individual (3, 8, 25). TILs isolated from HNC tumors were assessed for their phenotype, purity and viability by flow cytometry. The TILs were 99% viable (Supplementary Fig. S5) and predominantly CD3⁺ (91 $\pm$ 2% n= 3). 56 $\pm$ 4% of the CD3⁺ TIL were CD4⁺ while 35 $\pm$ 3% cells were CD8⁺ (n=3). The CD4⁺ TILs were 25 $\pm$ 4% CD4⁺CD25⁺CD127⁻ T_{reg} cells (n=3), while 82 $\pm$ 6% of the CD4⁺ TIL were CD4⁺CD45RO⁺CCR7⁻ T_{EM} cells (n=3) (3). The functionality of T cells depends on the intracellular Ca²⁺ signaling that is regulated by the activity of Kv1.3 channels (26). So far, the functional status and expression of Kv1.3 channels in T cells within the tumor and in the peripheral blood of cancer patients are not fully understood. Hence, we measured activity of Kv1.3 channels in TILs by electrophysiology and compared it to that of peripheral blood T cells (PBTs) from HNC patients (cPBTs) and healthy donors (hPBTs). We observed no differences in Kv1.3 activity in cPBTs and hPBTs, but the currents were highly suppressed in TILs as compared to those in cPBTs from the same individual (Fig. 1A). TILs also had a smaller capacitance than cPBTs (1.4 $\pm$ 0.2 pF, n=18, in TILs vs 1.8 $\pm$ 0.1 pF, n=34, in cPBTs; p=0.018) perhaps indicative of a reduced activation state (the cell capacitance is a measure of the cell size). There was no difference in cell capacitance between cPBTs and hPBTs (1.4 $\pm$ 0.1 pF, n=11, in hPBTs, p=0.24). The Kv1.3 currents in all cell types displayed C-type inactivation (characteristic of Kv1.3) and there were no differences in inactivation kinetics (463 $\pm$ 38 ms, n=9, in TILs vs 375 $\pm$ 18 ms, n=27, in cPBTs and 392 $\pm$ 38 ms, n=11, in hPBTs). On average, the Kv1.3 current density (number of channels/membrane unit surface) was the same in PBTs from healthy donors and patients, but TILs have a significantly lower number of channels (Fig. 1B). Moreover, several lymphocytes did not have detectable Kv1.3 currents (8.8 % and 33.3 % of cPBTs and TILs, respectively).

The decrease in Kv1.3 activity leads to a reduction in the Ca²⁺ influx in T cells (16, 18, 23, 26). Thus, we measured the store-operated Ca²⁺-response of hPBTs and cPBTs, and TILs. Representative recordings of intracellular Ca²⁺ levels in cPBTs and TILs from the same individual are shown in Fig. 1C: using the Ca²⁺ add-back method, the endoplasmic reticulum (ER) Ca²⁺-stores are emptied with TG (a SERCA pump inhibitor) in Ca²⁺ free medium (23, 27), which leads to the opening of the CRAC channels and influx of Ca²⁺ upon

re-addition of extracellular Ca^{2+} (Fig. 1C). Changes in intracellular Ca^{2+} concentration upon re-introduction of extracellular Ca^{2+} are TCR-independent. These recordings show that there was no change in the amount and kinetics of the Ca^{2+} release from the ER in cPBTs and TILs, but the influx of Ca^{2+} is reduced. The peak Ca^{2+} levels achieved during Ca^{2+} influx were significantly reduced by 32%, from 4.3 ± 0.5 fold-changes in intracellular Ca^{2+} concentration from the baseline value in cPBTs to 2.9 ± 0.2 in TILs ($n=4$, $p=0.04$; Fig. 1D). We also measured Ca^{2+} fluxes in hPBTs from three age-matched healthy donors and obtained a Ca^{2+} -fold change of 4.9 ± 1.1 , which was the same as for cPBTs ($p=0.64$, Fig. 1D). There was no change in the kinetics of peak Ca^{2+} decay indicating that there are no differences in Ca^{2+} re-uptake into the stores or efflux through the plasma membrane Ca^{2+} ATPase (PMCA) (data not shown).

These data show that the Kv1.3 channels expression and function is significantly attenuated in HNC TILs, which contribute to suppression of Ca^{2+} signaling. Still there is a variability of expression of Kv1.3 in TILs that warranted their further investigation (Fig. 1B). We thus determined the spatial distribution of TILs with variable Kv1.3 expression levels (and thus different Ca^{2+} handling capabilities) in relation to their proximity to cancer cells, as this would define, for CD8⁺ T-cells, the efficacy of the anti-tumor immune response.

CD4⁺ and CD8⁺ TILs preferentially localize in the tumor stroma

Histopathological analysis of the H&E stained tumor sections of 14 HNC patients revealed the presence of SCC tumor cells in 13 out of 14 sections (HDN 1036 sections had 0% tumor cells, Supplementary Table S1). One case (HDN 684) was excluded from subsequent analysis as the histopathology diagnosis of “squamous cell carcinoma with sarcomatoid features” (Supplementary Table S1) did not fit our inclusion criteria. IHC staining with anti-CD3 antibody was performed on the remaining 12 cases to determine TIL infiltration. FFPE sections from normal tissue resected adjacent to the tumor were stained as negative controls ($n=9$ cases), while sections from tonsil were stained as positive control (Supplementary Fig. S6). We observed that 9 out of the total 12 cases had “well-infiltrated” tumors which were further used for immunofluorescence (IF) studies, whereas 3 cases were “poorly infiltrated” (Supplementary Fig. S6) and therefore excluded from further experiments (Table 1).

To determine the tumor infiltration by CD4⁺ and CD8⁺ TILs, slides from well-infiltrated HNC cases were stained with anti-PCK (tumor cell marker), anti-Kv1.3 and either anti-CD8 (Fig. 2A) or anti-CD4 antibodies (Fig. 2B). Intratumoral and stromal areas were defined based on PCK staining. CD4⁺ or CD8⁺ TILs that were present in the tissue areas showing PCK staining were regarded as “intratumoral lymphocytes” (iTILs), whereas the rest of CD4⁺ or CD8⁺ TILs were defined as “stroma infiltrating lymphocytes” (sTILs). As shown in Fig. 2C and 2D, a higher number of CD8⁺ TILs ($p=0.05$, $n=9$ cases, Fig. 2C) and CD4⁺ TILs ($p<0.001$, $n=8$ cases Fig. 2D) are present in the tumor stroma compared to the tumor epithelium. Still, the functional status of TILs in the two compartments is not known.

Kv1.3 expression is lower in the intratumoral CD8⁺ but not in CD4⁺ TILs

As described above, electrophysiological experiments showed decreased Kv1.3 channel activity in TILs compared to cPBTs. These data revealed a widespread distribution in

channel density among the TIL population (Fig. 1B). IF was performed to determine whether sTILs and iTILs have different Kv1.3 expressions. We quantitated the Kv1.3 fluorescence intensity in the iTILs and sTILs and determined the percentage of Kv1.3^{high} CD4⁺ and CD8⁺ iTILs and sTILs (for definition of Kv1.3^{high} or Kv1.3^{low} TILs, see Materials and Methods and Supplementary Fig. S4). We found that CD8⁺ iTILs have lower Kv1.3 expression than sTILs ($p=0.02$, $n=9$ cases, Fig. 2E), whereas we did not observe any difference in Kv1.3 levels between the iTILs and sTILs in the CD4⁺ population ($p=0.080$, $n=8$ cases, Fig. 2F). These data indicate that CD8⁺ TILs not only fail to enter the tumor but also have reduced Kv1.3 expression. Since Kv1.3 channels control effector functions such as cytokine and GrB production, we determined whether the downregulation of Kv1.3 expression in CD8⁺ iTILs was associated with decreased effector functions.

Low Kv1.3 expression in CD8⁺ TILs is associated with low levels of Ki-67 and granzyme B

Tumor cells can be eliminated via secretion of GrB by cytotoxic CD8⁺ lymphocytes, which induces apoptosis in tumor cells (28). The GrB production and secretion depends on the Ca²⁺-signaling, which is regulated by Kv1.3 channels (19). Furthermore, Kv1.3 channels are responsible for the activation and proliferation of T cells (16, 20). To establish whether the decreased Kv1.3 expression in CD8⁺ TILs results in a suppression of activation and GrB production, we stained tissue sections from the 9 well-infiltrated HNC cases with DAPI and anti-PCK, anti-CD8, anti-Kv1.3, anti-GrB and anti-Ki67 (proliferation marker) antibodies (Fig. 3). CD8⁺ TILs were classified as either Kv1.3^{high} or Kv1.3^{low} based on the median Kv1.3 fluorescence. In these populations, the percentage of Ki-67^{high} and GrB^{high} cells (defined by the median Ki-67 and GrB fluorescence) were also calculated. We observed in all of the 9 patients that the percentage of proliferating (Ki67^{high}) cells is higher in Kv1.3^{high} as compared to Kv1.3^{low} CD8⁺ TILs ($p<0.001$, $n=9$ donors, Fig. 3B), whereas in 8 out of 9 cases, we observed that GrB level was lower in Kv1.3^{low} CD8⁺ TILs as compared to Kv1.3^{high} CD8⁺ TILs (Fig. 3C, $p=0.0505$ $n=9$; $p=0.00221$, $n=8$ without the “outlier” HDN1358). Overall these data indicate that the Kv1.3 expression in CD8⁺ cells is associated with a more active state, but unfortunately, these more active CD8⁺ cells preferentially localize in the stroma.

Discussion

Although controversy still exists as to the role that T-cell infiltrates play in cancer, it is generally accepted that a high number of cytotoxic and helper Th1 T cells in the tumors is of good prognostic value (8, 9, 29, 30). Yet, other features such as the location of TILs within the tumor and their functional state are crucial for TIL effectiveness in attacking and destroying cancer cells (8, 31, 32). Herein we have presented evidence that HNC TILs have decreased Kv1.3 function and reduced Ca²⁺ signaling compared to circulating T cells. Furthermore, CD8⁺ and CD4⁺ TILs preferentially localize in the tumor stroma as opposed to the tumor epithelium, and intratumoral CD8⁺ TILs have lower Kv1.3 expression which is indicative of reduced proliferative and cytotoxic abilities. Overall the data we have presented point to Kv1.3 channels in T lymphocytes as possible markers of the functionality of cytotoxic TILs in HNC.

The importance of Kv1.3 channels in T cell function is well established. Kv1.3 channels set the membrane potential and control the influx of Ca^{2+} through store-operated CRAC channels (16–18). In the current study, we show that Kv1.3 channels are suppressed in TILs of HNC patients and this defect contributes to their functional incompetence. The reduced ability of TILs to secrete cytokines and kill cancer cells is well-documented and here, we have provided a possible mechanism contributing to these deficiencies (3, 7). While TILs have reduced Kv1.3 currents compared to cPBTs, we observed that Kv1.3 currents in cPBTs are comparable to those recorded in age-matched hPBTs. This indicates no intrinsic defects of Kv1.3 in circulating T cells of HNC cancer patients although it has been reported that some of the defects of TILs, like increased apoptosis and reduced proliferation in response to anti-CD3 antibody, are also present in cPBTs (3). While there are no defects in Kv1.3 channels in resting/quiescent cPBT cells, we cannot exclude the possibility that cPBTs may fail to upregulate Kv1.3 upon activation or that signaling pathways that control Kv1.3 function upon activation may be altered in these cells (33). We observed not only a lower Kv1.3 channel density but also a significant fraction of TILs have no detectable Kv1.3 currents as compared to cPBTs. As there are no changes in current kinetics, it appears that there is a decrease in number of functional Kv1.3 channels. The reduction of Kv1.3 channels exclusively in TILs points to the TME as the culprit. Diminished viability due to the isolation process cannot be claimed responsible as the flow cytometry data indicated that TILs were 99% viable. Indeed, there are immune suppressive features of the TME that can influence the activity and expression of Kv1.3. Hypoxia reduced the activity and expression of Kv1.3 (20, 34). Other factors such as low pH in the TME may have the ability to inhibit Kv1.3 (35). Nevertheless, our data clearly indicate a reduction in Kv1.3 in TILs, which has repercussions on Ca^{2+} signaling. Furthermore, Kv1.3 downregulation could have an effect on the accumulation of intracellular K^+ that has been recently associated to the suppressed function of TILs in necrotic areas of the tumor (36). Interestingly, this study also reported that overexpressing Kv1.3 channels reduced tumor burden and improved survival of tumor bearing mice, thus underscoring the importance of these channels in tumor clearance and their therapeutic potentials (36).

TILs have reduced Ca^{2+} fluxes compared to cPBTs (which have Ca^{2+} fluxes similar to hPBTs). While we observed no differences in intracellular Ca^{2+} stores, the Ca^{2+} influx through the CRAC channels was decreased in TILs. The decreased intracellular Ca^{2+} levels of TILs observed is in agreement with what has been reported previously both in mice and humans: TILs have lower Ca^{2+} influx and consequently a remarkable decrease in $\text{IFN}\gamma$ production upon antigen stimulation (11, 37–39). In parallel with these findings, our data indicate that the defective Ca^{2+} influx in TILs can be attributed to the suppression of Kv1.3 channels. In contrast to what others have previously reported, we have conducted experiments using an experimental protocol that allows bypassing the TCR and measuring only the component of Ca^{2+} influx that depends on ion channels, transporters and pumps, thus providing the mechanistic evidence that the decrease in Ca^{2+} in TILs is, at least in part, is due to reduced Kv1.3 channel expression (22, 27). We cannot exclude the possibility that changes in other ionic pathways may contribute to the reduction in Ca^{2+} influx in TILs, but a previous study from our laboratory showed that a 70% reduction in Kv1.3 activity results in a 40% reduction in Ca^{2+} influx in PBTs, which coincides with our outcomes in TILs (23).

To further support our conclusion, it has been reported that TILs produce significantly higher IFN γ level when an increase in Ca²⁺ influx was induced by activating them with PMA and ionomycin, i.e. the restoration of an appropriate Ca²⁺ response corrected the functional defects of TILs (11). Furthermore, TILs were able to recover a Ca²⁺ response to TCR stimulation with time in culture indicating that disruption of the TME is sufficient to restore the functionality of TILs (37).

We have presented evidence that TILs are functionally impaired because of a defect in Kv1.3 channels. Yet, there is variability in Kv1.3 channel expression in TILs (in Fig. 1B it can be noted that one of the TILs has conserved the Kv1.3 current density of PBTs) that suggests a certain degree of functional variability. The anti-tumor importance of TILs depends on the number of T cells that infiltrate the tumor, their phenotype, where they localize within the tumor and whether they maintain a certain degree of functionality (measured by Kv1.3 expression). High levels of CD3⁺ T cell infiltration have been associated with positive prognostic outcomes in patients with HNC and many other solid malignancies (29, 30). Furthermore, the location within the tumor appears to be important in the resolution of the disease (40). HNC patients with high levels of CD3⁺ T lymphocytes in the proximity of malignant cells had an improved disease outcome whereas robust CD3⁺ T cell infiltration of the tumor stroma or periphery failed to have a prognostic value (40). We observed that both CD4⁺ and CD8⁺ TILs localize more in the stroma than in the tumor epithelia. This suggests that “forces” are in place to “trap” T cells in the stroma or “repel” T cells from the tumor epithelia. We also showed that the CD8⁺ TILs that enter the tumor mass have lower Kv1.3 expression than those that stay in the stroma. This suggests that, not only fewer cytotoxic cells come in contact with tumor cells, but also that they are functionally more incompetent than those located in the stroma. This is not true for CD4⁺ TILs whose Kv1.3 expression is not different regardless of their location within the tumor. The Kv1.3 expression in CD4⁺ iTILs is very variable which might reflect the heterogeneity of this cell population. Our findings show that 25% of the CD4⁺ TILs are T_{reg}. This high percentage of T_{reg} is in agreement with percentage reported before in HNC (3, 41).

Ki-67 and GrB are markers of CD8⁺ cell functionality, which are under the control of Kv1.3-regulated Ca²⁺ signaling and NFAT activity (19, 22, 23, 26). In cohorts of renal cell carcinoma, increased amounts of Ki-67 are associated with improved patient outcomes (29). One study in HNC patients reported an increased infiltration of CD8⁺GrB⁺ cells in the tumors but the infiltration had a limited effect on the patient outcomes (42). In patients with colorectal and ovarian carcinomas, the presence of increased number of CD8⁺GrB⁺ cells is associated with better survival rates (5, 29). Here we described that CD8⁺ TILs with higher Kv1.3 expression have higher GrB and proliferative capacities. Unfortunately, Kv1.3^{high} CD8⁺ TILs localize in the stroma more than in the tumor, which may be predictive for the recovery from HNC. Further testing of large cohort of patients are necessary to assess any prognostic clinical value to these findings.

Overall our data showed that suppression of Kv1.3 channels in TILs contributes to reduction in Ca²⁺ signaling, and decreased effector function of CD8⁺ cells thus raising the possibility that this channel may be used as a potential marker of functionally competent TILs in cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Kv1.3 expression and Ca²⁺ signaling are impaired in TILs

(A) Representative Kv1.3 currents recorded in whole-cell voltage clamp configuration upon a 50 mV depolarization step in a cPBT and a TIL isolated from the same patient. (B) Individual (empty circle) and average (triangle) Kv1.3 current density in hPBTs, (n=11 cells, 2 cases), cPBTs (n=34, 6 cases) and TILs (n=18, 4 cases) isolated from the same donor. Error bar shows SEM. (C) Typical Ca²⁺-response (shown as a ratio of Indo-1 fluorescence at 400 nm and 480 nm) recorded in cPBTs and TILs isolated from the same HNC patient. Cells were loaded with Indo-1 ratiometric dye and the fluorescence was recorded by flow cytometry (see Materials and Methods). (D) The average change of the peak Ca²⁺ level as expressed with the Ca²⁺-fold change (n=4 cases). Error bars represent SEM.

Fig. 2. Decreased Kv1.3 expression in intratumoral CD8⁺ but not CD4⁺ TILs

Representative confocal images of frozen HNC tumor sections stained for pan cytokeratin (tumor cell marker, PCK, yellow), nuclei (DAPI, blue), Kv1.3 channels (red) and CD8 (green, A) or CD4 (green, B). (C,D) Percentage of intratumoral lymphocytes (iTIL) and stroma infiltrating lymphocytes (sTIL) for HNC tumor sections stained for CD8⁺ TILs (C) (n= 9 cases, 10 fields/case) and CD4⁺ TILs (D) (n= 8 cases, 10 fields/case). Black full triangles represent the mean, and error bars the SEM for each group. (E,F) Percentage of CD8⁺ (E, n= 9 cases, 10 fields/case) and CD4⁺ (F, n= 8 cases, 10 fields/case) iTILs and sTILs with high (Kv1.3^{high}) and low (Kv1.3^{low}) Kv1.3 expression. For definition of “Kv1.3^{high}” cell, see Materials and Methods and Supplemental Fig. S4. The solid black triangles represent the mean for each population, error bars indicate SEM.

Fig. 3. Low Kv1.3 expression in CD8⁺ TILs is associated with low levels of Ki-67 and granzyme B
 (A) Representative confocal images of frozen HNC tumor sections from a single patient stained for cytokeratin (tumor cell marker, PCK, yellow), CD8 (green), Granzyme B (GrB, blue), Ki-67 (cell proliferation marker, magenta) and Kv1.3 (red). (B) Percentage of Ki-67 high cells (proliferating cells) in CD8⁺ TILs with low and high Kv1.3 expression. (C) Percentage of high GrB expressing cells in Kv1.3^{high} and Kv1.3^{low} CD8⁺ TILs. For (B) and (C), the solid black triangles represent the mean, while the error bars represent the SEM. Sections from 9 HNC patients were stained for Ki-67 and GrB, 10 fields were analyzed/case.

Table 1

Clinicopathological features of individual HNC cases (n=14)

#	Case ID	Age	Gender	Location of Squamous cell carcinoma	Pathological Stage	p16 status	CD3 ⁺ , infiltration
1	HDN 684	60	Male	Parotid	pT3N2bMx	+	N/A
2	HDN 731	68	Male	Oral cavity	rpT4N2BMx	N/A	Poor
3	HDN 733	65	Female	Oral cavity	pT3N0Mx	N/A	Good
4	HDN 741	54	Male	Larynx	pT4aN2cMx	N/A	Good
5	HDN 753	60	Male	Simonasal	pT4bN2bMx	+	Poor
6	HDN 972	50	Male	Oral Cavity	pT4aN2bMx	–	Good
7	HDN 974	58	Male	Cutaneous	pT3N0Mx	N/A	Good
8	HDN 1036	77	Male	Oral Cavity	pT4aN0Mx	N/A	N/A
9	HDN 1056	59	Male	Larynx	pT3N0Mx	N/A	Good
10	HDN 1260	64	Male	Oral Cavity	pT4aN0Mx	N/A	Poor
11	HDN 1331	74	Male	Oral Cavity	pT2N1Mx	–	Good
12	HDN 1351	72	Male	Parotid	Not given	N/A	Good
13	HDN 1358	49	Male	Hypopharynx	pT3N0Mx	N/A	Good
14	HDN 1459	62	Male	Oropharynx	pT1N0Mx	+	Good

Footnotes: N/A = Not available.

Table 2

Clinical data of HNC patients (n=14 cases)

#	Case ID	Survival	Adjuvant Radiation (Y/N)	Adjuvant Chemo (Y/N)	From Pathology Report Related to Surgical Resection				Perineural invasion (Y/N)	Lymphovascular invasion (Y/N)
1	HDN 684	Unknown	Y	Y	Extra-capsular spread (Y/N)	Positive Surgical Margins (Y/N)			N	Indeterminate
2	HDN731	Unknown	Y	N	Y	Y			Y	Y
3	HDN733	Alive	N	N	N	N			Y	N
4	HDN741	Unknown	Y	Y	N	N			N	N
5	HDN753	Deceased	Y	Y	Y	Y			N	Y
6	HDN972	Alive	Y	Y	Y	N			N	Indeterminate
7	HDN974	Alive	N	N	N	Y			N	N
8	HDN1036	Alive	Y	N	N	N			N	N
9	HDN1056	Alive	Y	N	N	N			N	Indeterminate
10	HDN1260	Alive	Y	N	N	N			Y	N
11	HDN1331	Unknown	Y	N	N	N			N	N
12	HDN1351	Alive	Y	N	N	N			N	N
13	HDN1358	Alive	Y	Y	N	Y			N	N
14	HDN1459	Alive	N	N	N	N			N	N

Footnotes: Unknown = lost to follow-up