Immune checkpoint proteins CTLA-4, PD-1 and PD-L1 in melanoma as predictive biomarkers to guide clinical decisions

Markus Tiemann
Institute for Hematopathology, Hamburg
Vera Samoilova
Institute of Hematophology, Hamburg
Dmitri Atiakshin
Voronezh State University Faculty of Medicine and Biology: Voronezskij gosudarstvennyj universitet Mediko-biologiceskij fakul'tet
Igor Buchwalow (buchwalow@pathologie-hh.de)
Institute for Hematopathology, Hamburg https://orcid.org/0000-0003-1142-7483

Research

Keywords: melanoma, T-lymphocytes, PD-1, PD-L1, CTLA-4

Posted Date: September 2nd, 2020

DOI: https://doi.org/10.21203/rs.3.rs-70003/v1

Abstract

Background

CTLA-4, PD-1 and PD-L1 are potential targets in cancer immunotherapy. However, to predict patients who are likely to respond to treatment with PD-1/PD-L1/CTLA-4 blockers still remains a challenge. Unfortunately, it is often ignored that CTLA-4 exists also in cells other than lymphocytes. This study aimed to assess the expression and diagnostic significance of predictive biomarkers for melanoma.

Methods

To address this issue, we performed an immunophenotyping of CTLA4/PD1/PD-L1-positive cells in melanoma employing multiple immunofluorescent immunolabeling. For immunolabeling, we used primary antibodies to PD-1 and PD-L1, a panel of anti–CTLA-4 antibodies of different clones and a panel of CD antibodies against diverse cell types (CD1a, CD3, CD8 and CD68).

Results

In contrast to the currently accepted view, immunolabeling with anti-CTLA-4 antibodies of different clones permitted to reveal CTLA-4 in cells other than lymphocytes. CTLA-4 and PD-L1 are both expressed in the tumor microenvironment cells including antigen-presenting cells ÷ macrophages and dendritic cells. PD-1 is expressed solely in T-lymphocytes. CTLA-4 and PD-L1 in melanoma are not expressed in malignant cells.

Conclusion

Accordingly, PD-L1 expression by tumor cells cannot serve as a biomarker of clinical response to checkpoint blockade in melanoma immunotherapy, but PD-L1 and CTLA-4 expressed in the tumor microenvironment cells are valuable biomarkers to guide clinical decisions. The expression of CTLA-4 also in cells other than T-lymphocytes suggests the involvement of this molecule in alternative CTLA-4 signaling pathways together with the well-known classical scheme for the regulation of T-lymphocyte activity.

Introduction

In 1992, the team of Tasuku Honjo in a screen for genes, involved in apoptosis, reported on a protein expressed on the surface of a subset of immune cells known as T cells and named this protein PD-1 (short for Programmed Death-1) [1]. In 1999, a ubiquitous anti-apoptotic receptor on cancer cells was reported from the Mayo Clinic [2]. Originally it was named B7-H1 but later renamed PD-L1, because it was identified as a ligand of PD-1. In 2002, Iwai et al. [3] found that blocking interaction between PD-1 and its

ligand enhanced immune activation, resulting in markedly stronger antitumor responses. Based on these findings, a fully human anti-PD-1 antibody was developed in 2005 by Ono Pharmaceutical Co. and Medarex (later acquired by Bristol-Myers Squibb). Development of therapeutic anti-PD-1/PD-L1 monoclonal antibodies leading to the reactivation of specific antitumor immune response has emerged as a promising strategy for cancer immunotherapy [4–9].

First reported in 1987[10], CTLA-4 (short for cytotoxic T-lymphocyte-associated protein 4) was later characterized as another negative regulator of T cell activation [11]. In 1996, Allison's team [12, 13] reported that CTLA-4 acts similar to PD-1 as an inhibitory molecule to restrict T cell responses and showed that the antibody blockade of CTLA-4, like the blockade of PD-1, could also lead to enhanced anti-tumor immune responses with tumor rejection [12, 14].

Used as targets in cancer immunotherapy, immune checkpoint proteins, PD-1, PD-L1 and CTLA-4, are also proposed as predictive biomarkers in clinical practice[2, 15, 16]. However, to predict patients who are likely to respond to treatment with PD-1/PD-L1/CTLA-4 blockers still remains a challenge [17]. To guide clinical decisions, further validation of predictive biomarker candidates is warranted [18]. The aim of or study was to analyze the phenotype and distribution pattern of predictive biomarkers in the human melanoma irrespective of the age of patients, degree of tumor development and localization of the tumor. To address this issue, we performed an immunophenotyping of CTLA4/PD1/PD-L1-positive cells in melanoma employing double and triple immunofluorescent immunolabeling [19–22].

Material And Methods

Patients

In all, 21 melanoma patients were included in this study. Melanoma tissue was obtained from five male and sixteen female patients. The age range of the patients was 30–82 years. Human tonsils were used as controls. Tonsil tissue was obtained from two male and four female patients (the age range of the patients was 25–50 years) undergoing tonsillectomy for recurrent tonsillitis. A written informed consent was obtained from each participant. The samples were retrieved from the files of the Institute for Hematopathology, Hamburg, Germany. These samples were redundant clinical specimens that had been de-identified and unlinked from patient information. Histological diagnoses were established according to the WHO classification [23]. This study was conducted in accordance with the principles of World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects" and approved by the Institutional Review Board of the Institute for Hematopathology, Hamburg, Germany.

Tissue probe stainings

Human melanoma and control tonsils tissue probes left over during routine diagnostic procedure were fixed in buffered 4% formaldehyde and routinely embedded in paraffin. PBS was used for all washings and dilutions. Paraffin tissue sections (1 µm thick) were deparaffinized with xylene and rehydrated with graded ethanols. For pathohistological analysis, tissue sections were routinely stained with H&E, covered

with permanent mounting medium and observed on an upright microscope (Zeiss Axio Imager.Z1) configured for *transmitted-light brightfield* microscopy and equipped with a microscope camera Zeiss Axio Cam HRc, color CCD.

Immunohistochemistry

For immunohistochemical assay, deparaffinised sections were subjected to antigen retrieval by heating the sections in a steamer with 10 mM sodium citrate buffer, pH 6.0, at 95 °C x 30 min. We reported previously that endogenous Fc receptors in routinely fixed cells and tissue probes do not retain their ability to bind Fc fragments of antibodies[24]; therefore, blocking the endogenous Fc receptors prior to incubation with primary antibodies was omitted. After antigen retrieval, sections were immunoreacted with primary antibodies. The list of primary antibodies used in this study is presented in the Table 1.

Table 1
Primary antibodies used in this study

Antibodies/Clone	Host	Source
Melan A (A103)	mouse monoclonal Ab	Ventana/Roche, Germany
SOX10 (SP267)	rabbit monoclonal Ab	Cell Marque, USA
CD1a (EP362R)	rabbit monoclonal Ab	Cell Marque, USA
CD3 (PS1)	mouse monoclonal Ab	AbCam, United Kingdom
CD3 (2GV6)	rabbit monoclonal Ab	Ventana/Roche, Germany
CD8 (SP57)	rabbit monoclonal Ab	Ventana/Roche, Germany
CD68 (KP-1)	mouse monoclonal Ab	Ventana/Roche, Germany
CD163	mouse monoclonal Ab	Cell Marque, USA
CTLA-4 (UMAB249)	mouse monoclonal Ab	Origene Technologies
CTLA-4 (AA 57–86)	rabbit polyclonal Ab	antikoerper-online, Germany
CTLA-4 (SP355)	rabbit monoclonal Ab	AbCam, United Kingdom
CTLA-4 (CAL49)	rabbit monoclonal Ab	AbCam, United Kingdom
PD-1 (NAT105)	mouse monoclonal Ab	Cell Marque, USA
PD-L1 (ZR3)	rabbit monoclonal Ab	Zeta Corporation, USA

Immunohistochemical visualization of bound primary antibodies was performed either with Ventana Slide Stainer or manually according to the standard protocol[19, 21]. For manually performed immunostaining, primary antibodies were applied in concentration from 1 to 5 µg/ml. PBS was used for all washings and dilutions. Bound primary antibodies were visualized using secondary antibodies (purchased from Dianova, Hamburg, Germany, and Molecular Probes, Darmstadt, Germany) conjugated

with Cy3, Alexa Fluor-488 or Alexa Fluor-647. For visualisation of primary anti-CTLA-4 antibodies, we used tyramide signal amplification (TSA) [21]. The list of secondary antibodies and other reagents used in this study is presented in the Table 2.

Table 2
Secondary antibodies and other reagents

Antibodies and other reagents	Source	Dilution	Label
Goat anti-mouse IgG Ab (#A21236)	Invitrogen, Darmstadt, Germany	1/100	Alexa Fluor 647
Goat anti-rabbit IgG Ab (#A21245):	Invitrogen, Darmstadt, Germany	1/100	Alexa Fluor 647
Goat anti-mouse IgG Ab (#115-165-166)	DIANOVA Hamburg, Germany	1/200	Cy3
Goat anti-rabbit IgG Ab (#A-11034)	DIANOVA Hamburg, Germany	1/200	Cy3
Goat anti-mouse IgG A (#A-11029	Invitrogen Darmstadt, Germany	1/200	Alexa Fluor 488
Goat anti-rabbit IgG Ab (#A-11034)	Invitrogen Darmstadt, Germany	1/200	Alexa Fluor 488
AmpliStain™ anti-Mouse 1-Step HRP (#AS-M1-HRP)	SDT GmbH, Baesweiler, Germany	ready-to-use	HRP
AmpliStain™ anti-Rabbit 1-Step HRP (#AS-R1-HRP)	SDT GmbH, Baesweiler, Germany	ready-to-use	HRP
4',6-diamidino-2-phenylindole (DAPI, #D9542-5MG)	Sigma, Hamburg, Germany	5 µg/ml	w/o
VECTASHIELD® Mounting Medium (#H-1000)	Vector Laboratories, Burlingame, CA, USA	ready-to-use	w/o
CC2 solution (# 950 – 223)	Ventana	ready-to-use	w/o
TSA Plus Fluorescein (#NEL741E001K)	PerkinElmer, Rodgau Germany	1/100	Fluorescein
TSA Plus Cyanine 3 (#NEL744E001KT)	PerkinElmer, Rodgau Germany	1/100	Cyanine 3
TSA Plus Cyanine 5 (#NEL745E001KT)	PerkinElmer, Rodgau Germany	1/100	Cyanine 5

For simultaneously detecting antigens from the same host species, we performed TSA with the subsequent heat elution treatment after each immunostaining step [25]. The bound primary/secondary antibody complex from the preceding immunolabeling step was thereby eluted with a citrate/acetate-based buffer, pH 6.0, containing 0.3% SDS (also available from VENTANA as CC2 solution, cat # 950 – 223) [26]. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 5 µg/ml in PBS) for 15 s, and the sections were then mounted using VectaShield (Vector Laboratories, Burlingame, USA).

Immunostained tissue sections were observed on a Zeiss Axio Imager.Z1 configured for fluorescence microscopy and equipped with a microscope camera Zeiss Axio Cam HRm, monochrome CCD.

Controls

Control incubations were omission of primary antibodies or substitution of primary antibodies by the same IgG species (Dianova, Hamburg, Germany) at the same final concentration as the primary antibodies. The exclusion of either the primary or the secondary antibody from the immunohistochemical reaction, substitution of primary antibodies with the corresponding IgG at the same final concentration resulted in lack of immunostaining. The TSA step alone did not contribute to any specific immunostaining that might have influenced the analysis. Moreover, the specific and selective staining of different cells with the use of primary antibodies from the same species on the same preparation is by itself a sufficient control of the specificity of immunostaining.

Image acquisition

Images were captured and processed with an AxioCam digital microscope cameras and the software AxioVision image processing (Carl Zeiss Vision, Germany). The images were imported as JPEG files into PhotoImpact 3.0 (Ulead Systems, Inc. Torrance, CA) and submitted with the final revision of the manuscript at 300 DPI.

Results

Because CTLA-4 was originally defined as a T-lymphocyte antigen [10], we first searched for a possible expression of CTLA-4 on T-lymphocytes. As a marker for T-lymphocytes, we used antibodies to CD3 and CD8. For immunolabeling of CTLA-4, we used five different anti-CTLA-4 antibodies of clones UMAB249, AA 57–86, SP355 and CAL49 (See Table 1).

In melanoma probes, double immunolabeling of T-lymphocytes vs CTLA-4 using anti-CTLA-4 antibodies of AA 57–86, SP355 and CAL49 clones revealed a presence of CTLA-4 protein on CD3-positive but not in CD8-positive lymphocytes. But immunolabeling of T-lymphocytes vs CTLA-4 in melanoma probes using anti-CTLA-4 antibody of the clone UMAB249 surprisingly revealed a lack of CTLA-4 protein both in CD3 and in CD8-positive lymphocytes (Fig. 1a,b).

In the probes of human tonsils used as a control, the lack of CTLA-4 protein on CD3-positive and CD8-positive lymphocytes was, like in melanoma, found when using anti-CTLA-4 antibodies of UMAB249

clone. The lack of CTLA-4 protein on CD3-positive and CD8-positive lymphocytes was also found with anti-CTLA-4 antibodies of AA 57–86 and SP355 clones, whereas combining CTLA-4 antibody o the clone CAL49 with CD3 or CD8 by immunofluorescence showed that CTLA-4-positive lymphocytes in tonsils were typically CD3-positive but extremely rare CD8-positive (Fig. 1c,d).

Further immunophenotyping of CTLA-4 positive cells in melanoma was performed using anti-CTLA-4 antibody of the UMAB249 clone in combination with markers of melanocytes, macrophages and dendritic cells. Malignant cells in melanoma were detected using two markers of melanocytes - Melan A (Fig. 2a,b) and SOX10 (Fig. 2c). Noteworthy, melanocytes were found not to express CTLA-4 (Fig. 2a). Double and triple immunolabeling of melanoma revealed that melanocytes immunolabeled with Melan-A antibody (Fig. 2b) or with SOX10 antibody (Fig. 2c) did not express PD-L1. PD-L1 was not also found in cells marked with CD3 (marker of T cells) (Fig. 2b) or with PD-1 (Fig. 2d).

We showed that PD-L1-positive cells co-express both CTLA-4 (Fig. 2e) and CD68 (Fig. 2f) or CD163 (not shown), which allowed us a conclusion that CTLA-4 in melanoma is expressed in macrophages. This conclusion was further supported by double immunolabeling for CTLA-4 and CD68 (Fig. 3a). Remarkably, CTLA-4 was found within the cytoplasm (Fig. 2e, 3a) like CD68 (Fig. 2f, 3a), while PD-L1 was found on the surface of these cells (Fig. 2e,f). Intracellular localization of CTLA-4 shown here is consistent with reports that a key feature of CTLA-4 is its rapid and constitutive endocytosis from the plasma membrane resulting in approximately 90% of CTLA-4 being intracellular [27].

CTLA-4 with a specific intracytoplasmic localization was also found in dendritic cell, labeled with CD1a antibody (Fig. 3b). Like macrophages, dendritic cells also co-expressed PD-L1 (Fig. 4a,b). This allowed us the conclusion that the immune checkpoint proteins PD-L1 and CTLA-4 are expressed in melanoma only in antigen-presenting cell - dendritic cells (Fig. 4a,b) and macrophages (Fig. 2e, 3a), but not in T-cells (Fig. 1, 2d) and not in melanocytes (Fig. 2a), whereas another immune checkpoint protein PD-1 was found to be localized exclusively in T-lymphocytes (Fig. 5).

Discussion

Given the complexity of the tumor microenvironment and the dynamic interaction between tumor and immune cells, PD-1/PD-L1/CTLA-4 regulatory pathways in tumors need a better understanding highlighting the need to investigate predictive biomarkers[28].

In malignant cells of various cancers, PD-L1 expression was graded from 0–50%[29]. Malignant cells of melanoma were previously reported to express PD-L1 [30]. However, we showed that malignant cell in melanoma lacked expression of PD-L1; this immune checkpoint protein was found only in antigen-presenting cells -macrophages (Fig. 2e,f, 3a) and dendritic cells (Fig. 3b, 4a,b). It means that PD-L1 expression by tumor cells cannot serve as an absolute biomarker of clinical response to checkpoint blockade in immunotherapy. This is in line with reports that patients, by which malignant cell in the tumor lack PD-L1 expression, also responded positively to PD-L1 checkpoint blockade therapies [29, 31, 32]. Correspondingly, patients with overexpressed PD-L1 in the tumor microenvironment, have improved

clinical outcomes with anti-PD-1/PD-L1-directed therapy[33]. It was earlier reported that PD-L1 is stronger presented in the tumor microenvironment then PD-1 [34] and PD-L1 has been proposed as a potential target in cancer immunotherapy in human clinic [33, 35]. Therefore, PD-L1 expression in the tumor tissue can be regarded as a more valuable biomarker than PD-1 to guide clinical decisions.

The precise mechanism by which CTLA-4 regulates the immune response is complex and not fully understood[36]. According to Pierre Golstein and colleagues, who were the first to identify CTLA-4[10, 37], CTLA-4 was originally defined as a T-lymphocyte antigen and later described as a T-cell surface receptor [11, 38–41], which, according to Allison's concept [12, 13], was later adopted as a target in melanoma immunotherapy. But we found that T-lymphocytes lacked the expression of CTLA-4, when using the anti-CTLA-4 antibody of the clone UMAB249, in human melanoma and in the tonsils also with the antibodies of AA 57–86 and SP355 clones (Fig. 1).

Most of the available data on CTLA-4 expression are limited to human T cells. Unfortunately, it is often ignored that CTLA-4 exists also in cells other than lymphocytes. When using the anti-CTLA-4 antibody of the clone UMAB249, we detected CTLA-4 in the melanoma in the tumor microenvironment cells, such as antigen-presenting cells ÷ macrophages (Fig. 2e,f, 3a) and dendritic cells (Fig. 3b, 4a,b), but not in T lymphocytes. In contrast, immunolabeling CTLA-4 with the antibody clone CAL49 vs T lymphocytes markers CD3, CD4, or CD8 showed that CTLA-4-positive T lymphocytes in human melanoma and tonsils were typically CD3 + and CD4+, but not CD8+ [42]. Different immunolabeling pattern with anti-CTLA-4 antibodies of different clones can be explained by generating anti-CTLA-4 antibodies that recognize different epitopes of this protein. Further studies on this plausible controversy are warranted.

Despite the huge success and efficacy of the response to anti-CTLA-4 therapy in patients with melanoma, subsequent clinical trials have shown that combination therapy targeting both CTLA-4 and PD-1 appeared to be even more effective [43]. This could be obviously explained by a massive T-lymphocyte presence in the tumor microenvironment observed in melanoma probes (Fig. 2b,d); most of CD3-positive cells co-expressed PD-1 (Fig. 5). Accordingly, better clinical outcomes with immune-checkpoint therapy in melanoma can also be awaited from combination therapy with the inclusion of PD-L1 as a target [44–46], while PD-L1 is strongly expressed in invaded macrophages in the melanoma tumor microenvironment (Fig. 2e,f) and in dendritic cells (Fig. 4a,b). This is in line with earlier reports that a high PD-L1 expression on immune cells, but not on tumor cells, is a favorable prognostic factor [29].

Conclusion

Taken together, our results indicate that CTLA-4 and PD-L1in melanoma are not expressed in malignant cells but are expressed in the tumor microenvironment cells. It means that PD-L1 expression by tumor cells cannot serve as a biomarker of clinical response to checkpoint blockade in melanoma immunotherapy. PD-1 is expressed exclusively in T-lymphocytes. Like PD-1 expressed on T lymphocytes, PD-L1 and CTLA-4 expressed in the tumor microenvironment cells are valuable biomarkers to guide clinical decisions. Better clinical outcomes with immune-checkpoint therapy in melanoma can be awaited

from combination of PD-1, PD-L1 and CTLA-4 as targets. The expression of CTLA-4 also in cells other than T-lymphocytes suggests the involvement of this molecule in alternative CTLA-4 signaling pathways together with the well-known classical scheme for the regulation of T-lymphocyte activity.

Abbreviations

PD-1 - Programmed Death-1);

PD-L1 - ligand for PD-1;

CTLA-4 - Cytotoxic T-Lymphocyte Antigen 4;

HRP - Horseradish Peroxidase;

DAB – 3,3'-diaminobenzidine

DAPI – 4',6-diamidino-2-phenylindole

TSA - tyramide signal amplification

Declarations

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

This study was designed and performed with the approval from the Ethics Committee of the Institute for Hematopathology, Hamburg, and a written informed consent was obtained from each participant.

Consent for publication

A written informed consent for publication was obtained from each participant.

Competing interests

The authors declare that they have no competing interests.

Funding

This research received no specific grants from any funding agency in the public, commercial or not-for-profit sectors.

Authors’ contributions

MT and IB designed the study, performed the analysis and draft the manuscript. VS, and DA collected the clinical data and did immunohistochemistry. All authors read and approved the final manuscript.

Acknowledgements

We thank Denis Morozov and Alexander Glomb for their perfect technical assistance and other colleagues from the immunohistology laboratory for sharing probes and reagents.

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Figures

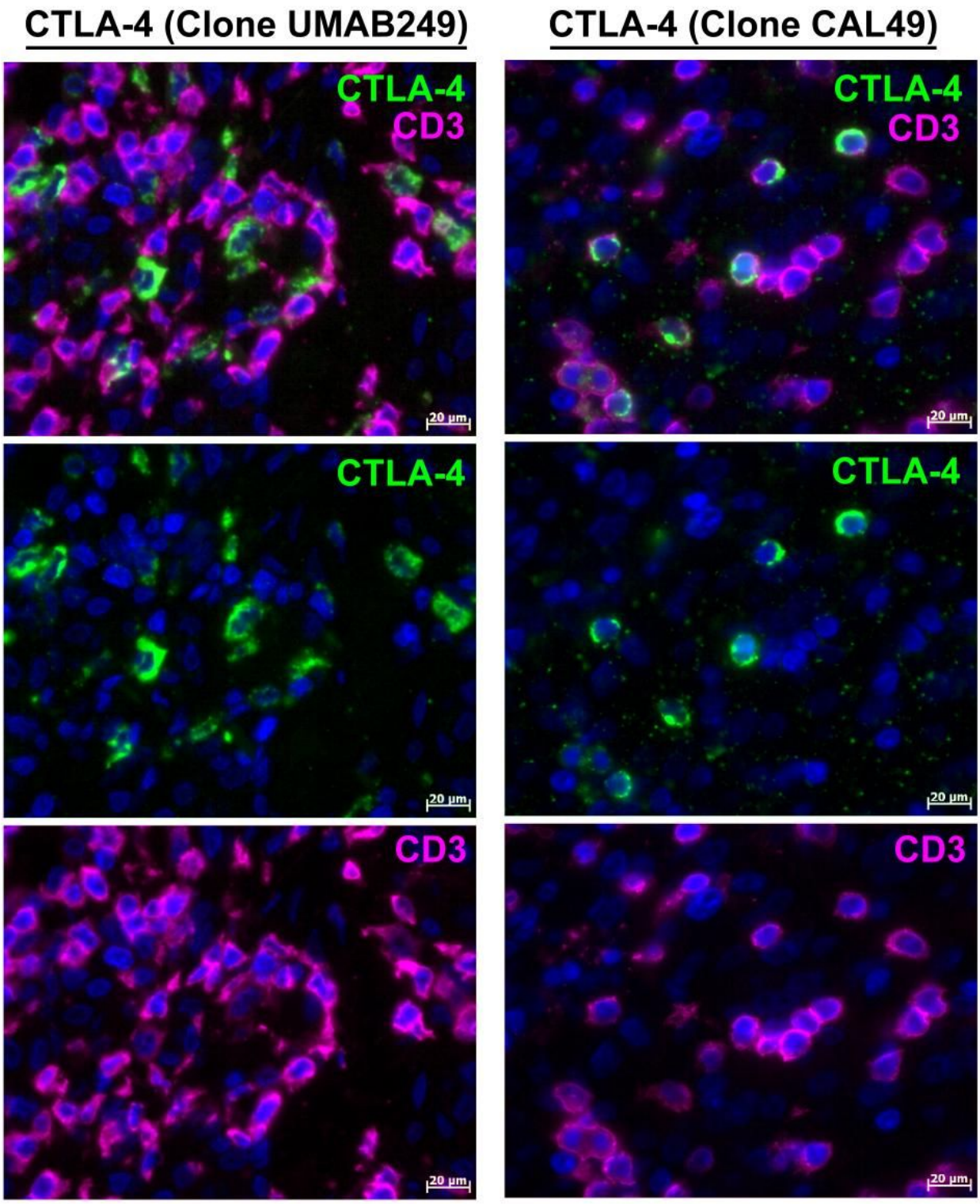

Figure 1

Expression of CTLA-4 antigen in T lymphocytes in the human melanoma and tonsils.

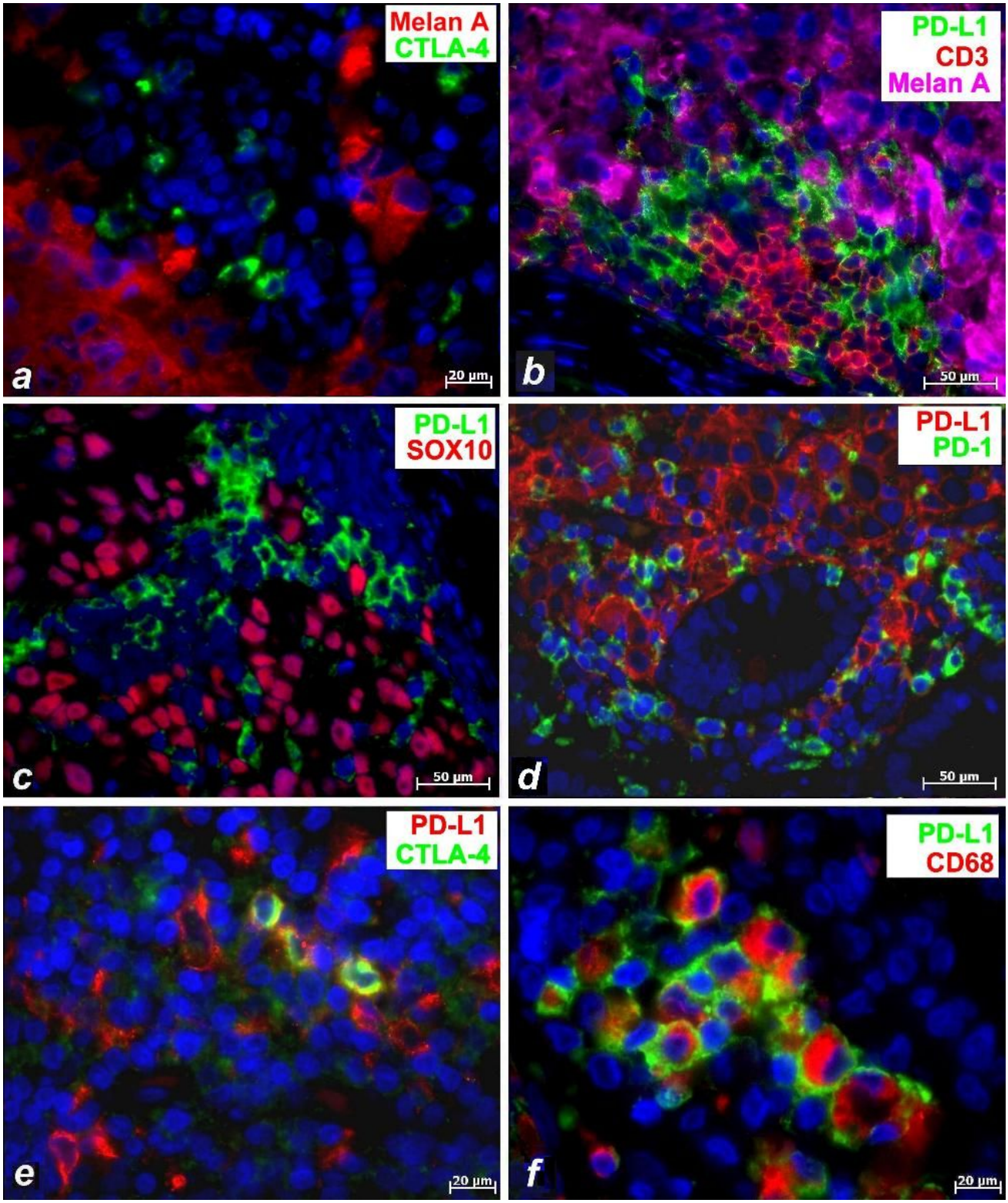

Figure 2

Human melanoma. a Melanocytes do not to express CTLA-4. b Immunofluorescent triple staining of CD3, PD-L1 and Melan A. Melan A, CD3 and PD-L1 and are expressed poles apart from each other. c Immunofluorescent double staining of SOX10 and PD-L1. SOX10 and PD-L1 are expressed poles apart from each other. d Immunofluorescent double staining of PD-1 and PD-L1. PD-1 and PD-L1 are expressed poles apart from each other. e Immunofluorescent double staining of CTLA-4 and PD-L1. CTLA-4 reveals

intracytoplasmic localization in some PD-L1-positive cells. f Immunofluorescent double staining of CD68 and PD-L1. CD68 reveals intracytoplasmic localization in some PD-L1-positive cells.

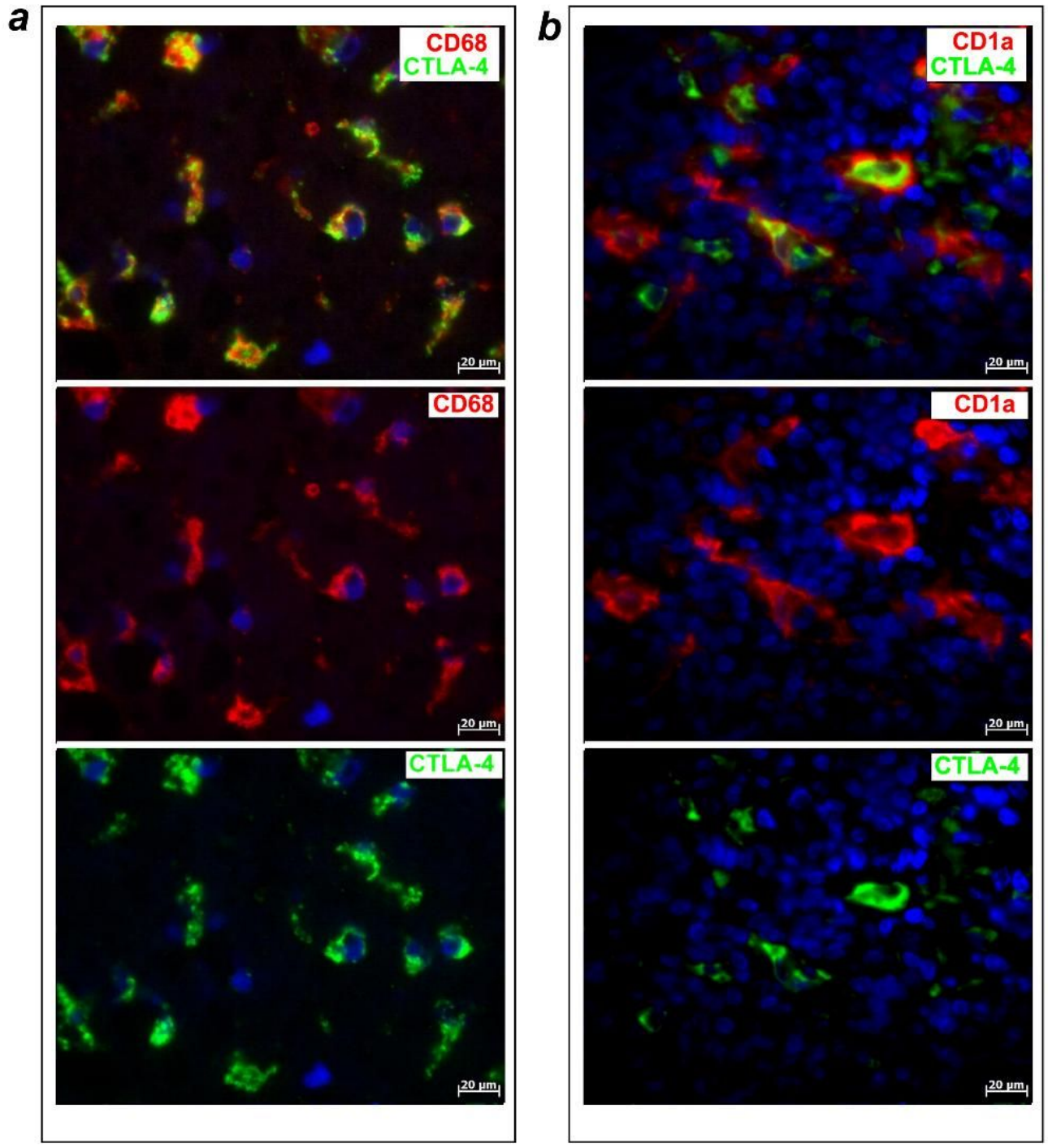

Figure 3

Human melanoma. a Immunofluorescent double staining of CD68 and CTLA-4 in the tumor microenvironment. b Immunofluorescent double staining of CD1a and CTLA-4 in the tumor microenvironment.

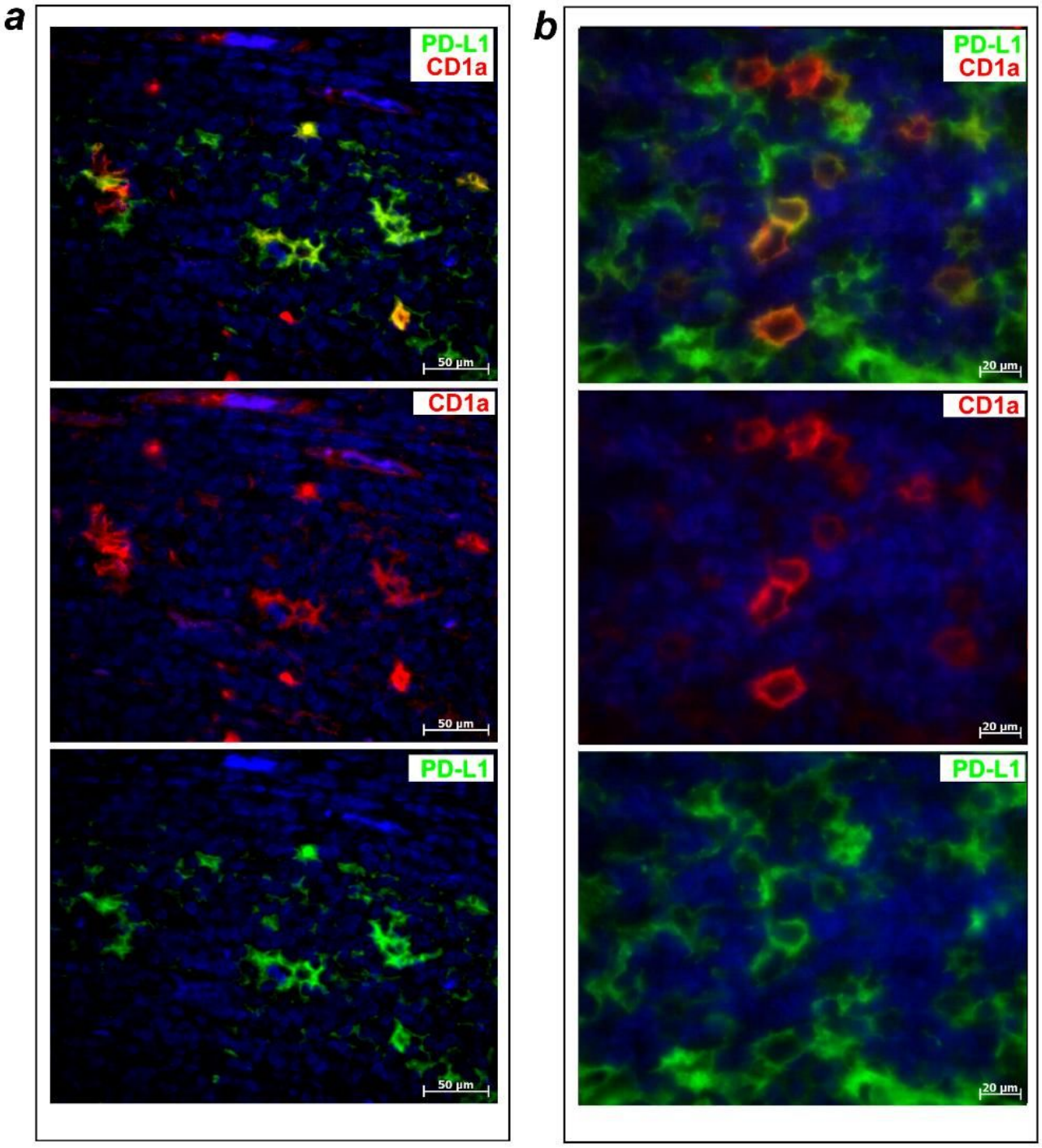

Figure 4

Human melanoma. a Immunofluorescent double staining of CD68 and CTLA-4 in the tumor microenvironment. b Immunofluorescent double staining of CD1a and CTLA-4 in the tumor microenvironment.

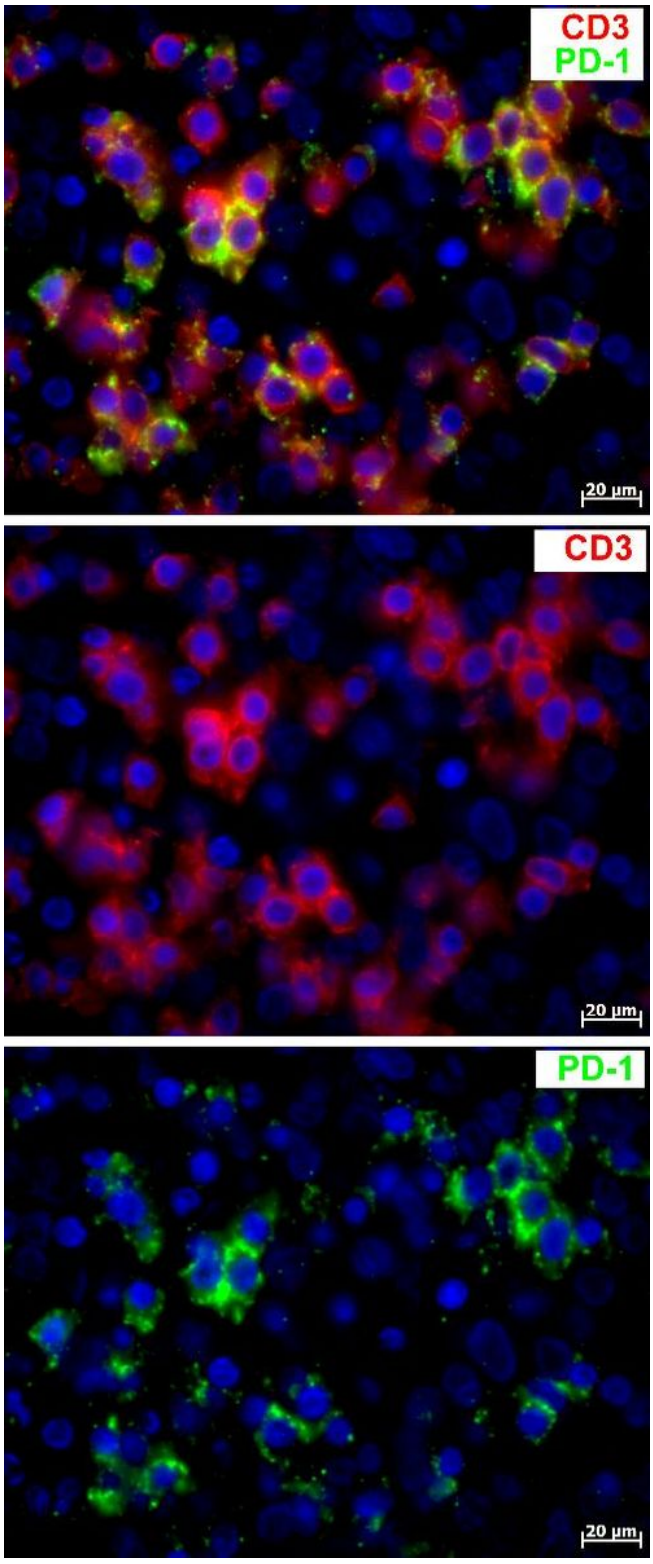

Figure 5

Human melanoma. Co-expression of PD-1 and CD3 in T lymphocytes.