Tumorigenesis and Neoplastic Progression

Human Renal Cell Carcinoma Induces a Dendritic Cell Subset That Uses T-Cell Crosstalk for Tumor-Permissive Milieu Alterations

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Tissue dendritic cells (DCs) may influence the progression of renal cell carcinoma (RCC) by regulating the functional capacity of antitumor effector cells. DCs and their interaction with T cells were analyzed in human RCC and control kidney tissues. The frequency of CD209⁺ DCs in RCCs was found to be associated with an unfavorable T_H1 cell balance in the tissue and advanced tumor stages. The CD209⁺ DCs in RCC were unusual because most of them co-expressed macrophage markers (CD14, CD163). The phenotype of these enriched-in-renal-carcinoma DCs (ercDCs) could be reiterated *in vitro* by carcinoma-secreted factors (CXCL8/IL-8, IL-6, and vascular endothelial growth factor). ErcDCs resembled conventional DCs in costimulatory molecule expression and antigen cross-presentation. They did not suppress cognate cytotoxic T-lymphocyte function and did not cause CD3 ζ down-regulation, FOXP3 induction, or T-cell apoptosis *in situ* or *in vitro*; thus, they are different

from classic myeloid-derived suppressor cells. ErcDCs secreted high levels of metalloproteinase 9 and used T-cell crosstalk to increase tumor-promoting tumor necrosis factor α and reduce chemokines relevant for T_H1-polarized lymphocyte recruitment. This modulation of the tumor environment exerted by ercDCs suggests an immunologic mechanism by which tumor control can fail without involving cytotoxic T-lymphocyte inhibition. Pharmacologic targeting of the deviated DC differentiation could improve the efficacy of immunotherapy against RCC. (Am J Pathol 2011, 179:436–451; DOI: 10.1016/j.ajpath.2011.03.011)

The mononuclear infiltrate in renal cell carcinoma (RCC) has been associated with the immunogenic nature of this tumor type and the clinical response rates achieved with immunotherapy.^{1,2} Paradoxically, the mononuclear cell infiltrate has also been negatively correlated with prognosis,³ suggesting limitations in the functional capability of the natural immune infiltrate to control tumor growth. Potential immune parameters that contribute to this phenomenon include the composition of the infiltrate, such as type and number of effector cells [T cells, natural killer (NK) cells, dendritic cells (DCs)], and the presence of regulatory cells (T-regulatory cells, macrophages, and myeloid-derived suppressor cells).^{4,5} With their bipartite functionality to induce immune activation and tolerance,⁶ DCs are well suited to set the balance between immune suppression and immune reactivity. We hypothesized that under the influence of the RCC tumor milieu, specific DC subtypes could de-

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velop, which may participate in regulatory networks responsible for silencing antitumor effector lymphocytes.⁷

DCs have been extensively studied in lymphoid organs, yet outside the lymphoid environment their effector biology is still poorly understood.^{8,9} Once in a tissue, phenotypic and functional diversifications can occur, depending on organ- and compartment-specific microenvironments.^{10,11} Indeed, plasticity and functional polarity are hallmarks of the DC/macrophage lineage, leading to considerable discussion concerning the subtype-specific nomenclature.^{8,9,12–14} Mouse models have been instrumental in deconstructing the diversity, yet comparable information for the human system is still sparse.^{8,9,13} Among peripheral organs, the lung, skin, and kidney have well-established DC biology. In murine kidney, DCs are a prominent cell type largely restricted to the tubulointerstitial region.^{15,16} It has been proposed that the murine kidney DCs exert regulatory functions that help protect the tubulointerstitium from immune cell injury.^{16,17} Clear cell RCCs arise from the renal tubular epithelial cells. If DCs within the carcinoma experience an environmental polarization with acquisition of similar functional characteristics, they could contribute to the silencing of the tumor's immune infiltrate.

Recent studies have attempted to link the presence of DCs to prognosis and response to therapy in human kidney disease^{11,18} and in RCC.^{5,19,20} Here, we document that RCCs harbor unusually differentiated DCs, referred to as enriched-in-renal-carcinoma DCs (ercDCs), which use the crosstalk with lymphocytes to cause milieu alterations associated with tumor promotion and reduced T_H1-polarized effector cell recruitment, promoting escape from the antitumor immune response.

Materials and Methods

Donors and Tissues

RCC tissues were clear cell RCCs from untreated patients (Table 1). Nontumor kidney cortices (NKC) were from tumor-free areas of tumor-bearing kidneys. Tumor peripheral areas were selected macroscopically and covered the tumor "pseudocapsule" and the adjacent regions of nontumor and malignant tissue (see Supplemental Figure S1A at <http://ajp.amjpathol.org>). Tissues were snap frozen after nephrectomy and stored at -86°C . Peripheral blood mononuclear cells were from healthy individuals. Sample collection was performed after informed consent and approved by the ethics committee.

Antibodies

Primary and secondary antibodies and their application are listed in Table 2.

IHC

Cryosections (5- μm thick) were stained using the alkaline phosphatase antialkaline phosphatase (APAAP) method²¹ or dual-labeling immunohistochemistry (IHC) (see Supplemental Figure S1, B and C, at <http://ajp.amjpathol.org>). Cryo-

Table 1. Clinicopathologic Features of Patients with Clear Cell RCC

Features	No. of patients
RCC	
Tumor size*	
pT1	15
pT2	5
pT3	22
pT4	1
Nodal status†	
pN0	39
pN1	0
pN2	4
Distant metastasis	
pM0	34
pM1	9
Histopathologic grading	
G1 (good)	2
G2 (moderate)	28
G3 (poor)	12
Gx (undetermined)	1
Tumor staging	
I	14
II	3
III	12
IV	14
Age, median (range), years	67 (34–87)
Sex	
Male	25
Female	18
Total	43
NKC	
Age, median (range), years	68 (38–83)
Sex	
Male	10
Female	7
Total	17

*Tumor staging was determined according to UICC (2002/2003).

†Nodal status includes only regional lymph nodes.

sections were incubated with primary antibodies [rabbit-anti-human CD3 and mouse-anti-human CD208/DC-Lamp, diluted in Tris-buffered saline with human serum (HS)] followed by secondary antibodies alkaline phosphatase-conjugated anti-rabbit immunoglobulin (Immunoresearch, West Grove, PA) and peroxidase-conjugated anti-mouse immunoglobulin (Immunoresearch). Detection was done with substrate Fast Blue followed by substrate aminoethylcarbazol (both from Sigma-Aldrich, Taufkirchen, Germany). Stained tissue sections were mounted using Immunomount (Vector Laboratories, Burlingame, CA). NK cell quantification in tissue was not possible by single-marker histologic analysis for the following reasons. The commonly used NK cell marker CD56 did not work reproducibly in cryosections. Moreover, CD56 is also expressed by a subset of cytotoxic T lymphocytes (CTLs)²² and thus gives cell counts not necessarily related to the NK cell number in a tissue.²³ NKp46 is a marker that is exclusively expressed by NK cells; however, it is subjected to regulation by the tumor milieu.²⁴ In particular, we observed down-regulation on a large proportion of NK cells of tumor-infiltrating lymphocytes (TILs) from RCC tissues using flow cytometry (P.P., unpublished observation). Therefore, the NK cell content in a tissue was determined as the percentage of NK cells (defined as CD3⁺CD56⁺) within TILs using flow cytometry.

Table 2. Antibodies and Applications

Antibody	Label	Clone	Species/isotype	Company	Application
Primary antibody					
BDCA-1		L161	Mouse	Immunotech	IHC, IF
BDCA-3		AD5-14H12	Mouse	Miltenyi	IHC, IF
CD3 ϵ		polyclonal	Rabbit	Dako	IHC
CD3 ϵ	PB	UCHT1	Mouse IgG1	Dako	IHC, IF, FC
CD3 ϵ		UCHT1	Mouse IgG1	Dako	FC
CD3 ζ		8D3	Mouse IgG1	BD Biosciences	IHC, IF, FC
CD3 ζ	FITC	G3	Mouse IgG2a	Serotec	FC
CD8		C8/144B	Mouse IgG1	Dako	IHC
CD8	PB	RPA-T8	Mouse IgG1	BD Biosciences	FC
CD14		RMO52	Mouse IgG2a	Immunotech	IHC, IF, FC
CD14	PB	M5E2	Mouse IgG2a	BD Biosciences	FC
CD15	FITC	HI98	Mouse	BD Biosciences	FC
CD16	FITC	3G8	Mouse IgG1	BD Biosciences	FC
CD40	FITC	5C3	Mouse IgG1	BD Biosciences	FC
CD45	PE-Cy7	HI30	Mouse	BD Biosciences	FC
CD56	APC	N901	Mouse IgG1	Beckman Coulter	FC
CD80	FITC	BB1	Mouse IgM	BD Biosciences	FC
CD83		HB15A	Mouse IgG2b	Immunotech	IHC
CD86	PE	2331/FUN-1	Mouse IgG1	BD Biosciences	FC
CD107a	FITC	H4A3	Mouse IgG1	BD Biosciences	FC
CD107b	FITC	H4B4	Mouse IgG1	BD Biosciences	FC
CD163		Ber-MAC3	Mouse IgG1	Dako	IHC, IF, FC
CD163	PE	GHI/61	Mouse IgG1	BD Biosciences	FC
CD209/DC-SIGN		DCN46	Mouse IgG2b	BD Biosciences	IHC, IF, FC
CD209/DC-SIGN	APC	DCN46	Mouse IgG2b	BD Biosciences	FC
CD274/B7-H1	FITC	MIH1	Mouse IgG1	BD Biosciences	FC
CX ₃ CR1		Polyclonal	Rabbit	eBioscience	FC
CD208/DC-LAMP		104.G4	Mouse IgG1	Immunotech	IHC
FOXP3		259D	Mouse IgG1	BioLegend	IHC, IF
Granzyme B	PE	GB11	Mouse IgG1	Serotec	FC
HLA-ABC/MHC-I	APC	G46_2.6	Mouse IgG1	BD Biosciences	FC
HLA-DR/MHC-II	FITC	L243	Mouse IgG2a	BD Biosciences	FC
HLA-DR/MHC-II	PE	G46-6	Mouse IgG2a	BD Biosciences	FC
IFN- γ	PE-Cy7	4S.B3	Mouse IgG1	BD Biosciences	FC
IL-2	APC	MQ1-17H12	Rat IgG2a	BD Biosciences	FC
Isotype	FITC	MOP-C21	Mouse IgG1	BD Biosciences	FC
Isotype	PE	MOP-C21	Mouse IgG1	BD Biosciences	FC
Perforin	FITC	δ G9	Mouse IgG2b	BD Biosciences	FC
TNF- α	A700	Mab11	Mouse IgG1	BD Biosciences	FC
Secondary antibody					
Anti-AP	AP		Mouse IgG1	Dako	IHC (APAAP)
Anti-mouse			Rabbit	Dako	IHC (APAAP)
Anti-mouse	HRP		Goat	Dianova	IHC (doublestain)
Anti-mouse IgG1	A488		Goat	Molecular Probes	FC
Anti-mouse IgG1	A568		Goat	Molecular Probes	IF
Anti-mouse IgG2a	A568		Goat	Molecular Probes	IF
Anti-mouse IgG2a	A647		Goat	Molecular Probes	IF
Anti-mouse IgG2b	A488		Goat	Molecular Probes	IF
Anti-rabbit	A488		Goat	Molecular Probes	FC
Anti-rabbit	AP		Goat	Dianova	IHC
Anti-rabbit	Cy5		Goat	Dianova	IF

A, Alexa Fluor; AP, alkaline phosphatase; APC, allophycocyanin; Cy, cyanin; FC, flow cytometry; HRP, horseradish peroxidase; IF, immunofluorescence; PB, Pacific blue; PE, phycoerythrin.

Multiparameter Immunofluorescence Histology, Confocal Microscopy, Subset Evaluation, and Contact Quantification

Cryosections 5- μ m thick were fixed in ice-cold 100% acetone and blocked with 2% bovine serum albumin (BSA) in PBS before incubation with primary antibody combinations, followed by corresponding combinations of secondary isotype- or species-specific fluorescent-labeled antibodies. Secondary reagents showed no cross-reactivity. All antibodies were diluted in 12.5% HS (Cambrex, East Ruther-

ford, NJ) in PBS and incubated at room temperature. The following antibody combinations were used: primary mouse-anti-human antibodies CD209/DC-SIGN, CD14, and CD163 followed by secondary antibodies anti-IgG2b-A488, anti-IgG2a-A568, and anti-IgG1-A647; primary mouse-anti-human antibodies CD209/DC-SIGN, CD14, and polyclonal rabbit-anti-CD3 ϵ followed by secondary antibodies anti-IgG2b-A488, anti-IgG2a-A568, and anti-rabbit-Cy5; primary mouse-anti-human antibodies CD209/DC-SIGN, CD163, and polyclonal rabbit-anti-CD3 ϵ followed by secondary antibodies anti-IgG2b-A488, anti-IgG1-A568, and anti-rabbit-

Table 3. Cell Lines

Cell line	Tissue/cell origin	Source or reference
RCC-26	Clear cell renal cell carcinoma of patient 26	22
RCC-53	Clear cell renal cell carcinoma of patient 53	23
A-498	Clear cell renal cell carcinoma	ATCC, HTB-44
CCA23	Clear cell renal cell carcinoma	27
HK-2	Proximal tubular epithelial cells	ATCC, CRL-2190
NKC-26	Normal kidney cells	22
Jurkat	T-cell leukemia cells	ATCC, TIB-153
Colo-357	Pancreatic cancer	Department of Clinical Pharmacology, LMU
DU-145	Prostate cancer	ATCC, HTB-81
Mel93.04A12	Melanoma (HLA-A2 ⁺ tyrosinase ⁺)	P. Schier, Leiden
LCL-26	B-lymphoblastoid cells of patient 26	22
CTL-JB4	Cytotoxic T cells, HLA-A2 allospecific	24
CTL-A42	Cytotoxic T cells, HLA-A2-restricted Melan-A/MART-1 peptide (AAGIGILT) specific	24

Cy5; primary mouse-anti-human antibodies CD209/DC-SIGN, CD14, and FOXP3 followed by secondary antibodies anti-IgG2b-A488, anti-IgG2a-A647, and anti-IgG1-A568; primary mouse-anti-human antibodies CD209/DC-SIGN and CD3 ζ , and rabbit-anti-CD3 ϵ followed by secondary antibodies anti-IgG2b-A488, anti-IgG1-A568, and anti-rabbit-Cy5. Detailed antibody information is in Table 2. After fixation [4% paraformaldehyde (PFA)] and nuclear staining with DAPI (Sigma-Aldrich), slides were mounted with Vectashield (Vector Laboratories). Fluorescence images were captured with a laser scanning microscope TCS SP2 (Leica Microsystems, Wetzlar, Germany) using HCX PL APO 63 $\times$ 1.40 oil immersion objective lens, pinhole 1.0 Airy units, 512 $\times$ 512 pixel image format, and four-frame averaging at a magnification of $\times$ 630. Sequential recording was applied to avoid fluorescence spillover, and Z-stacks were scanned to detect cells and intercellular contacts across different planes of the visual field. Image editing of contrast and brightness was applied to the whole image using Leica LCS Lite software.

CD209⁺ Subset Quantification

CD209, CD14, and/or CD163 multiparameter-stained tissue sections were used. CD209⁺ cells were identified in three different areas of RCC-inflicted kidneys: NKCs ($n = 8$) and two different tumor regions, the tumor center ($n = 11$) and the tumor periphery ($n = 6$). Tissues representing the three tissue areas were selected macroscopically. NKC tissues were derived from regions with the longest possible distance away from any tumor region. Histologic sections were microscopically free of malignant cells. Histologic sections of the tumor periphery were cross-sectional cuts that microscopically encompassed nontumor kidney, the "pseudocapsule" that surrounds the tumor, separating it from the nontumor kidney area, and the tumor region (see Supplemental Figure S1A at <http://ajp.amjpathol.org>). Myeloid cells located in the tumor parenchyma next to the capsule (ie, the tumor periphery) were evaluated. Tumor center tissue regions were macroscopically selected to be clearly away from the "pseudocapsule." The histologic sections showed no areas of nontumor kidney or the "pseudocapsule."

Of each tissue area, at least 10 nonoverlapping fields ($\times$ 630 magnification) containing CD209⁺ cells were evaluated. The frequency of CD209⁺ cells with co-expression of CD14 and/or CD163 was quantified by assigning CD14 and CD163 expression to each CD209⁺ cell.

Quantification of Contacts between T Cells and CD209⁺ Cell Subsets

Intercellular contacts between CD209⁺ cells and CD3⁺ or FOXP3⁺ cells were determined using multiparameter immunofluorescence-stained RCC or NKC sections. At least 10 nonoverlapping fields ($\times$ 630) containing CD209⁺ cells were evaluated. The frequency of contacts between CD209⁺CD14⁺ or CD209⁺CD163⁺ cells and CD3⁺ lymphocytes was assessed by selecting CD209⁺ cells and assigning co-expression of CD14 or CD163 and contacts with CD3⁺ lymphocytes. Intercellular contacts between FOXP3⁺ and CD209⁺CD14⁺ cells were quantified by selecting FOXP3⁺ fields.

CD3 ϵ and CD3 ζ Expression on T Lymphocytes in Relation to CD209⁺ Cells

CD3 ζ , CD3 ϵ , and CD209 multiparameter immunofluorescence-stained RCC sections were evaluated. Images containing CD209⁺ and CD3⁺ cells were captured at $\times$ 630 magnification. Fluorescence intensity of CD3 ζ and CD3 ϵ of T cells in close contact to CD209⁺ cells and those away from CD209⁺ cells was compared.

Cells and Cell-Conditioned Media

Cell-conditioned media were generated from cell lines^{25,26} (Table 3) by culturing 2×10^6 cells in 10 mL of serum-free AIM-V (Gibco/Invitrogen, Carlsbad, CA) for 10 days. Supernatants were centrifuged to remove cells. Cytotoxic T-effector cell clones CTL-JB4 (HLA-A2 alloreactive) and CTL-A42 (HLA-A2 restricted, MELAN/MART-1 specific) (Table 3) were cultured as described²⁷ and used at a resting state, generally between days 8 and 10 after the last restimulation, when they no longer secreted cytokines spontaneously. Both are T_H1-polar-

ized effector cells as evidenced by high expression of the chemokine receptor CXCR3 and CCR5.

Isolation of TILs

The TILs were isolated from fresh tissues immediately after surgical resection. Briefly, tissues were mechanically minced into small pieces and washed with HBSS buffer to remove contaminating blood lymphocytes. Intratumoral leukocytes were recovered from the tissue after two enzymatic digestions using collagenase IA (0.5 mg/mL) and DNase I type IV (0.19 mg/mL) (all from Sigma-Aldrich) with an intermittent step of 5 mmol/L EDTA in HBSS (without Ca^{2+} and Mg^{2+}). All incubations were performed for 30 minutes at room temperature.

In Vitro Generation of Myeloid Cell Subtypes

Monocytes were isolated from peripheral blood mononuclear cells using CD14^{+} microbeads (Miltenyi) and cultivated serum free ($5 \times 10^6/4$ mL of AIM-V) with IL-4 (400 U/mL; CellGenix, Freiburg, Germany) and granulocyte-macrophage colony-stimulating factor (GM-CSF/Leukine; 800 U/mL; Genzyme, Cambridge, MA) to generate CD209^{+} single-positive conventional DCs (cDCs). For tissue-conditioned cells, monocytes were cultivated with 20% cell-conditioned media or with CXCL8/IL-8 (7 ng/mL; PeproTech, Rocky Hill, NJ), IL-6 (1.9 ng/mL), and vascular endothelial growth factor (VEGF) (23.4 ng/mL) (both R&D Systems, Minneapolis, MN) or in combinations. The concentrations reflected those of RCC-26-conditioned medium. Functional analyses were performed with monocytes generated with RCC-26-conditioned medium. Myeloid cells within one experiment were derived from the same donor.

Generation of Microtumors and Monocyte Infiltration

Multicellular spheroids were generated as previously described.²⁸ In brief, 10^5 suspended cells from exponentially growing RCC-53 monolayers were cultured on 1% solid seaplaque agarose (Biozym, Wien, Austria) in 24-well plates. After 4 days, the tight aggregates were transferred to 20 μL of AIM-V containing 10^5 monocytes and cultured as hanging drops on the lid of a petri dish. After 24 hours, noninfiltrated monocytes were removed and the spheroids cultured for 3 more days. Thereafter, spheroids were dispersed in 5 mmol/L EDTA (mechanic disruption) and the single-cell suspension was analyzed by flow cytometry using LSRII (gated on CD45^{+} cells) (BD Pharmingen, San Diego, CA) and FlowJo (TreeStar, Ashlan, OR).

Macropinocytosis, Endocytosis, and Phagocytosis

For macropinocytosis, cells (3×10^5 cells/600 μL) were incubated with fluorescein isothiocyanate (FITC)-labeled BSA (1 mg/mL; Sigma-Aldrich) for 1 hour at 37°C or 4°C

(control) and analyzed by flow cytometry. Endocytosis involved FITC-labeled dextran (500 kDa; Sigma-Aldrich). For phagocytosis, the Vybrant phagocytosis assay (Molecular Probes/Invitrogen) was used.

Antigen Cross-Presentation

Antigen cross-presentation was performed as previously described.²⁹ The system involves the HLA-A2-restricted Melan-A/MART-1-specific CTL-A42 and the pep70-MART peptide, which is an extended 15mer peptide containing the HLA-A2-restricted T-cell epitope of the Melan-A/MART-1 antigen. The N-terminal extension prevents direct loading onto surface HLA-A2 molecules; thus, epitope presentation requires antigen uptake and processing by antigen-presenting cells (APCs) to achieve T-cell stimulation. T-cell stimulation results in interferon- γ (IFN- γ) secretion, which correlates with the amount of antigen cross-presented by the APCs.²⁹ Myeloid cells ($2 \times 10^4/100$ μL of AIM-V) were incubated with indicated concentrations of pep70-MART peptide for 1 hour at 37°C to allow uptake before addition of resting CTL-A42 ($4 \times 10^3/100$ μL of AIM-V, 24 hours, 37°C). IFN- γ in supernatants was measured by enzyme-linked immunosorbent assay (ELISA). Control samples, containing all components except the peptide, were used to determine IFN- γ background. Maximal IFN- γ secretion capacity of CTL-A42 was determined by co-culturing CTL-A42 with MEL93.04A12 ($15 \times 10^3/100$ μL), a melanoma cell line with endogenous expression and HLA-A2 presentation of the Melan-A/MART-1 antigen (Table 3).

Cell-Mediated Cytolysis

Cell-mediated cytotoxicity by CTL-JB4 was determined by a 4-hour chromium release assay as previously described.⁴

Flow Cytometry

Antibodies are listed in Table 2. For surface staining, 10^5 cells were incubated with antibodies in FACS buffer (PBS, 2% HS, 2 mmol/L EDTA, 0.1% NaN_3) (20 minutes, 4°C) and propidium iodide (Sigma-Aldrich). For intracellular staining ($\text{CD3}\zeta$), cells were incubated with 7-amino-actinomycin-D (7-AAD) (Sigma-Aldrich), fixed (1% PFA/PBS, 20 minutes, 4°C) and permeabilized (two consecutive washes with 0.1% and 0.35% saponin in 2% HS/PBS), then stained with antibodies. Apoptosis was detected by annexin V-FITC following the manufacturer's instruction (BioSource/Invitrogen).

Polychromatic flow cytometry was used to analyze TILs. The TILs from RCC tissues were stained with antibody combinations anti- CD45 -phycoerythrin (PE)-Cy7, anti- CD11c -FITC, anti- CD209 -APC, anti- CD14 -Pacific blue (PB), anti- CD163 -PE, anti- CD3 -AmCyan, and human leukocyte antigen (HLA)-DR-PE (for myeloid cell analysis) or anti- CD45 -PE-Cy7, anti- CD3 -PB, and anti- CD56 -APC (for NK-cell quantification). Dead cells were stained with 7-AAD. Myeloid cells were selected by gating on CD45^{+} 7-AAD $^{-}$ and CD11c^{+} cells. Within the myeloid gate, the

intensity of CD209, CD14, HLA-DR, and costimulatory molecules (CD40, CD80, CD86) was determined for the gated CD14⁺CD209⁻, CD14⁻CD209⁺, or CD14⁺CD209⁺ subpopulations. The frequency of NK cells among TILs was determined as the percentage of CD3⁻CD56⁺ cells among the gated lymphocyte population (CD45⁺, 7-AAD⁻, CD14⁻).

To assess maintenance of CTL function when exposed to myeloid APCs, CTL-JB4 was co-cultured with cDCs, ercDCs, or no APCs at a ratio of 1:0.3 in 300 μ L of AIM-V with 10% HS (6×10^5 CTLs) in polystyrene round-bottom tubes. After 24 hours, co-cultures were harvested and used to determine phenotypic markers (CD8, CD3 ζ , CD3 ϵ , perforin, and granzyme B), as well as cognate functional capacity (degranulation, cytokine production). For phenotype, 24-hour CTL co-cultures were surface stained with antibodies to CD45, CD8, and CD3 ϵ (Table 2) and 7-AAD in FACS buffer. Cells were fixed with 1% PFA followed by permeabilization with saponin and stained with antibodies to perforin and granzyme B. Data acquisition and analysis were performed with LSRII (BD Bioscience) and FlowJo (TreeStar). CTLs were selected by gating on CD45⁺ 7-AAD⁻ cells. The expression levels of markers were determined as the median fluorescence intensity of gated CTLs.

To assess CTL function, CTL-JB4 (3×10^5) (precultured with APCs) were mixed with cognate tumor cells (HLA-A2⁺ RCC-26) at a ratio of 1:1 in the presence of GolgiStop and Brefeldin-A (BD Biosciences, Franklin Lakes, NJ) in AIM-V with 10% HS. To detect degranulation, anti-CD107a-FITC and anti-CD107b-FITC antibodies were added during the stimulation phase. After 5 hours of stimulation, cells were stained for membrane markers (anti-CD45-AmCyan, anti-CD8-PB, anti-CD11c-PE) and 7-AAD, followed by staining for intracellular proteins [anti-IFN- γ -PE-Cy7, anti-IL-2-APC, and anti-tumor necrosis factor (TNF)- α -A700].

T-Cell, Target Cell, and Myeloid Cell Co-Culture

CTL-JB4 (3×10^3) and cognate HLA-A2-positive RCC-26 (15×10^3) were cultured for 24 hours in 96-well plates with or without HLA-A2-negative myeloid cells generated in parallel from monocytes of the same donor (0.5×10^3 or 3×10^3). Peripheral blood lymphocytes (PBLs) were cultured with allogeneic myeloid cells (ratio 48:1) in 96-well plates for 4 days. Supernatants were harvested for cytokine and chemokine measurements.

Cytokine and Chemokine Measurements

Cytokine and chemokine measurements were performed using the 27-Plex bead array (Bio-Plex; Bio-Rad, Hercules, CA) or ELISA (BD OptEIA, BD Pharmingen) according to the manufacturers' instruction.

MMP-9 Measurement

Gelatinase zymography was performed as previously described.²⁶ Culture supernatant of the human fibrosarcoma cell line HT1080 was used as a reference to detect

the position of the matrix metalloproteinase 9 (MMP-9) digestion area. Digestion areas were quantified using Image J software. Supernatants were from APCs ($5 \times 10^3/200 \mu$ L, 24 hours) and RCC lines cultured in medium with recombinant TNF- α (rTNF- α).

CTL Migration Using the Modified Boyden Chamber

CTL-JB4 (7.5×10^3), RCC-26 (37×10^3) expressing the cognate CTL-pMHC ligand, and either ercDCs or cDCs (7.5×10^3) (not expressing the cognate ligand) were co-cultured at a final volume of 500 μ L of AIM-V in 24-well tissue culture plates (Corning Costar, Cambridge, MA). Parallel cultures involved every combination with two cell types and each cell type alone. All cultures were prepared in duplicates. After 24 hours, Transwell inserts (6.5-mm diameter; 3.0- μ m pore size) (Corning Costar) were placed in each well, and carboxyfluorescein diacetate succinimidyl ester (CFDA-SE)-labeled CTL-JB4 (4×10^5 cells/100 μ L) was added to each insert and incubated at 37°C/6.5% CO₂. After 30 minutes and a brief shaking of the incubation plate, the inserts were removed and Counting Beads (CALTAG, Invitrogen, Camarillo, CA) were added to the lower chambers. Then cell suspensions were harvested from the lower chamber, and the amount of CFDA-SE⁺-CTL-JB4 was determined by flow cytometry (LSRII). Absolute CFDA-SE⁺-CTL counts were calculated with the help of the counting beads following the manufacturer's protocol. The migration index was determined as the ratio of each absolute cell number to the cell number in the control (medium). CTL-JB4 was labeled with 2 μ mol/L CFDA-SE (10^7 cells/mL) (Molecular Probes/Invitrogen) at 37°C for 10 minutes. The reaction was stopped by adding an equivalent volume of fetal calf serum (FCS) and subsequent washing with RPMI 1640 medium.

RCC Cell Proliferation

Proliferation of RCC cells was measured using the cell screen system (Roche Innovatis AG, Basel, Switzerland). It consists of an inverted phase microscope (Ix 50, Olympus, Hamburg, Germany) equipped with a motorized X-Y stage and a CCD camera. The cell density is determined by measuring the area in a well that is occupied by adherent cells using the pattern recognition software (PA V1.7; Roche Innovatis AG). CCA23 RCC cells³⁰ (4×10^3 cells in AIM-V/1% FCS) were seeded in 96-well plates. After 15 hours, medium was replaced by AIM-V/1% FCS supplemented with rTNF- α (0.5, 1, 5, 10, and 20 ng/mL) or CTL/APC co-culture supernatants (supplemented with 1% FCS). Each group was set up in six replicates. rTNF- α and co-culture supernatants were replenished after 48 hours. Cell proliferation was monitored over 4 days, each time scanning the full area of each well ($\times 4$ objective). Measurements started directly after the addition of rTNF- α or supernatants (0 hours) and were repeated after 24, 48, 72, and 96 hours. For blocking experiments, anti-human TNF- α rabbit immune serum (1:1000, gift of Dr. H. Engemann, Munich, Germany) or rabbit preimmune se-

Table 4. Immune Cell Composition in RCC Tumors

	CD209-cell ^{low} tumors		CD209-cell ^{high} tumors		<i>P</i> value*
	Median (range)	<i>n</i>	Median (range)	<i>n</i>	
Cells per HPF [†]					
CD209	6.0 (0–12)	24	20 (14–82)	18	<0.0001
CD3	32 (6–169)	22	60 (12–121)	18	0.018
CD8	17 (4–117)	21	25 (11–86)	17	0.038
% of lymphocytes [‡]					
NK cells	14 (2–41)	20	15 (2–37)	17	0.963
Ratio [§]					
CD3/CD209	6.7 (0.7–68)	22	2.6 (0.4–7.6)	18	0.001
CD8/CD209	3.0 (0.4–33)	21	1.1 (0.3–4.4)	17	0.002
NK/CD209	2.6 (0.2–20)	19	0.8 (0.1–2.3)	17	0.0002

*Statistical analysis according to Mann-Whitney *U* test.

[†]Numbers represent the median (range) of positive cells per HPF (400x magnification) in indicated number (*n*) of tissue samples. The median of positive cells in each sample was derived from at least 50 positive cells within 10 nonoverlapping HPFs, as assessed by light microscopy of APAAP-stained tissues. Only vital tissue areas were considered, excluding necrotic or damaged areas and immune cell clusters. Tumors were divided into those with high or low CD209⁺ cell count at a cutoff of 13 cells/HPF, according to receiver operating characteristic analysis.

[‡]Numbers represent the median (range) percentage of CD3⁺CD56⁺ cells within the lymphocyte population as assessed by polychromatic flow cytometry of TILs. NK cell quantification in tissue by single-marker histologic analysis was not possible (see *Materials and Methods*).

[§]Numbers represent the median (range) ratio of indicated markers. The ratios were determined for each individual tissue. The number of positive cells for the numerators CD3 and CD8 and the denominator CD209 were quantified by light microscopy of APAAP-stained tissue sections, as described above. The number of positive cells for the numerator NK was assessed by flow cytometry of TILs.

rum was added to 2 ng/mL of rTNF- α medium or ercDC/CTL co-culture supernatant and the proliferation was measured over 72 hours.

Statistical Analysis

Nonparametric statistical methods were used (Prism Windows 5.01; GraphPad, La Jolla, CA). For comparison of two unmatched groups, the two-sided Mann-Whitney *U*-test was applied. Contingency tables, composed of two pairs of categories, were analyzed by Fisher's exact test. Comparison of one parameter between more than two groups was performed with the Kruskal-Wallis test followed by Dunn's posttest. For analysis of multiple comparisons, the Mann-Whitney *U*-test was applied followed by corresponding *P* value corrections. Error bars are the mean deviation (MD) or as indicated. RCC tissues were divided into tumors with either low (CD209-cell^{low}) or high (CD209-cell^{high}) CD209⁺ cell content. The cutoff for CD209-cell^{high} tumors was set at 13 cells per high-power field (HPF) as determined by receiver operating characteristic analysis.

Results

RCCs with High CD209⁺ Cell Content Have Advanced Tumor Stages and Lower Fractions of CD8⁺ and NK Effector Cells

RCCs typically present with a prominent immune cell infiltrate, which includes CD8⁺ T cells, NK cells, macrophages, and DCs. Unlike what has been reported for other tumor types,^{31,32} the absolute number of intratumoral CD8⁺ T cells in RCC does not correlate with better prognosis. Rather, more intratumoral CD8⁺ cells are generally found in RCC tumors of higher stage and poor prognosis.³ We speculated that the composition of the infiltrate could be different in high- versus low-stage RCC

tumors. Using IHC, the DC subsets present in the tumors were characterized by applying antibodies specific for interstitial immature DCs (CD209) and mature DCs (DC-Lamp, CD83). As previously reported,²⁰ DC-Lamp⁺ and CD83⁺ cells were found to be rare and confined to lymphocyte clusters within the tumor parenchyma (see Supplemental Figure S1B at <http://ajp.amjpathol.org>). In contrast, CD209⁺ cells were numerous and regularly dispersed throughout the tumor parenchyma alongside T lymphocytes but were absent from clustered CD3 accumulations (see Supplemental Figure S1C at <http://ajp.amjpathol.org>). We then determined the presence of specific lymphocyte populations in tumors with high or low CD209⁺ cell content. CD209-cell^{high} tumors had more T cells than did the CD209-cell^{low} tumors but a similar number of NK cells (Table 4), indicating that an increase in one lymphocyte subset was not similarly paralleled by that of other subsets, thereby changing the composition of the infiltrate. To describe the infiltrate composition, the ratios of CD3⁺, CD8⁺, or NK cells to CD209⁺ cells were calculated for each tumor (Table 4 and Figure 1A). The ratios varied among individual tumors, indicating individual infiltrate compositions that were independent of the absolute numbers of each immune cell subset. Importantly, comparing tumors with high numbers of CD209⁺ cells to those with low numbers of CD209⁺ cells, it was observed that CD209-cell^{high} tumors had lower ratios of CD3⁺, CD8⁺, or NK cells relative to CD209⁺ cells than did CD209-cell^{low} tumors. Specifically, CD209-cell^{low} tumors harbored on average three times more CD8⁺ or NK cells for each CD209⁺ cell when compared with the CD209-cell^{high} tumors (Table 4). The difference between both groups was statistically significant (Figure 1 and Table 4). The change in infiltrate composition suggested a reduced level of cytotoxic effector cell recruitment in the CD209-cell^{high} tumors.

DCs can help mount immune responses against tumors,⁶ suggesting that a large DC content may correlate

Figure 1. Histopathologic characteristics of RCC tumors containing a high or low CD209⁺ cell infiltrate. RCC tissues ($n = 42$) were divided into tumors with either low (CD209-cell^{low}) or high (CD209-cell^{high}) CD209⁺ cell content (quantified by APAAP staining). The cutoff for CD209-cell^{high} tumors was set at 13 cells/HPF (details in Table 4). **A:** Quantified T_H1 cell infiltrate of CD209-cell^{high} or CD209-cell^{low} tumors. Depicted are the ratios of CD8⁺ cells (**left**) and NK cells (**right**) to the number of CD209⁺ cells in each tumor tissue. The number of CD209⁺ and CD8⁺ cells was derived from APAAP-stained tissues. NK cells were quantified by flow cytometry as the percentage of CD3⁺CD56⁺ cells among TILs (Table 4) because quantification by single-marker histologic analysis was not possible (see *Materials and Methods*). Each symbol represents one tumor. ** $P < 0.01$, *** $P < 0.001$ (Mann-Whitney U -test). **B:** Correlation between tumor classification and the extent of CD209⁺ cell infiltration. The bars depict the number of patients with tumors of either pT1N0M0 or pT2-4N0-2M0-1 classification divided according to CD209-cell^{high} or CD209-cell^{low} content (Fisher's exact test, $P = 0.057$, relative risk = 2.8, odds ratio = 4.2).

with an early tumor stage. However, setting the CD209⁺ cell content in relation to the TNM classification, a strong predictor of disease prognosis,³³ we observed that CD209-cell^{high} tumors rarely showed a TNM stage associated with good prognosis (pT1N0M0) but generally had prognostically poor tumor stages (pT2-4N0-2M0-1) (Figure 1B). By contrast, CD209-cell^{low} tumors were equally distributed among the two TNM categories. These results raise the possibility that the CD209⁺ DCs in RCC may have an adverse effect on tumor growth control.

Most CD209⁺ DCs in RCC Co-Express Macrophage Markers

CD209⁺ DCs were then characterized in RCC and NKC tissues using multiparameter immunofluorescence staining combining CD209 with the lineage marker CD14 for macrophages and CD163 for chronically activated macrophages and activated circulating DCs.¹⁰ Unexpectedly, conventional CD209⁺ DCs, which do not co-express these macrophage markers, were rarely found in RCC and NKC tissues. Rather, most CD209⁺ DCs co-expressed CD14, CD163, or both markers (Figure 2A). In RCC, the triple marker-positive CD209⁺CD14⁺CD163⁺ cells represented the dominant population among the CD209⁺ cells (mean, 62%; range, 26% to 80%), whereas they were significantly less frequent in NKC (mean, 19%;

range, 0% to 43%) ($P = 0.007$) (Figure 2B, upper panel). Most CD209⁺ cells in NKCs co-expressed CD163 without CD14 (mean, 70%; range, 45% to 95%), and the frequency of this subset was significantly higher than in RCC ($P = 0.006$). The CD209⁺CD14⁺ phenotype predominated in the tumor center (median, 71%; range, 51% to 83%) (Figure 2B, lower panel). In the tumor periphery, the tumor area directly adjacent to the tumor "pseudocapsule," an intermediate frequency of 46% (range, 37% to 61%) was found, whereas in NKCs only an 18% frequency (range, 0% to 88%) in this subset was observed (Figure 2B). This distinct subset distribution suggests that different regional milieus exist within renal tissue that determine marker co-expression. A histologic view depicting the different regions of RCC tumors is shown in Supplemental Figure S1A (available at <http://ajp.amjpathol.org>). Based on their predominance in RCC tissue, the CD209⁺CD14⁺ DCs (with or without CD163 expression) are referred to as ercDCs.

ErcDCs isolated from tumor tissues expressed the markers CD209 and CD14 at intensities similar to cells with single-marker expression (Figure 2C). This supports the proposition that the CD209⁺CD14⁺ ercDCs represent an additional subtype within the DC/macrophage differentiation spectrum. ErcDCs expressed HLA-DR and costimulatory molecules (CD40, CD80, CD86) at levels comparable or higher than cDCs (Figure 2D), and they were positive for the blood DC antigen 1 but not blood DC antigen 3 (not shown), indicating a DC lineage relationship.¹⁴

Soluble Tissue Factors Induce the Phenotypic Differentiation of ErcDCs in Vitro

Monocytes differentiate into macrophages or DCs, depending on the local tissue milieu.^{6,8,9} Because CD209⁺ cells with macrophage marker co-expression represented the dominant CD209⁺ population in tumor tissues, tumor tissue-related factors are probably responsible for the induction of this phenotype. Monocyte infiltration into tumors was mimicked by adding monocytes to RCC microtumors. Subsequent analysis revealed the induction of the ercDC phenotype on microtumor-infiltrating monocytes (Figure 3A, RCC-53 spheroid).

To determine whether soluble tissue-derived factors are sufficient to elicit the ercDC phenotype, monocytes were cultivated with cell-conditioned medium from cell lines, including RCC lines (RCC-26, RCC-53), proximal tubular epithelial cells (NKC-26, HK-2), pancreatic (Colo-357) and prostate (DU-145) carcinoma, Epstein-Barr virus transformed B cells (LCL-26), and T-cell leukemia (Jurkat) (Table 3). No additional cytokines were applied. Flow cytometry revealed that all cell-conditioned media, with the exception of that produced by LCL-26, induced co-expression of CD209, CD14, and CD163 (Figure 3A shows representative results achieved with indicated conditioned media). As expected, the IL-4/GM-CSF-exposed monocytes developed into a cell population only positive for CD209, reflecting monocyte-derived cDCs (Figure 3A). Monocytes cultured in LCL-26-conditioned

Figure 2. Renal micromilieu induce CD209⁺ cells co-expressing CD14 and CD163. **A:** CD209⁺ cell subsets in RCC and NKC. Confocal image of an RCC tissue (original magnification, $\times 630$; scale bar = 50 μm) showing single fluorescence channels for CD209 (green), CD14 (red), CD163 (blue), and nuclei (gray). **Arrowhead:** CD209⁺CD14⁻CD163⁻ cells; **triangular arrow:** CD209⁺CD14⁺CD163⁺ cells; **quadrangular arrow:** CD209⁺CD14⁺CD163⁺ cells. Images are stack merges of three z-planes (z-step = 0.8 μm). **B:** CD209⁺ subsets, quantified from multiparameter-stained tissues. Tumor areas were subdivided into tumor center and periphery (see Supplemental Figure S1A at <http://ajp.amjpatol.org>). Frequency of CD209⁺ subsets are shown in the **upper panel** ($n = 7$; values are means with MD; *** $P < 0.001$; Mann-Whitney U -test corrected after multiple comparisons) and **lower panel** ($n = 11$: tumor center, $n = 6$: tumor periphery; $n = 8$: NKC; values are medians with interquartile ranges; Kruskal-Wallis test with Dunn's posttest; ** $P < 0.01$). **C:** Histograms of flow cytometry of TILs depict relative expression levels of CD14 and CD209 among the three gated myeloid subsets. Shown is one example of at least 10 TIL analyses. Photomultiplier settings were optimized for lymphocytes within TILs. Thus, fluorescence profiles of the myeloid cells are shifted to the right due to their higher autofluorescence. **D:** Histograms depict the HLA-DR, CD40, CD80, and CD86 expression levels relative to isotype staining comparing the CD209⁺CD14⁺ ercDCs to CD209⁺CD14⁻ cDCs.

medium remained CD14 positive and negative for CD209 and CD163. The Jurkat-conditioned medium did not yield sufficient viable cells for analysis.

To define factors responsible for the co-induction of CD209, CD14, and CD163, the cytokine and chemokine profile of the cell-conditioned media was analyzed (Table 5). Three cytokines, IL-6, CXCL8/IL-8, and VEGF, were common to the cell-conditioned media that induced the triple-marker phenotype and absent in the LCL-26-conditioned medium, which did not induce this phenotype.

The T_H2 cytokines IL-4, IL-13, or IL-15, known inducers of CD209,^{34–36} or IL-10 were detected at very low concentrations in all conditioned media, including those that did not induce the ercDC phenotype. Transforming growth factor β was detected in most cell-conditioned media but was not present in the conditioned medium of Colo-357, which induced the ercDC phenotype.

A cocktail of IL-6, CXCL8/IL-8, and VEGF was subsequently shown to induce CD209 and CD163 on monocytes with maintenance of CD14 expression (Figure 3A). A combination of either CXCL8/IL-8 or IL-6 with VEGF only partially induced the ercDC phenotype (not shown),

demonstrating the importance of all three factors. Additional factors in the tumor cell-conditioned medium seem to support survival because cell yields were consistently higher when using the tumor cell-conditioned medium for differentiation of the ercDCs compared with the cytokine cocktail.

ErcDCs Express DC Markers and Have DC Functions

Surface marker analysis of *in vitro* generated single-CD209⁺ cDCs and triple-CD209⁺CD14⁺CD163⁺ ercDCs revealed that major histocompatibility complex class I and II, CD40, CD80, and CD86 expression was similar or higher on ercDCs (Figure 3C) differentiated *in vitro*, consistent with the expression pattern observed for the *in vivo* ercDC and cDC subsets from RCC tissues (Figure 2D). B7-H1, commonly associated with immunoinhibition,⁵ was lower on *in vitro* generated ercDCs, whereas CX₃CR1 was expressed at the same level (Fig-

Figure 3. *In vitro* generated ercDCs resemble cDCs in surface marker expression, endocytic activity, and antigen cross-presentation. **A:** CD209, CD14, and CD163 surface expression on *in vitro* generated myeloid cells. Cells are human blood monocytes after 7-day cultivation with IL-4/GM-CSF, cell-conditioned media (RCC-26, HK-2, Colo-357, LCL-26), or a cocktail of recombinant cytokines (CXCL8/IL-8, IL-6, VEGF) or from monocytes after infiltration into RCC-53 microtumors. **Solid or dotted lines** are specific and isotype stainings, respectively. One experiment of at least three is shown. **B:** Surface expression of costimulatory molecules and molecules associated with immunoinhibition on *in vitro* generated cDCs and ercDCs, assessed by flow cytometry. **Solid lines** represent the marker-specific staining; **dotted lines**, the corresponding isotype. **C:** Endocytic activity. cDCs and ercDCs were incubated with BSA-FITC at 37°C (**solid lines**) or 4°C (**dotted lines**) and analyzed by flow cytometry. **D:** Antigen cross-presentation. cDCs and ercDCs were incubated with indicated concentrations of pep70-MART peptide and then co-cultured with CTL-A42 for 24 hours. The extent of antigen cross-presentation was assessed by measuring the amount of IFN-γ secretion by the CTL-A42 using ELISA. Co-cultures of CTL-A42 with a melanoma line that endogenously expresses the MART-pMHC ligand served as control for CTL activity. Bars represent the mean of duplicate values + MD. All experiments are representatives of at least three independent experiments with similar results.

ure 3C). CD16 and CD15 were absent on both cell types (not shown).

In vitro generated ercDCs showed similar or higher activities of macropinocytosis, receptor-mediated endocytosis, and phagocytosis than cDCs, measured by uptake of BSA-FITC (Figure 3B), dextran-FITC, or FITC-labeled *Escherichia coli* BioParticles (not shown).

Endocytic activity is necessary for the cross-presentation of exogenous antigens to T lymphocytes. The capacity of cDCs and ercDCs in cross-presentation was compared using an established assay consisting of the CTL-A42 and an extended pep70-MART 15mer peptide, which requires uptake and processing by APCs for epitope presentation and T-cell stimulation.²⁹ ercDCs allowed stron-

Table 5. Cytokine Profile of Cell-Conditioned Media

Cell lines*	IL-4 [†]	IL-13	IL-10	TGF-β, pg/mL	IL-6	CXCL8	VEGF
RCC-26 [‡]	7	8	37	798	1599	7003	23m367
RCC-53 [‡]	9	9	46	2738	1884	1431	17,979
NKC-26 [‡]	1	3	7	1318	581	7003 [§]	721
HK-2 [‡]	16	7	25	729	>6000 [§]	>7000 [§]	6202
DU-145 [‡]	9	19	62	1201	126	>7000 [§]	>33,000 [§]
Colo-357 [‡]	9	30	82	0	17	>7000 [§]	>33,000 [§]
LCL-26	6	8	43	0	5	17	3927
Jurkat	1	0	0	0	0	2	0

*Cell-conditioned media of indicated cell lines (2.0 × 10⁶/10 mL) were harvested after 10 days.

[†]Cytokine concentrations were determined by 27-Plex bead array, except for TGF-β, which was determined by ELISA.

[‡]Cell lines that induced the CD209⁺CD14⁺CD163⁺ phenotype.

[§]Raw data for these values exceeded the upper limit of the standard curve range.

ger epitope recognition by CTLs than cDCs, as seen by higher CTL-IFN- γ secretion (Figure 3D). This confirms a key DC function (ie, antigen cross-presentation)¹⁴ for this cell population that co-expresses CD209 and the macrophage lineage marker CD14.

ErcDCs Are in Crosstalk with T Cells *in Situ*

CD209⁺ cells were found dispersed throughout the RCC parenchyma alongside T lymphocytes (see Supplemental Figure S1C at <http://ajp.amjpathol.org>). Contacts with T cells were frequent and equally involved CD209⁺CD14⁺ and CD209⁺CD163⁺ cells (Figure 4A, Table 6; see also Supplemental Figure S1C at <http://ajp.amjpathol.org>). Most of these cells are presumably CD209⁺CD14⁺CD163⁺ cells because they represent the predominant CD209⁺ subtype in RCC.

DCs can be immunosuppressive, causing T-cell inactivation or the induction of FOXP3⁺ T-regulatory cells.⁶ To identify functional consequences resulting from ercDC/T-cell contacts *in situ*, FOXP3⁺ cells and potential down-regulation of CD3 ϵ and CD3 ζ , representing signs of T-cell inactivation and preceding apoptosis,³⁷ were evaluated. CD3 ϵ and CD3 ζ expression was similar on T cells with or without contact to CD209⁺ cells (Figure 4B). FOXP3⁺ cells were infrequent in RCC or NKC tissues (confirming published results^{16,38}) and were rarely observed in close proximity to ercDCs (Table 6). Together, these data provided no evidence that the RCC-resident ercDCs would have a negative impact on the local T-cell infiltrate.

ErcDCs Do Not Suppress Cognate CTL Function *in Vitro*

The conclusion drawn from the *in situ* observation that ercDCs appear to not negatively affect the local T-cell infiltrate was supported by *in vitro* studies. In co-culture with T cells, *in vitro* generated ercDCs did not cause down-regulation of CD3 ϵ or CD3 ζ on T cells and there was no elevated T-cell apoptosis compared with cDC co-cultures (Figure 4C). In addition, ercDCs did not inhibit cognate stimulation of CTLs by tumor cells because similar amounts of IFN- γ were detected in co-cultures with ercDCs, with cDCs, or without APCs (Figure 4D). Moreover, cytotoxicity of CTLs against tumor cells was enhanced by both cDCs and ercDCs (Figure 4D). Maintenance of CTL effector function was also not negatively affected: cytokine response and degranulation were unchanged (Figure 5A) and lytic activity of CTLs that had been exposed to ercDCs or cDCs for 24 hours was higher than that of unexposed CTLs (Figure 5B). This higher lytic activity may result from the increased perforin and granzyme B observed in this context (Figure 5C).

ErcDCs Produce High Levels of MMP-9 and Regulate TNF- α and Chemokines in T-Cell Co-Culture

MMP-9 is a matrix degrading enzyme that can be produced by tumor cells or by tumor-infiltrating myeloid

Figure 4. ErcDCs are in contact with T cells without compromising CTL function. **A** and **B**: Confocal images of multiparameter-stained RCC tissue. **A**: Contacts of CD209⁺CD163⁺ cells (yellow, merge of CD209⁺ in green and CD163⁺ in red) with T cells (CD3⁺ blue) (arrows) (original magnification, $\times 630$, scale bar = 50 μ m). Image is a stack merge of 4 z-planes, z-step = 0.5 μ m. **B**: CD3 ϵ and CD3 ζ on T cells. Arrowhead indicates T cell in direct contact; arrow indicates T cell without contact (blue, CD209; green, CD3 ϵ ; red, CD3 ζ ; gray, nuclei). Image (original magnification, $\times 630$; scale bar = 25 μ m) is a stack merge of 10 z-planes (z-step = 0.7 μ m). **C**: CD3 ϵ and CD3 ζ expression on CTLs after co-culture with or without cDCs and ercDCs *in vitro*. Numbers are percentages of positive cells in respective quadrant. **D**: IFN- γ secretion and cytotoxicity of CTLs induced by target recognition in the presence of myeloid cells (generated from monocytes of the same HLA-A2⁺ healthy donor). **Left**: IFN- γ secretion of CTL-JB4 (3×10^3) stimulated for 24 hours with RCC target cells (15×10^3 , HLA-A2⁺) in the presence or absence of APCs (3×10^3 ; HLA-A2⁺). **Right**: Cytotoxicity of CTL-JB4 against cognate RCC cells determined by cell-mediated cytotoxicity, using a constant ratio of labeled RCC cells (2×10^3 , HLA-A2⁺) to CTLs (10×10^3) in the presence of titrated numbers of HLA-A2⁺ myeloid cells. Percentage of specific lysis values are the mean of duplicates $\pm$ MD. Shown is one of several experiments.

cells. Its expression is associated with more aggressive tumor growth.^{39,40} Using gelatinase zymography, ercDCs were found to secrete higher levels of MMP-9 (mean of 5 donors: 4.6-fold; range, 1.3–11.2) than did cDCs (Figure 6A).

The presence of ercDCs in CTL/tumor-cell co-cultures led to an increase in TNF- α (Figure 6, B and D), which did not occur in co-cultures containing cDCs. TNF- α is described as a key cytokine involved in tumor promotion

Table 6. Immune Cell Composition and Intercellular Contacts in Renal Tissue

	RCC		NKC		P value
	Median (range)	n	Median (range)	n	
Markers (cells per HPF)*					
CD209	11 (0–82)	42	5 (0.5–16)	17	0.01
CD14	25 (4–68)	15	7 (0–30)	10	0.02
CD163	40 (2–89)	15	8 (3–41)	10	0.09
CD3	44 (6–169)	39	12 (4–90)	16	<0.0001
FOXP3	1.0 (0–26)	13	0.5 (0–6)	6	0.19
Intercellular contacts, %					
CD209 ⁺ CD14 ⁺ contacting CD3 ⁺⁺	22 (0–35)	6	22 (0–47)	4	0.70
CD209 ⁺ CD163 ⁺ contacting CD3 ⁺⁺	28 (0–49)	6	31 (17–55)	4	0.60
FOXP3 ⁺ contacting CD209 ⁺ CD14 ⁺⁺	6.5 (0.9–12)	2	NA		NA

Statistical analysis was performed with the Mann-Whitney U-test.

*Numbers represent the median (range) of positive cells per HPF ($\times 400$ magnification) in indicated number (n) of tissue samples. The median of positive cells in each sample was derived from at least 50 positive cells within 10 nonoverlapping HPFs, as assessed by light microscopy of APAAP-stained tissues. Only vital tissue areas were considered, excluding necrotic or damaged areas and immune cell clusters.

[†]Numbers represent the median (range) percentage of CD209⁺ cells with macrophage marker co-expression in contact with CD3⁺⁺ cells. Quantification was performed by selecting 10 nonoverlapping CD209⁺ confocal images ($\times 630$ magnification) of tissues stained by multiparameter immunofluorescence. At least 50 CD209⁺ cells per tissue were evaluated.

[‡]Numbers represent the median (range) percentage of FOXP3⁺ cells in contact with CD209⁺CD14⁺ cells. Quantification was performed by selecting 10 nonoverlapping FOXP3⁺ fields ($\times 630$ magnification) of tissues stained by multiparameter immunofluorescence and assigning contacts with CD209⁺CD14⁺ cells. At least 50 FOXP3⁺ cells per tissue were evaluated.

NA, not assessed.

and angiogenesis, and it is also known to stimulate MMP secretion and proliferation in RCC tumors.⁴¹ Using rTNF- α , a concentration-dependent induction of MMP-9 secretion and proliferation of RCC was documented *in vitro* (see Supplemental Figure S2, A and B, at <http://ajp.amjpathol.org>). Furthermore, CTL/ercDC co-culture supernatants were shown to induce a stronger proliferation of an RCC line *in vitro* compared with co-culture supernatants from CTLs with cDCs (see Supplemental Figure S2C at <http://ajp.amjpathol.org>). Using a blocking anti-TNF- α antiserum, the proliferation induced by rTNF- α was completely abrogated. Using CTL/ercDC co-culture supernatants, the induced proliferation was attenuated but not completely blocked, suggesting that other factors in the ercDC co-culture supernatant also promote RCC cell proliferation (see Supplemental Figure S2D at <http://ajp.amjpathol.org>).

In addition to increased TNF- α , CTL/tumor cell co-cultures containing ercDCs showed a reduction in CXCL10/IP-10 and CCL5/RANTES (Figure 6, B and D). Other chemokines, such as CCL2/MCP-1, were present at similar levels in co-cultures containing ercDCs or cDCs (Figure 6D). CXCL10/IP-10 and CCL5/RANTES are important chemokines involved in the recruitment of T_H1-polarized effector cells and the antitumor immune response.⁴² Using the modified Boyden chamber assay and the T_H1-CTL clone JB4 (expressing the CXCL10/IP-10 receptor CXCR3), less CTL mobilization was observed when using CTL/tumor cell co-culture supernatants containing ercDCs (migration index, 2.6 ± 0.8) compared with supernatants of CTL/tumor cell co-cultures containing cDCs (migration index, 4.0 ± 1.4) (Figure 6C).

The changes in TNF- α and the chemokines moderated by ercDCs required co-culture with CTLs or PBLs because ercDCs or cDCs alone did not produce significant levels of these factors (see legend to Figure 6). Thus, ercDCs, although not compromising T-effector lymphocyte function, appear to use the crosstalk with T cells to

cause milieu alterations that favor tumor proliferation and can attenuate T_H1 responses.

Close contacts of ercDCs with T cells were observed in tissue sections. Thus, one might speculate that similar milieu alterations could result from these interactions *in vivo*. The CD209-cell^{high} tumors were of more advanced tumor stages and showed lower infiltration with CD8⁺ cells and NK cells (Figure 1). These lymphocytes are by majority CXCR3⁺⁴³ and equipped with lytic granules (P.P. unpublished) and, thus, are Th1-polarized cytotoxic effector lymphocytes responsive to CXCL10/IP-10. These observed characteristics of CD209-cell^{high} tumors could be a consequence of the modulatory effects evoked by the presence of ercDCs and their interaction with T cells *in situ*.

Discussion

DCs participate in networks that set the balance among immune activation, inhibition, and silencing.⁶ Together with CD8⁺ T and NK cells, DCs are part of the immune infiltrate seen in RCC tissues.^{5,19,20} We analyzed the CD209⁺ cells, which are considered to represent interstitial immature DCs, in RCC tissues and observed that tumors with a high content of CD209⁺ cells had an altered composition of the immune cell infiltrate with a shift toward lower fractions of T_H1 effector lymphocytes. These CD209-cell^{high} tumors also had advanced tumor stages with poor prognosis. Multiparameter fluorescence immunohistologic analysis revealed that most CD209⁺ cells in RCC were unusual in that they co-expressed the macrophage lineage marker CD14 and CD163, supporting the notion that a rigid demarcation between DCs and macrophages in tissues may not be possible using the current markers and functional attributes.^{9,44,45} This is also reflected by the inconsistencies in the denomination of CD209⁺ and CD209⁺CD14⁺ cells in the literature, where

Figure 5. Influence of *in vitro* generated ercDCs on the maintenance of CTL effector function. CTL-JB4 was co-cultured with cDCs, with ercDCs, or without APCs (generated from monocytes of the same HLA-A2⁺ healthy donor) and then assessed for effector cell function. **A:** Cognate cytokine secretion and degranulation of CTLs after co-culture with APCs. CTLs were co-cultured with cDCs or ercDCs or without APCs (ratio 1:0.3). After 24 hours, cells were harvested and HLA-A2⁺ RCC cells were added for stimulation (ratio 1:1). During the stimulation anti-CD107 antibodies, GolgiStop, and Brefeldin-A were present. After 5 hours, cells were harvested for intracellular cytokine detection by flow cytometry, and the percentage of CD107⁺ and cytokine⁺ CTLs (identified by gating on live CD45⁺ CD3⁺ cells) was determined. The graphs are the summarized results of three independent experiments, with the bars representing the normalized percentage of marker-positive CTLs using the values obtained for CTLs cultured without APCs as reference. Error bars are the SEM. The 24-hour co-culture conditions are indicated on the x-axis. **B:** Cognate cytotoxicity of CTLs after co-culture with APCs. CTLs were co-cultured for 24 hours with HLA-A2⁺ cDCs, with ercDCs, or without APCs at ratios indicated on the x-axis, then harvested and given to chromium⁵¹-labeled HLA-A2⁺ RCC cells. The percentage of specific lysis of RCC cells at a ratio of CTL:RCC of 5:1 is depicted on the y-axis. Shown is one representative experiment of two. **C:** Perforin and granzyme B content in CTLs after 24-hour co-culture with cDCs or ercDCs (ratio 1:0.5) or without APCs. Graphs depict the median fluorescence intensity determined by flow cytometry.

they are sometimes called DCs at other times macrophages.^{35,36,46,47} Because of their predominance in RCC tissues, we chose to call our CD209⁺CD14⁺CD163^{+/−} cells ercDCs.

In renal tissues, different structural areas exhibited distinct frequencies of ercDCs, with highest percentages found in the tumor center and lowest in the corresponding NKCs, suggesting that regional microenvironment dictate marker co-expression. A cocktail of three tissue factors, CXCL8/IL-8, IL-6, and VEGF, was found to be sufficient to induce the ercDCs *in vitro*. These factors are highly expressed in RCC^{5,48} and may thus promote the differentiation of ercDCs *in situ*. The identification of these factors was based on a study design that was distinct from those reported by others^{37,49,50}: cultivation supplements, IL-4, or GM-CSF, which are commonly used to differentiate DCs *in vitro* but do not represent a DC differentiation pathway *in vivo*,¹² were deliberately omitted. Thus, we believe that the used cultivation system better reflected the natural process of monocyte differentiation when entering the tissue milieu. Soluble factors (IL-4, IL-13, IL-15) previously reported to induce CD209^{34–36} or IL-10 were low in the tumor cell culture supernatants examined here. The involvement of IL-4 in the differentiation of ercDCs *in situ* appears unlikely because IL-4 transcript levels were low in the RCC and NKC tissues examined (not shown). In addition, IL-4 has been described to down-regulate CD14 concomitantly with the up-regulation of CD209.³⁴ Yet, CD209 and CD14 expression levels were high on ercDCs *in situ*.

In a murine study analyzing the effect of kidney stromal cells and their secreted factors on bone marrow-derived cells, it was found that IL-6 and VEGF together with unidentified factors produced by kidney stromal cells induced a specific DC subset that shares phenotypic and functional characteristics with the ercDCs described here.⁵¹ CXCL8/IL-8, identified here as a crucial factor for ercDC differentiation, is absent from the murine genome.⁵² Thus, functionally orthologous chemokines may substitute for this activity in the murine system. Reports of others^{15,16,53,54}: describing macrophage marker co-expression, high TNF-α, and good antigen presentation of murine renal kidney DCs further highlight the similarity between the human DC subset with murine renal DCs described here. The description of a human tissue DC resembling a murine subset and the factors driving its differentiation contributes to ongoing efforts of defining putative human/murine DC counterparts.^{9,12–14}

CD209⁺CD14⁺CD163⁺ DCs have been described in the human dermis,⁵⁵ in a murine leprosy model,³⁶ and in the decidua of early human pregnancy.⁴⁶ Their presence has been linked to deviated immune responses, allowing bacterial or embryo persistence. This parallels the situation of RCC tumors, which persist in the face of enriched presence of CD209⁺CD14⁺CD163⁺ DCs.

The immune control exerted by ercDCs does not appear to involve T-cell inhibitory mechanisms because there was no evidence *in situ* or *in vitro* of CD3ε or CD3ζ down-regulation, FOXP3 induction, T-cell apoptosis, inhibition of cognate CTL cytotoxicity, or CTL cytokine secretion. Thus, ercDCs deviate from the classic features of

Figure 6. ErcDCs secrete high levels of MMP-9 and regulate TNF- α and chemokines in co-culture with T cells. **A:** Gelatinase zymography of cDCs and ercDCs supernatants showing digestion area for MMP-9. Supernatant of the HT1080 cell line was used as control. **B:** TNF- α and chemokine concentrations in 24-hour co-cultures of CTL-JB4 and cognate cell line RCC-26 with cDCs or ercDCs (HLA-A2⁺). Bars represent the mean of duplicate or triplicate values $\pm$ MD. **C:** Migration of CXCR3⁺, T_H1-polarized, CTL-JB4 induced by CTL/RCC co-cultures containing either cDCs or ercDCs. Bars represent the mean of migration indices (calculated relative to medium) of two independent experiments. Error bars are the MD. Culture supernatants of cDCs or ercDCs alone induced very low CTL migration with migration indices of 1.9 ± 0.1 (cDCs) and 1.2 ± 0.4 (ercDCs), respectively (not shown). **D:** TNF- α and chemokine concentrations in 4-day co-cultures of allogeneic PBLs with APCs (ratio 48:1). Concentrations of chemokines were determined by 27-Plex bead array. Concentration of TNF- α was either determined by ELISA (CTL co-cultures) or by 27-Plex bead array (PBL co-cultures). Shown are representative results of at least three independent experiments with similar results. ErcDCs and cDCs cultured by themselves had undetectable levels of T_H1 (IL-2, IFN- β , TNF- α , IL-1 β), T_H2 (IL-4, IL-5, IL-13, IL-9) cytokine, IL-10, IL-12, IL-17, and CXCL10; low levels of IL-6 (~ 50 pg/mL), G-CSF (~ 25 pg/mL), TGF- β (~ 100 pg/mL), VEGF (~ 50 pg/mL), CCL5 (~ 100 pg/mL), and CCL2 (~ 200 pg/mL); but high levels of CXCL8 (>1000 pg/mL).

tumor-associated macrophages or myeloid-derived suppressor cells^{7,56} or the tumor-educated cells described by Kuang et al.³⁷ However, weak allostimulation and T_H1 polarization were observed originating from an absence of activatory signals (manuscript in preparation).

Although ercDCs apparently did not compromise T-effector lymphocyte function, they secreted high levels of MMP-9 and used the crosstalk with T cells to cause reduced levels of proinflammatory chemokines (CXCL10/IP-10, CCL5/RANTES) and high TNF- α levels. TNF- α and MMP-9 are well-established factors of epithelial tumor promotion,^{39–41} and TNF- α can additionally limit T-effector cell frequency.⁵⁷ CXCL10/IP-10 and CCL5/RANTES play pivotal roles in the recruitment of T_H1-polarized effector cells. Continuous recruitment of immune effector cells is considered crucial for effective tumor destruction⁵⁸ because immune cells become functionally quiescent within the tumor milieu.^{7,21,42,59} Other reports describe diminished numbers of immune cells expressing corresponding chemokine receptors (CXCR3⁺ and CCR5⁺ cells) in advanced RCC disease.⁵ Thus, interference with T_H1-effector cell recruitment through modulation of the chemokine milieu exerted by ercDCs may represent a component of tumor promotion in RCC by shielding the tumor from immune cell attack.

The interaction of ercDCs with T cells was an essential prerequisite for the modulation of TNF- α and the chemokines seen in our *in vitro* system. The resulting co-culture supernatants containing ercDCs induced stronger proliferation of an RCC line and were less efficient in mobilizing CTL migration *in vitro*. ErcDCs were found in close contact with T lymphocytes in tumor tissue. Similar milieu modulatory effects evoked by these interactions *in situ* can explain the lower infiltration with CD8⁺ and NK cells, which are T_H1-polarized effector lymphocytes in RCC as evidenced by expression of CXCR3 and CCR5⁴³ (P.P., unpublished data) and advanced tumor stages of CD209-cell^{high} tumors. Scenarios in which tumors shape their milieu to become tumor conducive through modulating myeloid cells and orchestrating the T-cell response without CTL inhibition were recently described in murine models.^{60,61} Our data now provide evidence for a similar situation in a human carcinoma.

Attenuating, rather than actively suppressing, the immune cell infiltrate fits with the immunobiology of RCC and the kidney, where similarly differentiated DCs were also found, albeit at lower frequency. CD8⁺ T cells are known to infiltrate the kidney and the RCC without causing major renal damage⁶² or tumor rejection. In murine models of nephritis, renal DCs can protect the tubulointerstitium from immune cell-mediated injury.^{16,17} The ercDCs described here can limit the local inflammatory T-cell response by mechanisms involving high TNF- α and reduction of T_H1-recruiting chemokines. RCC tumors are derived from the renal proximal tubular region. Upholding and exacerbating the ercDC differentiation program may thus represent a mechanism of RCC, enabling immune evasion and tumor persistence. In murine models, kidney inflammation can progress to tubular damage⁶² and RCC tumors are occasionally rejected,^{1,5} suggesting that the regulatory constraints on the T-cell response can be

overcome. In experimental inflammatory nephritis, tubulointerstitial damage has been shown to involve CCL5- and CXCL10-mediated CXCR3⁺ T_H1 cell recruitment and the activation of DCs.⁶² It will be of clinical value to determine whether pharmacologic intervention to moderate the differentiation and activity of eDCs can help restore antitumor immune competence and improve the efficacy of immunotherapy against RCC.

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