ONCOIMMUNOLOGY
2019, VOL. 8, NO. 10, e1636618 (6 pages)
https://doi.org/10.1080/2162402X.2019.1636618

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BRIEF REPORT

CD80 expression promotes immune surveillance in Barrett's metaplasia

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ABSTRACT

Esophageal adenocarcinoma (EAC) is the final step of a pathway starting with esophageal reflux disease, Barrett's metaplasia and Barrett's dysplasia. Positive costimulatory ligands such as CD80 have been suggested to contribute to anti-tumor T-cell efficacy. Here we report for the first time the role of CD80 in the inflammatory esophageal carcinogenesis and characterize the immune environment of EAC. Mucosa samples from cancer were obtained during esophagectomy from patients affected by EAC. Fresh biopsies were obtained from patients who underwent endoscopy for screening or follow-up. A rodent model of reflux induced esophageal carcinogenesis was created with an esophago-gastro-jejunostomy. CD80 expression was increased in epithelial cells during metaplasia in the inflammatory esophageal carcinogenesis cascade. *Cd80* null mice as well as WT mice that received antiCD80 antibodies showed a higher rate of dysplasia and KI-67+ cells. These results suggest that CD80 mediates an active immune surveillance process in early inflammation-driven esophageal carcinogenesis.

ARTICLE HISTORY
Received 21 January 2019
Revised 19 June 2019
Accepted 23 June 2019

KEYWORDS
Barrett's esophagus; esophageal dysplasia; immune surveillance; CD80; adenocarcinoma

Introduction

Esophageal adenocarcinoma (EAC) is an increasingly common cancer with a poor prognosis that almost always arises after a metaplasia-dysplasia-carcinoma sequence. The most important risk factor is metaplastic change of normal squamous epithelium called Barrett's esophagus (BE).[1,2] BE may be interpreted as a preneoplastic condition with reported risk of low grade dysplasia. However, results from surveillance cohorts indicate that most individuals with BE do not develop esophageal adenocarcinoma during endoscopic follow-up.[3–8] The inconsistency between the cumulative rate of dysplasia and the actual cancer incidence suggest the presence of a mechanism that can at least partially prevent malignant progression.

The immune system can specifically identify and eliminate tumor cells on the basis of their expression of tumor-specific antigens or molecules induced by cellular stress.[9,10] In this process, known as tumor immune surveillance or immunoediting, the immune system identifies cancerous and/or precancerous cells and eliminates them before they can cause harm. A successful elimination depends on an adequate T-cell priming and proper execution of the effector phase of the immune response. In each of these contexts, a potential role of T-cell costimulatory receptors has been implicated.[11] The lack of positive costimulatory ligands such as CD80 has been suggested to contribute to poor anti-tumor T-cell efficacy. Indeed, we have recently demonstrated that in colonic inflammatory carcinogenesis the progression from dysplasia to invasive cancer is controlled by an effective immune surveillance mechanism mediated by CD80 expression on epithelial cells that can completely clear preneoplastic lesions in a large proportion of cases.[12]

Despite improved knowledge about the interactions between immunity and cancer, regulation of the immune system during esophageal carcinogenesis is not yet fully understood. BE and EAC constitute interesting models for chronic inflammation associated with a (pre)malignant disease. Understanding interactions between tumor and the host immune system holds great promise to uncover biomarkers for targeted therapies and clinical outcomes. Therefore, the aim of our study was to evaluate CD80 expression and signaling in the context of inflammatory esophageal carcinogenesis.

Materials and methods

Patients

A prospective study of patients who underwent upper gastrointestinal endoscopy for dyspepsia (healthy controls) and for BE screening and follow up (BE or BE and esophageal dysplasia) or who had esophagectomy for EAC was designed. Biopsy samples of diseased mucosa were collected. The

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Supplemental data for this article can be accessed on the publisher's website.

study, which received institutional review board (Ethical Committee of the Veneto Institute of Oncology) approval (project MICCE1 IOV 2011/53), was performed according to the principles of the Declaration of Helsinki, and all those participating signed informed consent forms. The patients were diagnosed as follow: healthy controls, BE, BE and dysplasia, and EAC. Diagnosis was confirmed by clinical, radiological and histological parameters. Patients with gastritis and those who received neoadjuvant therapy were excluded. The characteristics of the patients and controls are outlined in Supplementary Table S1.

Flow cytometry analysis of the esophageal mucosa

Esophageal mucosa tissue samples were mechanically dissected and passed through a sterile Nylon Filter (BD Falcon, Heidelberg, Germany). The single cell suspension was pelleted, suspended in FACS buffer (PBS/2%FCS/0.02% sodium azide) and stained with the following fluorochrome-conjugated antibodies: anti-human CD80 FITC (clone 2D10.4), anti-human CD8a PE (clone HIT8a), anti-human CD28 FITC (clone CD28.2), anti-human HLA ABC FITC (clone W6/32) all from eBioscience and anti-pan Cytokeratin PE (clone C-11, Abcam). Staining was performed in FACS buffer for 30 minutes at 4°C after 20 min incubation with human Fc Receptor binding inhibitor (eBioscience). After two washes, samples results were acquired on a FACSCalibur based on CellQuest software (Becton Dickinson).

Quantitative real-time PCR

Total RNA was isolated using the SV Total RNA Isolation System (Promega), and reverse-transcribed into complementary DNA using the SuperScript™ VILO™ cDNA Synthesis Kit (Thermo Scientific). Specific mRNA transcripts were quantified with SYBR Green PCR Master Mix in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The expression of the target molecule was normalized to the expression of the *ACTB* housekeeping gene. Sequences of PCR primer pairs were for *PDCD1* (*PD-1*) fw 5′cgtggcctatccactcctca3′ rv 5′atcccttgtcccagccactc3′; *CD274* (*PD-L1*) fw 5′aaatggaacctggcgaaagc3′ rv 5′gatgagcccctcaggcattt3′; *ACTB* fw 5′ctggacttcgagcaagagatg3′ rv 5′agttgaaggtagtttcgtggatg3′.

Reflux induced esophageal carcinogenesis model

Animal experiments were performed according to Italian Law 26/2014 and European directive 2010/63/UE. This study was approved by the Ethical Committee of Padua University (Comitato Etico di Ateneo sulla Sperimentazione Animale CEASA, authorization n° 1121/2015-PR). In this study, an esophago-gastroduodenal anastomosis (EGDA) was

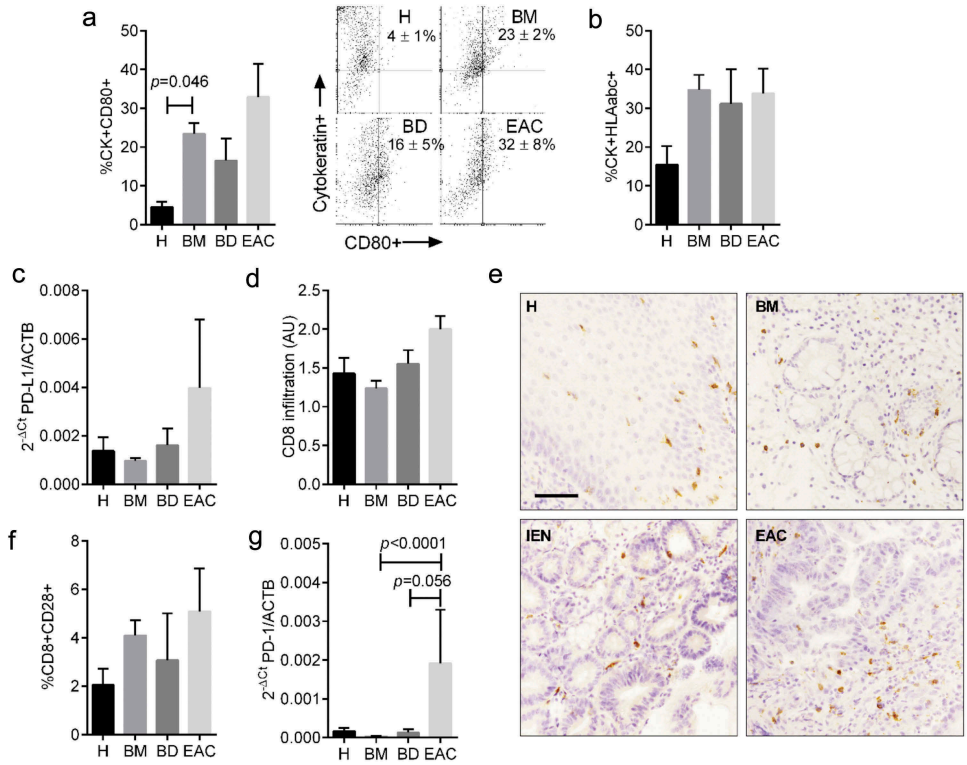

Figure 1. Characterization of CD80 expression and cytotoxic T cell in human inflammatory esophageal carcinogenesis.

Analysis of esophageal biopsies from healthy controls (H, n = 8), Barrett's metaplasia (BM, n = 55), Barrett's dysplastic esophagus (BD, n = 12) and esophageal adenocarcinoma (EAC, n = 15) for: (a) CD80 costimulatory molecule (b) HLA-ABC on esophageal epithelial cells (pan-cytokeratin+) by flow cytometry and (c) *PD-L1* immune checkpoint by Real Time qRT-PCR. (d) Immunohistochemical staining and quantification of CD8. (e) Representative immunohistochemical staining of CD8 in esophageal mucosa specimen. Magnification: 20x. (f) Flow cytometric analysis for CD28 on cytotoxic T cells (CD8+) and (g) Real Time RT-PCR quantification of *PD-1*. Statistical differences are indicated as *p* value (Dunn's multiple comparison test). IEN = intraepithelial neoplasia. Data are presented as mean ± SEM.

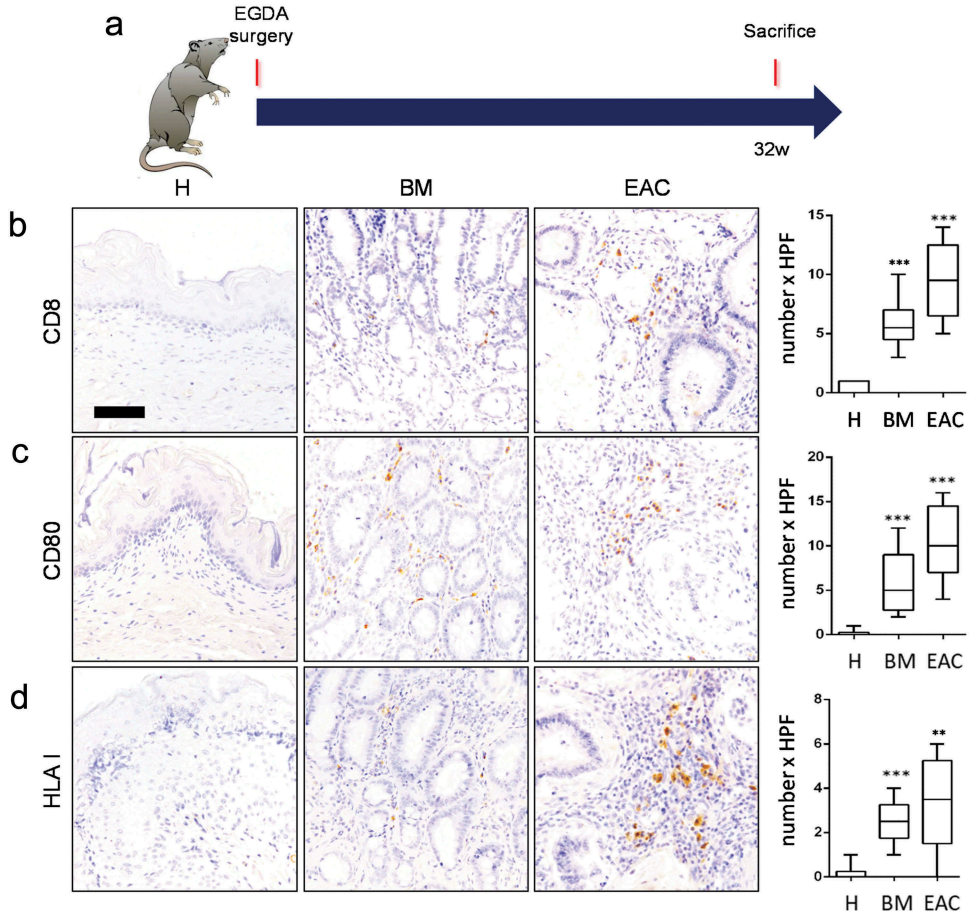

Figure 2. CD80 and immune microenvironment characterization in a rat model of reflux-induced esophageal carcinogenesis.
(a) Archival esophageal samples (n = 7) from male Sprague Dawley rats subjected to a surgical procedure to induce gastro-esophageal reflux and sacrificed 32 weeks after surgery were used. Representative immunohistochemical staining and quantification of (b) CD8, (c) CD80 and (d) HLA type I on esophageal samples of healthy (H), Barrett's metaplasia (BM) and esophageal adenocarcinoma (EAC) mucosa are shown. Graphs depicting number of positive cells x high power field (HPF) are shown for each staining. Statistical differences are indicated as *p* value (Dunn's multiple comparison test); **p < .01 and ***p < .001 vs healthy mucosa.

performed on 12 weeks old B6.129S4-Cd80 tm1Shr/J (CD80 null) mice and C57BL/6 mice purchased from Charles River accordingly to a previously published procedure.[13] Water and standard chow were given *ad libitum* before surgery. Water was permitted 2 hours after surgery and mouse chow was provided on the following day. Anesthesia was given using anesthetics pre-mixed in normal saline (80 mg/kg ketamine and 12 mg/kg xylazine, i.p.). The animals were given 5 mg/kg of Tramadol (Contramal®, Formenti, Verona, Italy) intraperitoneally immediately after the peritoneal incision. A side-to-side surgical EGDA was created between the first duodenal loop and the gastro-esophageal junction, with accurate mucosa-to-mucosa opposition, so that duodenal and gastric contents flowed back into the esophagus. After surgery, mice were divided into three groups: WT (reflux surgery, 15 male C57BL/6 mice), WT+anti-CD80 (reflux surgery, 15 male C57BL/6 mice, 200 µg/mouse injections of anti-CD80 antibody -clone 16-10A1, ATCC hybridoma no. HB-301- i.p. 24 and 28 weeks after surgery) and CD80 -/- (reflux surgery, 15 male CD80 null mice). The surviving animals were euthanized 32 weeks after surgery and the esophago-gastric specimens collected and analyzed in a blinded fashion.

Pathology

Immediately after death, the thoracic and abdominal cavities were examined and the esophagus, stomach, and jejunum were excised en bloc. The esophagus was opened longitudinally through the dorsal wall. With the mucosal surface uppermost, the margins of the specimen were fixed to a polystyrene plate with pins. Gross specimens were fixed in 10% neutral-buffered formalin for 24 hours. All specimens were examined grossly and cut serially (2–3 mm thick coronal sections). The tissue samples were routinely processed. Tissue sections (4 µm thick) were obtained from paraffin blocks and stained with hematoxylin & eosin (H&E). An experienced gastrointestinal pathologist (dr. Matteo Fassan) reviewed the slides in a blinded fashion.

Immunohistochemistry

Immunohistochemical (IHC) analyses were performed using standard procedures, and the resulting sections were evaluated by a single pathologist in a blinded fashion. Immunocomplexes were detected using the Dako Real Envision System Peroxidase and 3-3′ di-aminobenzidine tetrahydrochloride chromogen as

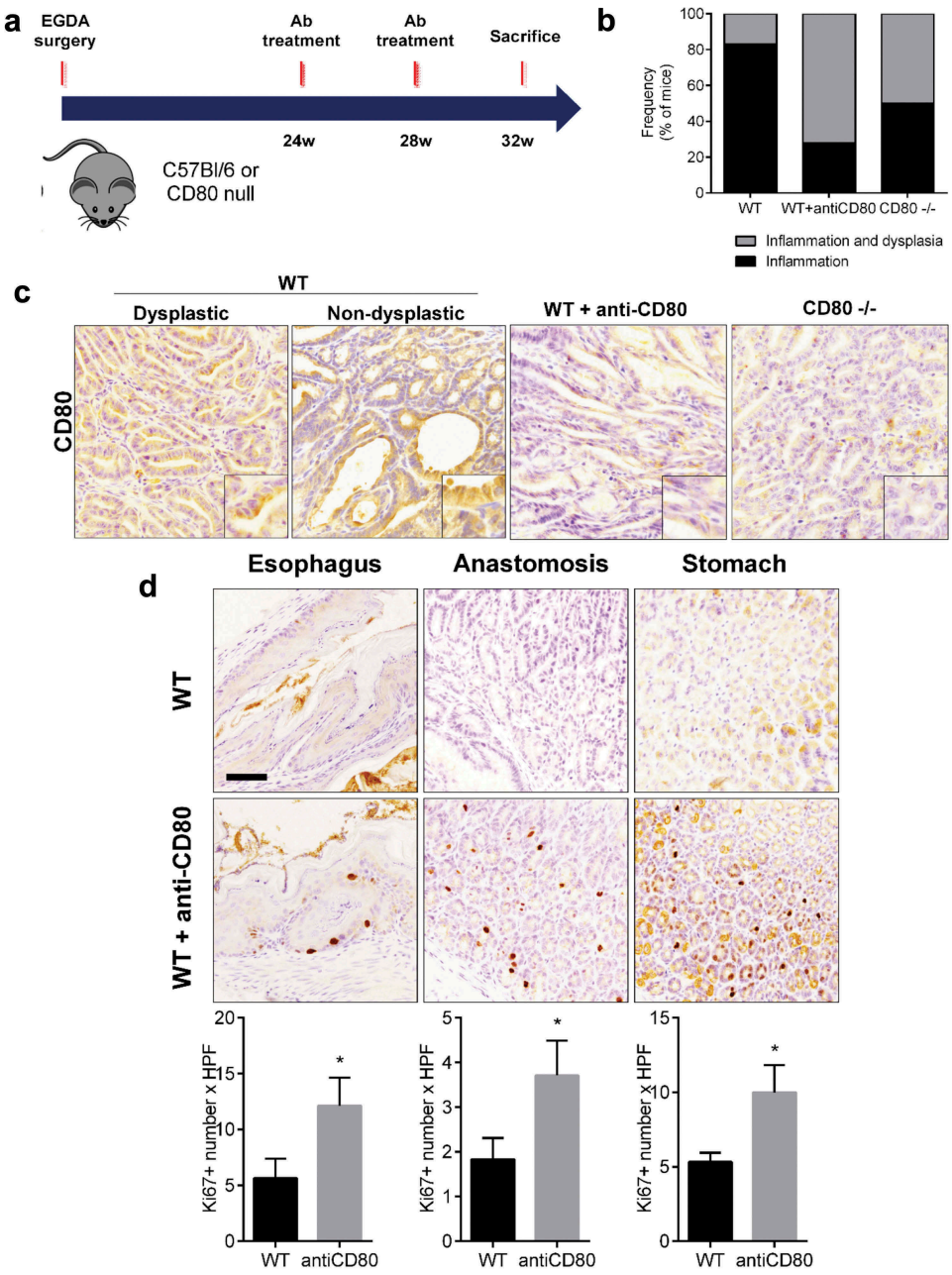

Figure 3. Effect of in vivo CD80 neutralization in a mouse model of reflux-induced esophageal carcinogenesis.

(a) Scheme for the experimental course of the esophageal carcinogenesis model. (b) Frequency of dysplasia in CD80-/- and WT mice with esophago-gastroduodenal anastomosis subjected to administration of IgG or anti-CD80 (n = 6–8 mice per group). (c) Representative immunohistochemical staining of CD80 expression in CD80-/- and WT mice with esophago-gastroduodenal anastomosis (n = 6–8 mice per group). (d) Representative immunohistochemical staining and quantification of Ki67 expression in the mid third of crypt on WT mice with esophago-gastroduodenal anastomosis subjected to administration of IgG or anti-CD80. Statistical differences are indicated as *p* value (Mann–Whitney's U test).

a substrate (Dako Denmark A/S) in formalin fixed paraffin embedded sections. IHC staining was performed using monoclonal antibodies for human CD8 (clone C8/144B, 1:50 dilution, DakoDenmark A / S), rat CD80 (clone MAB140, 1:800 dilution, R&D Systems), rat CD8 (clone M7103, 1:1300 dilution, Dako Denmark A/S), rat HLA type I (clone 66013–1-Ig, 1:1600 dilution, Proteintech Group) and mouse KI67 (clone SP6, M3062, 1:200 dilution, Spring Bioscience). The sections were lightly counterstained with hematoxylin. Moreover, positive cells were counted in five high power fields (HPF).

Statistics

Data are shown as mean +/− SEM. Statistical analysis was performed using GraphPad Prism Software 6.0 (GraphPad Software Inc., La Jolla, USA). The comparisons among the different step of the carcinogenesis were carried out with non parametric Kruskall Wallis'test and the post hoc comparison between groups was evaluated using Dunn's multiple comparison test. Mann–Whitney's U-test was used for comparisons of two groups. *P* values <.05 were considered significant for all the above-mentioned analysis.

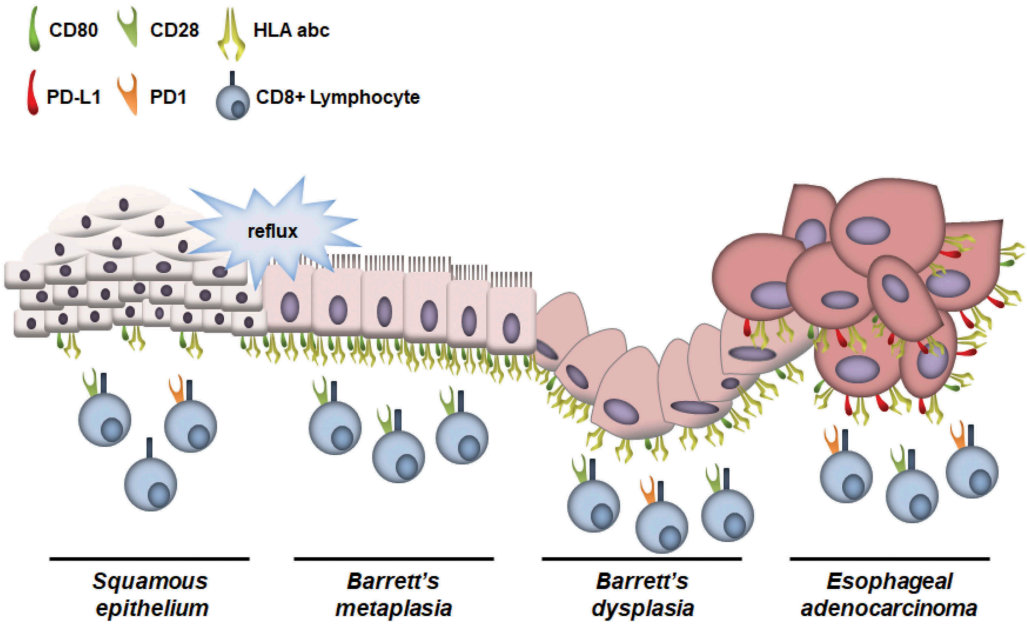

Figure 4. Mucosal microenvironment providing immune surveillance against esophageal carcinogenesis in Barrett's metaplasia.

Results

At first, we analyzed esophageal biopsies from healthy controls (n = 8), Barrett esophagus (n = 55), dysplastic esophagus (n = 12) and esophageal adenocarcinoma (n = 15) to evaluate the antigen presenting activity of epithelial cells and characterize the cytotoxic T lymphocytes in the microenvironment. The expression of the costimulatory molecule CD80 by esophageal epithelial cells augmented significantly during metaplasia in inflammatory esophageal carcinogenesis (Figure 1a) together with HLAabc expression (Figure 1b). On the other hand, the expression of the immune checkpoint *PD-L1* tended to increase just in the final step of esophageal carcinogenesis (Figure 1c). The infiltration of cytotoxic T lymphocytes remained substantially unchanged along the carcinogenesis cascade (Figure 1d), as well as the percentage of activated cytotoxic lymphocytes bearing CD28, the CD80 receptor (Figure 1e). Moreover, the expression of lymphocytes exhaustion marker *PD-1* was significantly lower in BE than in healthy controls and augmented in adenocarcinoma specimen (Figure 1f).

Immunostaining of normal, metaplastic and cancer archival esophageal tissues, obtained from rats subjected to a reflux model of esophageal carcinogenesis previously characterized by our group[13](Figure 2a), confirmed the same pattern of expression observed in human specimen: the number of CD80+, HLA type I+ and CD8+ cells significantly increased in Barrett's metaplasia compared to normal tissue (Figure 2 (b–d)). These data suggest these molecules are part of a shared immune surveillance mechanism among mammals.

To demonstrate the functional role of CD80 in immune surveillance during esophageal carcinogenesis progression, we used the same reflux model of esophageal carcinogenesis on CD80-/- or C57BL/6 mice, and subjected or not C57BL/6 operated mice to treatment with a neutralizing antibody against CD80 (Figure 3a). Consistent with a pivotal role for CD80 in esophageal cancer immune surveillance, 4 out of 8 CD80-/- mice and 5 out of 7 WT+anti-CD80 mice developed dysplasia in the fore stomach, at much higher compared to that observed in control WT mice (1/6) (Figure 3b). Immunostaining for CD80 revealed a moderate positive expression of the costimulatory molecule on dysplastic epithelial cells of WT mice, while there were low levels of expression in WT+antiCD80 group and no expression in CD80 knockout mice (Figure 3c). Moreover, expression of KI67, a marker of proliferative activity in malignant tumors, was significantly increased in the medium and upper third of the crypts in the esophagus, anastomosis and stomach mucosa of mice treated with anti-CD80 antibody compared to controls (Figure 3d), thus showing the protective role of CD80 in esophageal carcinogenesis progression.

Discussion

In this study, we characterized CD80 costimulatory molecule expression and demonstrated its protective role in inflammatory esophageal cancer progression. Indeed, BE rarely progresses to EAC, and a theory has recently been proposed that mucosal defenses in most patients with BE represent successful adaptations to the harsh intra-esophageal environment of chronic gastroesophageal reflux disease.[14] Several of these defenses have been identified, including the secretion of bicarbonate and mucous, expression of claudin 18 tight junctions, overexpression of defense and repair genes, and resistance to prolonged and repeated acid exposure.[15–18] In addition to these, for the first time we report evidence of an active immune surveillance process occurring in metaplasia involving CD80, possibly through the direct cross-talk between epithelial cells and cytotoxic lymphocytes.

The development of BE and EAC is associated with a relative increase of type 2 helper T cells[19] and the presence of an immunosuppressive (IL-4, IL-6, and IL-10) cytokine pattern[20] compared with gastroesophageal reflux induced esophagitis characterized by a type 1 helper T-cell immune response,

which is more appropriate for antitumor immunity. Our analysis of human esophageal specimen of the metaplasia-dysplasia -carcinoma cascade reveals that CD80 overexpression is occurring in metaplastic cells and that the immune microenvironment consists of cytotoxic lymphocytes responsive to CD80 co-stimulation, thus showing the presence of a microenvironment supporting immune surveillance in early esophageal carcinogenesis. This condition persists along the carcinogenesis cascade but at its final stages fails to work properly due to the increased expression of *PD-L1* and *PD-1* that favor immune escape in EAC (Figure 4). Moreover, our in vivo experiments with CD80-/- mice and WT mice treated with anti-CD80 indicate that the lack of CD80 favors the development of dysplasia, as shown by KI67 staining above the basal third of the mucosal crypts.[21] These data fix the time point when the CD80-CD28 crosstalk occurs, initiating the immune surveillance process in the esophageal carcinogenesis.

These findings represent a first step towards identifying those factors that may have a prognostic/predictive role and therefore allow the development of targeted therapies to improve patient outcomes. A fascinating working hypothesis is that BE patients with low CD80 expression might evolve towards dysplasia and thus, they should undergo a strict endoscopic follow up while those with high CD80 expression might skip part or all of it. Moreover, our human data suggest that the CD80 – CD28 crosstalk probably occurs even in EAC but its effect is inhibited by the PD-L1 – PD1 crosstalk.

Acknowledgments

The authors are extremely grateful to Professor Giuseppe Opocher, Scientific Director of the Veneto Institute of Oncology, for his constant support to this project.

Disclosure statement

The authors declare no conflicts of interest.

Funding

This work was supported by the Current Research Funding from Italian Ministry of Health to Veneto Institute of Oncology IOV-IRCCS to Carlo Castoro and then to Marco Scarpa; Finalized Research Funds (awarded in 2011 to MICCE1 project) from the Veneto Region to Carlo Castoro.

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