Immunobiology 222 (2017) 651–657

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Oral squamous cell carcinoma suppressed antitumor immunity through induction of PD-L1 expression on tumor-associated macrophages

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ARTICLE INFO

Article history:
Received 22 April 2016
Received in revised form 3 November 2016
Accepted 13 December 2016
Available online 14 December 2016

Keywords:
Oral squamous cell carcinoma
PD-L1
Macrophages

ABSTRACT

Oral squamous cell carcinoma (OSCC) is the most common solid tumor in the oral cavity. Development and progression of OSCC is associated with the elevated presence of inhibitory M2 type tumor-associated macrophages (TAMs). However, the underlying mechanism leading to the enrichment of M2 TAMs and the pathway through which TAMs foster tumor progression are still unclear. In this study, we harvested TAMs and tumor cells from primary OSCC resections of stage II and stage III patients. We showed that compared to peritumoral macrophages, TAMs presented upregulated expression of PD-L1 and elevated capacity in inducing T cell apoptosis. The level of PD-L1 expression directly correlated with the level of T cell apoptosis. Interestingly, peripheral blood monocytes with low initial PD-L1 level had upregulated PD-L1 expression and acquired the ability to induce T cell apoptosis, after incubation with primary tumor cells from OSCC patients. The PD-L1 expression by monocytes depended on interleukin 10 (IL-10), since blockade of IL-10 in the tumor-monocyte coculture abrogated PD-L1 upregulation. IL-10 mRNA expression in tumor cells and monocytes also preceded PD-L1 mRNA expression in monocytes. Furthermore, the IL-10 concentration in the tumor microenvironment directly correlated with the PD-L1 level on TAMs. Together, these results suggest that OSCC could directly suppress antitumor T cell immunity through conditioning TAMs.

1. Introduction

Oral squamous cell carcinoma (OSCC) is the most common type of oral cancer, which represents approximately 2% of all new cancer cases worldwide and contributes to an estimated number of over 222,000 deaths annually (Ferlay et al., 2010; Jemal et al., 2011). The frequent use of alcohol and tobacco, as well as human papilloma virus infection, has been shown to increase the risk of OSCC development (Moore et al., 2000; Nordfors et al., 2014). In addition, the status of antitumor immune responses in the host is implicated in the induction and development of solid tumors (Grivennikov et al., 2010).

Robust interferon-gamma-dominant T cell responses and extensive $CD4^+$ and $CD8^+$ T cell infiltrations have been shown to associate with better prognosis and longer disease-free survival time (Ikeda et al., 2002; Kim and Cantor, 2014; Klebanoff et al., 2006). However, one major challenge of developing effective antitumor T cell responses and T cell-based immunotherapies against solid tumors is the presence of multiple suppressive mechanisms in the tumor microenvironment, including those mediated by the tumor-associated macrophages (TAMs) (Gajewski et al., 2013; Mantovani et al., 2008; Quail and Joyce, 2013). Macrophages are crucial antigen-presenting cells during T cell activation, and have been shown to modulate the types of T cell response during inflammation (Adams and Hamilton, 1984; Italiani and Boraschi, 2014; Martinez and Gordon, 2014). The classically activated $iNOS^+$ M1 type macrophages secrete large amounts of IL-12, tumor necrosis factor (TNF)-alpha, reactive oxygen species, and reactive nitrogen intermediates, that favor the development of interferon (IFN)-gamma-producing Th1 responses and cytotoxic $CD8^+$ T cell responses. Whereas the alternatively activated $CD163^+$ M2 type macrophages present low IL-12 secretion, impaired nitric oxide induction, and high angiogenic factor expression, that favor the development of Th2 cells and suppressive Treg responses, and contribute to tumor progression and metastasis (Gordon and Martinez, 2010; Italiani and Boraschi, 2014). Enrichment of M2

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http://dx.doi.org/10.1016/j.imbio.2016.12.002
0171-2985/

type macrophages in TAMs has been demonstrated in multiple solid tumors (Biswas and Mantovani, 2010; Mantovani et al., 2002). Furthermore, the CD163$^+$ M2 type TAMs can be detected in stage I OSCC tumors and with elevated frequency in stage II OSCC tumors (Mori et al., 2011). Diagnostic incision biopsies could result in the polarization of TAMs toward the CD163$^+$ M2 type and have led to a worsening prognosis in OSCC patients (Weber et al., 2015). These results suggest that upregulation of suppressive TAMs are directly involved in the development of OSCC, but reason for the induction of suppressive TAMs in OSCC, and the pathway through which TAMs promoted tumor progression, are still not understood.

A previous report found that high PD-L1 expression in OSCC correlated with metastasis and poor progression (Lin et al., 2015). We therefore postulated that TAMs possibly mediated T cell apoptosis through the expression of PD-L1. Indeed, we observed elevated PD-L1 expression in TAMs compared to in peritumoral macrophages from freshly resected OSCC tumor. These TAMs also induced T cell apoptosis in a PD-L1 associated way. Interestingly, we found that monocytes harvested from peripheral blood with low initial PD-L1 expression had upregulated PD-L1 and acquired the ability to induce T cell apoptosis after incubation with primary tumor cells from OSCC patients. Further examination suggested that IL-10 production by tumor cells upregulated the PD-L1 expression in monocytes. Moreover, the IL-10 concentration in the tumor microenvironment directly correlated with the PD-L1 level on TAMs. Together, these results suggest that OSCC itself could directly suppress antitumor immunity.

2. Materials and methods

2.1. Human subjects

The study protocol was approved by the institutional review board of Xiangya Hospital. A total of 12 stage II and stage III OSCC patients who underwent surgical resections were recruited. Patient information was analyzed by DICAT (Vancouver, Canada). After receiving informed consent, the serum, peripheral blood and resected tumor samples were obtained from the patients. No patient received chemotherapy or radiation therapy before sample collection.

2.2. Tumor digestion

The tumor digestion protocol was adopted from a head and neck squamous cell carcinoma digestion method with minor modifications (Prince et al., 2007). Briefly, freshly resected tumor was washed with PBS + 1% penicillin-streptomycin (ThermoFisher Scientific). Peritumor tissues were separated from tumor by an experienced surgeon. Tumor was then cut into small pieces, minced carefully with a scapel, and then placed in Media 199 + 200 U/mL collagenase III (Sigma) at 2–4 g tumor per 10 mL medium for two rounds in a 37° C shaker, 1.5 h each. After digestion, the cells were pelleted, resuspended and filtered by a 40-μm mesh to obtain single cells, while the supernatant was frozen at −80° C for ELISAs.

2.3. Cell isolation and culture

Peripheral blood mononuclear cells (PBMCs), tumor mononuclear cells (TMCs) and peritumor mononuclear cells (PTMCs, if available) were harvested from blood or tumor/peritumor digestions by standard Ficoll centrifugation. Peripheral blood monocytes and T cells were negatively purified by using Human Monocyte Enrichment Kit and Human T Cell Enrichment Kit (STEMCELL Technologies), respectively. After removal of TMCs, primary tumor cells were seeded in a T25 flask and passaged when reaching 70–80% confluency. All cells were cultured at 10^6 cells/mL in RPMI 1640 supplemented with 1% penicillin-streptomycin, 1 × GlutaMax and 10% FCS, at 37° C in 5% CO_2. 1:1 ratio was used for all monocyte/macrophage-T cell and tumor cell-monocyte cocultures.

For the examination of T cell IFN-gamma production and apoptosis, monocytes and T cells were cocultured for 24 h, in the presence of 1 μg/mL each of anti-human CD3 (clone OKT3) and CD28 (clone CD28.2). For the tumor cells and monocytes cocultures, freshly isolated monocytes were either cultured alone or with autologous tumor 12 days, with the culture medium replaced every 3 days. The Human Monocyte Enrichment Kit was reapplied on monocytes after monocyte-alone culture or monocyte/tumor coculture so that purified monocytes could be used for coculturing with T cells.

2.4. Flow cytometry

All staining procedures were completed on ice. Before intracellular staining, cells were treated with 5 μg/mL brefeldin A for 5 h. If T cell apoptosis was examined, the Annexin V Apoptosis Detection Kit (eBioscience) was used prior to surface staining. Briefly, cells were resuspended in 1 × Binding Buffer and 5% fluorochrome-conjugated annexin V for 15 min at room temperature in dark. The cells were then washed and stained with combinations of surface antibodies, including anti-human CD3, CD14, CD16, CD19, CD56, CD68, and PD-L1, as well as Violet or Aqua viability dye for 30 min on ice. Cells were then washed and treated with CytoFix/CytoPerm (BD) for 15 min, after which anti-human IFN-gamma antibody was added for 30 min. Samples were acquired in BD FACSCanto and processed in FlowJo. All FACS figures shown were first gated to Violet-low or Aqua-low live cells and then to single cells based on forward scatter area vs. forward scatter height gating.

2.5. Reverse transcriptase quantitative PCR (RT-qPCR)

Total RNA was extracted by the RNeasy Mini Kit (Qiagen). cDNA was generated by the Superscript III Reverse Transcriptase kit (ThermoFisher Scientific). The following primer sets were used. PD-L1 sense: 5′-GGCATTTGCTGAACGCAT-3′; antisense: 5′-CAATTAGTGCAGCCAGGT-3′. IL-10 sense: 5′-GTGGAGCAGGTGAAGAATGC-3′; antisense: 5′-ATAGAGTCGCCACCCTGATG-3′. GAPDH sense: 5′-TGCACCACCAACTGCTTA-3′; antisense: 5′-GGATGCAGGGATGATGTTC-3′. qPCR was performed in a CFX96 cycler (Bio-Rad) with SYBR green PCR Master Mix (ThermoFisher Scientific).

2.6. Cytokine detection

The IL-10 and IFN-gamma levels in serum, tumor digestions and supernatants were measured using the Human IL-10 ELISA Ready-SET-Go and the Human IFN gamma ELISA Ready-SET-Go kits (eBioscience) following manufacturer's instructions.

2.7. Statistics

All statistical tests were performed in Prism 6. Two-tailed $P < 0.05$ was considered significant.

3. Results

3.1. TAMs presented elevated PD-L1 expression compared to peritumoral macrophages

Resected tumors and peritumoral tissues were digested into single cell suspensions, and were processed by Ficoll gradient centrifugation to obtain mononuclear cells (Prince et al., 2007).