GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Dendritic Cells in Barrett's Esophagus Carcinogenesis

An Inadequate Microenvironment for Antitumor Immunity?

Joan Somja,^{*†} Stephanie Demoulin,[†] Patrick Roncarati,[†] Michaël Herfs,[†] Noella Bletard,^{*} Philippe Delvenne,^{*†} and Pascale Hubert[†]

From the Department of Pathology,* University Hospital of Liege, Liege; and the Interdisciplinary Cluster in Applied Genoproteomics (GIGA),[†] Laboratory of Experimental Pathology, University of Liege, Liege, Belgium

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Address correspondence to
Pascale Hubert, Ph.D., Labora-
tory of Experimental Pathology,
University of Liege, Pathology
Tower B23, Sart-Tilman,
B-4000 Liege, Belgium.
E-mail: p.hubert@ulg.ac.be.

Barrett's esophagus corresponds to the replacement of the normal esophageal squamous epithelium by a columnar epithelium through a metaplastic process. This tissue remodeling is associated with chronic gastroesophageal reflux and constitutes a premalignant lesion leading to a 30- to 60-fold increase in the risk to evolve into esophageal adenocarcinoma. The present study aimed to investigate a possible immune evasion in Barrett's esophagus favoring esophageal adenocarcinoma development. We demonstrated that myeloid and plasmacytoid dendritic cells are recruited during the esophageal metaplasia-dysplasia-carcinoma sequence, through the action of their chemoattractants, macrophage inflammatory protein 3 α and chemerin. Next, we showed that, in contrast to plasmacytoid dendritic cells, myeloid dendritic cells, co-cultured with Barrett's esophagus and esophageal adenocarcinoma cell lines, display a tolerogenic phenotype. Accordingly, myeloid dendritic cells co-cultured with esophageal adenocarcinoma cell lines stimulated regulatory T cell differentiation from naïve CD4⁺ T cells. In agreement with those results, we observed that both metaplastic areas and (pre)malignant lesions of the esophagus are infiltrated by regulatory T cells. In conclusion, soluble factors secreted by epithelial cells during the esophageal metaplasia-dysplasia-carcinoma sequence influence dendritic cell distribution and promote tumor progression by rendering them tolerogenic. (*Am J Pathol* 2013, 182: 2168–2179; <http://dx.doi.org/10.1016/j.ajpath.2013.02.036>)

Barrett's esophagus (BE) is defined by the replacement of the normal esophageal squamous epithelium by a metaplastic columnar epithelium containing true goblet cells on histological examination.¹ BE is considered as an adaptive response after chronic gastroesophageal reflux.² BE can be classified histologically into four groups [no dysplasia, indefinite for dysplasia, low-grade dysplasia (LGBE), or high-grade dysplasia (HGBE)], depending on the presence or absence of dysplastic cells in the epithelium. In the general population, BE prevalence is estimated to be between 1.6% and 6.8%.² This tissue remodeling is associated with a 30- to

60-fold increase in the risk of developing an esophageal adenocarcinoma (EAC),² and EAC almost always arises after a metaplasia-dysplasia-carcinoma (MDC) sequence. BE remains an important health challenge. First, survival outcomes for patients with EAC remain poor despite recent

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diagnostic and therapeutic improvements, with community 5-year survival rates of <20%.^{3,4} Second, the incidence of BE has dramatically increased in the Western world during these past years, compared with other types of cancers, with an increment of >600%.^{4,5}

Despite improved knowledge about the interactions between immunity and cancer, regulation of the immune system during the MDC sequence is not yet fully understood. BE and EAC constitute interesting models for chronic inflammation associated with a (pre)malignant disease.⁴ For the most part, the development of BE and EAC is associated with a relative increase of type 2 helper T cells⁶ and the presence of an immunosuppressive (IL-4, IL-6, and IL-10) cytokine pattern,⁷ compared with gastroesophageal reflux-induced esophagitis characterized by a type 1 helper T-cell immune response, which is more appropriate for antitumor immunity.

Dendritic cells (DCs) are specialized antigen-presenting cells that provide a critical link between innate and adaptive immune responses. Human DCs are divided into two major intrinsically different subsets: myeloid DCs (mDCs), also called conventional DCs, and plasmacytoid DCs (pDCs), which differ from mDCs in their transcriptional program, phenotypic markers, and immunological functions.⁸ mDCs play a crucial role in the regulation of adaptive immunity by their unique ability to induce a primary immune response in resting naïve T cells.^{8,9} mDCs, characterized by the expression of the CD1a marker,^{10,11} arise from bone marrow-derived myeloid progenitors and circulate in the peripheral bloodstream as precursors that home to tissues where they reside as immature cells with high endocytic activity and low T-cell activation potential.¹² These mDCs express CCR6 and are attracted via this chemokine receptor by macrophage inflammatory protein (MIP) 3 α ¹³ in tissues. On exposure to danger signals [eg, lipopolysaccharide (LPS)], immature mDCs undergo maturation characterized by an up-regulation of costimulatory and antigen-presenting molecules.^{9,14–17} *De novo* CCR7 expression allows these mature cells to migrate to local lymph nodes, where they can trigger an effective T-cell response.¹⁷ Fully mature mDCs are characterized by the production of the IL-12 proinflammatory cytokine, which is required for the induction of an efficient T-cell response.¹⁸ pDCs, the second subset of DCs, represent a rare population of human blood cells characterized by a rapid and massive secretion of type I interferon on activation via a Toll-like receptor-dependent recognition of pathogenic agents or danger signals. Immature pDCs present plasma cell–like morphological characteristics and selectively express CD4, CD45RA, CD123, BDCA-2, and BDCA-4, but are CD11c negative.¹⁹ Under steady-state conditions, pDCs reside in secondary lymphoid tissues, from where they are mobilized to inflammatory sites²⁰ under pathological conditions, notably through the action of one of their chemoattractants, chemerin.²¹ Through their capacity to conjugate innate and adaptive immunity and to secrete soluble factors controlling cancer development, these cells are crucial actors in antitumor immunity.²²

mDC and pDC involvement in tumor immunity was shown to have a clinical impact because their infiltration in some primary tumor types has been associated with significant changes in patient survival and recurrent disease.^{9,23–29} It has been postulated that tumors may evade the immune system by an impairment of DC number and functions mediated by a local production of immunosuppressive cytokines, such as prostaglandin E₂ (PGE₂), receptor activator of NF- κ B ligand (RANKL), and IL-10, or by a modulation of chemokine expression.³⁰ Moreover, accumulating evidence suggests that mDCs and pDCs recruited to the tumor microenvironment often display tolerogenic properties by promoting regulatory T cell (Treg) expansion or proliferation.^{31–33}

In this study, we tested the hypothesis that impaired antitumor immunity in BE could promote the development and progression of EAC. First, we evaluated the differential density of mDCs, pDCs, and Treg cells in the MDC sequence associated with BE. Then, we evaluated the expression of chemokines (MIP3 α and chemerin) that could explain these changes of immune cell density in the MDC sequence. Finally, we performed *in vitro* studies to determine whether DC functionality is altered in the presence of soluble factors secreted by BE, HGBE, and EAC cell lines.

A better understanding of the factors involved in the transformation of BE into EAC through a deregulation of antitumor immunity could allow the development of new immunotherapeutic strategies leading to effective antitumoral responses.

Materials and Methods

Tissue Specimens

A total of 100 formalin-fixed, paraffin-embedded endoscopic biopsy or surgical specimens were obtained from the Tumor Bank of the University Hospital of Liege (Liege, Belgium). Samples were reviewed by two gastrointestinal pathologists (N.B. and J.S.) and included normal esophageal mucosa ($n = 20$), BE without dysplasia ($n = 20$), LGBE ($n = 20$), HGBE ($n = 20$), and EAC ($n = 20$). Only EACs arising in the esophagus in the presence of adjacent Barrett's metaplasia were considered. Each individual case was classified in accordance with the Vienna classification. We also analyzed the progression potential of these cases to more severe lesions in follow-up biopsy or surgical specimens. The protocol was approved by the Ethics Committee of the University Hospital of Liege, and all human subjects provided appropriate informed consent. Clinical and pathological data were available for each patient and are summarized in Table 1.

mDCs, pDCs, and Treg Cell Detection by IHC

Paraffin-embedded sections of esophageal biopsy specimens (4 μ m thick) underwent immunostaining using an automated

Table 1 Patients' Clinical and Pathological Features

Features	NE (n = 20)	BE (n = 20)	LGBE (n = 20)	HGBE (n = 20)	EAC (n = 20)
Age, means $\pm$ SD (years)	48 $\pm$ 17	61 $\pm$ 12	64 $\pm$ 11	64 $\pm$ 12	64 $\pm$ 12
Sex (% males)	45	85	80	70	90
Depth of invasion (%)					pT1 15 pT2 30 pT3 50 pT4 5
Nodal status (%)					Negative 35 Positive 65
Metastasis (%)					No 85 Yes 15
Differentiation (%)					Well 15 Moderate 65 Poor 20
Progression to higher grade (%)	0	20	30	50	
Survival (%)					<1 year 25 >1 year 75

pT, pathological tumor stage (7th American Joint Committee on Cancer).

immunostainer (Ventana Medical Systems, Tucson, AZ) with antibodies directed against human CD1a (Novocastra, Newcastle, UK), BDCA-2 (Dendritics, Lyon, France), or forkhead box P3 (FoxP3; eBioscience, San Diego, CA) (Table 2). An amplification kit (Ventana Medical Systems) and a detection system including diaminobenzidine (Dako, Glostrup, Denmark) as chromogen were used during the automated procedure. Archival lymph node sections were used as positive controls. For negative controls, the primary antibody was omitted. The mean number of mDCs, pDCs, and Treg cells in each patient was calculated by counting these cells (original magnification, $\times 400$) in the 10 most cellular microscopic fields, also called hot spots. Inflammatory

cells were quantified separately in the epithelium and lamina propria for CD1a-positive cells. Treg cells and pDCs were essentially limited to the lamina propria in the entire MDC sequence.

Double immunostaining with FoxP3 and CD1a was performed to analyze a possible colocalization of Treg cells with CD1a⁺ mDCs. Briefly, after FoxP3⁺ cell staining, sections were washed and antigens were retrieved with heating in a pressure cooker. Then, slides were incubated with anti-CD1a antibody for 1 hour. After rinsing, the sections were incubated with the secondary antibody, and antigen visualization was performed by using the EnVision G/2 system, Permanent Red (Dako).

Table 2 Antibodies Used for IHC and Flow Cytometry Analysis

Antibody	Dilution	Clone	Species	Company name
CD1a	Prediluted	MTB1	Mouse	Novocastra
FoxP3	1:50	236A/E7	Mouse	eBioscience
BDCA2	1:50	124B3.13	Mouse	Dendritics
MIP3 α /CCL20	1:100	NA	Rabbit	Abcam (Cambridge, MA)
Chemerin	1:1000	NA	Rabbit	Phoenix Pharmaceuticals (Burlingame, CA)
RANKL	1:20	70525	Mouse	R&D Systems (Minneapolis, MN)
CD1a-FITC	1:20	NA1/34	Mouse	Dako (Glostrup, Denmark)
CD14-PE	1:20	TÜK	Mouse	Dako
CD80-PE	1:10	2D10.4	Mouse	eBioscience
CD83-PE	1:20	HB15e	Mouse	BD Pharmingen (Franklin Lakes, NJ)
CD86-PE	1:20	c2331 (FUN-1)	Mouse	BD Pharmingen
HLA-DR-PE	1:20	AB3	Mouse	Dako
HLA-ABC-PE	1:20	W6/32	Mouse	eBioscience
CCR7-PE	1:20	150503	Mouse	R&D Systems
CD123-FITC	1:20	AC145	Mouse	Miltenyi Biotec, GmbH (Bergisch Gladbach, Germany)
CD11c-APC	1:20	B-ly6	Mouse	BD Pharmingen
BDCA-4-PE	1:20	AD5-17F6	Mouse	Miltenyi Biotec, GmbH
CD40-PE	1:20	5C3	Mouse	BD Pharmingen

APC, antigen-presenting cell; NA, not available.

MIP3 α , Chemerin, and RANKL Detection by IHC

Paraffin-embedded sections (4 μ m thick) were deparaffinized in xylene and rehydrated through a graded ethanol series, and antigen was retrieved with EDTA solution combined with heating in a pressure cooker for MIP3 α and citrate buffer, combined with heating in a microwave oven for RANKL. Antigen retrieval was unnecessary for chemerin. Slides were peroxidase blocked with peroxidase-blocking solution (Dako) for 10 minutes and then were incubated with the primary antibody anti-MIP3 α (Abcam, Cambridge, MA) or anti-RANKL (R&D Systems, Minneapolis, MN) (Table 2) for 1 hour and detected with the EnVision system (Dako) for 30 minutes. Other slides were incubated with primary anti-chemerin antibody (Phoenix Pharmaceuticals, Burlingame, CA) (Table 2) for 2 hours and detected with the LSAB2 System—horseradish peroxidase kit (Dako). Colorimetric detection was completed with diaminobenzidine (Dako) for 5 minutes. Slides were then counterstained with hematoxylin. MIP3 α , chemerin, and RANKL expression in the epithelium was evaluated, as previously described,³⁴ by a semiquantitative score of the intensity and extent of the staining according to an arbitrary scale.

mDC and pDC Generation

mDCs were generated from CD34⁺ hematopoietic progenitor cells, as previously described.³⁵ Briefly, CD34⁺ cells were isolated from cord blood and cultured for 7 days with 20 ng/mL human stem cell factor (Peprotech, Rocky Hill, NJ), 10 ng/mL thrombopoietin (Peprotech), 25 ng/mL fms-like tyrosine kinase 3 ligand (Flt3L) (Peprotech), 200 U/mL granulocyte-macrophage colony-stimulating factor (Amoytop Biotech, Xiamen, China), and 100 U/mL IL-4 (ImmunoTools, Friesoythe, Germany). Every 2 to 3 days, cultures were supplemented with 200 U/mL granulocyte-macrophage colony-stimulating factor and 100 U/mL IL-4. As previously described,³⁶ pDCs were generated from cord blood CD34⁺ hematopoietic progenitor cells cultured for 21 days in the presence of 10 ng/mL TPO, 100 ng/mL Flt3L, and 20 ng/mL IL-3 (Peprotech). All human samples were collected according to a protocol approved by the Ethics Committee of the University Hospital of Liège.

Co-Cultures of mDCs or pDCs in the Presence of Esophageal Cell Lines

Human SV40-immortalized normal esophageal (NE) squamous epithelial cell line (HET1A) was obtained from ATCC (Manassas, VA). Barrett's esophageal hTERT-immortalized cell lines CPA (nondysplastic metaplasia) and CPD (high-grade dysplasia) were kind gifts from Prof. Peter Rabinovitch (University of Washington, Seattle). The human esophageal adenocarcinoma cell line, JH Eso Ad1, was kindly provided by Prof. James Eshleman (Johns Hopkins

University, Baltimore, MD). Human esophageal adenocarcinoma cell line, OE33, was purchased from the European Collection of Cell Cultures (Salisbury, UK). Cell cultures were maintained following European Collection of Cell Cultures and ATCC reported methods. Co-cultures were initiated by seeding HET1-A, CPA, CPD, OE33, and JH-Eso Ad1 cell lines (2.5×10^4 cells/mL) or medium alone on 0.4- μ m pore size membrane inserts (Nunc, Roskilde, Denmark) in 6-well plates, into which mDCs or pDCs generated as previously described, were seeded at 0.5×10^5 and 0.5×10^6 cells/mL, respectively. For experiments using mDCs, inserts were extracted after 6 days of co-culture, and 1 μ g/mL LPS (Sigma-Aldrich, St. Louis, MO) was added to the wells for 24 hours to induce mDC maturation. For experiments using pDCs, inserts were removed after 24 hours of co-culture, and 12 μ g/mL of CpG oligodeoxynucleotide (ODN) 2216 [5'-ggGGGACGATCGTCgggggg-3' (lower-case letters represent phosphorothioate linkage); Eurogentec, Seraing, Belgium] was added to the wells to induce pDC maturation.

OPG Treatment

mDC co-cultures were either untreated or treated with 0.5 μ g/mL human recombinant osteoprotegerin (OPG; Abcam), an inhibitor of RANKL on days 1 and 3 of the co-culture. mDCs cultured alone or in the presence of OPG were used as controls.

Flow Cytometry Analysis

Flow cytometry studies were performed by using procedures published previously.^{36,37} For mDC studies, the following antibodies were used: CD1a—fluorescein isothiocyanate, CD14—phosphatidylethanolamine (PE), CD80-PE, CD83-PE, CD86-PE, HLA-DR-PE, HLA-ABC-PE, and CCR7-PE (Table 2). For pDC studies, the following antibodies were used: CD123—fluorescein isothiocyanate, CD11c—antigen-presenting cell, BDCA-4-PE, CD40-PE, CD83-PE, CD86-PE, CCR7-PE, and HLA-DR-PE (Table 2). Fluorescence intensity and positive cell percentages were measured on an FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ), and data were analyzed using FACSDiva software, version 6.1.2 (Becton Dickinson).

ELISA for IL-10, IL-12p70, and RANKL

Supernatants collected from co-culture experiments using mDCs were assessed for IL-10, IL-12p70, and RANKL concentrations by using ELISA kits, according to the manufacturer's instructions [IL-10 (Invitrogen, Carlsbad, CA), IL-12p70 (R&D Systems), and RANKL (Biomedica, Vienna, Austria)]. All assays were performed using duplicate samples.

Treg Cell Induction Assay

The assay was performed by culturing mDCs (stimulator cells) isolated from co-culture experiments with allogeneic CD4⁺ T cells (responder cells) sorted from peripheral blood mononuclear cells using the MACS CD4⁺ T Cell Isolation Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), according to the manufacturer's protocol. The stimulator-to-responder ratio corresponded to 1:10, and cells were placed in RPMI 5% human pooled AB serum (Invitrogen) in 6-well plates (Nunc) for 6 days. mDCs cultured alone were used as controls.

Quantitative FoxP3 Real-Time PCR

Total RNA (1 µg) extracted from Treg cell induction assays (RNeasy minikit; Qiagen, Valencia, CA) was reverse transcribed using Superscript II reverse transcriptase (Invitrogen), according to manufacturer's instructions. Quantitative real-time PCR was then performed using Power SYBR Green

Master Mix (Eurogentec) and the following primer sequences: FOXP3, 5'-CAGCACATTCCCAGAGTTCCTC-3' (forward) and 5'-GCGTGTGAACCAGTGGTAGATC-3' (reverse); and glyceraldehyde-3-phosphate dehydrogenase, 5'-ACCAGG-TGGTCTCCTCTGAC-3' (forward) and 5'-TGCTGTAGC-CAAATTCGTTG-3' (reverse) (Eurogentec). All of the experiments were performed in duplicate using the ABI-Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), and negative controls (master mix without any cDNA) were added in each run. The results were analyzed by comparative C_T values and reference gene C_T values and normalized by glyceraldehyde-3-phosphate dehydrogenase as an internal control.

Statistical Analysis

Statistical analyses were performed by using GraphPad Prism software, version 4.00 (GraphPad Software, San Diego, CA). The data were not normally distributed. Nonparametric tests, such as the Kruskal-Wallis and *U*-test were then used, and

Figure 1 mDC, pDC, and Treg densities increase along the MDC sequence. CD1a, BDCA-2, and FoxP3 immunostaining in BE and EAC specimens. **A** and **B**: BE displays a low density of CD1a⁺ cells in the lamina propria compared with EAC, which is associated with a relative increased density of DCs. **D** and **G**: BE shows few BDCA-2⁺ and FoxP3⁺ cells. These cells are almost exclusively localized in the lamina propria. **D**, **E**, **G**, and **H**: BDCA-2⁺ and FoxP3⁺ cell numbers are higher in EAC compared with BE. **C**, **F**, and **I**: Evaluation by IHC of the number of CD1a⁺, BDCA-2⁺, and FoxP3⁺ cells per 10 high-power fields (HPFs) in BE, LGBE, HGBE, and EAC specimens. **C**: We observed modifications of CD1a⁺ cell densities within the sequence MDC. The development of EAC was associated with an increased density of mDCs compared with BE. **F** and **I**: BDCA-2⁺ and FoxP3⁺ cell numbers increased during the MDC sequence. Original magnification, ×200 (**A**, **B**, **D**, **E**, **G**, and **H**). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 compared with BE. Statistical significance was calculated using a *U*-test.

differences were considered as statistically significant when $P < 0.05$.

Results

mDC, pDC, and Treg Density Is Increased Along the MDC Sequence

To evaluate the immune cell infiltration, focusing on mDCs, pDCs, and Treg cells in BE carcinogenesis, the density of CD1a⁺, BDCA-2⁺, and FoxP3⁺ cells was evaluated by immunohistochemistry (IHC). Examples of representative IHC staining are presented in **Figure 1**. BE displayed a low density of CD1a⁺ cells in the lamina propria with an irregular distribution between the glands (**Figure 1A**). EAC was associated with a relatively increased density of CD1a⁺ cells compared with BE ($P < 0.001$) (**Figure 1B**). Semiquantitative analysis showed modifications of CD1a⁺ mDC density within the MDC sequence (**Figure 1C**). HGBE was associated with a significant increase in the density of CD1a⁺ mDCs compared with BE ($P < 0.05$). The development of EAC was also associated with a relatively increased density of CD1a⁺ mDCs compared with LGBE ($P < 0.001$) and HGBE ($P < 0.05$). BDCA-2⁺ pDCs showed a significant increase during the MDC sequence compared with BE ($P < 0.01$) (**Figure 1, D–F**). FoxP3⁺ Treg cell number also increased during the MDC sequence and was significantly different from BE, HGBE ($P < 0.01$), and EAC ($P < 0.001$) (**Figure 1, G–I**).

Increased Expression of MIP3 α and Chemerin in the MDC Sequence

By using IHC, BE, LGBE, HGBE, and EAC specimens were evaluated for MIP3 α and chemerin expression. BE was often associated with weak MIP3 α immunoreactivity in the epithelium (**Figure 2A**). In LGBE (**Figure 2C**), MIP3 α expression increased significantly compared with BE ($P < 0.05$). The MIP3 α score was statistically higher in HGBE (**Figure 2E**) ($P < 0.01$) and EAC (**Figure 2G**) ($P < 0.001$) than in BE. Semiquantitative evaluation of MIP3 α intraepithelial expression is shown in **Figure 2I**. In the same way, chemerin was detected in the cytoplasm and nucleus of epithelial cells, often associated with diffuse stromal expression. Chemerin immunoreactivity was weak and sporadic in BE (**Figure 2J**). Intense immunostaining for chemerin was observed in epithelial EAC samples (**Figure 2H**), with a significant increase compared with BE (**Figure 2B**) ($P < 0.001$). However, chemerin expression in BE and HGBE (**Figure 2F**) showed a significant decrease compared with NE ($P < 0.01$) (data not shown). We also observed a significant increase of chemerin expression from BE to LGBE ($P < 0.01$), BE to HGBE ($P < 0.01$), and HGBE to EAC ($P < 0.05$).

mDCs Present a Tolerogenic Phenotype in the Presence of BE, HGBE, and EAC Cell Lines

To evaluate the effects of the soluble factors released by epithelial cells on mDCs in BE carcinogenesis, mDCs were co-cultured in the presence of NE, BE, HGBE, and EAC cell lines, with transwell membranes avoiding cell-to-cell contact. After 6 days, mDCs were matured with LPS. BE, HGBE, and EAC cell lines inhibited mDC maturation. Indeed, mDCs generated in the presence of CPA (BE cell line) showed a lower expression of CD80, CD83, and CD86, as did other molecules involved in antigen presentation (HLA-ABC and HLA-DR) compared with control mDCs (**Figure 3, A–F**). A similar reduced expression of CD80 was observed when mDCs were cultured in the

Figure 2 MIP3 α and chemerin expression is increased in the MDC sequence. Representative IHC examples of MIP3 α and chemerin expression in the epithelium of BE (**A** and **B**), LGBE (**C** and **D**), HGBE (**E** and **F**), and EAC (**G** and **H**). **I** and **J**: Semiquantitative analysis of MIP3 α and chemerin expression shows a significant increased expression in LGBE, HGBE, and EAC compared with BE. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with NE. Statistical significance was calculated by using a *U*-test. Original magnification, $\times 200$ (**A–H**).

Figure 3 mDCs display an immature IL-10^{High}IL-12^{Low} tolerogenic phenotype in the presence of BE and EAC cell lines. **A–F:** Analysis of mDC phenotype after co-culture with normal esophageal squamous cells (HET-1a), Barrett's esophagus (CPA), high-grade Barrett's esophagus (CPD), and esophageal adenocarcinoma (OE33 and JHEsoAd1) cell lines. After 6 days of co-culture and 24 hours of incubation with LPS, analysis of the cell surface expression of CD80, CD83, CD86, HLA-DR, HLA-ABC, and CCR7 was performed using flow cytometry. Mean percentages of positive cells and SDs obtained in 12 different experiments are reported. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with control mDCs (CTRL). Statistical significance was calculated by using a Kruskal-Wallis test. **G and H:** Secreted cytokine levels (IL-10 and IL-12p70) in culture supernatants of mDCs co-cultured with normal esophageal squamous cells (HET-1a), Barrett's esophagus (CPA), high-grade Barrett's esophagus (CPD), and esophageal adenocarcinoma (OE33 and JHEsoAd1) cell lines. The secretion levels were measured by ELISA after 6 days of co-culture and 24 hours of incubation with LPS. mDCs cultured alone were used as controls (CTRL DC). Data are presented as means and SDs of 10 independent experiments performed in duplicate. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with controls. Statistical significance was calculated by using a *U*-test.

presence of CPD (HGBE cell lines). In addition, OE33 and JhEsoAd1 (EAC cell lines) were also associated with a reduced expression of CD80, CD83, and CD86 in co-cultured mDCs. In contrast, no significant difference was observed when HET-1a (normal esophageal squamous cell line) was used. Consistent with their reduced immunostimulatory capacity, mDCs generated in the presence of BE and EAC cell lines produced higher levels of IL-10 and lower amounts of IL-12 compared with control mDCs (Figure 3, G and H). HET-1a cells did not alter the secretion of these two cytokines. The fact that BE and EAC cell lines inhibit dendritic cell maturation and IL-12p70 secretion, while enhancing IL-10 secretion in response to LPS, suggests a tolerogenic mDC phenotype. On the contrary, pDC maturation status was not significantly modified after they were co-cultured with NE, BE, HGBE,

and EAC cell lines, compared with pDCs cultured alone (data not shown).

mDCs Co-Cultured with BE, HGBE, and EAC Stimulate Treg Cell Differentiation from Naïve CD4⁺ T Cells

By using a Treg cell induction assay, we showed that mDCs co-cultured with BE, HGBE, and EAC cell lines stimulate the differentiation of naïve CD4⁺ T cells into Treg cells.

However, only mDCs co-cultured with EAC cell lines (JH Eso Ad1 and OE33) induce a significant increase in the expression of *FoxP3* mRNA ($P < 0.05$) in T cells when compared with *FoxP3* induction in T cells exposed to mDCs cultured alone. These results confirmed that mDCs differentiated in the presence of EAC cell lines are able to induce a tolerogenic response (Figure 4A).

Figure 4 mDCs co-cultured with BE, HGBE, and EAC induce Treg differentiation *in vitro* and colocalize with Treg cells *in vivo* in EAC. **A:** mDCs isolated from previously described co-culture experiments were seeded with allogeneic CD4⁺ T cells for 6 days. mDCs cultured alone were used as controls. Quantitative real-time PCR analysis was performed to assess FoxP3 expression in the samples collected. We observed that EAC cell lines significantly increase the ability of mDCs to stimulate Treg cell differentiation compared with mDCs cultured alone ($n = 3$). Statistical significance was calculated by using a *U*-test. * $P < 0.05$. **B:** CD1a⁺ and FoxP3⁺ cells in an EAC surgical specimen. CD1a⁺ cells are stained red, whereas FoxP3⁺ cells are visualized in brown. A close localization between CD1a⁺ cells and FoxP3⁺ cells is observed in the peritumoral area. Original magnification, $\times 400$.

mDCs and Treg Cells Colocalize in EAC

Double immunostaining with CD1a and FoxP3 revealed the existence of a direct contact between FoxP3⁺ Treg cells and CD1a⁺ mDCs. These cells formed several clusters within the lamina propria in the four tested specimens of EAC. An example of these CD1a⁺ mDCs and FoxP3⁺ Treg cell clusters is presented in Figure 4B.

RANKL Expression Increases during the MDC Sequence

RANKL expression was investigated using IHC. EAC and HGBE epithelium showed a higher and more diffuse RANKL immunoreactivity compared with BE ($P < 0.01$ and $P < 0.001$, respectively). In LGBE, RANKL immunoreactivity was weaker and detected in fewer cells (Figure 5, A–D). ELISA analysis in BE, HGBE, and EAC

culture media confirmed that RANKL is secreted by these epithelial cell lines (Figure 5E). RANKL expression was significantly higher ($P < 0.01$) in OE33 (EAC cell line) than in CPA (BE cell line), as shown in Figure 5F. However, RANKL inhibition by OPG, a RANKL antagonist, in co-cultures was not sufficient to significantly reverse the tolerogenic phenotype of mDCs (Figure 5, G–L).

Discussion

BE is a premalignant lesion presenting a high risk of developing an EAC. The malignant transformation of BE could result from the presence of immune alterations that would prevent the development of an efficient antitumor immune response. Metaplastic and/or inflammatory cells could generate an immunosuppressive environment and promote malignant transformation in metaplastic areas by altering antitumor immunity.³⁸ Indeed, Moons et al⁶ showed that the mean proportion of CD8⁺-effective T cells in BE is less than in NE. Moreover, higher IL-10 mRNA levels were reported in BE,³⁹ suggesting a decreased immunosurveillance during the esophageal MDC sequence.

Because previous reports showed that DCs can present an altered functionality and/or tolerogenic activities in a tumor setting,^{40–43} we investigated whether these cells play a role in the evolution of BE to AEC. First, we demonstrated that mDCs and pDCs are present in esophageal tissues during the MDC sequence. In all BE samples, CD1a⁺ mDCs were found to be widely distributed around metaplastic glands. The number of CD1a⁺ mDCs in BE was significantly less than that in NE (data not shown). In contrast, the development of EAC from LGBE was associated with a relatively increased density of mDCs. These data suggest an initial decrease of mDC homing in BE compared with NE, followed by an increased infiltration from LGBE to EAC. The increased amount of mDCs along the MDC sequence could be due to a sequestration of immature mDCs within the tissue and/or a tumor-induced maturation stop of tumor-infiltrating mDCs. Interestingly, we also demonstrated that the number of BDCA-2⁺ pDCs was significantly increased in the MDC sequence. The changes in mDC and pDC densities may probably be explained by the high expression in the same areas of their respective chemotactic factors, MIP3 α and chemerin. Unexpectedly, despite the few infiltrating pDCs, IHC analyses showed a high expression of chemerin in the NE (data not shown). Even if the role of chemerin in the NE is still unknown, it can be speculated that, under homeostatic conditions, chemerin may represent a mechanism allowing a rapid attraction of effector cells on the generation of pathogenic signals, as suggested in normal skin by Albanesi et al.⁴⁴ In addition, proteinases required for chemerin activation are likely to be present in BE, LGBE, HGBE, and EAC. Indeed, BE and EAC are often associated with an important inflammatory infiltrate, which likely contains chemerin-activating proteases, such as

Figure 5 RANKL expression increases in the MDC sequence, but its inhibition is not sufficient to reverse the tolerogenic profile of mDCs. Representative immunostaining examples of RANKL expression in the epithelium of BE (A), low-grade Barrett's esophagus (B), high-grade Barrett's esophagus (C), and EAC (D). Original magnification, $\times 200$ (A–D). E: Semiquantitative analysis of RANKL expression showing a significant increased expression in HGBE and EAC compared with BE. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with BE. Statistical significance was calculated using a *U*-test. F: Secreted RANKL levels in culture supernatants of Barrett's esophagus (CPA), high-grade Barrett's esophagus (CPD), and esophageal adenocarcinoma (OE33 and JHEsoAd1) cell lines. The secretion levels were measured by ELISA. Data are presented as means and SDs of four independent experiments performed in duplicate. $^{**}P < 0.01$. Statistical significance was calculated by using a *U*-test. G–L: Analysis of mDC phenotype after co-culture in the presence of Barrett's esophagus (CPA), high-grade Barrett's esophagus (CPD), and esophageal adenocarcinoma (OE33 and JHEsoAd1) cell lines either untreated or treated with human recombinant OPG. After 6 days of co-culture and 24 hours of incubation with LPS, analysis of the cell surface expression of CD80, CD83, CD86, HLA-DR, HLA-ABC, and CCR7 was performed using flow cytometry. Mean percentages of positive cells and SDs obtained in four different experiments are reported herein. Statistical significance was calculated by using a *U*-test. There was no significant difference between untreated and treated cell lines.

urokinase plasminogen activator.⁴⁵ Increased MIP3 α expression could be explained by the fact that bile acid, probably the most important toxic component of the refluxate linked to BE, could actively induce its expression via the activation of the bile acid receptor (alias farnesoid X receptor). These results suggest an important role for biliary reflux in immature mDC homing.

The many mDCs in dysplastic BE and EAC could be intuitively associated with the induction of a functional antigen-presenting stimulation and, thus, to the development of an antitumor immune response. However, the microenvironment and the characteristics of local mDCs vary for

each type of tumor. Because mDCs could have an altered maturation in the MDC sequence, a tolerogenic immune response could occur and promote immune evasion in Barrett's carcinogenesis. Production of immunosuppressive molecules may be an important mechanism by which esophageal cells from the MDC sequence could escape from immunosurveillance. Therefore, we studied the effect of soluble factors secreted by cultured human BE, HGBE, and EAC cell lines on mDC and pDC functionality. We demonstrated that these cell lines alter mDC, but not pDC, maturation phenotype. In parallel, mDCs co-cultured with BE and EAC cell lines showed a tolerogenic profile of

secreted cytokines characterized by increased IL-10 secretion, an anti-inflammatory cytokine that inhibits a type 1 helper T-cell response,⁴⁶ and decreased IL-12p70 secretion, a proinflammatory cytokine required for type 1 helper T-cell responses. Indeed, mDCs with an IL-10^{high} and IL-12^{low} secretion profile have been reported to promote Treg cells.^{47,48}

Esophageal cell lines used in this study may release different signals that could negatively affect the function of mDCs, making them functionally deficient and/or tolerogenic. Studies on several different neoplastic entities have shown that, in patients with cancer, mDCs present in the blood, tumor tissues, and/or draining lymph node are functionally defective and possess a poor T-cell stimulatory ability.^{9,49–52} By using a Treg cell induction assay, we showed that mDCs co-cultured with BE, HGBE, and EAC stimulate Treg cell differentiation from naïve CD4⁺ T cells, confirming that the microenvironment notably encountered in EAC is tolerogenic. In agreement with those results, we observed that both metaplastic areas and (pre)malignant lesions of the esophagus are infiltrated by Treg cells, and a colocalization was observed between mDC and Treg cells in those areas. One possible explanation for this colocalization could be that their presence around (pre)malignant epithelial cells reflects an ongoing antigen-specific immune response, and localization of these clustered cells close to (pre)malignant cells could represent a tertiary lymph node tissue leading to the local generation of Treg cells.⁹ Thus, MIP3 α expression in the MDC sequence could be linked to the increased number of FoxP3⁺ Treg cells observed in the same areas. Interestingly, a selective recruitment of Treg cells through MIP3 α expression was recently described in hepatocellular carcinoma.⁵³

Among the immunosuppressive factors potentially responsible for mDC alterations in the MDC sequence, IL-10⁵⁴ and RANKL^{55–57} represent interesting candidates. Indeed, IL-10-mediated inhibition of maturation has been reported to render mDCs tolerogenic.⁵⁸ However, supernatants obtained from the esophageal cell lines used in this study showed an absence of IL-10 secretion (data not shown). RANK and its ligand, RANKL (alias OPG ligand), are members of the tumor necrosis factor superfamily and were first described as key regulators of bone homeostasis, mammary gland formation, and lymph node development.⁵⁹ Several studies showed that the RANK-RANKL interaction has a crucial role in the regulation of the immune system.^{55–57} RANKL has been observed in keratinocytes of inflamed skin, and its overexpression has been linked to functional alterations of epidermal DCs and a systemic increase of Treg cells.⁵⁵ Together with our data showing a significant increase of FoxP3⁺ Treg cells in the MDC sequence and the studies suggesting that epithelial RANKL controls Treg cells numbers via the activation of mDCs,^{55,60} the results of these experiments suggest a possible link between epithelial RANKL expression and the regulation of FoxP3⁺ Treg by mDCs in the MDC sequence. We

confirmed that RANKL is expressed in the cell lines and showed, for the first time to our knowledge, a significant *in vivo* increase of RANKL expression in LGBE, HGBE, and EAC compared with NE. To determine whether RANKL expression in the MDC sequence is responsible for the acquisition of an altered tolerogenic phenotype by mDCs, we added OPG, a RANKL antagonist,⁶¹ to the co-cultures. Because the *in vitro* treatment of mDCs with human recombinant RANKL was associated with a reduced expression of mDC costimulatory molecules, which was reversed after OPG addition (data not shown), we expected to reverse the alteration of the mDC phenotype when OPG was added to our co-cultures. However, despite a tendency to reverse the inhibitory effect of BE and EAC cell lines, OPG addition to the co-cultures did not induce a significant reversion of the mDC phenotype, suggesting that RANKL may not be the main mediator responsible for the mDC functional alteration in the MDC sequence. Because of the fact that many soluble factors are present in the tumor microenvironment, it is not surprising that the sole inhibition of RANKL was not sufficient to reverse the maturation and function of mDCs observed after their co-culture with the esophageal cell lines.

These findings suggest that additional secreted epithelial factors are involved in the induction of tolerogenic mDCs. It is also possible that the action of all secreted factors from the tumor microenvironment is required to elicit a significant effect. Other factors, such as PGE₂, IL-6,⁶² gangliosides,⁶³ mucin 1 (MUC1),⁶⁴ transforming growth factor- β , retinoic acid, thymic stromal lymphopoietin, and cobalt protoporphyrin,¹² have been shown to play a role in the inhibition of mDC maturation. Among these, MUC1 and PGE₂, which are expressed in BE⁶⁵ and EAC,⁶⁶ are potential candidates. Indeed, MUC1 is highly expressed by several epithelial tumors, including breast, colon, kidney, lung, esophageal, stomach, and ovarian cancers.⁶⁷ Moreover, tumor-derived MUC1 has been shown to interact with differentiating monocytes and to inhibit mDC maturation and IL-12p70 secretion, while enhancing IL-10 secretion.⁶⁸ On the other hand, mDCs differentiated in the presence of PGE₂ also display a tolerogenic phenotype.⁴⁸ Finally, programmed death 1 and its ligand (PD-1/PD-L1) could also play a role in the immune escape observed in the Barrett MDC sequence. Accumulating data demonstrated that PD-1/PD-L1 interaction inhibits T-lymphocyte proliferation, survival, and function, induces apoptosis of tumor-specific T cells, and promotes the differentiation of CD4⁺ T-naïve cells into regulatory T cells in the tumor microenvironment. A high expression of PD-L1 has been described in several histological types of cancers. Interestingly, in a recent article, Loos et al⁶⁹ showed an aberrant PD-L1 expression in Barrett cells. Moreover, in a multivariable analysis performed on 101 patients, these authors showed that high tumor PD-L1 expression is significantly associated with an increased risk of death from Barrett adenocarcinoma.⁶⁹

Consequently, these results suggest that PD-L1 could represent both a potential target in the treatment of esophageal adenocarcinoma and another mechanism implicated in Barrett's carcinogenesis immune escape.

In conclusion, a better understanding of the microenvironment modifications associated with the esophageal MDC sequence and of their implication in the alterations of mDC functionalities may be crucial to elaborate new immunotherapies able to induce an effective antitumoral response and, indirectly, to reverse the MDC sequence.

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