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Brief Report

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LAG3 and TIGIT Expression on Tumor-Infiltrating Lymphocytes in Cutaneous Melanoma

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Keywords
Melanoma · Tumor-infiltrating lymphocytes · Immune checkpoint proteins · Multiplex immunofluorescence staining · Spatial gene expression pattern

Abstract

Background: Melanoma is widely recognized to be an immunogenic tumor that often contains tumor-infiltrating lymphocytes (TILs) in the tumor microenvironment. During cancer progression, expression of ligands that bind immune checkpoint (IC) proteins, such as PD-1, expressed on the surface of TILs, hinder them from exerting their antitumor functions. TILs consist of a heterogenous group of immune cells and their presence is associated with an improved overall survival in melanoma patients. Introduction of IC inhibitors has revolutionized management and prognosis of advanced melanoma. Unfortunately, the response rates have continued to be limited, resulting in growing interest in characterizing novel IC proteins, and developing combination therapy that includes inhibitors against multiple IC proteins. ***Methods:*** In a regional cohort of 166 patients diagnosed with cutaneous superficial spreading melanoma with different degree of TILs, we investigated the tumor immune-associated gene expression profile using NanoString Technology. We used multiplex immunofluorescence (mIF) staining in a subset of tumors (N = 7), combining IC proteins T-cell immunoglobulin and ITIM domain (TIGIT) and LAG3 with a melanoma cell marker (SOX10) and immune cell markers (CD8 [cytotoxic T cells], CD4 [T helper cells], FOXP3 [regulatory T cells/Tregs], PAX5 [B cells], and CD56 [NK/NKT cells]) and IC protein PD-1. ***Results:*** We found upregulation of 91 differentially expressed genes, including IC proteins, LAG3 and TIGIT in melanomas with brisk TILs compared to tumors where TILs were absent. mIF staining revealed LAG3 and TIGIT expression in the majority of CD8+ T cells. Only few Tregs and CD4+ T cells expressed LAG3, whereas majority of them expressed TIGIT. LAG3 and TIGIT were expressed in a small fraction of the NK/NKT cells and lacked in the B cells. The majority of PD-1+ cells co-localized with LAG3 and TIGIT. ***Conclusion:*** We report a variable expression of LAG3 and

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TIGIT on TILs subtypes and a coeval occurrence with PD-1. This knowledge places LAG3 and TIGIT in spatial and cellular context in melanoma. The data suggest that targeting multiple IC proteins might help overcome the current challenges with IC therapies.

Introduction

Melanoma is widely recognized to be an immunogenic tumor that often contains tumor-infiltrating lymphocytes (TILs) in the tumor microenvironment (TME). TILs consist of a heterogenous group of immune cells and their presence is associated with an improved overall survival in melanoma patients. However, during cancer progression, TILs express ligands that bind immune checkpoint (IC) proteins, which hinder them from exerting their antitumor functions. Therefore, introduction of IC inhibitors that block these interactions and re-establish TIL antitumor activity has revolutionized management and prognosis of advanced melanoma with dramatic improvements in outcomes for a subset of patients. Unfortunately, the limited clinical efficacy and the emergence of resistance to therapy highlight the need for new targets, and discovery of additional IC proteins has the potential to expand our ability to treat advanced melanoma. Several reports, including machine learning studies, have revealed that clinicopathological and molecular biomarkers may have a role in predicting prognosis as well as invasiveness in melanoma [1–3].

High expression level (IC) proteins such as programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) have emerged as markers of T-cell dysfunction. Co-expression of newer IC proteins, such as lymphocyte-activation gene 3 (LAG3) and T-cell immunoglobulin and ITIM domain (TIGIT) with PD-1 has been reported. These cells mark dysfunctional T cells with decreased cytokine production and degranulation capacities [4–6]. Co-expression of IC proteins on T cells correlates positively with progressive dysfunction, supporting the rationale behind combining TIGIT or LAG3 inhibitor with a PD-1/PD-L1 inhibitor in order to restore additional effector functions of T cells [7, 8]. Previous studies in melanoma have primarily investigated LAG3 and TIGIT expression on immune cells by flow cytometry which lacks information about the spatial context in tissue [9–14]. In the current study, we used a previously collected cohort of melanomas to evaluate the TILs infiltration in the tumors. We investigated the immune-related gene expression and performed multiplex immunofluorescence (mIF) stainings to characterize the expression pattern of LAG3 and TIGIT on TILs subtypes and to study the co-expression with PD-1 in situ for the first time in human skin melanoma tissue.

Materials and Methods

Patients

The melanoma cohort was described in detail in recently published study [15]. Briefly, the cohort comprises 166 cutaneous superficial spreading melanoma (SSM) diagnosed in Region Zealand, Denmark, between 1995 and 2015, and includes tumors with Breslow thickness <1.0 mm or >4.0 mm (without presence of regression or ulceration). TILs were scored as absent, nonbrisk (infiltrating focally), and brisk (infiltrating the entire tumor or entire base of the tumor). Extensive clinical and histopathological data were retrieved for each patient, including progression and death. The study was approved by the Regional Ethics Committee (SJ-742) and the Danish Data Protection Agency (REG-066-2019).

Gene Expression Studies Using NanoString nCounter Platform

RNA isolation from entire tissue sections, RNA quantification, and gene expression analysis in this cohort has been previously described [15]. We used the NanoString nCounter™ gene expression platform consisting of sample preparation station, digital analyzer and nSolver™ 4.0 software (NanoString, Seattle, WA, USA) to analyze 770 genes in the PanCancer IO 360™ panel. This panel provides a comprehensive overview of gene expression related to tumor, microenvironment and immune response. In addition, 20 custom genes of interest were added to the panel (online suppl. Table S1; for all online suppl. material, see https://doi.org/10.1159/000533932).

Data and Statistical Analysis

T test was used to identify differentially expressed genes (DEG). Two-fold change, $p < 0.05$ and $q < 0.05$ were used as cut-off. Qlucore Omics Explorer v. 3.5 (Qlucore AB, Lund, Sweden) was used to analyze and visualize data, by applying heatmaps and semi-unsupervised 2-way hierarchical clustering. Smaller heatmaps were also made in Morpheus (https://software.broadinstitute.org/morpheus). In order to identify biological pathways involved in the differentiation of the groups, we conducted Kyuto Encyclopedia of Genes and Genomes (KEGG) pathway analysis in Enricher (https://maayanlab.cloud/Enrichr/enrich#).

Immunohistochemical Staining

Formalin-fixed paraffin-embedded sections (3 μm) were stained on the fully automated Ventana discovery (Roche Diagnostics, Basel, Switzerland/Ventana, AZ, USA). Sections were deparaffinized, exposed to antigen retrieval using Cell Conditioning Solution, CC1 pH 8.5 (Roche Diagnostics, cat #950-500) and heated for 48 min at 100°C. Slides were incubated with rabbit anti-LAG3 (clone D2G40, dilution 1:50, Cell signaling, Danvers, MA, USA, cat # 15372) and rabbit anti-TIGIT (clone E5Y1W, dilution 1:25, Cell signaling, cat# 99567) for 32 min at 36°C. Antibodies were diluted in Dako diluent (Agilent, Santa Clara, CA, US, cat #S2022). After washing and blocking of endogenous peroxidase activity, the reactions were detected using Goat anti-

rabbit-HQ/anti-HQ (Roche Diagnostics, cat #760-4815/760-4820) and visualized using ChromoMap DAB Kit (Roche Diagnostics, cat #760-159) according to the manufacturer's instructions. Finally, slides were counterstained with Hematoxylin II (Roche Diagnostics, cat #790-2208) and mounted with Pertex.

mIF Staining

For subtyping of TIGIT and LAG3 positive cells, triple immunofluorescence staining was applied on seven cases (two with nonbrisk and five with brisk TILs) using the Ventana Discovery Ultra platform (Roche Diagnostics/Ventana). TIGIT- and LAG3-specific antibodies were used in combination with SOX10 and either CD8, CD4, FOXP3, PAX5, CD56, or PD-1 antibodies.

Briefly, sections were deparaffinized and subjected to heat-induced epitope retrieval in Discovery Cell Conditioning 1 reagent (pH 8.5) (Ventana, #950-500) for 48 min at 95°C, followed by quenching of endogenous peroxidase activity using Discovery Inhibitor (Ventana Medical Systems, #760-4840) for 8 min (36°C). Three-plex panel mIF was performed using the following antibodies: SOX10 (clone BS7, dilution 1:1,000, Nordic Biosite, Täby, Sweden, #BSH-7959-1), TIGIT (clone E5YIW, dilution 1:15, Cell Signaling Technology, Danvers, MA, USA, #99567S), LAG3 (clone D2G40, dilution 1:50, Cell Signaling technology, #153725), CD8 (clone C8/144B, dilution 1:600, Agilent/DAKO, Glostrup, Denmark, #M7103), CD4 (clone EP204, dilution 1:25, Cell Marque, Rocklin, CA, USA, #AC-0173), FOXP3 (clone 236A/E7, dilution 1:25, Thermo Fisher, Waltham, MA, US, #14-4777-82), PAX5 (clone DAK-PAX5, dilution 1:25, Agilent/DAKO, #M7307), anti-CD56 (clone MRQ-42, dilution 1:400, Cell Marque, #156*R*-96), PD-1 (clone NAT105, dilution 1:25, Cell Marque, #315M-96). Antibodies were incubated for 32 min at 36°C. For each biomarker, detection was performed using either Omnimap goat anti-mouse horseradish peroxidase (HRP) (Ventana, #760-4310) or Omnimap goat anti-rabbit HRP (Ventana, #760-4311) for 12 min (36°C) and reactions were visualized using Tyramide Signal Amplification (TSA)-conjugated fluorochromes for 8 min (36°C): DCC/Aqua (Ventana Medical systems, #760-240), Red610 (Ventana Medical systems, #760-245), and FAM/Green (Ventana Medical systems, #760-243). Finally, sections were counterstained with DAPI and coverslipped.

The mIF process involved 3 sequential rounds of staining, one for each biomarker. To prevent cross-reactivity between the individual sequences, a neutralization step using Discovery Inhibitor for 20 min (36°C) was inserted between the first and second round and a heat deactivation (HD) step using Cell Conditioning 2 solution (Ventana Medical systems, #950-223) for 8 min (100°C) between the second and third round. SOX10 (DCC/Aqua) was always detected in first round, LAG3 or TIGIT (Red610) in second round and CD8, CD4, FOXP3, PAX5, CD56, or PD-1 (FAM/Green) in the last sequence (online suppl. Table S2).

Nikon Eclipse 80 fluorescence microscope with standard filters for each fluorochrome was used to evaluate the slides. Images were visualized on a live histogram in the image acquisition software (NIS-Elements D3.2) and captured with Nikon camera (DS-5Mc Color Cooled Digital/MQA15000). Photoshop (23.3.1) was used to merge images.

In order to test for potential cross-reactivity between immuno-reagents, we performed two control experiments (online suppl. Table S3). First, a neutralization experiment was conducted to ensure that the enzyme activity of introduced HRP molecules in the prior sequence was completely eliminated. Using the same incubation times/temperatures as described above, we stained for SOX10 (mouse antibody) and detected the reactions with Omnimap goat anti-mouse HRP but omitted the TSA-DCC/Aqua step. After a neutralization step (Discovery Inhibitor), we applied TSA-conjugated fluorochrome Red610 – no nuclear reactions should be seen in neoplastic cells of the melanomas. Second, HD was performed to ensure that there was no cross-reactivity between immuno-reagents. HD controls were performed for each marker in the two first sequences (SOX10, TIGIT, and LAG3) to unravel if cross-reactivity occurs with third round immuno-reagents. Antibodies and immuno-reagents were applied as described above and reactions were detected using either Omnimap goat anti-mouse HRP or Omnimap goat anti-rabbit HRP, depending on the host of the primary antibody and the TSA-DCC/Red610 step was omitted. After a heat deactivation step using Cell Conditioning 2 for 8 min at 100°C, the primary antibody of the next sequence was also omitted followed by detection with either OmniMap goat anti-mouse HRP or OmniMap goat anti-rabbit HRP and TSA-conjugated with the fluorochrome FAM – no nuclear staining (SOX10) should be seen in the neoplastic cells of the melanomas. As expected, no staining for TIGIT or LAG3 was observed.

Results

Our recent analysis of melanoma samples (1–4 mm thickness with no ulceration or regression from the Danish Pathology Register collected during a 20-year period (1995–2015), and the associated clinical data, led to the insight that presence of TILs in the primary tumors significantly improved survival [15]. Here, we expanded that study to characterize the immune response in these tumors. We extracted RNA from all samples in the cohort, which included 166 patients diagnosed with cutaneous SSM, and analyzed the gene expression of 790 immune-associated genes using the NanoString Technology. We compared cases with different immune responses defined by the grade of TILs infiltration (brisk TILs, non-brisk TILs, and absent TILs) (online suppl. Table S4–S6; Fig. S1). The comparative analysis of tumors with brisk infiltrates (N = 47) to those with absent infiltrates (N = 46) revealed 91 DEG (q = 0.05, FC >2) that were upregulated in melanomas with brisk infiltrates (Fig. 1a). Functional enrichment analysis confirmed, as expected, genes involved in the immune system such as cytokine-cytokine receptor interaction and T-cell receptor signaling pathway (Fig. 1b). The DEG also identified IC genes (*CTLA-4*, *PD-1*, *PD-L1*, *BTLA*, and *IDO1*), and characteristic immune cell markers (CD3, CD79A, FOXP3, and CD28), as well as chemokines (CXCL9, CXCL10, and CXCL11) and cytokines (LTB, TNF, and IL-32) (Fig. 1c–e). Additionally, DEG analysis identified KLRD1/CD94, a protein expressed mainly by

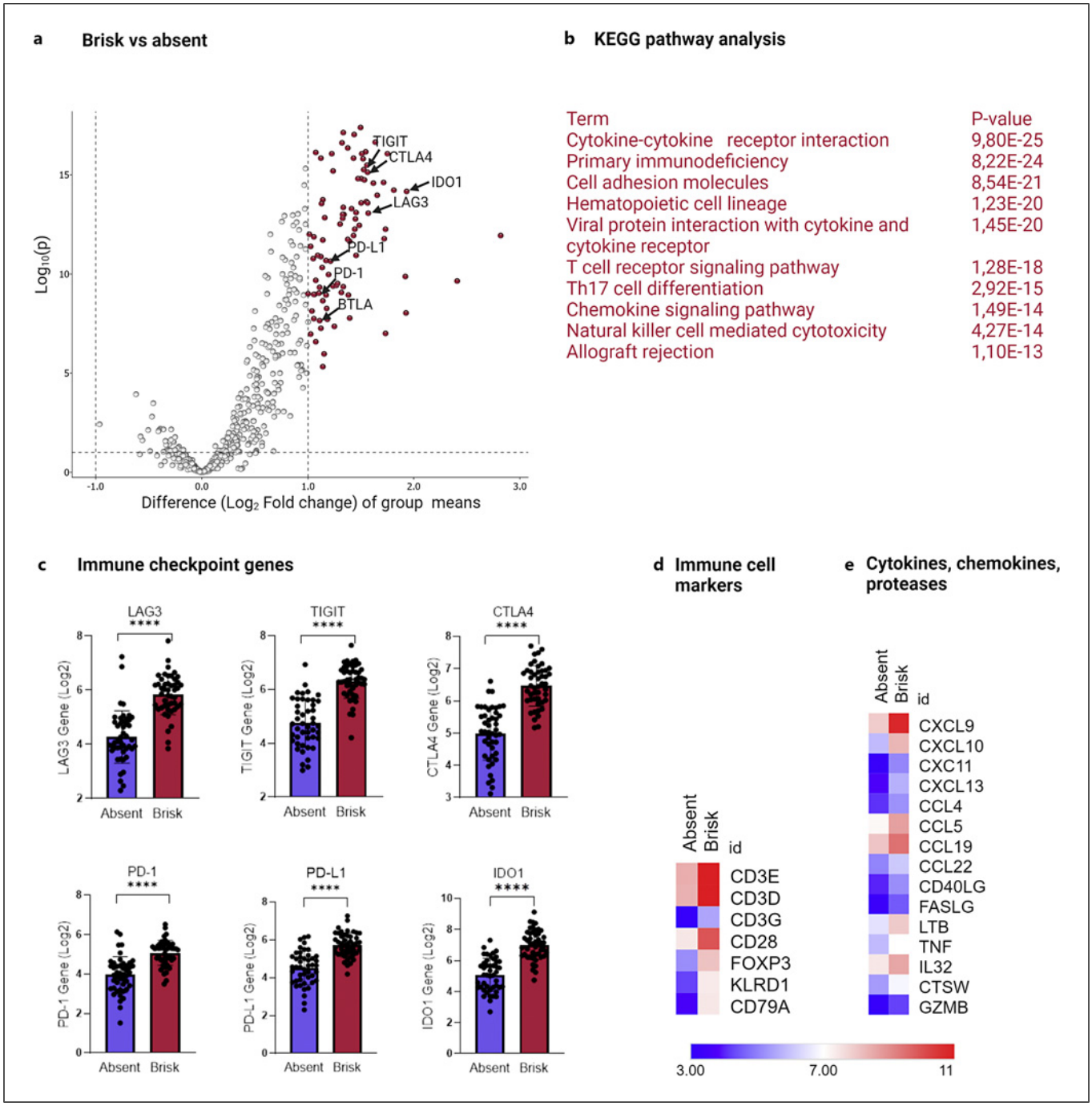

Fig. 1. FGene expression analysis reveals upregulation of genes involved in the immune response. **a** Differential expression analysis of genes between brisk TILs and absent TILs resulted in 91 upregulated genes (q value <0.05, fold change >2. Seven IC genes are highlighted. **b** Functional enrichment with KEGG pathway analysis of DEG identify involvement in various immune-related processes. The KEGG pathway terms are sorted by p value ranking. **c** Expression level of the novel IC genes (*LAG3* and *TIGIT*) and other IC genes (*CTLA4*, *PD-1*, *PD-L1*, and *IDO1*) in brisk TILs compared to absent TILs. **** indicate $p < 0.0001$ by unpaired T test of normalized, log2 transformed mRNA counts. Results are presented as mean ± SD. **d**, **e** The heatmaps show the mean normalized count of immune cell-specific genes and cytokines, chemokines, and proteases that were differentially expressed between brisk TILS and absent TILs. The gene expression values are indicated on the color bar.

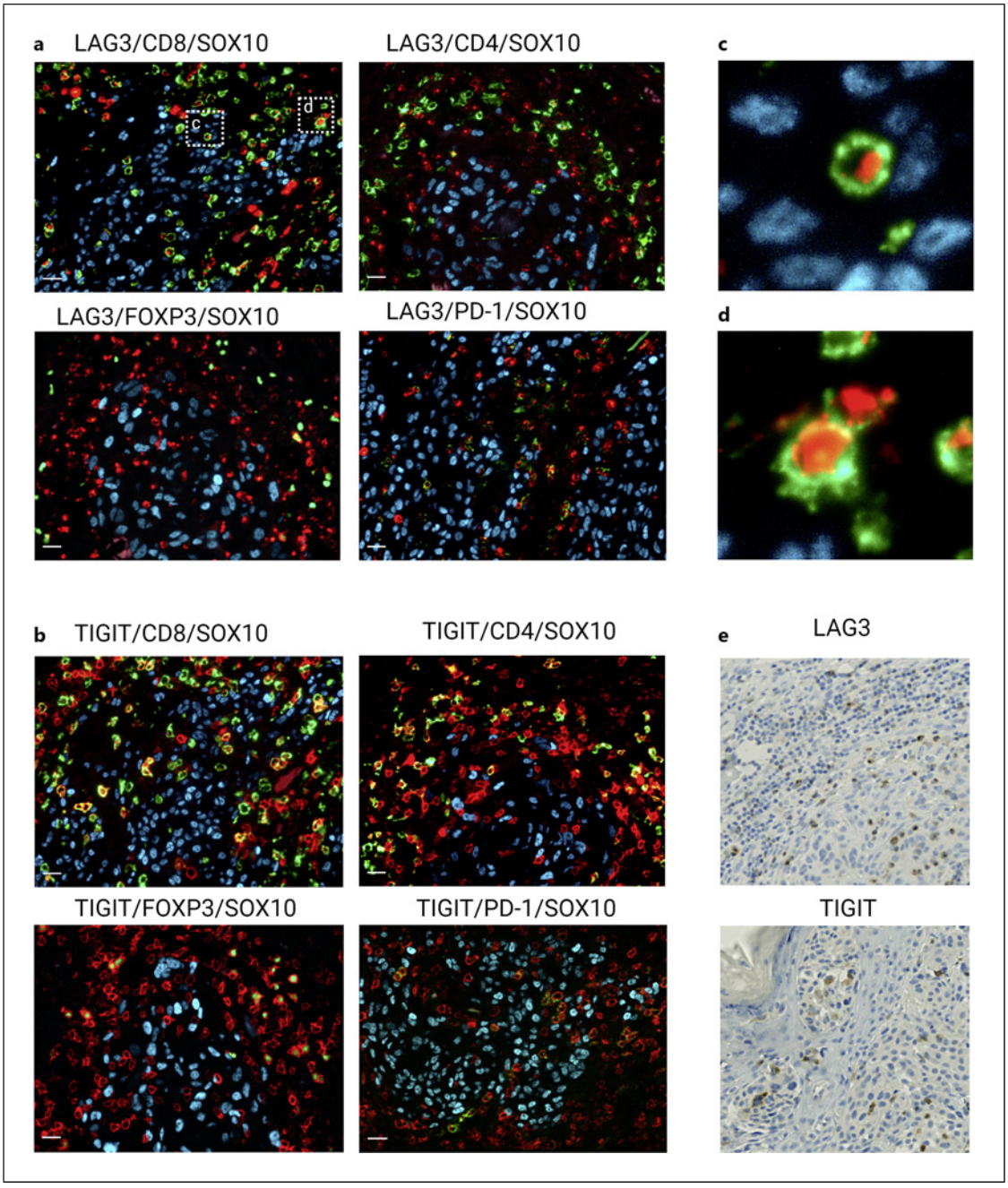

Fig. 2. Multiplex immunofluorescence (mIF) staining displays the expression of LAG3 and TIGIT on different subtypes of immune cells (CD8+, CD4+, FOXP3+) and on PD-1+ cells. The red staining represents TIGIT or LAG3, the green staining highlights the different immune cells or PD-1+ cells and aqua staining (SOX10) label the melanoma cells. Although LAG3 and TIGIT are primarily considered to be transmembrane proteins, a substantial proportion of both are found intracellularly by mIF. **a**, **b** LAG3 and TIGIT are expressed on a large fraction of CD8+ T cells. A minor fraction of FOXP3+ cells and CD4+ T cells express LAG3, while majority co-express TIGIT. PD-1+ cells co-express both LAG3 and TIGIT. Original magnification ×40. Scale bar = 50 μm. **c** High magnification of a CD8+ T cell with green membranous stain and co-expression of LAG3 (red staining). **d** High magnification of a CD8 + T cell with co-localization of LAG3 on the cell membrane. The green and red fluorescence in the same compartment is presented by a yellow color. **e** LAG3 and TIGIT immunolabelled TILs in a skin melanoma. Scale bar = 20 μm.

natural killer (NK) cells that may act both as activating and inhibitory receptor on TILs, as well as cathepsin W and granzyme B, two proteases known to induce target cell apoptosis [16].

Interestingly, we noticed that LAG3 and TIGIT were among the highly expressed genes in melanomas with brisk TILs (log2 FC 1.57 and 1.56, respectively, Fig. 1e). These two proteins were recently reported as novel IC targets [17, 18], although not much is known about their distribution and function within melanoma, TILs and TME. To address this question, we used mIF staining in a subset of tumors (N = 7), combining TIGIT and LAG3 antibodies with a melanoma cell marker (SOX10) and immune cell markers including CD8 (cytotoxic T cells), CD4 (T helper cells), FOXP3 (regulatory T cells/Tregs), PAX5 (B cells), and CD56 (NK/NKT cells) and in addition an IC protein, PD-1 (Fig. 2). We observed LAG3 and TIGIT expression in a large subset of $CD8^+$ T cells. We noticed that only few Tregs and CD4+ T cells expressed LAG3, whereas majority of them expressed TIGIT. As opposed to other studied immune cells, Tregs are known to suppress the immune system by inhibiting other immune cells, and expression of LAG3 and TIGIT enhances their suppressive functions upon ligand binding. LAG3 and TIGIT were expressed in a small fraction of the NK cells and were devoid in the B cells (online suppl. Fig. S2). Importantly, the majority of PD-1 positive cells co-expressed LAG3 and TIGIT (Fig. 2).

Discussion

Although TILs are associated with better survival, immunosuppression is evident through upregulation of IC genes *LAG3* and *TIGIT* in patients with Brisk TILs. Multiplex IF staining demonstrated high level of LAG3 expression on TILs, with highest expression on CD8+ T cells and to a lesser degree on CD4+ T cells, Tregs, and NK/NKT cells. These results are in line with previous reports of LAG3 expression on cell membranes of cytotoxic T cells, T helper cells, NK/NKT cells, Tregs and Dendritic cells in mice, and cell culture studies (Everett et al. [19]; Workman et al. [10, 11]). An IHC analysis in cutaneous melanoma reported LAG3 and TIGIT expression on TILs, but the TILs subtypes were not characterized further [20]. In non-small cell lung cancer (NSCLC) LAG3 was expressed on CD4+ TILs and mIF staining in esophageal adenocarcinoma reported LAG3 expression in CD4+ and CD8+ TILs [21, 22].

We observed no expression of LAG3 on B cells, which were sparsely present in TME. PAX5 is a nuclear transcription factor that plays an important role in B-cell differentiation [23]. PAX5 expression is increased during B-cell development and retained throughout maturation [24]. B cells participate in both humoral and cellular immunity, but their roles in antitumor immunity remain controversial [25]. Among the 91 DEG, CXCL13, a chemoattractant for B lymphocytes was identified. CXCL13 is associated with favorable prognosis in melanoma [26]. Previous reports of LAG3 expression on B cells are conflicting, as one study failed to detect LAG3 on B cells in human blood and tonsils samples, while another detected LAG3 on B cells in splenocyte culture from mice [9, 27].

Multiplex IF staining with TIGIT demonstrated positive expression on the majority of Tregs, on a large fraction of CD8+ T cells, and on a minor fraction of CD4+ T cells. This is in line with reports of highest expression of TIGIT on Tregs and to a lesser degree on activated T cells and NK cells [13, 28–30]. TIGIT was not expressed on B cells. However, in flow cytometry, a subclass of memory B cells exerted immunosuppression through expressing TIGIT and other inhibitory molecules [31]. Blessin et al. [32] investigated the expression pattern of TIGIT in colorectal and lung cancers using mIF. They detected TIGIT in CD8+ T cells, CD4+ T cells, FOXP3+ Tregs and NK cells, but not in CD11c+ dendritic cells, CD68+ macrophages, and CD20+ B lymphocytes. In the same study, TIGIT+ TILs were detected in tissue microarrays of 86 different cancers, including melanoma, but the expression pattern on TILs subtypes was not explored further.

For both LAG3 and TIGIT co-expression with PD-1 was detected. In melanoma co-expression of LAG3, TIGIT, and PD-1 has been associated with unfavorable outcomes in cutaneous melanoma [20]. The mechanism of action of LAG3 and TIGIT is distinct from that of PD-1. PD-1 blockade primarily reactivate T cells through co-receptor signaling, while LAG3 and TIGIT may directly regulate signaling through the TCR [33]. Simultaneous blocking of LAG3 and PD-1 has potential to enhance antitumor immunity with less side effects due to their distinct mechanism [8]. Furthermore, TIGIT suppresses tumor immune response primarily through Tregs and to a lesser degree through CD8+ T cells [34]. This finding is supported by an almost 100% TIGIT expression on FOXP3+ Tregs in our mIF staining. The high expression of LAG3 and TIGIT on TILs, and the co-occurrence with PD-1 in cutaneous SSM we identified here, suggests that adding LAG3 and TIGIT targeted treatment to current IC

inhibitors has the potential to restore additional effector functions of T cells in this context. This suggestion is further supported by emerging mechanistic understanding that points to convergence of TIGIT and PD-1 pathways [35] and preclinical and clinical results suggesting that dual targeting of PD-1 and TIGIT is a more effective strategy for treatment of glioblastoma [36] and non-small cell lung cancer [37]. Similar insights are emerging for LAG3 [38].

As we only used 6 cases to study LAG3 and TIGIT in situ expression, our results should be confirmed in a bigger cohort. Evaluation of the spatial context of immune response in individual cancers may add value in designing personalized treatment.

Key Message

BRAF V600E expression in melanoma predicts better prognosis.

Statement of Ethics

The study protocol was reviewed and approved by the data protection agency in Region Zealand, Denmark, approval number (REG-066-2019) and by the Ethics Committee in Region Zealand, Denmark, approval number (SJ-742). The approval from the Ethics Committee in Region Zealand also included exemption from informed consent.

Conflict of Interest Statement

Lise Mette Rahbek Gjerdrum has received funding from NanoString Technologies. The remaining authors state no conflict of interest.

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Author Contributions

Lise Mette Rahbek Gjerdrum and Beatrice Dyring-Andersen designed the research; Soraya Naimy, Michael Bzorek, and Jens Ole Eriksen performed experiments; Thomas Litman, Soraya Naimy, Lise Mette Rahbek Gjerdrum, Beatrice Dyring-Andersen, Jens Ole Eriksen, and Marianne Bengtson Løvendorf analyzed the data; Soraya Naimy, Lise Mette Rahbek Gjerdrum, Beatrice Dyring-Andersen, Michael Bzorek, and Marianne Bengtson Løvendorf wrote the paper. All authors read and approved the manuscript.

Data Availability Statement

Dataset related to this article has been deposited in the Gene Expression Omnibus (GEO) with accession number GSE193802. Further inquiries can be directed to the corresponding author.

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