The Immune Microenvironment in Clear Cell Renal Cell Carcinoma : The heterogeneous immune contextures accompanying CD8+ T cell infiltration in clear cell Renal Cell Carcinoma

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"For it would seem
that we write,
not with the fingers,
but with the whole person.
The nerve which controls the pen
winds itself about every fiber of our being,
threads the heart,
pierces the liver."

Virginia Woolf, Orlando.

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Summary

To decipher the potential mechanisms linking increased CD8+ T cell infiltrations with an adverse clinical outcome in ccRCC, in this study we determined: 1) the prognosis associated with the expression of immune checkpoints and its coordination with dendritic cell (DC) and CD8+ cell infiltration, and 2) the phenotypic traits of tumor infiltrating lymphocytes (TIL). The prognosis associated with CD8+ and DC infiltrations, in addition to the expression of immune checkpoints were investigated in a cohort of 135 ccRCC by quantitative immunohistochemistry. We found that the densities of CD8+, PD-1+ and LAG-3+ cells were closely correlated, and independently associated with decreased PFS and OS. In addition, patients whose tumors presented both high densities of PD-1+ cells and PD-L1+ and/or L2+ tumor cells (>5% positive cells), displayed the worst clinical outcome. High densities of immature DC isolated in the tumour stroma were associated with high expression of immune checkpoints and patients' poor clinical outcome. In contrast, the presence of mature DC within Tertiary Lymphoid Structures identified, among the tumours with high CD8+ TIL densities, those with low expression of immune checkpoints and prolonged survival. To functionally characterize the CD8+ T cell infiltrates, we investigated the phenotype of freshly isolated TIL in 21 ccRCC by flow cytometry. We found a group of tumors (8/21) characterized by the over-expression of inhibitory receptors (PD-1 and TIM-3) and activation markers (CD69 and CD38), the expansion of the effector memory cell subpopulation (CCR7-CD45RA-), and a trend toward more aggressive features. In summary, we demonstrated that the infiltration with CD8+ TIL in ccRCC is accompanied by the enhanced expression of immune checkpoints and a poorly coordinated immune response in a subgroup of aggressive tumors. This immune profile defines a poor prognosis group of patients that should be suitable to receive immune checkpoint inhibitors.

Abbreviations

CRC: Colorectal Cancer.

TME: Tumour Microenvironment.

CIN: Cervical in-situ Neoplasia.

DC: Dendritic Cells.

TIL: Tumour Infiltrating Lymphocytes.

MSI: Microsatellite Instability.

TLS: Tertiary Lymphoid Structures.

Treg: Regulatory CD4+ T cells.

IM: Invasive Margin.

TAM: Tumour Associated Macrophage.

NSCLC: Non-small Cell Lung Cancer.

HCC: Hepatocellular Carcinoma.

LPS: Lipopolysaccharide.

MHC: Major Histocompatibility Complex.

DC: Dendritic Cells.

ccRCC: Clear Cell Renal Cell Carcinoma.

VHL: Von Hippel-Lindau.

TT: Target Therapy.

VEGF: Vascular Endothelial Growth Factor.

PDGF: Platelet Derived Growth Factor.

TGF: Transforming Growth Factor.

GLUT: Glucose Transporter.

TKI: Tyrosine Kinase Inhibitors.

mTOR: Mammalian Target Of Rapamycin.

PB: Peripheral Blood.

MDSC: Myeloid Derived Suppressor Cells.

ROS: Reactive Oxygen Species.

RCC-LM: Renal Cell Carcinoma Lung Metastases.

CRC-LM: Colorectal Cancer Lung Metastases.

nccRCC: Non-Clear Cell Renal Cell Carcinoma.

NTLS-DC: Non-Tertiary Lymphoid Structures Dendritic Cells.

TLS-DC: Tertiary lymphoid Structures Dendritic Cells

AM: Activation Markers.

InR: Inhibitory Receptors.

EM: Effector Memory.

CM: Central Memory.

EMRA: Effector Memory CD45RA+.

Introduction

Chapter 1 - The Anti-Tumour Immune Response

Bullet points

- A chronic inflammatory microenvironment promotes the development of cancer.
- Once a tumour has emerged, an inflammatory microenvironment also promotes malignant cells growth, tumour spread and metastasis.
- The adaptive immune response controls tumour growth and elimination.
- The major cellular mediators of the anti-tumour immune response identified so far are the CD8+ T cells and Th1-oriented CD4+ lymphocytes, and their increased densities are associated with good clinical outcome in most tumours.
- There are few exceptions to this rule, including Renal Cell Carcinoma and Hodgkin Lymphoma, where an increased CD8+ cell infiltration has been associated with patient's poor prognosis.
- Tumour cell often develop mechanisms that modulate and/or inhibit the immune response, which are associated with patient's poor prognosis.

Inflammation and Cancer

Several lines of evidence have established an association between chronic inflammation and cancer (1). First, approximately 20% of the tumours are linked to inflammation-inducing infectious organisms (2), including Helicobacter pylori and gastric cancer (3), Hepatitis B and C viruses and hepatocellular carcinoma (4) and human papilloma virus and cervical and head/neck cancers (5) (6). Second, chronic noxious stimuli or inflammatory diseases can favor neoplasia, such as cigarette smoke and asbestos/silica for lung carcinoma (7), gastroesophageal reflux for cancer of the esophagus (8), inflammatory bowel disease for colorectal cancer (CRC) (9), chronic pancreatitis for pancreatic cancer (10) and pelvic inflammatory disease for ovarian cancer (11). Third, the chronic intake of nonsteroidal anti-inflammatory drugs inversely correlate with CRC incidence, and recent studies indicate a negative effect of aspirin consumption on tumour growth (12). Finally, the neutralization of inflammatory

mediators (e.g. cytokines and pro-inflammatory transcription factors) decreases the incidence and spread of tumours both in mice and in humans (13) (14). Table 1 lists cancers where a chronic inflammation has been implicated in their pathophysiology.

Table 1. Cancers associated with chronic inflammatory conditions

Inflammatory Process	Associated Neoplasia
Infectious Etiology	
Human Papilloma Virus	Cervical Cancer and Head/Neck Cancer
Hepatitis B and C virus	Hepatocellular Carcinoma
Epstein-Barr virus	Nasopharynx Cancer and Lymphoma
Human herpes virus type 8	Kaposi's Sarcoma
Helicobacter pylori	Gastric Cancer
Schistosoma haematobium	Bladder Cancer
Opisthorchis viverrini and Clonorchis sinensis	Hepatocellular Carcinoma
Chronic Noxious Stimuli	
Tobacco smoke	Lung Cancer, Esophageal Cancer, etc.
Silica	Lung Cancer
Asbestos	Mesothelioma
Alcohol intake	Esophageal Cancer
Chronic pelvic inflammatory disease	Ovarian Cancer
Aflatoxins	Hepatocellular Carcinoma
Chronic Inflammatory diseases	
Gastroesophageal reflux and Barret's metaplasia	Esophageal Cancer
Type A Gastritis	Gastric Cancer
Chronic Pancreatitis	Pancreatic Cancer
Inflammatory Bowel Disease	Colorectal Cancer
Chronic Osteomyelitis	Bone Cancer
Hashimoto's thyroiditis	Thyroid Lymphoma
Thyroiditis	Papillary Thyroid Cancer
NASH, Hemochromatosis	Hepatocellular Carcinoma

The mechanisms directing inflammation-induced tumourigenesis are well known. DNA damage and extra cellular matrix disruption by inflammatory mediators [e.g. through the production of reactive oxygen species (15) and matrix metalloproteinases (16), respectively], in addition to the stimulation of tumour cell growth by cytokines [e.g. IL-1B for gastric carcinoma (17) and IL-8 for melanoma (18)], are the main recognized tumour-promoting mechanisms.

In several situations in which the pre-cancerous stages can be studied, a shift from an immunological pattern with a Th1 orientation to a pro-inflammatory tumour microenvironment (TME) has been reported. It is illustrated in cervical carcinoma in which high expression of genes encoding Th1 cytokines is found in cervical in-situ neoplasia (CIN), whereas IFN-γ expression is lost, and the expression of pro-inflammatory cytokine-genes and macrophage infiltration is high in invasive and aggressive cervical carcinoma (19) (20). A similar shift has been described in pancreatic cancer, where there is a decrease in the density of CD8+ T cells and mature (DC) cells from low-grade premalignant lesions into invasive ductal adenocarcinoma and also during transformation of benign to malignant head and neck cancer (21). In cervical cancer, a change towards a Th2-type cytokine pattern has also been

reported in the evolution from intraepithelial neoplasia to invasive carcinoma (Bais 2005).

Not surprisingly, once a tumour has emerged, an inflammatory microenvironment can also promote malignant cells growth, resulting in neo-angiogenesis, acquisition of new mutations, extracellular matrix disruption, tumour cell migration and finally metastasis (22). The main cellular mediators of this process are macrophages (and to less extent neutrophils) that are, by far, the major immune cellular component of tumour infiltrates (23). These cells produce high quantities of IL-1β, IL-6, IL-23 and TNF-α, the key cytokines mediating the inflammatory-induced tumour igenesis (reviewed in (22) and (23)).

Immune Control and Tumour Escape

In addition to the link between inflammation and tumourigenesis, other cellular and molecular mediators of the immune system can contribute to control of tumour growth and elimination. Several epidemiological observations support this fact, including scarce reports of spontaneous cancer regression (24), the augmented incidence of cancer in immunosuppressed individuals (25) and the association between increased tumour infiltrating T cell (TIL) and favorable clinical outcome (26) (27).

The fact that tumour cells express antigens encoded by mutated genes (28) often renders them targets of the immune cells. Indeed, autologous TIL can induce tumour cell lysis in vitro and in vivo (29) and tumour-specific lymphocytes are often detected in patients with cancer. This phenomenon has been well characterized in colorectal cancers, where microsatellite instability (MSI, a genetic defect that impedes DNA mismatch repair) fosters the expression of thousands of new antigens on tumour cells. Characteristically, MSI+ tumours have a prominent CD8+ T cell infiltration and are associated with favorable clinical outcome (30).

The major cellular mediators of the anti-tumour immune response are the CD8+ T cells, in addition to the Th1-oriented CD4+ lymphocytes. The first are in charge of the elimination of tumour cells through the production of apoptosis-inducing molecules or cytotoxic granules (e.g. granzymes, perforin and granulysin) (31), while the latter can provide help to the CD8+ T cells and foster the anti-tumour response by the secreting major cytokines, including IFN-γ (32). Several lines of evidence suggest that mature dendritic cells (DC) orchestrate the T and B cells anti-tumour immune response. Characteristically, these cells are present in highly organized peri-tumour immune cellular aggregates, called Tertiary Lymphoid Structures (TLS) (33) (discussed in next section).

The major antitumour immune response cytokines and chemokines are IFN-γ, IL-12, CXCL9 and CXCL10, mainly involved in CD8+ T cell recruitment (CXCL9 and CXCL10) and activation (IL-12 and IFN-γ) (34) (35).

All these processes submit tumour cells to a significant selective pressure. In fact, tumour cells can develop mechanisms that modulate and/or inhibit the immune response, including: first, the production of immunosuppressive molecules (e.g. IL-10 and TGF-β) that hamper the cytotoxic and proliferative capacity of T cells (36); and, second, the expression of ligands for inhibitory receptors expressed on the TIL (37). Of particular relevance, PD-1 is a molecule expressed on activated and exhausted T cells that diminishes the strength of the cellular immune response upon binding to its ligands (PD-L1 and PD-L2) (38). Under physiological conditions, the expression of PD-L1 and PD-L2 is highly regulated and it is limited to dendritic cells, macrophages, activated T cells (PD-L1 only) (39) and certain tissues where immunomodulation is required (e.g. syncytiotrophoblast in the placenta). Nevertheless, certain tumour cells can express these ligands, and subsequently inhibit T cell activity. Similar mechanisms have been reported, including the expression of the ligands for TIM-3 and LAG-3, two additional inhibitory receptors expressed on T cells (reviewed in (37)). Ultimately, this microenvironment induces the development of suppressive immune cells, including regulatory T cells CD4+ (Treg) and myeloid derived suppressor cells (36), that sustain self-tolerance against tumour antigens.

This complex interconnected network of myeloid and lymphoid cells, endothelial and lymphatic vessels and stromal cells –named the tumor microenvironment (TME) (26) (40)– has been largely studied in the last decade. Its influence on patient's clinical outcome and tumour progression has been of particular interest: patients with tumours that develop immunosuppressive mechanisms have the worst prognosis, and their tumours will often display a higher histologic grade characterized by dedifferentiation, neo-vascularization and an inflammatory infiltrate.

The Immune Microenvironment as a Prognostic Tool

Many studies have described the distribution of the inflammatory and immune infiltrate within different tumours. Overall, the macrophages, mast cells and granulocytes are mostly found infiltrating or surrounding the tumour nests both in the invasive margin and the core of the tumour. On the contrary, the lymphoid infiltration is more precisely distributed, and some locations are enriched in certain cell types: NK cells are mostly found in the stroma and are not in contact with tumour cells; B cells are mostly found in the invasive margin (IM) of the tumours within lymphoid aggregates; and T cells, particularly CD8+ T cells, are mainly located in the IM, but can also infiltrate the tumour core (26) (41).

The analysis of the immune microenvironment in retrospective cohorts across different tumours has established a clear correlation between the density of infiltrating immune cell and patient's clinical outcome.

Tumour associated macrophages

Several studies have found that the clinical outcome associated with increased numbers of Tumor Associated Macrophages (TAM) is mainly determined by their functional orientation, and is heterogeneous across tumor types (42). In fact, while the augmented densities of this population are associated with favorable clinical outcome in colorectal (43) (44) (45) (46) (47), gastric (48), non-small cell lung cancer (NSCLC) (49) (50) (51) (52), hepatocellular carcinoma (HCC) (53) (54), prostate (55) and cervical cancer (56), it has the opposite association in endometrial (57), esophageal (58), gastric (59) (60) (61) (62), urothelial (63), oral (64) (65), HCC (66) (67), melanoma (68), breast (69) (70) (71) (72) (73) (74), ovarian (75), bladder (76) (77), NSCLC (78), thyroid (79), pancreatic neuroendocrine (80) and endometrial (81) tumours.

The heterogeneous association between TAM densities and the clinical outcome across different tumours might reflect the plasticity of this immune population. Overall, two different subtypes of macrophages have been described (82) (83):

1. M1: activated by Toll-like receptor ligands [e.g. Lipopolysaccharide, (LPS)] and IFN-γ; they preferentially express pro-inflammatory cytokines in addition to inducible nitric-oxide synthase, an overall they potentiate the inflammatory and immune response.
2. And M2: stimulated by IL-4 or IL-13; they express arginase 1, CD206, IL-4r, TGF-β1 and PDGF. This population is rather implicated in wound repair, promoting fibroblast proliferation and extracellular matrix deposition.

A protective role in tumourigenesis has been proposed for M1 macrophages (through mechanisms including the activation of the Th1 response and by antagonizing the suppressive activities of regulatory immune cells), while M2 have shown to promote tumour growth, invasion, metastasis, stroma remodeling and angiogenesis (84).

The absence of M1 and M2 specific markers has been the major obstacle in the assessment of the clinical impact of each of theses subtypes. Up to date, this task has been accomplished using CD11c or NOS2 for M1 TAM, and CD163, CD204 or CD206 for M2 TAM. Interestingly, increased M1 TAM densities seem to be associated with a favorable clinical outcome in NSCLC (51) (52), ovarian (85), colorectal (86) and gastric cancer (87), while those of M2 are linked to poor prognosis in several tumours, including: NSCLC (88) (89) (90), mesothelioma (91), esophageal (92), gastric cancer (61) (93), pancreatic (94) (95) (96) (97) (98) (99), CRC (100), HCC (101), Hodgkin lymphoma (102), renal (103) (104) (105), urothelial (106), breast (107), endometrial (108), ovarian (109), melanoma (68), and squamous oral carcinoma (110). Additionally, some studies have demonstrated that, when associated with poor clinical outcome, CD68+ cells are often correlated with the tumour microvessel density, in

addition to HIF, VEGF (63) and matrix metalloproteinases expression (62) (69) (111) (112) (76), suggesting they might have an M2 phenotype.

Although not yet conclusive, the evidence linking the infiltration with TAM and the patient's clinical outcome suggests that high densities of M2-oriented cells are associated with more advanced tumour stages and patient's poor prognosis. In addition, it also indicates that the biological value of measuring the sole densities of CD68+ cells should be revisited, as it does not provide information of the TAM function or polarization.

NK cells

Natural Killer cells is another major cellular population mediating the anti-tumour immune response (113). These cells express an array of receptors (including activating, inhibitory, adhesion and cytokine receptors) that enable them to identify tumour cells. Overall, the integration of these signals determines whether or not NK cells become activated, and eliminate its target. The two most important mechanisms of cancer cell recognition by NK cells is the down-regulation of major histocompatibility complex (MHC) class I and the expression of stress-induced ligands to NK activation receptors (e.g. MICA or MICB, which bind to NKG2D expressed on the NK cell) by tumour cells.

The prognostic impact of NK infiltration has been studied in some tumours, and their increased densities have been consistently associated with favorable clinical outcome. This association has been demonstrated in CRC (114) (115) (116), gastric (117) (118) (119), vulvar squamous cell (120), esophageal (121), renal (122) (123), HCC (124), NSCLC (125) (126) (127), in addition to CRC and RCC lung metastases (128). Nevertheless, other studies in NSCLC have not found an association between NK infiltration and prognosis (129), where they display an inhibited phenotype and decreased functional capacities (130) (129). More studies assessing the prognostic impact associated with NK infiltration are needed.

Dendritic cells

Upon encounter with an antigen and in the presence of danger signals, immature DC go through a process called maturation, that allows them to migrate into the lymph node, where they can prime naïve CD4+ and CD8+ T cells. The phenotype of the mature DC plays an important role in determining the orientation and strength of the subsequent immune response, and it is determined by the cytokine microenvironment, in addition to the type of antigen being processed (131). The TME takes advantage of the DC plasticity, and can induce a pro-inflammatory and/or tolerogenic DC, or block their maturation at different stages.

Due to the plasticity and heterogeneous DC phenotype, the quantification of infiltrating DC has been a difficult task. The more relevant markers that have been used for their quantification are S-100, CD83, DC-LAMP or CD1a, in addition to CD207 for Langerhans cells, and their clinical impact has been reported in a variety of human solid tumours. Overall, several studies support that the augmented infiltration with DC is associated with increased overall survival in many tumours types, including melanoma (132) (133) (134) (135), HCC (136) (137), gallbladder (138) (139), oral (140), esophageal (141) (142) (143), gastric (144) (145) (146) (119), NSCLC (147) (127) (148), colorectal (149) (150) (151) (152), bladder (153), breast (154) (155) (155) (156) (157), endometrial (158) (159) and ovarian cancer (160) (161). Nevertheless, the infiltration with CD123+ plasmacytoid DC has been associated with shorter overall survival in breast cancer (162) (163), as for the presence of CD208+ and CD1a+ DC in colorectal (164) and gastric cancer (165).

Tertiary lymphoid structures and associated mature dendritic cells

Interestingly, lymphoid aggregates can be detected in the invasive margin of most tumours. Some of them exhibit properties of active immune sites that resemble those arising in other tissues upon infection, or secondary to autoimmune or chronic inflammatory diseases (33). Characteristically, they exhibit a T cell zone (composed mainly of CD4+/CD62L+/CD45RO+ CM an in less extend CD8+/CD62L+/CD45RA+ naïve T lymphocytes) with embedded mature DC, germinal centers with proliferating B cells and they are surrounded by high endothelial venules (166). In addition, laser microdissection of these structures reveled they are enriched in genes associated with T cell chemoattraction molecules, such as CCL19, CXCL13, CCL21, IL16, CCL22 and CCL17 (166).

In view of their similarities with germinal centers in lymph nodes, it has been hypothesized that these Tertiary Lymphoid Structures (TLS) represent a site where in situ antigen presentation and lymphocyte activation can occur under a protected environment (33). Indeed, studies in primary melanoma and NSCLC have correlated the densities of mature DC (DC-Lamp+) within TLS with a strong infiltration with activated T cells and a Th1-oriented response, respectively (132) (167). Moreover, the higher densities of these structures correlated with favorable clinical outcome in NSCLC (168) (167) (169), colorectal (151) (170), melanoma (171) and breast cancer (172). The mechanisms underlying the neogenesis of these structures are still unclear in human tumours.

CD4+ and CD8+ T cells

Overall, a high infiltration by CD8+ T lymphocytes is associated with good clinical outcome in many tumour types, including lung, liver, stomach, CRC, breast, esophageal, bladder, melanoma, ovarian and prostate cancers (reviewed in (26)). However, there are exceptions to this rule, including diffuse

large B cell lymphoma (173), Hodgkin lymphoma (174), RCC (175) and potentially head and neck cancer (176), where high densities of tumour-infiltrating CD8+ T and/or Th1 cells have been associated with poor prognosis. Overall, Th1 CD4+ T cells show a similar clinical impact to that of CD8+ T cells; nevertheless, there are again exceptions to this rule, including head and neck cancer (177) and RCC (178) (179). The infiltration by other T cell subsets (Th2, Th17 and Treg) is less clear, and seems to be dependent on the cancer type (26).

In view of the clinical impact of infiltrating CD8+ T cells in cancer, sustained efforts are being made to validate and promote their quantification in the routine clinical setting; this approach that has been called Immunoscore (180). The development of automatized software that quantify the densities of immune cells after immunohistochemical staining is promoting the gradual change from semi-quantitative approaches to quantitative, and more powerful, methods.

B lymphocytes

Evidence assessing the clinical impact of tumour infiltrating B cells is scarce. In inflammatory settings other than cancer, B cells enhance T cell responses by producing antibodies, stimulatory cytokines and chemokines, serving as local APCs, and organizing the formation of tertiary lymphoid structures that sustain the immune response. Although studied in less detail, the potential mechanisms of action of B cell in tumours have been divided into a direct (antibodies production and direct cytolitic activity) and an indirect (by presenting antigens to T cells or activating them) (181) effect. In addition, recent evidence suggests that tumor-infiltrating B cells can play an immunomodulatory role through the production of IL-10, among other cytokines (182). Indeed, the role of B cells in cancer is suggested by the fact that the majority of human cancer patients mount tumour-specific antibody responses (183), they often are organized within TLS where they undergo somatic hypermutation (184) (185) (186) (169), and they often correlate with the functionality of T cells (187) (188) (116).

In accordance with this presumption, several studies have reported a positive correlation between the B cell densities and the clinical outcome in different cancers, including NSCLC (189) (169), primary cutaneous melanoma (190), breast cancer (185) (191) (72) and ovarian cancer (192).

The Immune Microenvironment and Other Histopathologic Features

The link between the tumour immune infiltrate and other pathology/clinical parameters has been assessed in independent studies, and there is not yet a consensus on this matter. Overall, tumours poorly infiltrated with CD8+ T cells often display a higher histologic grade, characterized by dedifferentiation, prominent vascularization and inflammation. Two independent studies in large cohorts of melanoma lesions established a correlation between an increased lymphocyte infiltration (known as

higher TIL grade) and thinners lesions (Clark level), smaller radial growth phase, lower stage and negative sentinel lymph nodes (193) (194) (195). A similar picture has been described in CRC, where there is an inverse correlation between the CD8+ and CD45RO+ (memory T lymphocytes) cell densities and the tumour stage (196) and perineural invasion (197). Moreover, the density of innate cells increases, whereas that of most other T cell subsets decreased along with tumour progression in this pathology (116).

Nevertheless, this is not the case for all tumour types. Breast cancer deserves particular attention, because in the basal subtype an increased lymphocytic infiltration has been related with advanced histologic grades (198) (199) (200).

Therapies that modulate the tumour microenvironment

In view of the important immune processes taking place within tumours, many therapies to boost the local immune response and diminish the inflammatory or suppressor molecules are been currently developed.

One of the first successful immunotherapies used in the clinical setting was recombinant IL-2, whose aim was to activate and expand the intra-tumour T lymphocyte (201). The treatment of thousands of patients in the late 80s and 90s established that only metastatic melanoma and metastatic RCC responded to this therapy, and complete response rate was limited to 10% (201). Because of the high rate of adverse effects, this therapy was replaced over the years, but it set a precedent for the development of other immunotherapies: boosting the T cell response could mediate complete destruction of large, vascularized and invasive cancers in humans.

Other immunotherapies used in similar clinical scenarios are IFN-α and anti-angiogenic drugs (e.g. Sunitinib and Bevacizumab). In metastatic RCC, Sunitinib as monotherapy has shown high objective response rates (up to 50%) and currently is the first-line treatment option for metastatic RCC patients (202). In addition to normalizing the tumour vascularization, this drug promote anti-tumour immunity through different mechanisms (203).

New therapies based on the recent understanding of the immune-suppressive cells and T cell inhibitor pathways are being tested. The term checkpoint blockade describes the injection of monoclonal antibodies specific for inhibitory receptors expressed on the surface of lymphocytes (anti-PD-1, anti-CTLA-4 and anti-LAG-3), or their ligands on tumour or other suppressive immune cells (PD-L1 and PD-L2) (37) (204). Several trials on increasing number of malignancies are ongoing; overall they have shown exceptional results in some cancer including melanoma (205) (206), RCC (207) (208) (209), lung cancer (207), Hodgkin lymphoma (210) and bladder cancer (211).

Indeed, the analysis of the TME is becoming a powerful tool to predict the response to immunotherapies. Interestingly, preliminary data from clinical trials of PD-1 blockade suggest that the presence of: 1) infiltrating CD8+ or PD-1+ T cells (212) and/or 2) PD-L1+ tumour (213) (207) (210) or immune cells (211) (214), are the more sensitive parameters to predict the patients' response to treatment (215).

Chapter 2 - Renal Cell Carcinoma

Bullet points

- RCC is the 13th most prevalent tumours worldwide, and the clear cell RCC (ccRCC) histological subtype accounts for 70% of cases.
- 70% of the ccRCC displays a lost of function of the von Hippel-Lindau (VHL) gene, which induces the expression of molecules related to cellular hypoxia.
- Patients bearing advanced-stage tumours display a very poor clinical outcome (less than 20% for stage IV).
- RCC is resistant to chemo and radiotherapy, and currently surgical excision is the first-line treatment for localized disease.
- In turn, Target Therapies (TT) are the first-line treatment for advanced RCC, but all tumours will eventually develop resistance.
- Checkpoint blockade is becoming a promising treatment for advanced RCC, but no theranostic markers to predict patient's response are currently available.

Epidemiology and Pathophysiology

Renal cell carcinoma is the 13th most prevalent tumour worldwide (with an estimated incidence of 209 000 new cases and 102 000 deaths per year), and represents approximately 2.5% of all neoplasias (216). The identified risk factors for developing this cancer are obesity (body max index >30), active or passive cigarette smoking and hypertension. Other factors not conclusively associated are end-stage renal failure, acquire renal cystic disease, exposure to asbestos or trichloroethylene and some dietary habits (216). The risk is similar throughout all races, but it is slightly higher in males than females and the peak of incidence is between the 6th and 7th decade of life (217).

Approximately 2-3% of the RCCs are familiar and expressed as a dominant trait. Notably, the von Hippel-Lindau syndrome is the most prevalent hereditary RCC (1/36 000) and is characterized by the development of several vascular tumours including ccRCC, hemangioblastoma of the central nervous system and pheochromocytoma. In most of the cases, the mutation has been localized in the VHL gene. Physiologically, VHL targets hypoxia inducible factor 1 (HIF-1, a transcription factor involved in the cell response to low oxygen microenvironments) and induces its degradation. The loss of

function of the VHL protein promotes the intra-cellular accumulation of HIF-1, and the subsequent transcription of several hypoxia-related molecules such as Vascular Endothelial Growth Factor (VEGF), Platelet-Derived Growth Factor (PDGF), Transforming Growth Factor alfa (TGF-α) and Glucose Transporter 1 (GLUT-1). More than 300 different mutations have been described in the hereditary form of VHL disease (218).

The rest of the RCC cases (97-98%) are sporadic. The analysis of more than 400 RCC by the TGCA group (219) confirmed that between 55-82% have mutations in the VHL gen (220) (219) (221), while genetic abnormalities on the PBRM1 and SETD2 accounted for 33% and 11% of cases, respectively (219).

Classification

Histologically, RCC has been divided in 6 entities, each deriving from different parts of the nephron and possessing distinct genetic abnormalities (217). The most common –accounting for 70-80% of cases– is the clear cell disease (ccRCC), characterized by the accumulation of lipids in tumour cell cytoplasm that, upon being dissolved during histological preparation, leaves an empty (clear) space. This tumour type often displays a deletion of the chromosome segment 3p, inactivation of the VHL promoter by mutations or hypermethylation or, less frequently, a gain of chromosome 5q (222).
The second most common variant is the papillary subtype that has a frequency of 10-15% among RCC tumours. The lesions with this histology frequently display genetic abnormalities related to trisomy of chromosomes 7 and 17, duplications involving the gene MET or lost of chromosome Y. It has an overall good prognosis (222).

In order of frequency these two phenotypes are followed by the chromophobe, oncocytoma and collecting duct carcinoma (217).

Clear cell renal cell carcinoma

ccRCC probably arises form proximal tubular epithelial cells, as suggested by the shared expression of CD10, villin and intracellular adhesion molecule by tumour and tubular cells (223). Recently, it has been proposed that ccRCC cancer cells arise from primitive regenerating epithelial tubular cells that display a dedifferentiation towards a mesenchymal origin (Vimentin+, E-cadherine) (224). Metabolically, ccRCC tumour cell in ccRCC are characterized by over-active glycolytic and glycogenic pathways, in addition to a deficient lipolysis, that lead to the accumulation of glycogen and lipids in the cell cytoplasm (225). Biallelic loss of VHL function occurs in 90% of the ccRCC, and several other mutations have been reported. The deletion of the 3p locus tumour suppressor gene

cluster (containing, among others, the VHL, PBRM1 and BAP1 genes) is the most common origin of VHL lost in ccRCC (225).

The comprehensive genome-scale mutational and epigenetic analyses of ccRCC have demonstrated that there are at least 4 molecular subtypes of ccRCC (226) (219), with different impacts in patients' prognosis. Nevertheless, there is not yet a published consensus on the molecular sub-classification of ccRCC.

Diagnosis

The classic presentation of RCC is hematuria, flank pain and palpable abdominal mass. Nevertheless, this clinical triad is rarely present (around 10% of patients), and currently more that 50% of patients with RCC are incidentally detected when abdominal imaging studies for other pathologies are being carried out. When advanced, this tumour can often cause symptoms derived from paraneoplastic syndromes including hyperparathyroidism, hypereninism and erythrocytosis (217). Currently, the diagnosis is mainly made through computed tomography (enhancing renal mass). Urinalysis, on the other hand, has shown a limited utility.

The staging of RCC masses follows the next parameters (217) (227):

- **T1:** Tumours ≤ 7cm and confined to the kidney.
- **T2:** Tumours >7cm and confined to the kidney.
- **T3:** Tumour invades the adrenal gland (3a), the renal vein (3b) or into the vena cava (3c), but no beyond Gerota's Fascia.
- **T4:** Tumour invades beyond Gerota's Fascia.

- **N1:** Metastasis in one regional lymph node.
- **N2:** Metastasis in more than one regional lymph node.

- **M1:** Distant metastasis present.

Stage I and II (including T1/T2, N0 and M0) are associated with good clinical outcome, with a 5-year survival of 95% and 88%, respectively. Stage III (T3N0/1M0, or T1/2N1M0) is associated with a 59% 5-year survival. Finally, stage IV RCC (T4NxMx or TxNxM1) is associated with very poor clinical outcome, with a 5-year survival inferior to 20% (222) (AJCC Cancer staging Manual. 7th Edition. 2010).

Treatment

Nephrectomy and other surgical approaches

RCC is highly resistant to chemotherapy, with only a 4-6% response rate (228). This is probably due to the expression of multidrug resistant transporter in proximal-tubule cells (cells from which clear-cell and papillary renal-cell carcinoma may originate).

Overall the treatment of most renal masses is surgical excision (through partial or total nephrectomy), although is currently accepted to follow a conservative treatment with masses smaller that 3cm, understanding that surgery can carry serious short and long-term complications. In fact, some studies have found that only 20% of the small renal masses (<4cm) are potential aggressive tumours (229) (230). Additionally, in very selected cases –such as small tumours in patients that cannot be candidate for conventional surgery– thermal ablation is gaining acceptance in clinical setting.

Regarding the choice of the surgical procedure, partial nephrectomy is preferred over total nephrectomy because it carries less risk of chronic kidney disease in the long term (231). Partial nephrectomy is therefore recommended in all tumours measuring between 4-7cm. For local advanced disease, a "radical extirpate" surgery is strongly recommended, with the aim to achieve total tumour surgical excision. With this aggressive approach, up to 40-60% of patients will display a durable tumour remission (232) (233) (234). In addition, complete resection of either synchronous or metachronous solitary metastases from RCC is justified and can contribute to a long-term survival in a selected group of patients (235).

Therapeutic approaches in locally advanced and metastatic RCC

Target therapies

Medical therapies are also generally recommended for locally advanced or metastatic RCC, and those tumours where surgery is not advisable. The growing understanding of the physiopathological mechanisms of RCC has allowed the development of drugs that interfere with specific molecules involved in tumours spread and progression (TT). Until now, therapies targeting the VHL–VEGF pathway have been the main focus of clinical research. Three main categories have been tested in the clinical setting: 1) Tyrosine kinase inhibitors (TKI), 2) mTOR inhibitors and 3) monoclonal antibodies against VEGF.

Apart VEGF receptor 1-3, TKI have multiple targets, including PDGF-receptor, c-KIT, and FLT-3. Four of these drugs are currently available in the United States, including Sunitinib, Sorafenib, Pazopanib, and Axitinib. Sunitinib is the molecule more frequently used in the clinical practice, and multiple clinical trials have shown a significantly benefit on PFS as compared to interferon alpha (202)

(236). Currently, it is the first-line treatment in advanced and stage IV RCC (237). The survival impact of other inhibitors of the VEGF pathway has been analyzed in clinical trials and overall has shown a benefit on OS (238).

The second class of TT used in RCC are the inhibitors of mammalian target of rapamycin (mTOR), a signaling pathway regulating cell growth, proliferation, metabolism and angiogenesis. The currently available mTOR inhibitors include Everolimus and Temsirolimus. A phase 3 trial established the benefit of Everolimus as mono-therapy over placebo in patients with advanced RCC after progression on Sunitinib and/or Sorafenib (239), and it is approved for Sunitinib/Sorafenib resistant tumours. Also noteworthy, a phase III clinical trial found a beneficial effect on OS and PFS for patients treated with Temsirolimus (another inhibitor of mTOR pathway named) alone versus interferon in advanced ccRCC (240). This agent is currently recommended as therapy for poor prognosis RCC (237).

Finally, Bevacizumab is a humanized anti-VEGF monoclonal antibody that has shown a benefit on OS and PFS in different clinical trials, and is another first-line therapy option, rarely used in clinics (241) (242) (243) (244).

It is worth noting that targeted therapies are not cytotoxic but cytostatic and all tumours will eventually develop resistance and progress. This fact highlights the necessity to develop curative therapies for advanced RCC.

Immunotherapies

The high frequency of chemo/radio-resistant RCC encouraged the development of other therapies to treat the advanced disease. The first medications that displayed a significant impact on tumour recurrence and overall survival in metastatic RCC were IL-2 and IFN-α. The treatment of thousands of patients in the late 80s and 90s with high-dose IL-2 established that complete response rate was limited to 10% patients (201). Because of the high rate of adverse effects, this therapy was replaced over the years. Similarly, treatment of advanced RCC with IFN-α reported response to treatment in up to 14-29% of cases, and an improvement of survival of 3.8 months (245) (222), with a median duration of six months. Currently, it is the drug of choice to use in combination with other agents in experimental approaches, and is recommend as first-line therapy in combination with Bevacizumab.

New therapies based on the recent understanding of the immune-suppressive cells and T-cell inhibition pathways in cancer are being designed, with the ultimate aim of breaking tolerance and boosting anti-tumour immunity.

The term checkpoint blockade describes the administration of monoclonal antibodies that are specific for inhibitory receptors expressed on the surface of lymphocytes (e.g. anti-PD-1, anti-CTLA-4 and anti-LAG-3) or the PD-1 ligands expressed on tumour or other immune cells (PD-L1 and PD-L2) (37) (204). Metastatic RCC has been one of the tumours that have shown high response rates to

checkpoint blockade, specifically with antibodies blocking the PD-1 axis. In a phase 1 study, 9 out of 33 patients (27%) with advanced RCC receiving anti-PD1 antibody (nivolumab 0.1 to 10.0mg every 2 weeks, up to 12 cycles) had an objective response (complete of partial). Of the 8 patients under treatment for 1 year or more, 5 had a sustained response (207). A phase I dose escalating trial with an anti-PD-L1 antibody (BMS-936559) was also recently conducted on 17 RCC, from whom 2 had an objective response with a duration of 4 and 17 months (208). Several ongoing clinical trials are evaluating the efficacy of PD-1 blockade in metastatic RCC (https://www.clinicaltrials.gov/).

Theranostic markers

TT and checkpoint blockade are becoming the mainstay of treatment for advanced RCC. Nevertheless, the efficacy of to these therapies is highly heterogeneous across patients, and no markers to predict the response to treatment are currently available.

Although several studies have assessed the utility of different biomarkers to predict the response to TT (reviewed in (246)), there is not yet a consensus on which should be used in the clinical setting.

Nevertheless, the analysis of the TME is becoming a powerful tool to predict the response to checkpoint blockade. Indeed, preliminary data from clinical trials of PD-1 blockade suggest that the presence of: 1) infiltrating CD8+ or PD-1+ T cells (212) and/or 2) PD-L1 expression on tumour (213) (207) (210) or immune cells (211) (214), are the more sensitive parameters to predict the patients' response to treatment (215).

Chapter 3 - The Tumour Microenvironment in Renal Cell Carcinoma

Bullet points

- RCC often displays prominent inflammatory microenvironment that is associated with tumour progression and metastasis.
- The inflammatory features in RCC are probably driven by the tumour cells, and amplified in a second instance by tumour-associated macrophages, and probably other innate immune cells.
- The adaptive immune response in RCC is characterized by functional defects in dendritic-cell and T-lymphocytes, presumably related to the inflammatory TME.
- The T cell dysfunction in RCC is characterized by exhaustion rather than defective recruitment.
- Scarce publications had reported an association between increased infiltrations of CD45RO+, CD4+, non-proliferating CD8+ T cell and poor clinical outcome in RCC.
- The characteristics of the immune microenvironment associated with a Th1 and CTL immune response in RCC and their impact on patient's clinical outcome remains poorly understood.

Renal cell carcinoma: an inflammatory neoplasia

A large amount of evidence indicates that RCC often displays prominent inflammatory features, characterized by the presence of almost all types of chemokines, cytokines and other inflammatory mediators. The strongest evidence supporting this notion comes from gene expression studies in large cohorts of RCC-bearing patients, which have highlighted the central role of molecules such as IL-6, C1q, C1r, GRO1 and MMP9 in the initiation of an intra-tumour inflammatory cascade (247) (248) (249). The presence of several of these cytokines in the supernatant of RCC primary cultures (in addition to IL-8, IL-10, TGF-β, GM-CSF, TNF-α and VEGF) suggests that tumour cells probably orchestrate the inflammatory environment (250) (251) (252) (253) (254). In addition, the fact that some of them can be found in the serum of RCC-bearing patients (e.g. TGF-β, IL-1β, TNF-α and MCP-1), and not healthy individuals (255) (256), supports their active production throughout RCC natural history.

The genetic/epigenetic background inducing the expression of inflammatory mediators by RCC tumour cells is poorly understood. Nonetheless, as the VHL mutation (found in almost 70% of RCC) causes the overexpression of VEGF, PDGF and TGF-α, it seems plausible that it could also promote the transcription of other inflammatory molecules by direct or indirect means (257). Additionally, some studies have suggested that tubular cells (RCC origin cell) have the tendency to acquire a mesenchymal

and highly pro-inflammatory phenotype under stressful conditions (258), a characteristic that tumour cells could possibly retain (259) (260) (261).

Interestingly, the production of inflammatory cytokines in RCC is clinically relevant. Thus, the presence of an inflammatory gene signature in RCC (247) (248) and the plasma concentration of C-reactive protein in RCC-bearing patients (262) (263) (264) (265) (266) (267), are linked to a higher tumour grade, positive metastatic status at diagnosis and poor prognosis. Other markers of systemic inflammation also predict poor survival in patients with RCC, including an augmented erythrocyte sedimentation rate, leukocytosis and thrombocytosis, in addition to the increased plasmatic concentrations of IL-6 and TNF-α (268) (269) (270) (271) (272) (273) (274) (275). In vitro studies reinforce this concept, since several pro-inflammatory cytokines (e.g. IL-6, TNF-α, hypoxia-inducible factor-α and matrix metalloproteinase-2) are preferentially produced by the RCC cell lines exhibiting the highest malignant potential (276) (277) (277) (260).

In view of the correlation between inflammation and adverse clinical outcome in RCC-bearing patients, various clinical trials using drugs that target key molecules in the inflammatory cascade have been carried out. Two sequential Phase II trials using Infliximab (anti-TNF-α monoclonal antibody) after progression with cytokine treatment were conducted in 2007, and induced a partial response or stable disease in 41% of the patients (278), indicating that TNF-α might be implicated in RCC growth and pathogenesis. Nevertheless, the combination of this drug with Sorafenib did not increase the efficacy of Sorafenib alone (279), and therefore clinical trials using this antibody were discontinued. Similarly, a phase I/II clinical trial using Siltuximab (an anti-IL-6 monoclonal antibody) showed response rates of >50% in previously progressive metastatic RCC (280), but similarly no clinical trials assessing its efficacy are currently being conducted.

The mechanisms of tumour promotion associated with the RCC inflammatory cascade are diverse, and similar to those described in other types of cancer [discussed in the section "Cancer and Inflammation" (Page 13)]. Other mechanisms specifically described in RCC include: 1) the TNF-α and CSF-1 induction of tumour cell proliferation/migration (281) and Epithelial-Mesenchymal Transition (EMT) (277) (282); 2) the IL-1β-dependent up-regulation of metalloproteinases (283); 3) the IL-6 and IL-8 promotion of angiogenesis; and 4) the TGF-β-dependent stimulation of tumour cell migration and invasion (284). The autocrine effect of tumour-produced molecules in RCC is depicted in Figure 1.

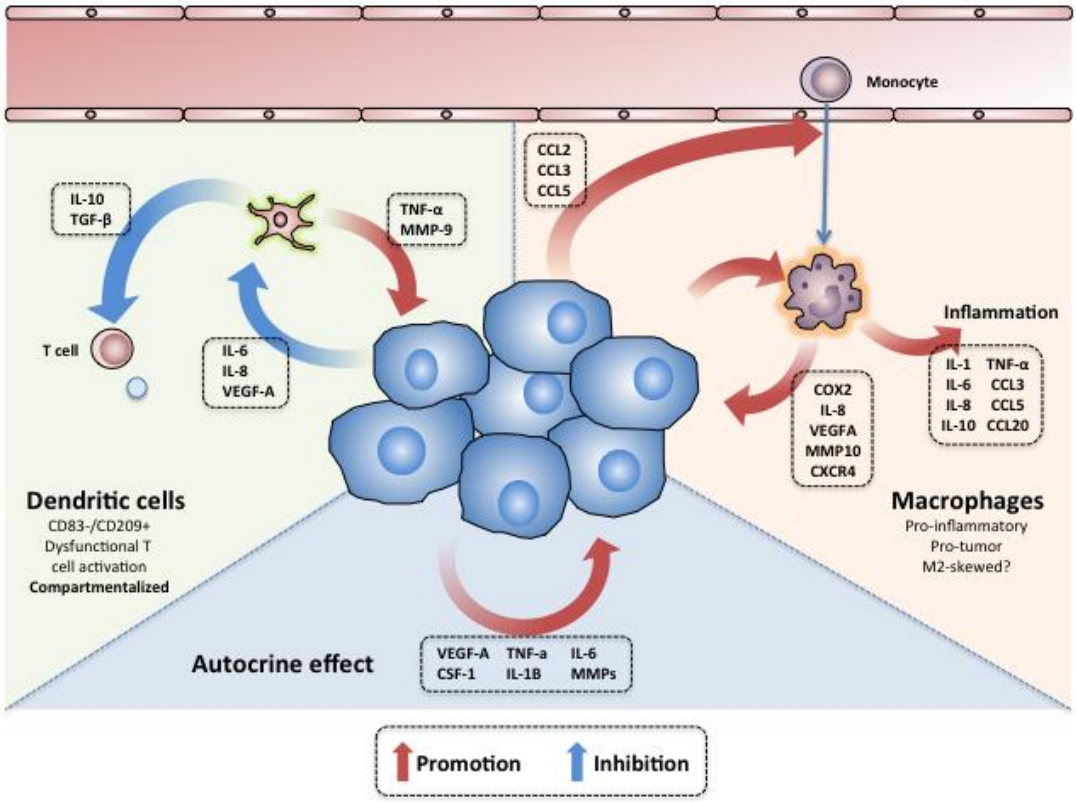

Figure 1. The autocrine and paracrine effects of tumor secreted molecules in RCC microenvironment. Cartoon depicting the inter-talk between the tumour cells, dendritic cells and macrophages in ccRCC. In the right, the tumour cells can express molecules that induce the infiltration with monocytes; once inside of the tumour, the monocytes/TAM can express a wide arrange of molecules that amplify the inflammatory cascade and promote tumour cell growth and invasion. In the left, several molecules produced by the tumour cells can also render the infiltrating DC tolerogenic, inhibitory and pro-tumoral. In the bottom, distinct cytokines secreted by the tumor cells can have an autocrine effect inducing cell proliferation and spreading.

Tumour associated macrophages

RCC tumour cells produce a wide range of molecules with monocyte-chemoattractive properties (e.g. CCL2, CCL3 and CCL5); not surprisingly, these tumours are often infiltrated with copious amounts of monocytes/TAM (285) (286) (285) (247) (287) (288) (289). In addition, several studies have described that the RCC microenvironment can prompt the differentiation of TAM into highly pro-inflammatory cells, characterized by the production of IL-1, IL-6, IL-8, IL-10, TNF-α, CCL2, CCL3, VEGFA and eicosanoids (290) (291) (292). The mechanisms skewing the RCC TAM into this phenotype are probably related with the tumour cell expression of TNF, PTGS2, IL-6 and VEGF-A (293).

The overactive monocyte recruitment and activation in RCC turns into a vicious cycle that ultimately promotes tumour inflammation, growth and spreading. A recent work by Chittezhath et al. described the molecular profile of monocytes/TAM in RCC-bearing patients (293); overall, both

peripheral blood (PB) monocytes and TAM exhibited highly inflammatory characteristics (e.g. over-expression of TNF, IL1A, IL1B, IL6, CCL3, CCL5 and CCL20), produced tumour-promoting molecules (e.g. COX2, IL8, VEGFA, MMP10 and CXCR4) and induced angiogenesis (293). In other studies, and consistently with these findings, increased RCC TAM densities have been associated with higher plasma VEGF levels (294), increased tumour microvessels densities (295) (69) (296), presence of necrosis, advanced tumour stages (297) and poor prognosis (104) (298). Altogether these data provide strong evidence supporting the role of RCC monocytes and macrophages in tumour promotion and spreading.

RCC TAM are also capable of producing a series of immunomodulatory molecules that can hamper the cytotoxic anti-tumour immune response. In fact, some studies suggest that RCC TAM are skewed towards an M2-phenotype, characterized by the expression of CD163, interferon regulatory factor 4, fibronectin 1 and IL-10 (103) (104) (299). This phenotype could induce Treg expansion (292), in addition to PD-1 and TIM-3 expression on T lymphocytes (104).

The interaction between the TAM and tumour cells in RCC is depicted in Figure 1.

Myeloid derived suppressor cells

Myeloid derived suppressor cells (MDSC) represent a heterogeneous and complex population of immune cells. Although their function and phenotype has been mainly investigated in animal models, it is now well accepted that they infiltrate human tumours, and play an important role in hampering the T cell responses (300). Several mechanisms of immunoregulation by MDSC have been described, including the depletion of nutrients necessary for T cell function, the production of reactive oxygen species (ROS), the obstruction of T cell trafficking into lymph nodes and the induction of Treg (300). MDSCs originate from immature myeloid circulating cells, and their recruitment is induced by several molecules, including VEGF, TGF-β, GM-CSF, IL-6, IL-10, gangliosides and prostaglandins (300).

Despite the fact that most of the molecules that induce the accumulation of MDSC are abundant in the RCC microenvironment (301), very few studies have assessed the role of this population in the anti-tumour immune response inhibition in this pathology. Nevertheless, evidence suggesting that Sunitinib and other TKI inhibitors induce tumour regression by a mechanism related to the MDSC depletion (discussed below) highlights the possible relevance of this population in RCC pathogenesis. Currently, the only two described mechanisms of MDSC-induced immunosuppression in human RCC are the depletion of L-arginine (302) (303) (304) and the overproduction of ROS (305).

Dysfunctional dendritic cells and defects in T cell priming

Upon encounter with an antigen (and in the presence of danger signals), immature DC go through a process called maturation, in which significant phenotypic and morphological changes occur, including the lose of endocytic activity, the up-regulation of MHC–peptide complexes and costimulatory molecules and the acquisition of migratory capacity towards the regional lymph nodes. Once there, mature DC activate naïve CD4+ and CD8+ T cells, and induce their expansion and differentiation (131). Many cytokines present in the TME (particularly IL-6, GM-CSF and VEGF) can impair the process of DC differentiation/maturation (306) (290) (307) (308), or render them inhibitory and protumoural (309) (131). Since most of these cytokines are enriched in RCC microenvironment, it is not unreasonable to suspect that this milieu could easily induce an abnormal maturation of the intra-tumour DC.

Several studies have assessed the DC biology in RCC, and some agree that these cells can be found in two main locations: isolated in the tumour stroma or within immune aggregates in the invasive margin of the tumour (310). Interestingly, the two types of DC display different phenotypes and capacities of T cell priming, presumably due to the distinctive microenvironments in which they develop. On the one hand, isolated/tumour stroma DC display an immature phenotype (CD80-, CD86-, CD83- and DC-Lamp-) (310) (311), express molecules that potentially promote tumour development (e.g. TNF-α and MMP-9) (312) and are unable to activate lymphocytes in vitro (313) (312). On the other hand, DC within immune aggregates express maturation and activation markers (310) (314), and can potentially prime T cells in situ.

The facts that the phenotypic abnormalities of DC are: 1) restricted to those cells embedded in the tumour stroma, and 2) potentially reversible when cells are removed from the tumour, strongly suggest that RCC microenvironment is enriched in molecules that hamper DC activity and maturation. Indeed, conditioned media from RCC primary cultures or cell lines can induce in vitro tolerogenic and dysfunctional DC (HLA-DR^{dim}, CD80-/CD86-, IL-10+/TGF-β+) (315) (316) (317) by mechanisms dependent on IL-6, IL-8 and VEGF (313) (312). The dysregulation of DC maturation and migration might be responsible of the low density of TLS in RCC in comparison to other tumour types (128), and could be involved in the dysfunctional priming of T cells and defective elimination of tumour cells in RCC. The interaction between infiltrating DC, the tumour cells and T lymphocytes in RCC is depicted in Figure 1.

T Lymphocytes: Rather a functional defect

In other neoplasias, similar inflammatory mechanisms as those described for RCC have been associated with three main alterations in T cell function, namely: dysfunctional priming, deficient

recruitment and diminished cytotoxic/proliferative potential (318). The deleterious impact of the three phenomena has been demonstrated in numerous neoplasias, where they have been linked with poor tumour control and adverse clinical outcome. Nevertheless, the defective tumour cell elimination in RCC is probably not related to defects in T-cell recruitment; in fact, those tumours with the highest T cell densities usually display more malignant features and are often associated with patient's worst clinical outcome. Consequently, in RCC the dysfunctional T cell responses must originate either from defects in the T cell priming and/or their direct inhibition.

The clinical impact of T cell infiltration and activation in RCC

RCC express a wide range of molecules that no only attract inflammatory cells but also T lymphocytes into the tumour. Between the T cells chemoattractants produced by RCC cell lines it is worth noting CCL4, CCL5, CXCL9-11 and CXCL16 (319) (320) (321), since their respective receptors (CCR5, CXCR3 and CXCR6) are commonly expressed by RCC TIL (322) (319) (323). Once T cells have breach into the tumour, they become activated, as suggested by the overrepresentation of cells with an effector memory phenotype expressing activation markers (CD69, HLA-DR and FAS-L) on TIL, when compared to PBL (324) (200) (325) (326) (327) (328) (104).

Although abundant and often displaying an activated phenotype, the biologic consequences of an augmented T-cell recruitment in RCC has been enigmatic, because contrary to most tumours where increased densities Th1 and CD8+ TIL are associated with a favorable clinical outcome (26), few studies have described the opposite association in RCC. Nakano et al. studied a cohort of 233 RCC, and reported that high CD8+ TIL densities were associated with poor prognosis by univariate, but not by multivariate, analysis (175). Interestingly, in the same cohort, increased infiltrations with CD8+/Ki67+ double-positive cells were associated with the opposite clinical outcome (175). More recently, Hotta et al. described that the increased densities of CD45RO+ cells in RCC were correlated with advanced TNM stages, increased CRP plasma concentrations, and higher risk of patient's progression or death (329). Nevertheless, in the latter and similar studies (178) (179), only the increased densities of CD4+ TIL (and not those of CD8+ TIL) have been associated with poor clinical outcome.

Although conflicting regarding the prognostic significance of CD8+ TIL infiltration, all these studies agree in two things: first, they have consistently found a positive correlation between the densities of CD8+ TIL and the tumour nuclear grade (330) (175) (329); and second, they agreed in that the T-cell infiltration is not associated with improved survival in RCC. Nevertheless, whenever proliferating, augmented densities of CD8+ cell could be potentially associated with good prognosis. And, although this result needs confirmation, it indicates that only when CD8+ RCC TIL are poorly

functional (either due to their direct inhibition or to defects in their priming/activation) they could predict an adverse clinical outcome.

In order to determine if the neutral or poor prognosis associated with increased CD8+ TIL densities in RCC depended on the organ where the neoplasia developed (the soil) or it was related to an effect of the tumour cells (the seed) on the CTL recruitment/inhibition, our group assessed the prognosis associated with the CD8+ TIL infiltration in a cohort of 52 RCC lung metastases (RCC-LM) (128). In the latter, increased densities of CD8+ T cells correlated with shorter overall survival (contrary to colorectal cancer-LM), suggesting that RCC tumour cells induce a dysfunctional CTL response, independently of the organ where they develop. Interestingly, we also found that the infiltration with CD8+ T cells RCC-LM correlated with the expression of genes associated with chronic inflammation, angiogenesis and CTL suppression (128).

The RCC TIL dysfunction: priming, recruitment or inhibition?

Some studies have found that the T cell responses in RCC are characterized by a low amount of expanded clones (326) (331) (332) (333). Whether this finding is associated with 1) a dysfunctional T cell priming, 2) a recruitment of low-affinity T cells (with a subsequent impairment in the T cell expansion) or 3) the inhibition of T cell activation/proliferation, is currently unknown but it seems feasible that the three mechanisms could be playing a role. The phenotypic abnormalities of the infiltrating DC (discussed above), the virtual absence of central memory TIL and TLS (334) (335) (128) and the studies demonstrating that TIL clones isolated from RCC are characterized by poor antitumour cytotoxic function (336) (326), support the first two hypotheses.

Supporting the third hypothesis, there is solid evidence demonstrating that RCC TIL display a highly inhibited phenotype and function. The first well documented defect in RCC TIL was their diminished proliferative capacity (337) (338), that is related to alterations in the production of IL-2 (339) (340) (341) (338) and its intracellular signaling pathway (252) (253) (342) (343) (344) (345) (338) (346) (347) (347) (348) (348) (349) (350) (350). Other defects in RCC TIL have been described, and include their diminished cytotoxic potential (325) (351) (352), the poor development of polyfunctional responses after PMA-ionomycin activation (334), and the increased expression of inhibitory receptors (immune checkpoints).

The PD-1/PD-L1 axis in RCC

PD-1

PD-1 is an inhibitory receptor of the CD28/B7 superfamily, upregulated on lymphocytes upon TCR or BCR engagement or γ-chain cytokine-mediated activation (353) (354) (355). This molecule is normally downregulated during the contraction phase of the primary immune response. However, its expression remains elevated in pathologic conditions characterized by persistent antigen stimulation (356), such as chronic infections and cancer; in these contexts, PD-1+ cells often exhibit alterations in the production of several cytokines (e.g. IL-2, IFN-γ and TNF-α) and defective proliferative capacities (39), a phenomenon known as 'T lymphocyte exhaustion'. Recently, this concept has been challenged since evidence suggests that PD-1+ T cells probably fulfill specialized functions in the anti-tumour immune response (204). For instance, CD8+PD-1+ TIL in melanoma are enriched in clones capable of lysing autologous tumour cell lines (357) as compared to CD8+PD-1– cells. In addition, the fact that a strong anti-tumour immune response can be restored by blocking the interaction between PD-1 and its ligands in some tumours (207), reinforces the concept that these cell are circumstantially inhibited rather than exhausted, and could potentially fulfill an important role in the anti-tumour immune responses. In addition, studies in chronic infectious models have demonstrated that PD-1+ lymphocytes express similar or increased levels of cytotoxic molecules (but not IFN-γ or TNF-α) (358) (359) when compared to their PD-1– counterparts.

A relevant amount of evidence has implicated the expression of PD-1 and their ligands in the RCC pathogenesis. Indeed, RCC TME is often enriched in PD-1-expressing T cells (333) (104) (360), and their increased densities are associated with a higher nuclear grade, advanced TNM stage and patient's poor prognosis (361) (362). Similarly, the increased percentages of PB CD4+/PD-1+ and CD8+/PD-1+ T cells correlate with advanced tumour stages (363). Our understanding of the mechanisms inducing the PD-1 up-regulation in RCC TIL is limited, and it is still unclear if it could be related to a TCR-dependent activation in the context of low affinity interactions (354), or if it could be the consequence of the abundant inflammatory cytokines in the RCC microenvironment (355).

PD-L1 and PD-L2

Two ligands for PD-1 have been identified (PD-L1 and PD-L2). PD-L1 has a more wide range of expression, not only restricted to immune cells (including APC, macrophages and lymphocytes), but also comprising nonhematopoietic tissues (e.g. placenta), and it has been classically described to be induced by type I and II IFN (364). In turn, the expression of PD-L2 is restricted to activated DC and macrophages (39), and it can be induced by IFN-γ, GM-CSF and IL-4 (365).

To date, the role of the PD-1 ligands in RCC pathogenesis has mainly focused on the expression of PD-L1 by tumour cells. Some studies have found that approximately 20-30% of the primary ccRCC

express this molecules (using 5% as cut-off) (366) (367) (368) (369), and compared to their negative counterparts, these tumours present higher TNM stages and nuclear grades, and are associated with patient's poor survival (366) (367) (368).

Three main mechanisms of PD-L1 induction have been proposed in RCC: 1) a VEGF-dependent mechanism, as suggested by the diminution of PD-L1 expression after VEGF-targeted therapy in metastatic ccRCC (320); 2) a cytokine-induced mechanism, under the rationale that some of most abundant molecules in RCC microenvironment (e.g. TNF-α, IFN-γ and IL-4) can induce PD-L1 expression in other tumours (370) (371) (320) (369); and 3) a hypoxia-induced mechanism, insinuated by some studies where PD-L1 expression can be induced in RCC cell lines under hypoxic conditions trough a HIF-α-dependent pathway (372) (373).

Although studied in less detail, some reports have also implicated the expression of PD-L1 by immune cells in the inhibition of T cell responses in RCC (366) (367) (368) (369).

In contrast to PD-L1, the role of PD-L2 expression in RCC has not been studies in detail. Nevertheless, a couple of observations from clinical trials support that other PD-1 ligands besides of PD-L1 could be expressed in RCC: on the one hand, the response rates among patients with advanced RCC goes from 10% when using drugs inhibiting just PD-L1 (208) to 25% when inhibiting the PD-1 receptor (207); and, on the other hand, although very few, there are PD-L1 negative tumours that respond to anti-PD-1 treatment (207). To date, only one study has assessed the expression of PD-L2 in RCC, and found that its expression was limited to 1 out of 6 tumours. The biological function of PD-L2 in RCC is still to be determined (213).

Other inhibitory receptors

The role of other inhibitory receptors in RCC TIL has not been assessed in detail. Similarly to PD-1, few studies haves demonstrated an expansion of the TIL expressing TIM-3 and/or LAG-3 compared to autologous PBL (333) (104). The role of other immune checkpoint, including CTLA-4, CD137, OX40 and A2aR in RCC has not been assessed to date. Nevertheless, a phase II clinical trial with Ipilimumab (anti-CTLA-4 antibody) in metastatic RCC showed a partial response of approximately 10% of patients (374), suggesting the potential role of this molecule in tumour development.

CD4+ T-cell orientation in RCC: Th2 and Treg

CD4+ T helper cells are divided into different subtypes, including Th1, Th2, Th17 and Treg; each sub-population accomplishes specific roles in the anti-tumour immune response. Overall, a Th1-oriented response antagonizes the tumour growth and is often associated with good clinical outcome. The role of Th2, Th17 and Treg is less clear, but are often associated with poor prognosis in different

tumours (26). Multiple studies have a demonstrated that the RCC-infiltrating CD4+ T cell compartment often acquires functional orientations that obstruct or inhibit the anti-tumour immune response.

Th2 cells

Some studies have found that RCC-infiltrating CD4+ T cells preferentially express cytokines associated with a Th2 response (e.g. IL-10 and IL-4) (336) (375) (325) (376) (377) (378) (379) (380). Indeed, it has been suggested that the high concentrations of TGF-β, IL-6, IL-4, COX2-PGE2 and gangliosides in the RCC microenvironment could be implicated in the preferential development of Th2 responses (381) (348) (382). In addition, the fact that primary RCC tumour supernatants can inhibit the expression of IFN-γ in T cells, but not the production of type 2 cytokines (e.g. IL-4, IL-5, and IL-10) (347) (348), suggests the central role of the tumour cells in this phenomenon.

The clinical relevance of the Th2 skewing in RCC is currently unknown. Nevertheless, a small study by Cozar et al. (n=24) suggested that CD4+ TIL preferentially display a Th1 orientation in early-stage RCC, while they exhibited a Th2 skewing in more advanced disease (383).

Regulatory T cells

Treg are a subpopulation of CD4+ T lymphocytes that mediate peripheral tolerance and help in the maintenance of the immune homeostasis. They exert this effect by suppressing the effector cell responses through different mechanisms, including: 1) the production of inhibitory cytokines (e.g. IL-10, TGF-β and IL-35); 2) the direct cytolysis of CD8+ T cells (through the secretion of granzyme A and perforin); 3) the disruption of the metabolic microenvironment (e.g. by cytokine deprivation or adenosine production); and 4) the suppression of DC development and maturation (384).

Some studies have implicated the Treg subpopulation in the CTL inhibition in RCC (385) (319) (362). Their recruitment and in situ expansion seem to be induced by the CCL22/CCR4 and TGF-β/VEGF-A axes (386) (387) (388). Interestingly, the expansion of Treg subpopulation within PBL and TIL in RCC-bearing patients has been associated with advanced TNM stages (104) (256) (389), denser tumour microvascularization (390) and poor survival (391) (392) (393) (362).

NK cells

NK lymphocytes contribute to tumour elimination by detecting the MHC I down-regulation and/or the expression of NK-activation ligands on the tumour cells. This is one of the few immune populations in RCC whose presence has been correlated with a favorable clinical outcome (383) (394)

(123) (128). Often, these cells represent a relevant percentage of the RCC TIL (325) (383), and they express activation (CD69+) (325) (395) and inhibitory markers (CD94/NKG2A) (396). Although they exhibit diminished cytolytic capacities compared to PB NK cells (395), some RCC-infiltrating NK lymphocytes are still capable of lysing MHC Class I negative tumour cell lines after a short-term activation with IL-2 (396).

Cytokines shaping the RCC immune microenvironment

As described above, RCC microenvironment is characterized by the presence of copious amounts of cytokines and chemokines. But, contrary to other scenarios where a delicate balance between the immune activation and counterregulation allows the antigen elimination without major collateral damage, RCC often displays a disorganized immune contexture, enriched in cytokines that hamper the immune function.

IL-10

IL-10 is conspicuously found in the RCC microenvironment. In physiological conditions, this cytokine has been implicated in T cell dysfunction by indirect mechanisms involving: 1) the inactivation of macrophages and DC, with a consequent inhibition the expression of MHC class II and co-stimulatory molecules (397); 2) activation of tolerogenic pathway in DC through the up-regulation of IL-1RA, TGF-β and HLA-G; and 3) promotion of Treg differentiation and survival (398).

In RCC, the overproduction of IL-10 has been related to M2-macrophage polarization (298) (292), the development of dysfunctional DC (290), the expansion of Treg (317) and abortive activation of naïve T cells (399). Three different sources of IL-10 in this pathology have been described: 1) the tumour cells (253), 2) the Treg/Th2 CD4+ TIL (400) (401) (341) (401) (402) (334) (104) and 3) TAM/DC (290) (291) (292) (293).

Regarding the clinical impact of the increased expression of IL-10 in RCC, scattered studies have found a correlation between its in situ expression, poor tumour differentiation and increased metastasis risk (403) (404). A similar picture has been described when analyzing the PB of RCC-bearing patients, where the IL-10 serum concentrations have been linked with higher tumour grades (341) (405).

TGF-β

TGF-β is another molecule that has been widely implicated in the inhibition of the DC and T-cell responses in RCC (250) (251) (252) (253) (254) (315) (317) (256). In physiological conditions, TGF-β regulates T cell homeostasis by promoting survival of low-affinity T cells and by inhibiting TCR-driven

activation of high-affinity T cells (406). In addition, it inhibits the differentiation, activation and effector functions of Th1, Th2 and cytotoxic T cells, while it promotes the development of Treg and Th17 cells (407). In RCC, the elevated concentration of TGF-β (probably originating in tumour cells or DC) has proven to induce the development of dysfunctional DC (290) and the expansion of Treg (317), among others. Not surprisingly, some studies have described an association between the expression of TGF-β, advanced tumour stages and poor prognosis in this pathology (408) (409) (410).

CCL2 and CCL5

CCL2 and CCL5 are chemokines extensively produced by RCC tumour cells. In addition to their implication in macrophage recruitment, it has been proposed that these cytokines can also promote the tumour growth and immune-suppression by different mechanisms, including the induction of an inflammatory microenvironment, the promotion of angiogenesis, and the generation of metastatic sites (411).

Other mechanisms of immunomodulation: MHC Class I and II

One of important mechanisms of immunomodulation in cancer is the lack of the tumour recognition by CTLs through the down-regulation of MHC class I in the neoplastic cells (412). Nevertheless, this phenomenon has never been confirmed in RCC, and in fact the majority of these tumours express high quantities of MHC class I and II molecules as compared to many other neoplasias (413) (414). The biological significance of this phenomenon is still poorly understood. In fact, some works have shown that the overexpression of MHC by tumour cells does not necessarily translate into an efficient antigen presentation, since all the processing machinery is dysfunctional (415) (416). In addition, it has been suggested that the overexpression of MHC class I molecules could also be skewed towards inhibitory molecules (e.g. HLA-G and HLA-E) (417) (418) (419), and in addition it could suppress the tumour recognition by NK cells (420) (421).

A similar picture has been described for MHC Class II molecules in RCC (422) (423) (424), with the interesting observation that the peptidome analysis of the tumour cells displayed the enrichment of MHC class II-restricted peptides (425) (426). Whether the increased expression of these molecules translates into an effective presentation of tumour-associated MHC-peptides to CD4+ cells, and how it coordinates with the expression of homolog MHC class II molecules, such as PD-L1 and PD-L2, also remains elusive.

Shaping the RCC immune microenvironment: Lessons we have learned from immunotherapies?

The widespread use of immunotherapies in RCC-bearing patients has helped us to comprehend the relevance of different immune pathways and cellular populations in the tumour growth and spreading. Numerous clinical trials have shown that the response to many of these therapies is heterogeneous among patients, and could be determined by the delicate balance between the induction of an anti-tumour and pro-tumour immune response (427) (428) (429).

rIL-2 and IFN-α

The first immunotherapy ever used in patients with advanced RCC was rIL-2, a treatment associated with a response rate of 10-20% and a high incidence of adverse effects (201). Although its efficacy is restricted, rIL-2 treatment has induced a complete tumour regression in dozens of patients with advanced RCC (430) (201). Extensive efforts were made in order to understand the mechanism of action of rIL-2, as well as the immune features preferentially induced in the responder patients. The clinical studies using rIL-2 in advanced RCC showed that this treatment induced the infiltration with activated-mature DC and functionally active CD8+ T cells (431) (427) (432) (433), but also could increase the densities of MDSC (434) and Treg (429).

Similarly, IFN-α has also been used in the treatment of advanced RCC, based of the same rationale of boosting the otherwise inhibited immune response. In RCC-bearing patients, IFN-α also induces the shrinkage of the Treg (as well as most other immune populations, except CD8+ T cells) and concomitantly promotes the diminution of the VEGF intra-tumour concentrations (435).

Our understanding of the mechanisms inducing a strong anti-tumour immune response in RCC with cytokines-based treatment is still limited. Some studies suggest that the response to these agents largely depends on the pre-therapy patient's immune status. Remarkably, individuals with higher densities of tumour infiltrating dendritic cells and low percentages of PB Treg were more likely to response to rIL-2 and IFN-α treatment, respectively (76) (436) (437). On the contrary, increased plasma concentrations of IL-6 and TNF-α could predict poor responses (438). In addition, the response to treatment also seems to depend on the expansion of activated immune cell populations after the treatment administration (439) (440).

These findings provide strong evidence supporting the central role of a dysfunctional CTL response in the RCC pathogenesis. Furthermore, it highlights the importance of the balance between the anti-tumour and pro-tumour immune response, which could be potentially skewed with these therapeutic approaches. The major problematic behind the cytokine-based therapies in RCC was their unpredictable effect, and unfeasibility to generalize a regimen due to the inter-patients heterogeneity and

the intra-tumour complexity. With evidence showing the poor specificity of these treatments in addition to their side effects, rIL-2 and IFN-α have been steadily abandoned from the clinical practice.

Target Therapy

The first line therapy for advanced RCC are drugs named TT, named like that as they interfere with specific molecules involved in tumour growth, spread and progression. Sunitinib (a TKI inhibitor) is the molecule more frequently used in the clinical practice, holding partial or complete response rates of almost 50%.

Studies in mouse models have suggested that, beside of inducing normalization of the vasculature, this molecule can potentially modulate the immune microenvironment by reducing the amount of infiltrating MDSC and Treg, and therefore enhance the Th1 responses (441) (442) (443) (444). It has also been described that the treatment of tumour-bearing mice induces a down-regulation immune checkpoint (CTLA-4 and PD-1) expression in T cells, and its ligands (PD-L1) in MDSCs and DC (441) (445) (320).

In humans, the treatment with Sunitinib in RCC induces a reduction in peripheral blood MDSC and Treg, and increases the Th1 and CTL responses (436) (446) (443). In addition, some studied suggest that the extent of shrinkage of the myeloid DC and Treg populations correlate with the overall survival after Sunitinib therapy (447) (448) (449). Similar studies evaluating the immune parameters after treatment with other anti-angiogenic drugs such as Sorafenib (388) (386), Pazopanib (450), Imatinib and Dasatinib (451)and Bevacizumab (452) have displayed similar results. These results provide solid evidence suggesting that the myeloid compartment, and probably the Treg, play an important role in the pathogenesis and natural history of advanced RCC.

So far, little is known about the effect of TT on T cell responses in RCC. An interesting study quantitatively measuring the number of T cell clones in different tumour regions after Everolimus treatment vs. placebo, determined that this drug induced the intra-tumour clonal expansion of T cells, and augmented the inter-regional heterogeneity of T cell clones (332), suggesting that this drug could boost the tumour-specific T cell response.

Objectives

In the view of the potential poor prognosis associated with high densities of CD8+ T cell and a Th1 immune response in RCC, we designed a project to solve the next question:

What are the characteristics of the immune microenvironment associated with a Th1 and CTL immune response and how do they predict the patients' clinical outcome in ccRCC?

To unravel this question, we established two main objectives in the project:

I. **To determine whether the density and location of ccRCC infiltrating mature DC and CD8+ T cells predict patient's clinical outcome.**
II. **To determine the expression pattern of PD-1, LAG-3, PD-L1 and PD-L2 in tumours with high and low densities of CD8+ T cells, and their significance as prognostic markers.**

Our hypothesis was that, if associated with a poor clinical outcome, CD8+T cells would display a suppressed phenotype (characterized by the expression of immune checkpoint), in addition to signs of a dysfunctional immune response; simultaneously, the TME would provide the appropriate microenvironment to induce this phenotype.

In the first part of our work, we could determine that the increased densities of CD8+ T cells were associated with poor clinical outcome, but interestingly this link was largely modulated by the expression of the immune checkpoints and the localization of mature DC in the TME. The tumours associated with worst prognosis were characterized by a suppressive microenvironment, characterized by the expression of immune checkpoints and the absence of functional mature DC and TLS.

These results lead us to characterize in detail the cellular immune response and TME of ccRCCs with a "suppressive-like" contexture. Therefore, the third objective of this work was the next:

III. **To characterize the phenotype of ccRCC TIL, and its relation with the expression of immunomodulatory molecules in the TME.**

Results

Article 1: <<Orchestration and Prognostic Significance of Immune Checkpoints in the Microenvironment of Primary and Metastatic Renal Cell Cancer>>

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This article tries to solve the first two objective of my thesis, and was published in Clinical Cancer Research in February 2015.

Summary of the results in Article 1:

Contrary to most tumours, few studies had reported an association between the increased infiltration of CD8+ T cells and poor clinical outcome in primary ccRCC. In this article, we collected a retrospective cohort of 135 primary ccRCC and 51 ccRCC lung metastases, with their respective pathological and clinical characteristics and follow-up. The prognosis associated with CD8+ and DC infiltrations, in addition to the expression of immune checkpoints was investigated by quantitative immunohistochemistry (IHC). We confirm some of the results using the gene expression dataset published by the TCGA group on 496 ccRCC primary tumours.

As reported previously in the literature, increased densities of CD8+ TIL were linked with poor clinical outcome in ccRCC. Interestingly, this correlation was largely modulated by the localization and phenotype of DC in the TME; while the intra-tumour DC were associated with a poor clinical outcome, high densities of DC within lymphoid aggregates (TLS-DC) identified a group of patients with prolonged survival and increased CD8+ TIL densities. The association of CD8+ T cells and poor prognosis was further confirmed by gene expression analyses, since increased CTL- and Th1-associated transcripts were correlated with shorter OS in the TCGA cohort.

In view of the high sensitivity of RCC to PD-1 blockade therapies, we hypothesized that the expression of inhibitory receptors in TIL could modulate the prognosis associated with CD8+ TIL

infiltration. To probe this hypothesis, we analyzed the expression of immune checkpoint in the tumour with the highest CD8+ T cell densities (n=40) as compared to those with lowest (n=40). The densities of PD-1+ and LAG-3 were closely associated with the CD8+ infiltration grade, and we could confirm be IF that several CD8+ T cells co-expressed both receptors. Nevertheless, we demonstrated that patients whose tumours exhibited both high densities of PD-1+ lymphocytes and PD-L1+ and/or PD-L2+ tumour cells had the worst prognosis.

Article 1

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Biology of Human Tumors

Clinical Cancer Research

Orchestration and Prognostic Significance of Immune Checkpoints in the Microenvironment of Primary and Metastatic Renal Cell Cancer

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Abstract

Purpose: Clear cell renal cell carcinoma (ccRCC) has shown durable responses to checkpoint blockade therapies. However, important gaps persist in the understanding of its immune microenvironment. This study aims to investigate the expression and prognostic significance of immune checkpoints in primary and metastatic ccRCC, in relation with mature dendritic cells (DC) and T-cell densities.

Experimental Design: We investigated the infiltration and the localization of $CD8^+$ T cells and mature DC, and the expression of immune checkpoints (PD-1, LAG-3, PD-L1, and PD-L2) in relation with prognosis, in 135 primary ccRCC tumors and 51 ccRCC lung metastases. RNA expression data for 496 primary ccRCC samples were used as confirmatory cohort.

Results: We identify two groups of tumors with extensive $CD8^+$ T-cell infiltrates. One group, characterized by high expression of immune checkpoints in the absence of fully functional mature DC, is associated with increased risk of disease progression. The second group, characterized by low expression of immune checkpoints and localization of mature DC in peritumoral immune aggregates (tertiary lymphoid structures), is associated with good prognosis.

Conclusions: The expression of the immune checkpoints and the localization of DC in the tumor microenvironment modulate the clinical impact of $CD8^+$ T cells in ccRCC. *Clin Cancer Res; 21(13); 3031–40. ©2015 AACR.*

Introduction

A solid tumor is an intricate and dynamic ecosystem containing tumor and immune cells, fibroblasts, blood, and lymphatic vessels (1). The density and composition of the immune microenvironment is heterogeneous among patients and tumor types, and it is becoming a robust tool to predict postsurgical recurrence and death (1, 2). In the vast majority of cancers, tumor infiltration by $CD8^+$ memory cytotoxic T cells and Th1 cells is associated with good clinical outcome (1). In addition, it has been reported that an organized local immune reaction, characterized by the presence of mature dendritic cells (DC) localized in tumor-associated tertiary lymphoid structures (TLS), is necessary to orchestrate this cytotoxic and Th1 immune contexture and is associated with good clinical outcome (3–8) and response to therapeutic vaccines (9, 10).

However, several recent findings challenge this concept of the unequivocal relation of effector memory $CD8^+$ T-cell infiltration with a good clinical outcome in cancer. First, in diffuse large B-cell lymphoma (11), Hodgkin lymphoma (12), and clear cell renal cell carcinoma (ccRCC; ref. 13) high densities of tumor-infiltrating $CD8^+$ T cells have been associated with poor prognosis in some studies. Second, a recent publication from our group showed that the infiltration of lung metastases from ccRCC by $CD8^+$ T cells was correlated with poor overall survival (OS) whereas it was a factor of good prognosis in lung metastases from colorectal carcinoma (14). Finally, in non–small cell lung cancer (NSCLC) in which $CD8^+$ T cells densities correlate with good prognosis, a low density of TLS-DC associated with high $CD8^+$ T-cell infiltration identifies a group of patients with high risk of death, suggesting a functional impairment of intratumoral $CD8^+$ T cells in this situation (4).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Clear cell renal cell carcinoma (ccRCC) is an enigma of the tumor microenvironment studies because, in contrast with most malignancies, high densities of $CD8^+$ T cells correlate with poor clinical outcome. We characterize the microenvironment of ccRCC primary tumors and lung metastases associated with an extensive $CD8^+$ T-cell infiltrate. In one group of patients, a T-cell exhausted microenvironment characterized by high expression of immune checkpoints, and the absence of fully functional mature dendritic cells (DC) was found and correlated with poor prognosis. In the second group, low expression of immune checkpoints and DC localized in peritumoral immune aggregates were found and conversely associated with good prognosis. Our data provide novel prognostic biomarkers highlighting the central role of PD-L2 and LAG-3 in the immunomodulation of ccRCC. The combination of these immune profiles could guide the selection of patients who are likely to respond to checkpoint blockade therapies.

Immune checkpoints on infiltrating T cells are key regulators of immune escape in cancer. In primary ccRCC, several studies have shown that the expression of the inhibitory receptor PD-1 on the immune cells (15, 16) or its ligand PD-L1 on tumor cells (17–19) is associated with a poor clinical outcome. Interestingly, antibodies that block the PD-1 axis have yielded a 20% to 30% response rate in metastatic ccRCC (20, 21) that seems to be related to the expression of PD-L1 by the tumor cells (20, 22). Another inhibitory molecule that has gained recent attention is the lymphocyte activation gene-3 (LAG-3), which is coexpressed with PD-1 on $CD8^+$ tumor-infiltrating lymphocytes in melanoma (23), and together with PD-1 synergistically regulates T-cell function (24). We expected immune checkpoints high ccRCC patients to have a worse prognosis than the immune checkpoint low.

In these newly described scenarios in which $CD8^+$ T-cell infiltration correlates with poor prognosis, it is important to define the combination of immune-based biomarkers that will predict patients' prognosis and further guide immunotherapeutic approaches. This study aims to investigate the expression and prognostic significance of immune checkpoint receptors and paired ligands on primary and metastatic ccRCC in relation with TLS-DC and T-cell densities.

Materials and Methods

Patients

A cohort of 135 primary ccRCC human tumors and another of 51 ccRCC lung metastases were collected. The primary cases derived from specimens of radical nephrectomy operated between 1999 and 2003 at the hospital Necker-Enfants Malades (Paris, France). The ccRCC lung metastases cohort resected at the Hotel Dieu hospital (Paris, France) or Hôpital Européen George Pompidou (HEGP, Paris, France) between 1992 and 2010 was already described in ref. (14). This research was conducted according to the recommendations outlined in the Helsinki declaration and approved by the medical ethics boards of all participating institutions, and with the agreement of the Ile-de-France II ethics committee (no. 2012-0612). The demographic characteristics of the cohorts are depicted in Supplementary Table S1 (14).

In addition, expression data for 496 primary ccRCC samples with complete follow-up were downloaded from The Cancer Genome Atlas' (TCGA) KIRC study, using version 2 of the normalized RNA sequencing data. Corresponding clinical data (updated on 2013-04-06) were downloaded from (25).

Clinic and pathologic features

The original histologic diagnosis was confirmed on archival hematoxylin and eosin–stained slides, and histopathologic features such as histologic subtype (26), tumor size, regional lymph node invasion, distant metastases at surgery, Fuhrman nuclear grade (27), and sarcomatoïd features were collected. All tumors were pathologically staged according to the TNM (tumor–node–metastasis) classification (28). The duration of follow-up was calculated from the date of the surgery (nephrectomy or metastasectomy) to the date of cancer progression, last follow-up or death.

Immunohistochemical and immunofluorescence staining

Serial 5-μm formalin-fixed paraffin-embedded (FFPE) tissue sections from primary and metastatic ccRCC were stained using autostainerPlus Link 48 (Dako). Antigen retrieval and deparaffinization were carried out on a PT-Link (Dako) using the EnVision FLEX Target Retrieval Solutions (Dako). The antibodies used in this study for IHC and immunofluorescence (IF) are listed in Supplementary Table S2. IF-stained slides were scanned after secondary antibody incubation and mounting. For the IHC staining, peroxidase activity was detected using 3-amino-9-ethylcarbazole substrate or Novared and alkaline phosphatase using alkaline phosphatase substrate III (Vector Laboratories).

Tests of the specificity and sensitivity of PD-1, PD-L1, PD-L2 and LAG-3 monoclonal antibodies for IHC experiments were performed using generated FFPE cell pellets from transfected 300.19 cells (for PD-1, PD-L1, and PD-L2; ref. 29) and CHO cells (for hLAG-3), whereas parental untransfected cells served as negative controls (Supplementary Fig. S1; Costim Pharmaceuticals). Multiple organs ($n = 32$), human TMA (FDA999a, US Biomax), and malignant cancer tissues ($n = 10$) from different oncology indications were also tested using the above mentioned protocols. Normal human FFPE tonsil sections for PD-1, LAG-3, PD-L2, and normal placenta for PD-L1 were used as positive controls (Supplementary Fig. S1).

Immunohistochemical quantification

Stained slides were scanned with a Nanozoomer (Hamamatsu) and analyzed with Calopix software (Tribvn). For quantification purposes, tissues were divided into Invasive Margin (IM) and Tumor Center (TC) as previously described (30), and the density of positive cells was calculated in the whole tumor region (IM and TC). Because of the small size (or absence) of TC region in the majority of the metastases, the analysis on this cohort was done not discriminating between the two regions. The percentages of tumor cells stained positive for PD-L1 and PD-L2 were quantified by two independent reviewers (A. Lupo and N.A. Giraldo) without prior knowledge of patient outcome, and the tumors above 5% tumor cell expression were considered as positive in accordance with studies in other types of cancer (20, 31).

Statistical analysis

Comparisons among the demographic and pathologic features, immune marker densities, and PD-L1 and PD-L2 expressions were

evaluated by using χ^2, Fisher exact, and Wilcoxon rank-sum tests. Association of variables to prognosis was assessed using the Kaplan–Meier method, univariate and multivariate Cox regression analyses. To segregate patients in two groups based on numerical variables (cell density or gene expression), Log-rank *P* values of each possible cutoff were computed. The cutoff that minimized the *P* value of a log-rank test for DFS was retained, and the corresponding *P* value was corrected using the method published by Altman and colleagues (32). These cutoffs were later used to segregate patients into two groups, and their associated Kaplan–Meier curves are displayed throughout the figures. To further confirm and validate the prognosis association of the cell densities, we determined the median and third quartile cutoff and calculated the corresponding univariate Cox-regression *P* values (Supplementary Table S3). Only those variables univariately associated with prognosis were included in the multivariate Cox regression analysis. The duration of follow-up was calculated from the date of nephrectomy or metastasectomy to the date of death or last follow-up.

Results

Tumor infiltration by CD8$^+$ T lymphocytes and expression of Th1-associated genes correlate with poor prognosis in ccRCC

The density of CD8$^+$T cells in the IM of the primary tumors was heterogeneous in the cohort of 135 primary ccRCC. On the basis of the Optimal *P* value cutoff for DFS (OPv; 630 cells/mm^2) we found that the CD8^{High} ($n = 41/135$, 30%) group had a shorter disease-free survival (DFS, $P = 0.0001$, Fig. 1A) and OS ($P = 0.001$, Fig. 1A). The density of CD8$^+$ T cells in the TC had no prognostic impact (Supplementary Table S4). The density of CD8$^+$ T cells in the IM correlated with the Fuhrman grade (Supplementary Fig. S2), but not with any other pathologic variables, including TNM (data not shown).

An independent cohort of lung metastases of ccRCC, in which the negative impact of high densities of CD8$^+$T cells on OS had been previously described using semiquantitative counting techniques (14), was reanalyzed using a quantitative approach on 51 cases. The OPv for CD8$^+$ T cells density was 490 cells/mm^2, and the CD8^{High} ($n = 14/51$, 27%) group displayed a shorter OS ($P = 0.001$, Fig. 1B). This result confirms our previous observations (14).

From TCGA public database of 496 primary ccRCC, we analyzed the expression of 844 immune-related genes, from which we extracted data concerning seven genes expressed in a Th1- and CD8$^+$ T-cell–oriented response according to Galon and colleagues (30). We found that the expression of most of the genes associated with this cell signature correlated with poor prognosis: *CD8A* $P = 0.04$, *TBX21* $P = 0.03$, *IRF1* $P = 0.01$, *GZMB* $P = 4.4 \times 10^{-5}$, and *IFNG* $P = 3.17 \times 10^{-7}$ that displayed the lowest *P* value (Fig. 1C). On the basis of the OPv for *IFNG*, patients that had high quantities of intratumoral mRNA for this gene displayed a shorter OS ($P = 0.006$, Fig. 1D).

Simultaneous expression of immune checkpoints in primary and metastatic ccRCC identifies patients with poor clinical outcome

Primary tumors. To investigate the impact of immune checkpoint molecules on the negative prognostic impact of the CD8^{High} group, we analyzed the protein expression of PD-1, LAG-3, PD-L1, and PD-L2 molecules in this group of tumors (we could analyze 40/41 tumors) and a randomly matched group of the same size ($n = 40/94$) coming from the CD8^{Low}.

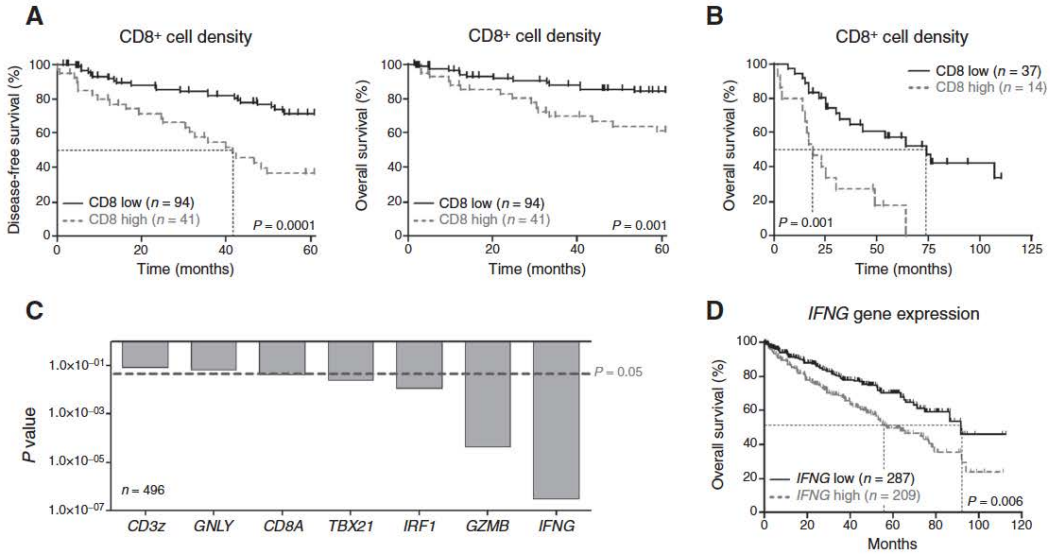

Figure 1.
CD8$^+$ T cells and CD8/Th1 gene signature are associated with poor prognosis in ccRCC. OS and DFS according to the presence of a high or low density of CD8$^+$ T cells in the IM of primary ccRCC (A) and ccRCC lung metastases (B). The *P* values by univariate Cox regression analysis for OS on seven Th1-related genes are displayed, $P = 0.05$ dotted black line, HR >1.0 gray columns (C). OS according to *IFNG* gene expression in primary ccRCC (D).

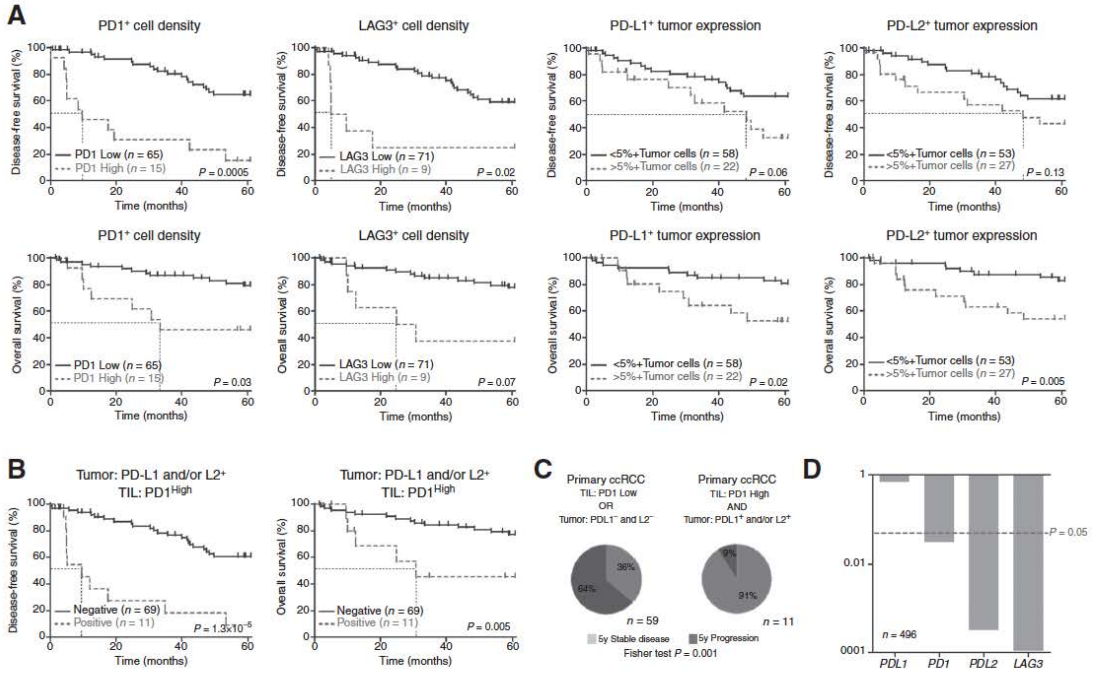

Figure 2.
Expression of immune checkpoints correlates with unfavorable clinical outcome for patients with primary ccRCC. OS and DFS according to the presence of a high or low density of PD-1$^+$ and LAG-3$^+$ cells, and the expression of PD-L1 or PD-L2 by >5% of the tumor cells in primary ccRCC (A). OS and DFS (B) and pie chart (C) representing the percentage of patients that had progressed after 5 years of surgery according to the expression of PD-L1 and/or PD-L2 on tumor cells related with high densities of PD-1$^+$ lymphocytes. The *P* values by univariate Cox regression analysis for OS on four genes are displayed, $P = 0.05$ dotted gray line, HR >1.0 gray columns (D).

On the basis of the OPv cutoff, 15 tumors of 80 were considered as PD-1^{High} in the IM and they displayed a shorter DFS ($P = 0.0005$, Fig. 2A) and OS ($P = 0.03$, Fig. 2A). The density of PD-1$^+$ cells in the TC was not significantly associated with prognosis (Supplementary Table S4). The Fuhrman grade was associated with the PD-1$^+$ cell density in the IM (Supplementary Fig. S2).

Of the 80 patients, 9 were considered as LAG-3^{High} (subdivided by the OPv cutoff) in the IM and they displayed a shorter DFS ($P = 0.02$, Fig. 2A), and did not reach significance for the OS ($P = 0.07$, Fig. 2A). The density of LAG-3$^+$ cells in the TC was not significantly associated with patients DFS or OS (Supplementary Table S4). Representative pictures of the IHC staining of highly and poorly PD-1– and LAG-3–infiltrated lesions are shown in Supplementary Fig. S3A and S3B, respectively.

On the basis of the 5% cutoff generally used in clinical trials with anti-checkpoint antibodies (20), 22 of 80 (27%) and 27 of 80 (34%) patients were PD-L1$^+$ and PD-L2$^+$, respectively (Supplementary Fig. S3C and S3D); among them, 9 tumors were double positive. Patients with PD-L1$^+$ tumors had a shorter OS than those with less than 5% positive tumor cells ($P = 0.02$, Fig. 2A); a trend toward shorter DFS was also found ($P = 0.06$, Fig. 2A). Univariately, patients with PD-L1$^+$ tumors were 2.9 times more likely to die in the 5 years post-nephrectomy than patients with less than 5% PD-L1$^+$ tumor cells [OS risk ratio, 2.87; 95% confidence interval (CI), 1.2–7.1; $P = 0.02$, Supplementary Table S4]. Patients with PD-L2$^+$ tumors displayed a shorter OS compared with those with less than 5% positive tumor cells ($P = 0.005$, Fig. 2A), but no impact on the DFS was found ($P = 0.13$, Fig. 2A). Univariately, patients with PD-L2$^+$ tumors were 3.4 times more likely to die than those with negative tumor cells (risk ratio, 3.4; 95% CI, 1.4–8.5; $P = 0.005$, Supplementary Table S4).

Patients with tumors exhibiting both high densities of PD-1$^+$ lymphocytes in the IM and PD-L1$^+$ and/or PD-L2$^+$ tumor cells ($n = 11$) had the worst prognosis, as assessed by DFS and OS (DFS, $P = 1.3 \times 10^{-5}$; OS, $P = 0.005$; Fig. 2B). Patients that met these criteria had 6.1 times more risk to progress after resection than patients who did not (risk ratio, 6.08; 95% CI, 2.4–15.0; $P < 0.001$, Supplementary Table S4). Strikingly, 91% of the patients having a PD1^{High} infiltrate and tumor cells expressing PD-L1 and/or PD-L2 progressed in the subsequent 5 years of surgery versus 36% in the negative group (complete 5-years follow-up $n = 70$, Fisher exact test $P = 0.001$, Fig. 2C). In the TCGA public datasets of 496 primary ccRCC, the gene expression of *PD-1* (*PDCD1*), *LAG-3*, and *PD-L2* (*PDCD1LG*) was associated with shorter OS ($P = 0.03$, $P = 0.0001$, and $P = 0.0003$, respectively) whereas *PD-L1* (CD274) was not ($P = 0.67$; Fig. 2D).

It has been reported that IFNγ can induce PD-L1 expression on tumor cells (27). We found a significant positive correlation between the gene expression of *IFNG* with *PD-L1* ($R = 0.13$,

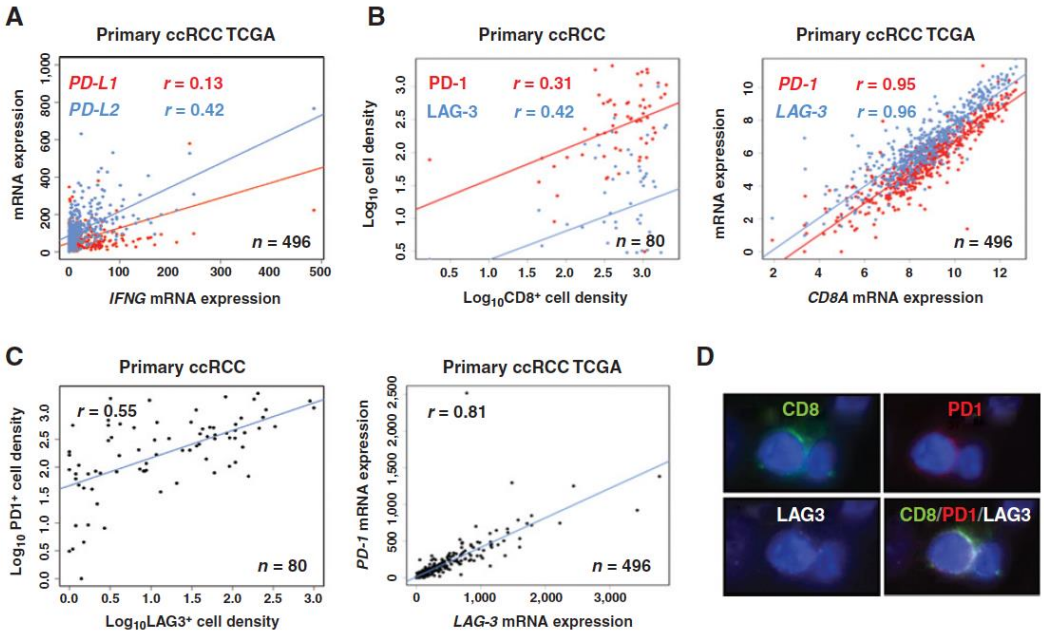

Figure 3.
Expression of immune checkpoints correlates with CD8$^+$ T cells infiltration in primary ccRCC. Dot plot of the gene expression of *PD-L1* (red dots) and *PD-L2* (blue dots; *y*-axis) against *IFNG* (*x*-axis; A). Dot plot of the Log_{10} cell density and gene expression of PD-1 (red dots) and LAG-3 (blue dots; *y*-axis) against CD8 (*x*-axis; B). Dot plot of the log_{10} cell density and gene expression of PD-1 (*y*-axis) against LAG-3 (*x*-axis; C). Pearson *r* value and the number of samples for each correlation are displayed. IF staining on 1 paraffin-embedded ccRCC showing the colocalization of CD8 (green), PD-1 (red), and LAG-3 (white) proteins in lymphocytes (D); nuclei are stained with DAPI.

$P = 0.004$) and with *PD-L2* ($R = 0.42$, $P = 2.2\times10^{-16}$) in the TCGA cohort (Fig. 3A). In addition, a significant positive correlation was found between the densities of PD-1$^+$ and CD8$^+$ T cells ($R = 0.31$, $P = 0.004$), LAG-3$^+$ and CD8$^+$ T cells ($R = 0.42$, $P = 0.001$) and PD-1$^+$ and LAG-3$^+$ cells ($R = 0.55$, $P < 0.0001$) in the IM of the 80 primary ccRCC (Fig. 3B and 3C). An even stronger correlation was found at the gene-expression level between *PD-1* and *LAG-3* ($R = 0.81$, $P = 0.002$), *PD-1* and *CD8A* ($R = 0.95$, $P < 0.001$), and *LAG-3* and *CD8A* ($R = 0.96$, $P < 0.001$) in the TCGA cohort of 496 primary tumors (Fig. 3B and C). In accordance with all these observations, we also detected the presence of triple-positive CD8$^+$/PD-1$^+$/LAG-3$^+$ cells in 6 of 7 cases from the CD8^{High} group of primary tumors by IF on paraffin sections (representative pictures are displayed in Fig. 3D).

Metastases. Metastatic ccRCC has been one cancer in which antibodies inhibiting the PD-1 axis have induced remarkable tumor regression in some patients (20), highlighting the necessity to define prognostic and predictive immune-based biomarkers in this disease. Of the 51 patients with ccRCC lung metastasis, 13 were considered as PD-1^{High}, and the latter displayed a shorter OS ($P = 0.008$, Fig. 4A). Seven of 51 were considered as LAG-3^{High}, and again they displayed a shorter OS ($P = 0.048$, Fig. 4A).

On the basis of a 5% cutoff, 5 of 51 (10%), and 15 of 51 (29%) metastases were PD-L1$^+$ or PD-L2$^+$, respectively; of them, 3 were double positive. Whereas patients with PD-L1$^+$ metastases did not have a significantly worse clinical outcome ($P = 0.12$, Fig. 4A), the expression of PD-L2 on tumor cells was associated with a shorter OS ($P = 0.03$, Fig. 4A). Univariately, patients with PD-L2$^+$ metastasis were 2.2 times more likely to die compared with patients with PD-L2- ones (risk ratio, 2.17; 95% CI, 1.03–4.35; $P = 0.04$, Supplementary Table S4).

Patients with high densities of PD-1$^+$ lymphocytes and PD-L1$^+$ and/or PD-L2$^+$ tumor cells in their ccRCC lung metastases ($n = 12$) had the worst prognosis as assessed by OS ($P = 0.003$, Fig. 4B). The patients that met these criteria had 3.1 times more risk to die after metastasectomy than patients who did not (risk ratio, 3.1; 95% CI, 1.28–6.66; $P = 0.003$, Supplementary Table S4). Strikingly, 100% of the patients having a PD1^{High} infiltrate and metastases simultaneously expressing one or both of its ligands (PD-L1 or PD-L2) died in the subsequent 5 years after surgery versus 57% in the negative group (Fisher tests, *P* value = 0.004, Fig. 4C).

A significant and strong correlation was found between densities of PD-1$^+$ and CD8$^+$ T cells ($R = 0.51$, $P = 0.0001$), LAG-3$^+$ and CD8$^+$ T cells ($R = 0.4$, $P = 0.004$; Fig. 4D) and PD-1$^+$ and LAG-3$^+$ cells ($R = 0.71$, $P < 0.0001$).

In conclusion, the combined analysis of the expression of PD-1, PD-L1, and PD-L2 identified a group of patients with a high risk of progression and death in primary and in an independent cohort of metastatic ccRCC.

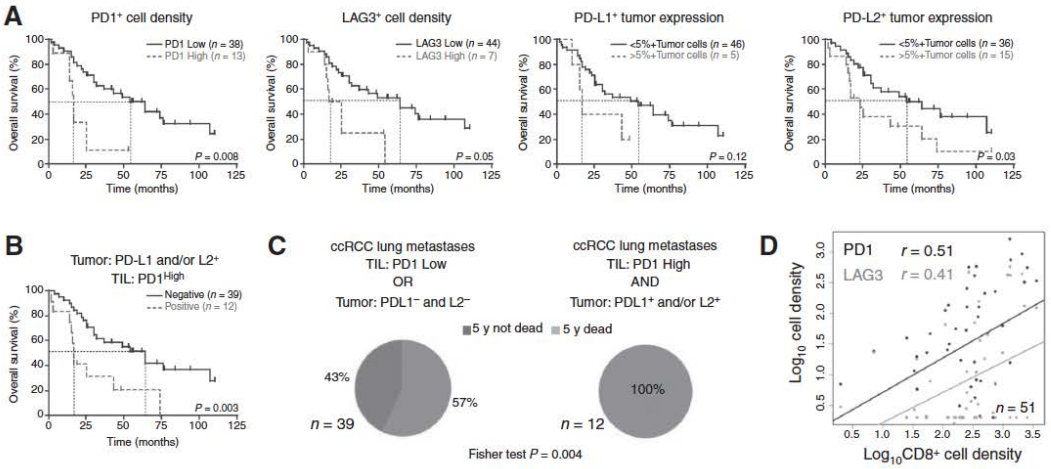

Figure 4.
Expression of immune checkpoints correlates with unfavorable clinical outcome for patients with ccRCC lung metastases. OS according to the presence of a high or low density of PD-1$^+$ and LAG-3$^+$ cells, and the expression of PD-L1 or PD-L2 by >5% of the tumor cells in ccRCC lung metastases (A). OS (B) and pie chart (C) representing the percentage of patients that had died 5 years after the metastasectomy according to the expression of PD-L1 and/or PD-L2 on tumor cells related with high densities of PD-1$^+$ lymphocytes. Dot plot of the Log_{10} density of PD-1$^+$ (black dots) and LAG-3$^+$ cells (gray dots; *y*-axis) against CD8 (*x*-axis; D); Pearson *r* value for each correlation is displayed.

Opposite correlations of TLS-DC and NTLS-DC with prognosis and their association with immune checkpoints

It has been shown that TLS-DC orchestrate intratumoral CD8$^+$ cytotoxic T cells and Th1 responses in NSCLC (3, 4). In the primary ccRCC cohort ($n = 135$), DC-Lamp$^+$ cells were detected within TLS (TLS-DC) in the IM. They coexpressed CD83 and high amounts of MHC Class II, and were localized in the vicinity of PNAd$^+$ high endothelial venules (HEV; Fig. 5A) as described in other tumor types (3). There was a trend where high densities of TLS-DC were associated with longer DFS (OPv = 0.09), but not with OS (Supplementary Table S4). TLS-DC densities in the TC were not associated with prognosis. Interestingly, high densities of TLS-DC (based on the OPv cutoff) in the IM identified a group of patients with good prognosis among the CD8^{High} group for both DFS ($P = 0.001$, Fig. 5A) and OS ($P = 0.03$, Fig. 5A).

However, in contrast with NSCLC where most of the DC-Lamp$^+$ cells are localized within TLS (4), in ccRCC a large percentage (79% ± 20%) of DC-Lamp$^+$ cells with DC morphology was found isolated and outside TLS (NTLS-DC, Fig. 5B). They colocalized with CD34$^+$ blood vessels, but not with PNAd$^+$ HEV, expressed low amounts of MHC class II and were CD83 negative (Fig. 5B). The majority of NTLS-DC was localized in the IM (71±14%) and their high densities (based on the OPv cut-off) were associated with poor clinical outcome in primary ccRCC patients (DFS $P = 0.006$, OS $P = 5.1\times10^{-5}$, Fig. 5B).

The opposite influences of TLS-DC and NTLS-DC on prognosis prompted us to explore their relationships with the expression of PD-1 and its ligands in the CD8^{High} group of patients. A negative correlation was found between the densities of TLS-DC and PD-1$^+$ cells ($r = -0.23$, $P = 0.04$, Fig. 5C). Tumors containing PD-L1$^+$ and/or PD-L2$^+$ tumor cells exhibited less TLS-DC ($P = 0.014$; Supplementary Fig. S4), but similar densities of CD8$^+$ T cells ($P = 0.96$). In contrast, the density of NTLS-DC was associated with the tumor expression of PD-L1 (OR, 6.54; 95% CI, 1.23–45.45; $P = 0.012$) and PD-L2 (OR, 2.7; 95% CI, 1.2–18.1; $P = 0.04$). Most of the patients that were PD-1^{High} and PD-L1$^+$ and/or L2$^+$ (8 of 11) were also CD8^{High} and TLS-DC^{Lo} in primary ccRCC (OR, 15.02; 95% CI, 1.66–72.7; $P = 0.004$), and the presence of one or both patterns correlated with shorter DFS ($P < 0.0001$) and OS ($P = 0.001$, Fig. 5D).

Identification of a group of patients with worst prognosis in primary and metastatic ccRCC

To define the independent prognostic significance of the previously mentioned immune profiles (CD8^{High} and TLS-DC^{Low} or PD-1^{High} and PD-L1$^{+and/or}$L2$^+$), we included them in a multivariate Cox regression analysis with the other significant prognostic clinical variables (TNM and Furhman grade). The strongest independent poor prognostic factors in primary ccRCC for DFS were to have a CD8^{High} and TLS-DC^{Low} ($P = 0.001$, Table 1) or PD-1^{High} and PD-L1$^{+and/or}$PD-L2$^+$ ($P = 0.03$, Table 1) immune profile. For the metastatic cohort, we found that the strongest independent worst prognostic variable for OS was being CD8^{High} ($P = 0.004$, Table 1) or having a tumor with PD-1^{High} and PD-L1$^+$ and/or PD-L2$^+$ immune profile ($P = 0.02$, Table 1).

Discussion

Compared with other neoplasia, the immune microenvironment in ccRCC has not yet been studied in detail. However, it is of paramount importance to understand its regulation in view of the paradoxical correlation of CD8$^+$ T-cell infiltration with poor prognosis (13, 14) and the recent advances of immunotherapy with anti-checkpoint antibodies (anti PD-1 and anti PD-L1; refs. 20, 21).

ccRCC has been described as a proinflammatory neoplasia where tumor cells produce several cytokines (such as VEGF, IL6 and TGFβ; refs. 33–35) that may lead to the recruitment and

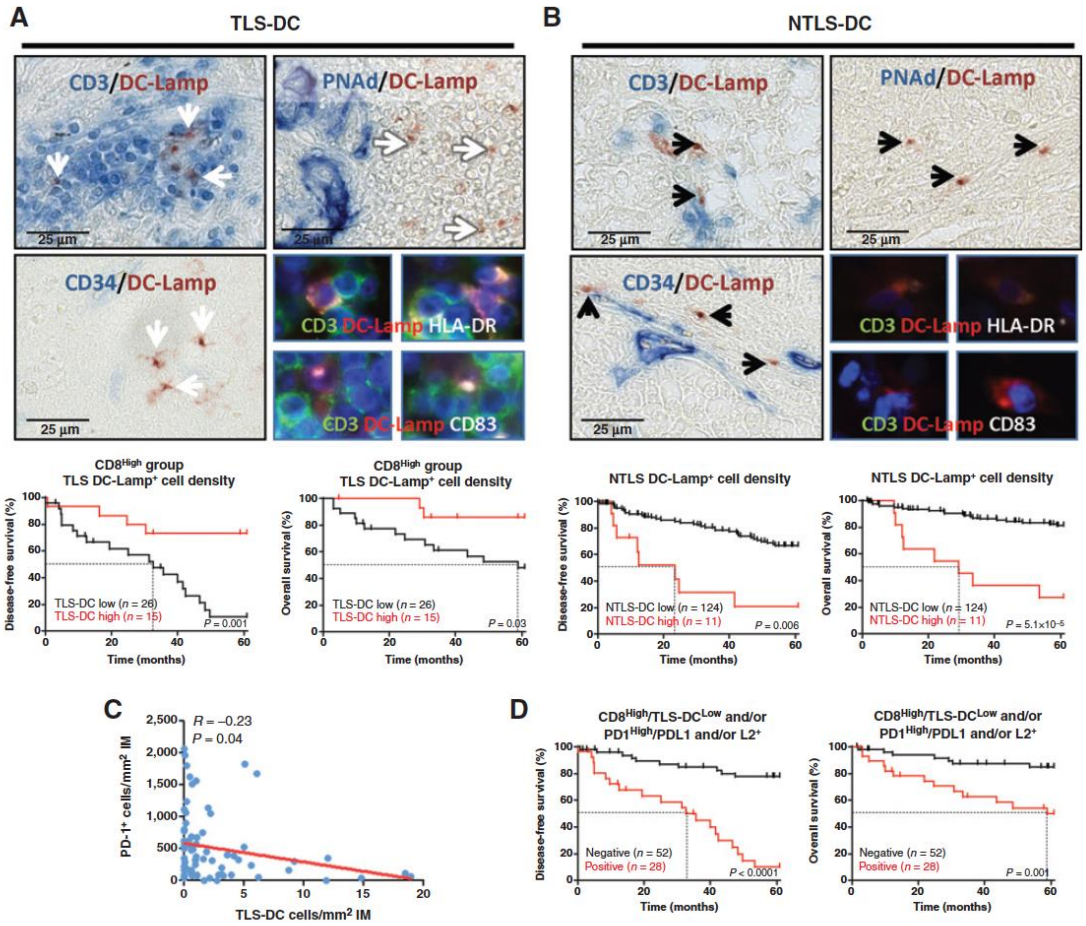

Figure 5.

Characteristics of TLS-DC and NTLS-DC and their relationships with prognosis and immune checkpoints. IHC photomicrographs of DC-Lamp(Red)/CD3(Blue) illustrating the presence of mature DC in TLS (A, white arrows) or outside TLS (B, black arrows); DC-Lamp(Red)/PNAd(Blue) and DC-Lamp(Red)/CD34 (Blue) illustrating TLS-DC near HEV (A) and NTLS-DC near endothelial cells (B) in primary ccRCC. IF staining showing the colocalization of CD3 (green), DC-Lamp (red) with HLA-DR or CD83 (white) expression in TLS-DC (A), but not in NTLS-DC (B); nuclei are stained with DAPI. OS and DFS according to the presence of a high- or low-density TLS-DC in the $CD8^{High}$ group (A, bottom) or NTLS-DC in the entire cohort (B, bottom). Dot plot of the $PD\text{-}1^{+}$ against $TLS\text{-}DC^{+}$ cell densities (blue dots; C); Pearson *r* value is displayed. OS and DFS according to the presence of a $CD8^{High}$ and $TLS\text{-}DC^{Low}$ and/or $PD\text{-}1^{High}$ and PD-L1 and/or $L2^{+}$ immune profiles (D).

activation of polyclonal $CD8^{+}$ T cells (36–39). Our data suggest that these recruited $CD8^{+}$ T cells could only be locally educated when high densities of TLS-DC are present, and in only in these cases their density correlates with favorable prognosis. ccRCC is the first tumor type where a large proportion of $DC\text{-}Lamp^{+}$ cells outside TLS structures have been observed (NTLS-DC). Interestingly, we found that these cells do not express activation and costimulatory markers, and they are probably recruited directly from the blood into the tumor stroma—contrary to the usual path that $DC\text{-}Lamp^{+}$ cells follow in other type of tumors (5, 40). Accordingly, several studies have shown that the ccRCC microenvironment can induce a dysfunctional DC maturation, a downregulation of costimulatory molecules and tolerogenicity (34, 41, 42), whereas DC in the TLS are likely to be protected from these effects (43). Altogether, our results suggest that the particular proinflammatory milieu initiated by tumor cells induces the recruitment of $CD8^{+}$ T cells that—due to the low number of fully functional mature DC present in specialized T-cell–priming sites, or the presence of DC with suppressive phenotype—are not able to mount an effective antitumor immune response, but rather express exhaustion/inhibition molecules.

This work reinforces the concept that T-cell exhaustion/inhibition (44) plays an important role in ccRCC pathogenesis. It has been described that the density of $PD\text{-}1^{+}$ cells (15, 16) and the tumor expression of PD-L1 (17–19) in primary ccRCC are associated with a poor clinical outcome. In this study, we confirm

Table 1. Multivariate Cox regression analysis for DFS and OS of the pathologic and immune variables in primary and metastatic ccRCC

	Multivariate Cox regression analysis DFS		Multivariate Cox regression analysis OS	
Primary ccRCC	**HR (95% CI)(lower–upper)**	***P***	**HR (95% CI)(lower–upper)**	***P***
$CD8^{high}$ TLS-DC^{low} (yes vs. no)	3.19 (1.42–3.83)	0.001	1.57 (0.45–1.82)	0.41
PD-L1 or L2 5%–100% + $PD\text{-}1^{high}$ (yes vs. no)	1.88 (1.56–2.89)	0.03	2.22 (0.80–1.16)	0.24
Fuhrman grade	1.90 (0.99–2.00)	0.06	2.68 (0.99–2.16)	0.32
TNM stage (I–IV)	1.59 (0.18–2.33)	0.12	2.74 (1.01–3.05)	0.002

	Multivariate Cox regression analysis OS	
Metastatic ccRCC	**HR (95% CI)(lower–upper)**	***P***
CD8 group (high vs. low)	2.86 (1.21–3.11)	0.004
PD-L1 or L2 5%–100% + $PD\text{-}1^{high}$ (yes vs. no)	1.41 (1.19–2.48)	0.02
Lymph node invasion (yes vs. no)	0.72 (0.35–1.42)	0.47

these findings and extend them to ccRCC lung metastases. This information is highly relevant because metastatic ccRCC-treated patients have shown one of the highest objective durable response rates to PD-1 blockade (approximately 30%; ref. 20) and many efforts are being dedicated to define theranostic tools in this pathology. Furthermore, we describe for the first time the prognostic significance of PD-L2. This molecule seems to be expressed in a higher proportion of tumors than PD-L1, and up to 30% of them might express it solely according to our results. This might be of clinical relevance because—although in two different nonrandomized cohorts—the anti–PD-L1 treatment alone seems to have a lower response rate than the anti–PD-1 (12% and 27%, respectively; refs. 20, 21). Furthermore, there are PD-L1–negative tumors that respond to anti–PD-1 treatment (22), suggesting that indeed there are other molecules beside PD-L1 implicated in the PD-1 inhibition axis of ccRCC. Few publications have reported PD-L2 mRNA or protein expression in other tumors, including primary mediastinal large B-cell lymphoma (29), NSCLC (45), ovarian (46), and esophageal (47), where it has shown a limited impact of patients' prognosis.

To our knowledge, this is the first report on the poor prognostic impact associated with high densities of $LAG\text{-}3^+$ cells in human tumors. Furthermore, we provide clear evidence that the expression of PD-1 and LAG-3 is highly correlated in ccRCC. Some studies on mouse models have shown a synergistic effect of the inhibition of both pathways in boosting antitumor immune response (24). Therefore, our data support the rationale of dual blockade of these molecules in ccRCC.

Although recent works have emphasized that PD-L1 is preferentially upregulated by IFNγ (31, 48), whereas PD-L2 is regulated by IL4 (49), the weak association of *PD-L1* mRNA with *IFNG* might suggest an important role for posttranscriptional regulation of PD-L1 expression in ccRCC as for other aggressive tumors (50). Furthermore, it is highly suggestive that IFNγ can also induce PD-L2 upregulation in tumors, as in immune cells (51), and supports the rationale to use therapeutic antibodies targeting this ligand. Taken together, our results suggest that the expression of these molecules is related with a chronic inflammatory and highly suppressive process that is unselectively recruiting $CD8^+$ and NTLS-DC cells from the circulation, and overall is associated with a poor prognosis.

Another characteristic of ccRCC is the lack of prognostic significance of the immune cells in the TC. Although $CD8^+$, $PD\text{-}1^+$, $LAG\text{-}3^+$, and NTLS-DC densities in the IM of the primary tumors were associated with poor prognosis, they had no prognostic significance when present in the TC. In colorectal carcinoma, both regions are important to define the best immune score, which correlates with patient's longer survival (30). Whether this dichotomy between ccRCC and colorectal carcinoma reflects different tumor tissue organization or relates to other factors of the tumor microenvironment remains to be clarified.

Recent unsupervised gene clustering of stage IV primary ccRCC showed that tumors with high inflammatory immune infiltrate (approximately 15%) also have a high expression of *PDCD1* (PD-1) and its ligands, and correlate with the worst prognosis (52). Indeed, this inflammatory/proangiogenic profile (probably originated from the ccRCC tumor cells) found in ccRCC primary tumors and in ccRCC lung metastases (14), is not found in colorectal carcinoma lung metastases (14), highly supporting that it may contribute to local immunosuppression process, the absence of fully mature DC, and high expression of immune checkpoints (53).

In summary, we identify a subset of primary and metastatic ccRCC patients characterized by (i) an extensive $CD8^+$ T-cell infiltrate, (ii) expression of immune checkpoints, and (iii) the absence of fully functional DC, which is associated with poor clinical outcome. Our results highlight novel independent prognostic factors in ccRCC based on the concomitant quantification of densities of DC, $CD8^+$, $PD\text{-}1^+$, and $LAG\text{-}3^+$ lymphocytes in addition to PD-L1/PD-L2 expression by tumor cells. These immune profiles should guide the selection of suitable patients to receive immunotherapies and need to be further validated in larger and independent cohorts. Because this choice depends on both the extent of $CD8^+$ T-cell infiltration and the maturation and localization of DC, it invites revision of the idea that intratumoral $CD8^+$ T cells are always associated with favorable prognosis in human tumors.

Disclosure of Potential Conflicts of Interest

Y. Vano reports receiving other research grants from Novartis and is a consultant/advisory board member for Novartis and Pfizer. G.J. Freeman has ownership interest (including patents) in Amplimmune, Boehringer-Ingelheim, Bristol-Myers Squibb, EMD Serono, Merck, Novartis, and Roche and is a consultant/advisory board member for Novartis and Roche. S.M. Oudard reports receiving speakers bureau honoraria from and is a consultant/advisory board member for Janssen, Novartis, Pfizer, Roche, and Sanofi. W-H. Fridman is a consultant/advisory board member for Costim. No potential conflicts of interest were disclosed by the other authors.

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Supplementary data

Supplementary Tables

Primary ccRCC	
Number of Patients	135
Males No. (%)	98 (73%)
Age (years)	61 ± 14
Overall survival time (months)	47 ± 21
Disease Free Survival (months)	40 ± 40
Tumor size major axis (cm)	5.1 ± 3.1
Sarcomatoid variant	18 (13%)
TNM Stage	
I	49 (36%)
II	10 (7%)
III	53 (39%)
IV	20 (15%)
Unclassified	3 (2%)
Fuhrman Grade	
I	5 (4%)
II	33 (24%)
III	78 (58%)
IV	19 (14%)
ccRCC Lung Metastases	
Number of Patients	51
Males No. (%)	42 (82%)
Age (years)	64 ± 10
Overall survival time (months)	42 ± 32
Fuhrman Grade	
I	4 (8%)
II	22 (33%)
III	23 (37%)
IV	12 (21%)

Table S1. Demographic and clinical characteristics of the analyzed patients are depicted (Mean {plus minus} SD)

Antibody	Specie	Clone	Source	Concentration (ug/mL)	pH Antigen Retrival	Secondary Antibody
CD8	Mouse IgG1	C8/144B	Dako	3.14	High	Anti-mouse EnVision FLEX/HRP labeled polymer
CD68	Mouse IgG3	PG-M1	Dako	0.6	Low	
Alpha Smooth Muscle Actin	Mouse IgG2a	1A4	Dako	0.7	Low	
CD34	Mouse IgG1	Qbend-10	Dako	0.24	Low	

Table S2. Antibodies used for the IHC and IF studies. Antibodies and conditions used for the IHC and IF studies

Cell population	Cut-off	Cut-off values (cells per mm2)	P value DFS	P value OS
		Primary ccRCC		
CD8+ cells IM	Minimum P value	630	0.0001*	0.001*
	Median	371	0.17	0.23
	1st-3rd quartile vs 4th quartile	892	0.0003	0.01
NTLS DC	Minimum P value	6.1	0.006*	<0.0001*
	Median	0.79	0.13	0.09
	1st-3rd quartile vs 4th quartile	2.19	0.01	0.002
PD-1+ cells IM	Minimum P value	626	0.0005*	0.03*
	Median	235	0.48	0.94
	1st-3rd quartile vs 4th quartile	519	0.0016	0.012
LAG-3+ cells IM	Minimum P value	156	0.02*	0.07*
	Median	10.7	0.81	0.88
	1st-3rd quartile vs 4th quartile	70.9	0.08	0.04
		Metastatic ccRCC Cohort		
Cell population	Cut-off	Cut-off values (cells per mm2)	P value DFS	P value OS
CD8+ cells IM	Minimum P value	490	NA	0.001*
	Median	278	NA	0.02
	1st-3rd quartile vs 4th quartile	581	NA	0.0003
PD-1+ cells IM	Minimum P value	172	NA	0.008*
	Median	24.3	NA	0.78
	1st-3rd quartile vs 4th quartile	172	NA	<0.0001
LAG-3+ cells IM	Minimum P value	121	NA	0.048*
	Median	1.70	NA	0.76
	1st-3rd quartile vs 4th quartile	21.9	NA	0.02

*Corrected for multiple-comparisons

Table S3. Survival analysis according to various cut-offs. Log-rank test p-values for CD8+, PD-1+ and LAG-3+ cell densities at the optimal, median and third quartile cut-offs

Table S4	Univariate Coxregression Analysis OS				Univariate Cox regression Analysis DFS			
	Univariate Coxre		95% CI				95% CI	
Primary ccRCC	HR	P value	Lower	Upper	HR	P value	Lower	Upper
Sex (male vs. female)	1,4	0,41	0,6	3,0	0,90	0,73	0,5	1,6
Age	1,00	0,12	1,0	1,0	1,00	0,86	1,0	1,0
CD8+ cell density IM	**3,41**	**<0.001**	1,1	5,1	**3,21**	**<0.001**	1,2	11,3
CD8+ cell density TC	1,85	0,54	0,2	14,0	0,97	0,98	0,2	5,8
DC-Lamp+ cell density IM	4,51	0,08	0,8	2,5	1,12	0,90	0,2	6,9
DC-Lamp+ cell density TC	1,17	0,92	0,0	34,0	0,65	0,79	0,0	14,1
TLS-DC density IM	0,72	0,53	0,2	2,3	0,78	0,57	0,3	1,9
TLS-DC density TC	1,00	0,59	0,7	1,2	1,00	0,85	1,0	1,0
NTLS-DC density IM	**2,21**	**<0.001**	1,0	12,5	**3,21**	**0,002**	1,4	7,8
NTLS-DC density TC	0,95	0,89	0,7	1,4	1,00	0,99	1,0	1,0
CD8High TLS-DCLow (yes vs. no)	**4,35**	**<0.001**	2,0	9,2	**5,79**	**<0.001**	2,8	9,4
PD-1+ density IM	**6,40**	**0,02**	1,3	31,0	**5,79**	**0,005**	1,7	20,0
PD-1+ density TC	2,94	0,29	0,3	30,0	3,67	0,15	0,6	22,0
LAG3+ density IM	3,86	0,15	0,6	24,0	**17,06**	**<0.001**	3,2	91,0
LAG3+ density TC	2,70	0,36	0,3	23,0	4,46	0,10	0,8	26,0
PD-L1+ Tumor cells 5-100% (yes vs. no)	**2,87**	**0,02**	1,2	7,1	1,96	0,06	1,0	4,0
PD-L2+ Tumor cells 5-100% (yes vs. no)	**3,40**	**0,005**	1,4	8,5	1,71	0,13	0,8	3,5
PD-L1+ and/or L2+ 5-100% (yes vs. no)	2,10	0,20	0,7	6,3	1,60	0,25	0,7	3,6
PD-L1+ and/or L2+ 5-100% AND PD1 High (yes vs. no)	**4,30**	**0,005**	1,4	13,0	**6,08**	**<0.001**	2,4	15,0
CD8High/TLS-DCLow and/or PD-1High/PD-L1andorL2+ (Yes vs No)	**4,06**	**0,001**	1,6	10,0	**6,38**	**<0.001**	3,0	14,0
Tumor size	**1,20**	**<0.001**	1,1	1,3	**2,00**	**<0.001**	1,5	2,6
Metastasis (yes vs. no)	**6,84**	**<0.001**	3,6	13,0	**4,39**	**<0.001**	2,5	7,6
Positive Lymph nodes (yes vs. no)	**3,47**	**0,002**	1,7	7,1	**1,82**	**0,002**	1,1	3,0
TNM Stage	**2,73**	**<0.001**	1,8	4,1	**2,73**	**<0.001**	1,8	4,1
Fuhrman Grade	**4,03**	**<0.001**	2,4	6,9	**2,89**	**<0.001**	1,9	4,3
Blood neutrophils before surgery	1,02	0,44	1,0	1,1	1,04	0,08	0,9	1,1

			95% CI	
ccRCC Lung Metastasis	HR	P value	Lower	Upper
CD8+ cell density	**3,86**	**0,05**	1,0	15,0
PD-1+ cell density	**6,28**	**0,01**	1,6	25,0
LAG3+ cell density	**5,31**	**0,01**	1,6	18,0
PD-L1+ Tumor cells 5-100% (yes vs. no)	2,32	0,13	0,8	6,7
PD-L2+ Tumor cells 5-100% (yes vs. no)	**2,17**	**0,04**	1,0	4,4
PD-L1+ and/or L2+ 5-100% AND PD1 High (yes vs. no)	**3,06**	**0,003**	1,3	6,7
DFI	0,99	0,13	1,0	1,0
Positive lymph nodes (yes vs. no)	**1,97**	**0,04**	1,7	4,1
Fuhrman Grade	1,16	0,50	0,8	1,8
Blood neutrophils before surgery	1,02	0,82	0,9	1,2

Table S4. Univariate Cox regression analysis for OS and DFS in primary and metastatic ccRCC. P-values and HR of significant variables are highlighted in bold font.

Supplementary Figures

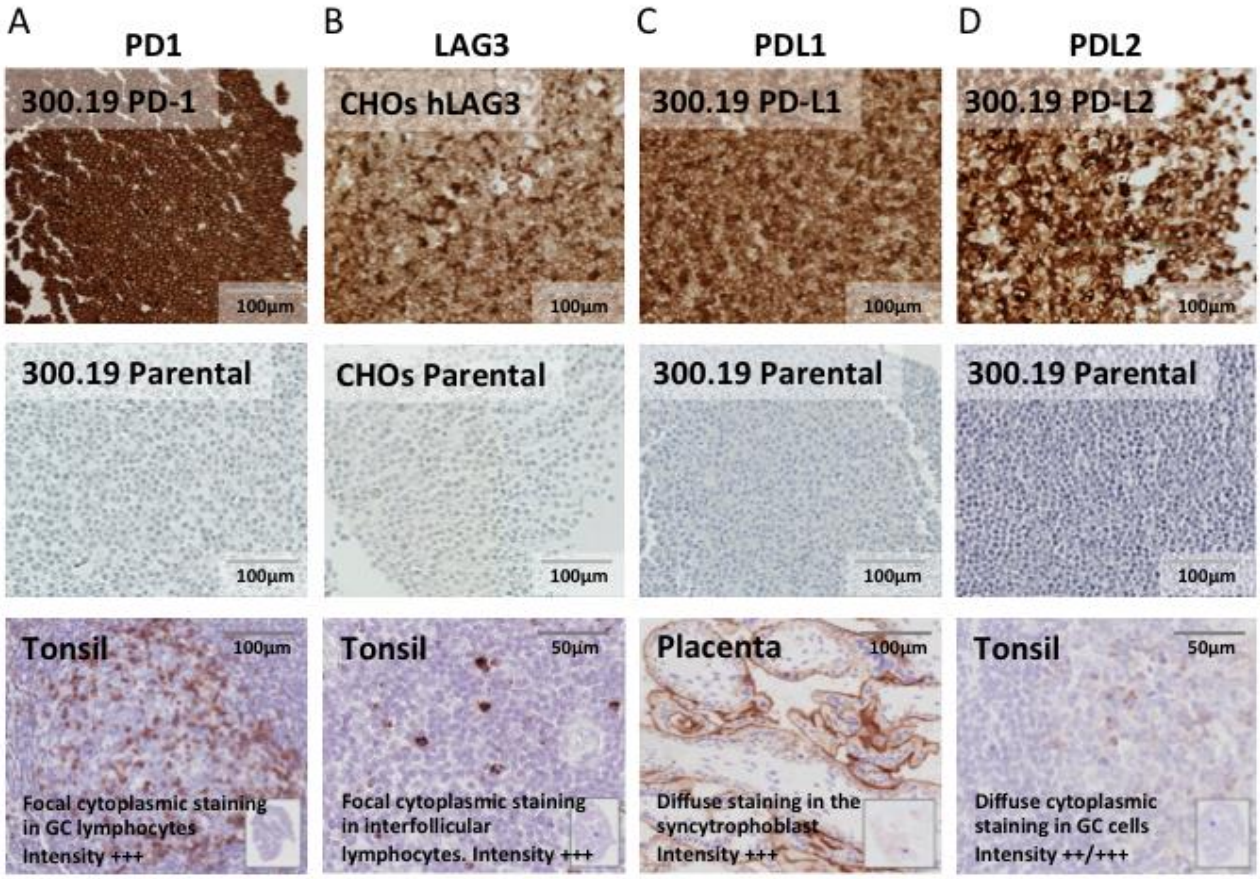

Figure S1. Test of the specificity of anti-immune checkpoints : Immunohistochemical staining of immune checkpoints on sections from paraffin embedded cell pellets of untransfected and transfected cell lines used as negative and positive controls respectively and from paraffin embedded tonsils and placenta.

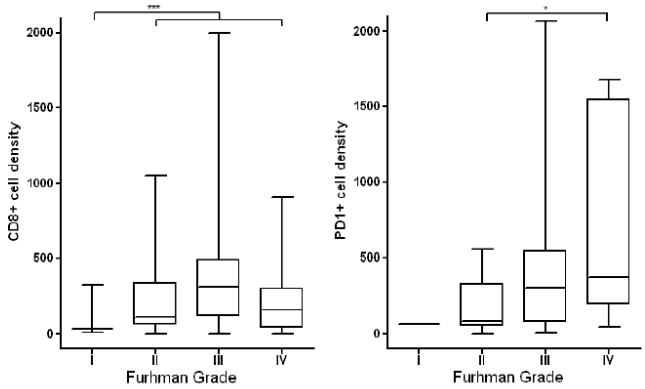

Figure S2. CD8+ and PD1+ cell density and Furhman Grade in primary ccRCC

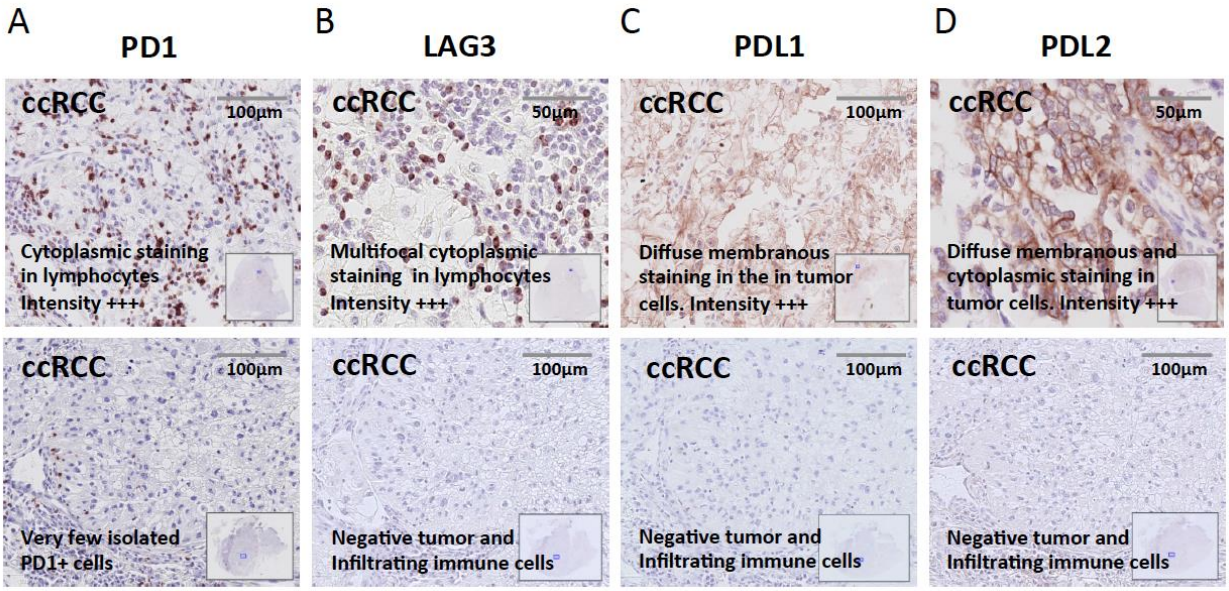

Figure S3. Representative IHC staining of immune checkpoints in ccRCC : Immunohistochemical staining of immune checkpoints on sections from paraffin embedded ccRCC tumors illustrating highly and poorly PD-1 (A) and LAG-3 (B) infiltrated lesions and PD-L1 (C) and PD-L2 (D) positive and negative tumors

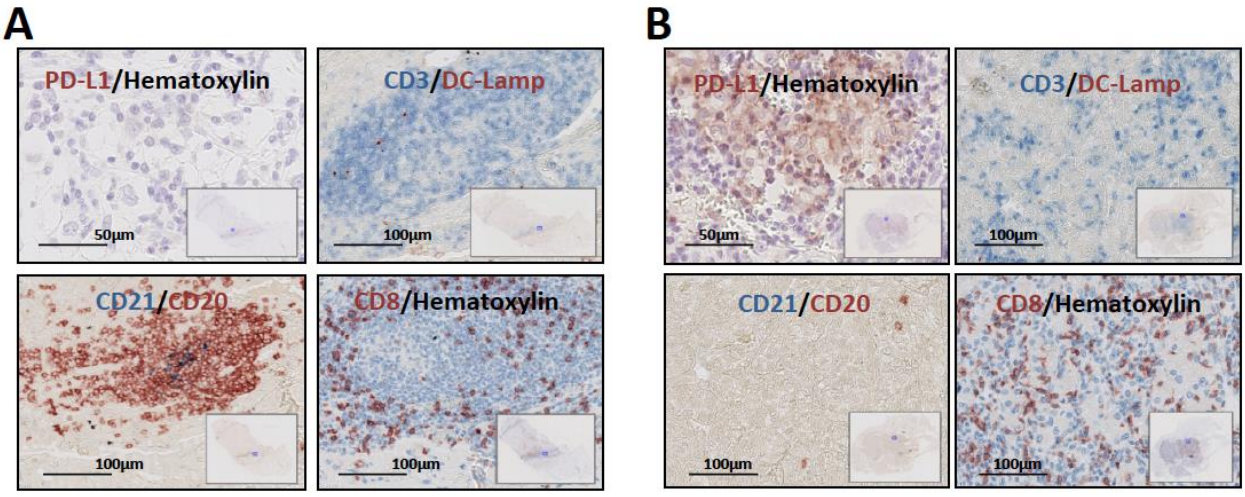

Figure S4. Inverse relation between TLS and immune checkpoints in primary ccRCC : photomicrographs of immunohistochemistry staining of PD-L1(red)/hematoxylin and TLS structures as revealed by CD3(Blue)DC-Lamp(Red), CD21(Blue)/CD20(Red), and of CD8(red)/hematoxylin in PD-L1+and/orPD-L2+ (A) and PD-L1-/PD-L2- (B) primary ccRCC

Article 2: << The immune contexture of primary and metastatic human tumours>>

Nicolas A. Giraldo, Etienne Becht, Romain Remark, Diane Damotte, Catherine Sautes-Fridman and Wolf H. Fridman.

This article is a comprehensive review of the literature on tumour immunology in primary and metastatic tumours, published in Current Opinion Immunology in April 2014.

Summary of the results in Article 2:

The topics addressed throughout the text were:

1. The immune contexture as a tool to predict patient's clinical outcome.
2. Chronic inflammation, cancer development and tumour growth.
3. The few exceptions to the dogma that an adaptive immune response predicts longer survival in all cancer.
4. The influence of tumour-originating factors on the immune contexture.
5. The variations in the immune contexture from the primary to the metastatic lesions.

Article 2

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Current Opinion in Immunology

The immune contexture of primary and metastatic human tumours

Nicolas A Giraldo[1,2,3,5], Etienne Becht[1,2,3,5], Romain Remark[1,2,3], Diane Damotte[1,2,3,4], Catherine Sautès-Fridman[1,2,3] and Wolf H Fridman[1,2,3]

A tumour grows in a complex microenvironment composed of stromal cells, lymphoid and myeloid cells, vascular and lymphatic vessels, and the resultant cytokine and chemokine milieu. In most primary tumours, a strong Th1/cytotoxic T cells infiltration correlates with a longer survival. This beneficial effect can be hampered by the presence of M2 polarized macrophages and high VEGF production. Recent studies revealed that the pattern of the tumour microenvironment remains a major prognostic factor even in the metastatic lesions, while been reproducible between the primary and metastatic tumour. Nevertheless the prognostic impact of the Th1/cytotoxic T cell infiltrate could be different according to the origin of the primary tumour. This model highlights a novel tumour cell-dependent immune contexture that predicts patient's clinical outcome and has implications in the use of immunotherapies.

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Current Opinion in Immunology 2014, **27**:8–15

This review comes from a themed issue on **Tumour immunology**

Edited by **Philip K Darcy** and **David S Ritchie**

For a complete overview see the Issue and the Editorial

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Introduction

The complex network of epithelial and endothelial cells, vascular and lymphatic vessels, as well as myeloid and lymphoid elements present within tumours has been named the tumour microenvironment. More than being just a passive niche where tumour develops, this complex ecosystem impacts tumour evolution and influences clinical outcome [1,2••]. Cancer natural history follows a complex multistep process [3] in which an organ-specific precancerous lesion, often undetected, becomes a primary tumour that invades the surrounding tissue, while malignant cells penetrate the blood and lymphatic vessels or migrate along sympathetic nerves to form distant metastases [4]. During these different steps the malignant cells interact with their microenvironment, in the primary tumour site, the draining lymph nodes and in the distant organs where they metastasize [4]. The current best available tool to decipher the role of the diverse elements of the tumour microenvironment in humans is the characterization of the different cellular populations, their location and densities, their functional orientation, their chemokine production and their correlation with patient's clinical outcome [2••,5–7,8•]. The study of large cohorts of human cancers gene expression arrays strengthens these analyses [9–12]. Furthermore, novel data from immunotherapy trials allow researchers to correlate a particular modulation of the immune contexture with the clinical responses to treatment [13••].

Indeed the density and the composition of the immune microenvironment are heterogeneous among patients and tumours. It is now well established that in general the tumour infiltration with M2-phenotype macrophages and myeloid derived suppressor cells (MDSC) promotes tumour progression [14•,15] whereas infiltration of memory cytotoxic and Th1 T lymphocytes are often associated with good clinical outcome [2••,5,16,17] and good response to immunotherapy [18–20]. The clinical impact of other lymphoid and myeloid cell populations is less consistent and seems dependent on the tumour type and stage [2••]. Finally, although the vast majority of analyses focus on primary tumours, recent reports shed light on the dialogue between malignant cells and their microenvironment at distant metastatic sites [21•,22••], allowing to potentially re-visit the paradigm describing the interactions between tumours and their microenvironment at the different steps of local invasion and metastasis.

The present review analyses recent data from human primary and metastatic tumours in order to propose an integrated view of the '*in situ*' dialogue between malignant cells and the immune and inflammatory microenvironment, and its effect on patient's clinical outcome.

Inflammation and immunity: foes and friends

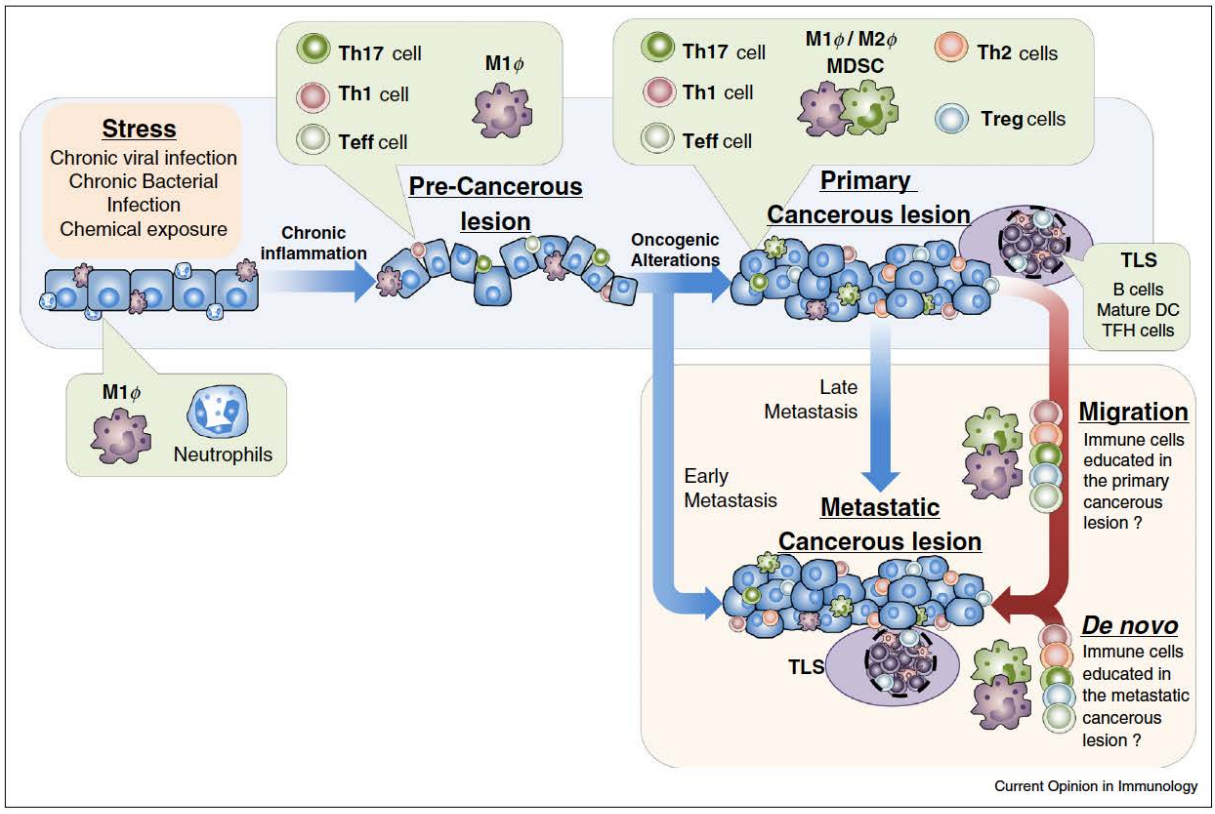
The link between inflammation and tumour development has been extensively recognized [1]. At precancerous stages, the presence of chronic inflammation in an organ helps promoting cancer outbreak and growth. The inflammatory context may be the result of viral infections

such as HPV in cervical [23] and head and neck carcinoma [24] or HBV and HCV in hepatocellular carcinoma [25]; external bacterial infections, such as *Helicobacter pylori* in gastric cancer [26] and non-Hodgkin gastric lymphoma [27] or the augmented permeability of the epithelial barrier to commensal microbiota promoting the development of colorectal cancer [28]; or the presence of noxious chemicals such as smoke for lung cancer [29] (Figure 1).

The inflammatory milieu can promote tumour growth through the production of cytokines such as IL-6, IL-1 or TNF-α [1], in addition to angiogenic molecules such as VEGFA, transforming growth factor-β (TGF-β), adenosine, prostaglandin E2 [30•] and suppressor myeloid or T cells attracting chemokines, including CXCL1, CXCL5, CCL2 and CCL12 [6,14•]. Indeed, preventive treatments with anti-inflammatory agents such as aspirin or Cox-2 inhibitors have shown efficacy in decreasing the incidence of colorectal cancers [31]. More strikingly, the antibiotics cure of *H. pylori* infections and the vaccination against HPV and HBV have significantly reduced the incidence of gastric cancer [32], cervical carcinoma [33] and hepatocellular cancer [34], respectively.

Once a primary cancerous lesion is established, inflammatory cells can also act as tumour promoting elements. Accordingly, the density of tumour infiltrating macrophages or a CSF-1 response gene signature correlates with poor prognosis and metastasis in breast [35], lung [36], thyroid [37] and liver cancer [38]. Moreover, it has been suggested that tumour-associated macrophages are implicated in the metastatic process in human breast cancer [39]. In contrast, inflammation may also induce cancer regression as evidenced by the exceptional spontaneous remission of patients presenting recurrent cervical sarcomas and acute bacterial infection, which had led Coley to

Figure 1



Major events and immune players in cancer natural history. A cartoon depiction of the development of a cancerous lesion in which the normal tissue is exposed to stress that leads to an influx of inflammatory cells, mainly from the innate immunity. If chronic inflammation continues, there are prominent changes in the infiltration that will then be characterized by M1 macrophages, Th1 and Th17 cells and T effector (Teff) cell presence. The presence of chronic inflammation and a noxious stimulus could lead to the development of a precancerous lesion. The transition from a premalignant lesion to cancer is characterized by a shift of this previous immune contexture to a Th2, Treg, M2 macrophages and MDSC dominant response. The metastatic spread of the malignant cells can occur in the early or late stages of the tumour development. The primary tumour and metastatic lesion have analogous immune contextures, and probably the infiltrating cells can arrive either from cells educated in the tertiary lymphoid structures (TLS) of the primary lesion or educated '*de novo*' in those from the metastatic tumour.

propose with some success the use of bacterial extracts to treat cancers; indeed, BCG is still the state of the art adjuvant therapy of superficial bladder cancer [40].

A long lasting matter of debate, it is now recognized that the adaptive immune response contributes to the control of tumour development and progression. Thus the incidence of cancers is higher in primary and acquired immunodeficiencies, such as in HIV+ patients [41] or transplant recipients treated with immunosuppressive drugs [42]. Moreover, once a cancer is established, the density of tumour infiltrating lymphocytes (TIL) is positively associated with favourable prognosis. A recent meta-analysis of 20 different cancer types has revealed a consensus establishing that high densities of intratumoral memory cytotoxic/Th1 T cells are associated with longer disease free and overall survival [2••]. The clinical impact of other T cell subsets is less consensual and Th2, Th17 and Treg infiltrations correlate with poor prognosis in half of the cases, and with favourable or no impact in the other half [2••]. Nevertheless, the transition from precancerous to invasive stage in cervical carcinoma [43] and pancreatic [44] cancers parallels a shift from Th1 to Th2 immune microenvironment. On the other hand, an interesting observation has been reported in ovarian [30•] and colorectal [45] cancers where high levels of VEGFA abolish the positive influence of infiltrating Th1/CD8 T cells suggesting a complex interplay between angiogenesis and immunity [30•].

A striking characteristic of the tumour microenvironment is the presence of lymphoid islets, adjacent to the tumour nests and absent in the distant non-tumoral tissue. These structures are thus likely induced by the tumour, and were first reported in non-small cell lung cancer [46] and subsequently in breast [47] and colon cancers [7] as well as in melanoma [48]. They exhibit properties of active immune sites, with a T cell zone (where T cells contact mature dendritic cells, DC), a B cell zone with follicular DC and proliferating B cells, and are surrounded by high endothelial venules (HEV) [47,49] and podoplanin positive lymphatics [46]. It is postulated that these islets can act as tertiary lymphoid structures (TLS) in which naïve T cells penetrate through HEV, are activated and educated by mature DC. They can then migrate toward the tumour attracted by CCL19, CCL22 and CXCL13, or into the lymphatic vessels attracted by CCL21 to circulate in the periphery as central memory T cells [49]. We hypothesized that these circulating cells could later initiate a secondary immune response in the metastatic sites [49]. Interestingly, the density of TLS and mature DC correlates with a memory Th1/cytotoxic phenotype of TIL and a favourable clinical outcome [48,50].

The status of immunity and inflammation in metastatic sites

The vast majority of the studies that analysed the tumour microenvironment have been performed on primary tumours. Only scarce publications have reported that immune cells can infiltrate metastatic sites and may have a clinical impact. It is not a trivial question since one could hypothesize that metastatic cells have escaped immune destruction by editing immunoresistance [51] through their genome plasticity [52••]. However, it has been reported that the density of CD8 T cells in the invasive margin of liver metastases of colorectal cancers were predictive of response to chemotherapy and overall survival [21•]. More recently, the analysis of the immune microenvironment of lung metastases from colorectal (CRC) and renal cell carcinoma (RCC) revealed interesting characteristics [22••]: firstly, although there might be quantitative differences, there is a correlation between the immune microenvironments (densities of mature DC, CD8+ T cells and natural killer (NK) cells in the primary colorectal or renal cell tumours) and their subsequent lung metastasis and finally, the densities of mature DC, CD8+ T cells and NK cells are similar in synchronous metastatic sites and between a surgically removed and a recurrent metastasis in the same patient. The immune microenvironment is therefore likely to be shaped during the development of a primary tumour and is a hallmark of the dialogue between the tumour and the host during the different stages of the disease. These recent data are compatible with the hypothesis that the metastatic process might begin early in the development of the primary malignant lesion [53] and can therefore preserve several characteristics of the immune microenvironment.

Another striking and unexpected observation of this work was that a high density of infiltrating mature DC and CD8+ T cells in the metastatic sites correlated with prolonged overall survival in colorectal cancer and with reduced overall survival in renal cancer [22••]. The quantification of these immune cells in primary tumours of the same patient confirmed previous reports in CRC and RCC and yielded the same contrasting effect on overall survival of the patients [5,54]. In an attempt to understand what could be responsible for these differences, a gene expression analysis was performed and revealed that the T cell infiltrates correlated with coordinated Th1 expression in both cases [22••]. However, expression of genes associated with angiogenesis (i.e. VEFGA), immunosuppression, inflammation and Th2 orientation were higher and positively correlated with CD8 and DC densities only in renal cell cancer when compared with primary or metastatic colorectal carcinoma [22••].

These data support the concept that the clinical outcome of the patients is governed by the complex interaction between immunity and inflammation at the different steps of tumour development rather than by their sole respective influences (Figure 1). Since many of these interactions occurred by the time of clinical presentation, analysis of tumour samples, although essential for defining prognostic

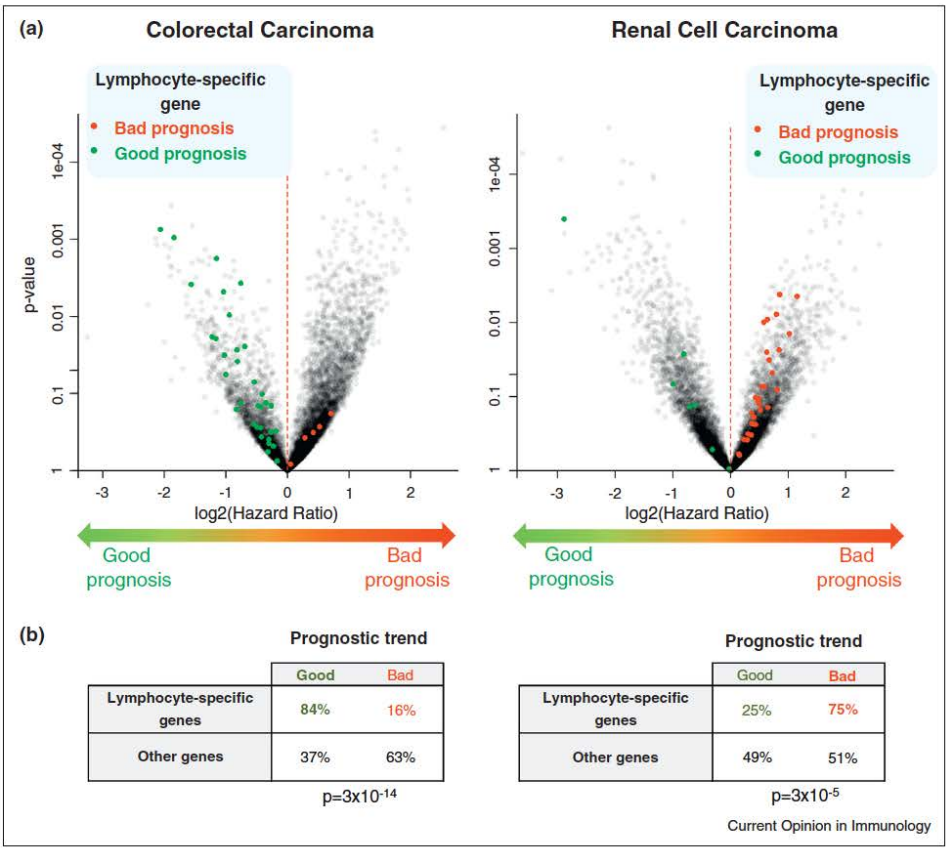
and theranostic markers, may not fully reflect the process behind its development.

The colorectal and renal cell model of tumour immunology and inflammation: a new paradigm?

Using transcriptomes from purified immune cells and cancer cell lines, we generated a list of genes specifically expressed in B or T cells and not expressed by malignant cells or other immune cell types. By running Cox regression analyses, we assessed the prognostic value for overall survival of the expression of these lymphoid specific genes and all the other genes (E Becht *et al.*, unpublished data). Representative results obtained on the RCC dataset from The Cancer Genome Atlas [55] and from the CRC dataset from the Moffit Cancer Center [55] (GEO: GSE17536) are shown in Figure 2. This analysis reveals a clear shift of the prognostic value associated to the expression of lymphocyte specific genes from favourable in CRC datasets to detrimental in RCC datasets, compared to the distribution of all the other genes ($P = 10^{-14}$ for CRC and $P = 10^{-5}$ for RCC, Fisher's exact test).

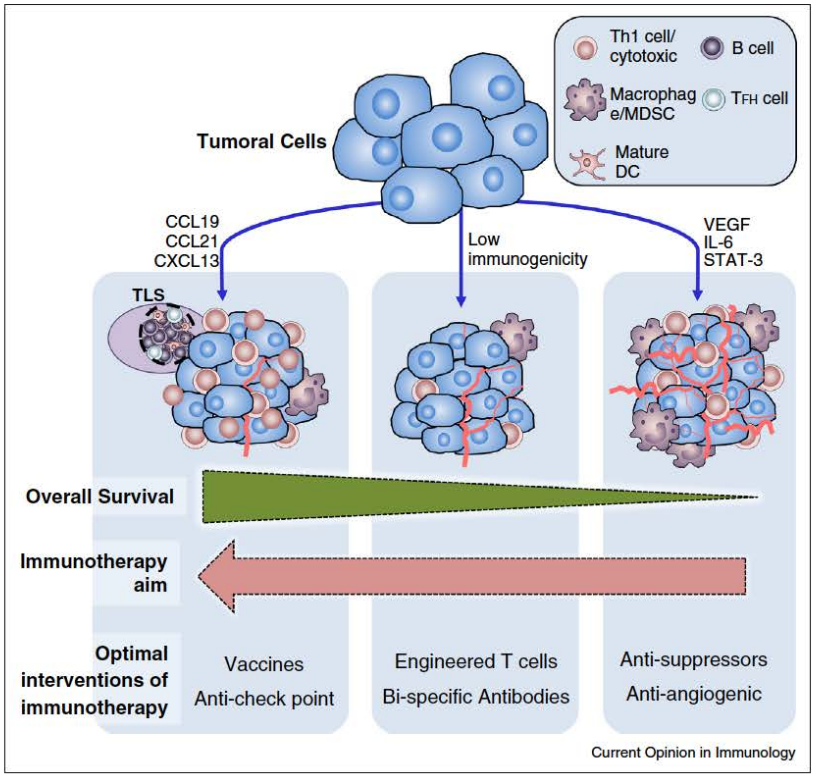
Therefore the cellular and molecular analyses of these two cancer prototypes refine our previous model explaining the impact of the immune and inflammatory microenvironment in the evolution of human cancers [9]. In most cancer types, the CRC paradigm applies [2••]: a strong infiltration of memory Th1/cytotoxic T cells, likely educated in adjacent TLS, correlates inversely with VEGFA, IL-6, STAT-3 expression [7,22••] and is associated with favourable prognosis. In RCC, another paradigm applies, where a strong infiltration of CD8+ T cells, potentially educated in other sites with scarce adjacent TLS, are associated with a strong expression of VEGFA, STAT-3, IL-6, TNF-α, TGF-β and IL-10 and poor survival [22••]. Whether the RCC situation is unique or

Figure 2



Prognostic trend associated to the expression of lymphocyte-specific genes in CRC and RCC. **(a)** Volcano plots showing the Hazard Ratio and *P*-value associated to the expression of genes in two representative public datasets, as assessed by Cox Proportional Hazards Regression analyses. Lymphocyte-specific genes are pictured by green dots in the left half (good prognostic trend) and red dots in the right half (bad prognostic trend). **(b)** Contingency tables representing the percentages of genes in the left and the right halves of the volcano plots for both lymphocyte-specific genes and other genes.

Figure 3



Cancer immune contexture scenarios and personalized medicine for immunotherapy. Cartoon depiction of the major immune contexture scenarios for cancer. A strong immune response (left) can be induced by the presence of CX3CL1, CXCL9 and CXCL10 and is characterized by Th1/cytotoxic T cells infiltration and the presence of tertiary lymphoid structures (TLS), while associated with a highest overall survival. In the other extreme, those tumours associated with high expression of IL-6, VEGF-A and STAT-3 induce a rather inflammatory and pro-angiogenic environment (right) associated with poor overall survival. Knowing the possible scenarios of the immune contexture allows to use the optimal immunotherapy treatment in each case, to lastly induce an appropriate immune response in the tumour microenvironment.

can apply to other cancer types remains to be further explored. Indeed, a recent publication by Scott *et al.* identified a gene signature where the overexpression of CD68, IL15RA, STAT1, IFNG and genes associated with cytotoxic T and NK cells, correlated with poor prognosis in terms of OS in classical Hodgkin Lymphoma. This is another example that reinforces the validity of the new model [56].

Altogether, these recent findings strongly support the hypothesis that the interactions between tumour cells and their microenvironment imprint the latter during all stages of the disease with a similar architecture and clinical impact. This new paradigm also has important implications for the design of novel immunotherapies aimed to properly activate T cells or modulate inflammatory and immunosuppressive elements. The aim of these immunotherapies would be to modify the contexture of immune, inflammatory and angiogenic elements to favour a strong Th1 cytotoxic microenvironment in primary and metastatic tumours [13••,57] (Figure 3). Anti-angiogenic treatments which downregulate MDSC [14•] and Treg [59] at the primary tumour site represent a model of these therapies but also they apply to anti-check point antibodies [58•], inhibitors of indoleamine-2,3-dioxygenase [60], bi-specific antibodies [61] or chemokines and chemokine-receptor antagonists. They should be investigated in combined clinical trials [62••] based on the immunoscore [63•] and the characterization of the full immune contexture.

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- • of special interest
- •• of outstanding interest

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This manuscript sets the worldwide effort to establish the clinical routine use of the immunoscore.

Unpublished results

We have been collecting data to accomplish the third objective of this project:

3. To characterize the phenotype of ccRCC TIL, and its relation with the expression of immunomodulatory molecules in the TME.

Summary of the Unpublished Results:

To functionally characterize the $CD8^+$ T cell infiltrates in ccRCC, we investigated the major phenotypic traits of freshly isolated lymphocytes from 21 tumours obtained by partial or radical nephrectomy. Non-supervised analyses of the $CD8^+$-TIL phenotype revealed two different types of tumours: The first group or "inhibited group" (7/21) was characterized by the over-expression of inhibitory receptors (PD-1 and TIM-3) and activation markers (CD69 and CD38), and the expansion of the effector memory cell subpopulation ($CCR7^-CD45RA^-$) in the $CD8^+$-TIL; the second group (13/21) was characterized by the expansion of the $CD8^+$EMRA subpopulation ($CCR7^-CD45RA^+$) in addition to a reduced expression of activation or inhibitory receptors. Preliminary data suggest that the "inhibited group" of tumours is characterized by more advanced tumour stages, higher Fuhrman grades and increased $CD8^+$ cell densities (as determined by IHC), and thus shares major characteristics with tumours associated with patients' worst clinical outcome in the retrospective cohort.

Material and Methods

Patients

A cohort of 21 primary ccRCC human tumours were collected between 01-2014 and 06-2015, from specimens of radical or partial nephrectomy, operated at the hospital Institut Mutualiste Montsouris (Paris, France). We obtained pre-operative peripheral blood samples (n=19) and adjacent non-tumour kidney tissue (n=19) in some patients. This research was conducted according to the recommendations outlined in the Helsinki declaration and approved by the medical ethics boards of all participating institutions, and with the agreement of the ethics committee (no. CEPAR- 2014-001). The demographic characteristics of the cohorts are depicted in Table 2.

Table 2. Demographic and clinical characteristics of the analyzed patients are depicted

ccRCC patients	
Number of patients	21
Males (%)	14 (67%)
Age (years)	63 ± 14
Tum. size major axis (cm)	3.8 ± 0.8

T stage	
I (%)	14 (67%)
II (%)	2 (10%)
III (%)	5 (23%)

Fuhrman Grade	
G1 (%)	1(5%)
G2 (%)	11(52%)
G3 (%)	6 (29%)
G4 (%)	3 (14%)

Tumour processing and surface staining

Tumours were dilacerated and incubated for 1h at 4°C with Cell Recovery Solution (Fisher Scientific); mixtures were filtrated and TIL separated with Ficoll-Paque PLUS (GE Healthcare Life Science). TIL were then stained with the monoclonal antibodies: CD45RA-ECD (2H4, Beckman coulter), CD3-AF700 (UCHT1, BD), CD4-BV 605 (OKT4, Biolegend), CD8-BV 650 (RPA-T8, Biolegend), CD69-PE (FN50, BD), CD38-PercpeF710 (HB7, eBioscience), CD40L-APC-Cy7 (Biolegend), ICOS-FITC (Isa-3, eBioscience), GITR-APC (AITR, eBioscience), PD-1-APC-Cy7 (EH12.2H7, Biolegend), TIM 3-BV421 (F38-2E2, Biolegend), CTLA-4-APC (L3D10, Biolegend), LAG-3-FITC (17B4, Enzo) and TIGIT-PercpeF710 (MBSA43, eBioscience). Samples were acquired in a FACS Fortessa cytometer with FACSDiva software (BD Bioscience), and data analyzed with FlowJo 7.9.4 software (Tree Star, Inc. Ashland, OR, USA).

Results

CD4+ and CD8+ RCC TIL express higher but heterogeneous levels of activation markers and inhibitory receptors as compared to PBL

We characterized the phenotype of CD4+ and CD8+ TIL from 21 primary tumours, and compared them that of autologous-PBL (n=19). We measured the cell surface expression of lineage markers CCR7 and CD45RA, inhibitory receptors (InR) PD-1, TIM-3, LAG-3, GITR, CTLA-4 and TIGIT and activation markers (AM) CD69, CD38, ICOS and CD40L.

CD4+ TIL displayed lower frequencies of cells with a central memory (CM) and naïve phenotype, but higher fractions of effector memory (EM) cells in comparison to PBL (Figure 2A). In addition, CD4+ TIL exhibited an enhanced expression of AM (CD69, CD40L and ICOS) and InR (PD-1, TIM-3, LAG-3 and GITR), but not CD38, CTLA-4 or TIGIT, as compared to PBL (Figure 2A). CD69 was the receptor most overexpressed in CD4+ TIL (35.0±17.4), followed by PD-1 (35.0±17.4), TIM-3 (11.0±9.7) and GITR (10.4±10.4) (Figure 2A). Interestingly, the frequencies of CD4+ TIL expressing CD69 (3-62%), PD-1 (1-35), TIM-3 (1-34) and GITR (0-35%) were highly heterogeneous across tumours. The analysis of the co-expression of AM demonstrated a substantial expansion of CD4+ TIL co-expressing ≥2 AM (cumulative frequency 14.4±13.9) in comparison to PBL (0.8±0.9) (Figure 2C). Moreover, the fraction of CD4+ TIL co-expressing ≥2 InR was also higher than in PBL (5.3±5.0 vs. 0.3±0.4) (Figure 2C). Among CD4+CD69+ TIL, 22±10% expressed PD-1 and 10±8% TIM-3 (Figure 2D). At the same time, 51±24% and 21±15% of the CD4+PD1+ TIL expressed CD69 or TIM-3, respectively (Figure 2D). The expression of CD69, PD-1 or TIM-3 was enhanced in EM (38.7±4.5, 17.5±2.3 and 26.5±5.5) and CM populations (21.7±3.48, 16.5±2.9 and 19.4±5.3, respectively), in comparison to naïve (6.3±1.9, 6.3±4.7 and 2.8±1.1) and EMRA (10.9±2.4, 4.7±1.3 and 4.2±1.0) CD4+ TIL. The fraction of CD4+ TIL expressing AM and InR in relation to their differentiation status is depicted in Figure 2E. Representative density plots of the co-expression of AM and InR in CD4+ PBL and TIL are depicted in Figure 2B.

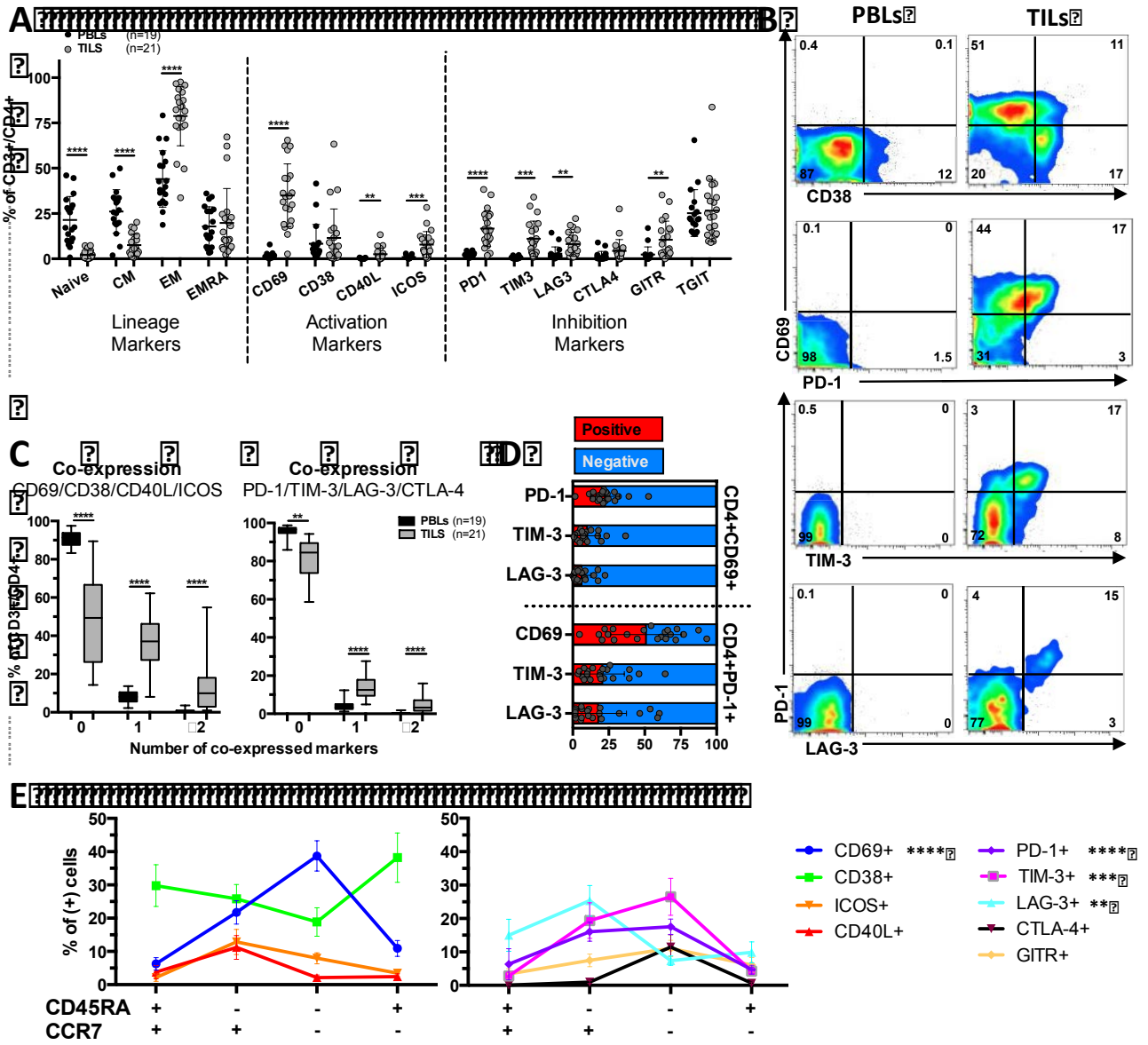

Figure 2. CD4+ ccRCC TIL exhibit particular phenotypic characteristics compared to PBL (**A**) Percentages of CD4+ PBL (black dots) and TIL (grey dots) expressing lineage, activation or inhibitory receptors (mean ± SD) in ccRCC-bearing patients (n=21). (**B**) Density plots on the co-expression of CD69, CD38 and PD-1 (upper panel), in addition to PD-1, TIM-3 and LAG-3 (lower panel) on the PBL and TIL from 1 representative patient. The cell percentage in each quadrant is displayed. (**C**) Percentages of PBL (black box) and TIL (grey box) expressing 0, 1 or ≥ 2 activation (up to 4, left panel) or inhibitory (right panel) markers. (**D**) Frequencies (mean and SD) of PD-1+, TIM-3+ and LAG-3+ cells among the CD4+CD69+ TIL (top); frequency of CD69+, TIM-3+ and LAG-3+ cells among the CD4+PD-1+ TIL (bottom). (**E**) Mean values (and SEM) of activation and inhibitory receptor expression in relation to the differentiation status of CD4+ TIL; colour code is displayed. **P < 0.01, ***P < 0.001, ***P < 0.0001, Mann-Whitney or Kruskal-Wallis test.

CD8+ TIL displayed lower frequencies of cells with a CM and naïve phenotype, but higher fractions of EM cells than PBL (Figure 3A). In addition, CD8+ TIL exhibited an enhanced expression of AM (CD69, CD38, CD40L and ICOS) and InR (PD-1, TIM-3 and LAG-3) but not GITR, in comparison to PBL (Figure 3A). Interestingly, we found reduced percentages of CD8+/TIGIT+ among TIL as compared to PBL (Figure 3A). CD69 was the receptor most expressed in CD8+ TIL compared with PBL (43.0±22.5), followed by CD38 (34.6±24.5), PD-1 (31.7±26.4) and TIM-3 (23.3±22.6) (Figure 3A). The analysis of the co-expression of AM demonstrated a substantial expansion of CD8+ TIL co-expressing ≥2 AM (cumulative frequency 25.1±20.8) in comparison to PBL (0.3±0.3) (Figure 3C). In addition, the fraction of CD8+ TIL co-expressing ≥2 InR was also higher than in PBL (46.3±28.4 vs. 10.3±8.7) (Figure 3C). Among CD8+CD69+ TIL, 35±24% expressed PD-1, 23±19% TIM-3 and 7.2±8.5% LAG-3 (Figure 3D). Meanwhile, 57±19%, 43±26% and 9.2±8.9% of the CD8+PD1+ TIL expressed CD69, TIM-3 or LAG-3, respectively (Figure 3D). The expression of CD69, CD38, PD-1 or TIM-3 was enhanced in EM (46.6±4.7, 37.6±5.5, 38.0±5.9 and 11.4±2.1) and CM populations (39.0±6.8, 31.9±6.7, 26.6±6.0 and 15.6±3.9, respectively), in comparison to naïve (7.8±2.1, 19.5±6.3, 0.6±0.3 and 3.7±1.7) and EMRA (15.9±2.6, 22.2±5.4, 4.0±0.9 and 11.1±3.4) CD8+ TIL. The fraction of CD8+ TIL expressing AM and InR in relation to their differentiation status is depicted in Figure 3E. Representative density plots of the co-expression of AM and InR in CD8+ PBL and TIL are depicted in Figure 3B.

We also analyzed the phenotype of KL. As compared to PBL, KL displayed an expansion of the CD4+ T cells demonstrating an EM phenotype, a contraction of the CM and Naïve compartments, a overexpression of AM (CD69, CD40L and ICOS) and InR (PD-1, TIM-3, LAG-3 but not GITR). No major differences were found compared to CD4+ TIL. Consistently, the CD8+ T KL displayed an expansion of the EM, a diminution of the CM and Naïve cells, an overexpression of AM (CD69 and CD40L) and InR (PD-1 and TIM-3). Compared to CD8+ TIL, CD8+ KL displayed a sub-expression of TIM-3n (data not shown).

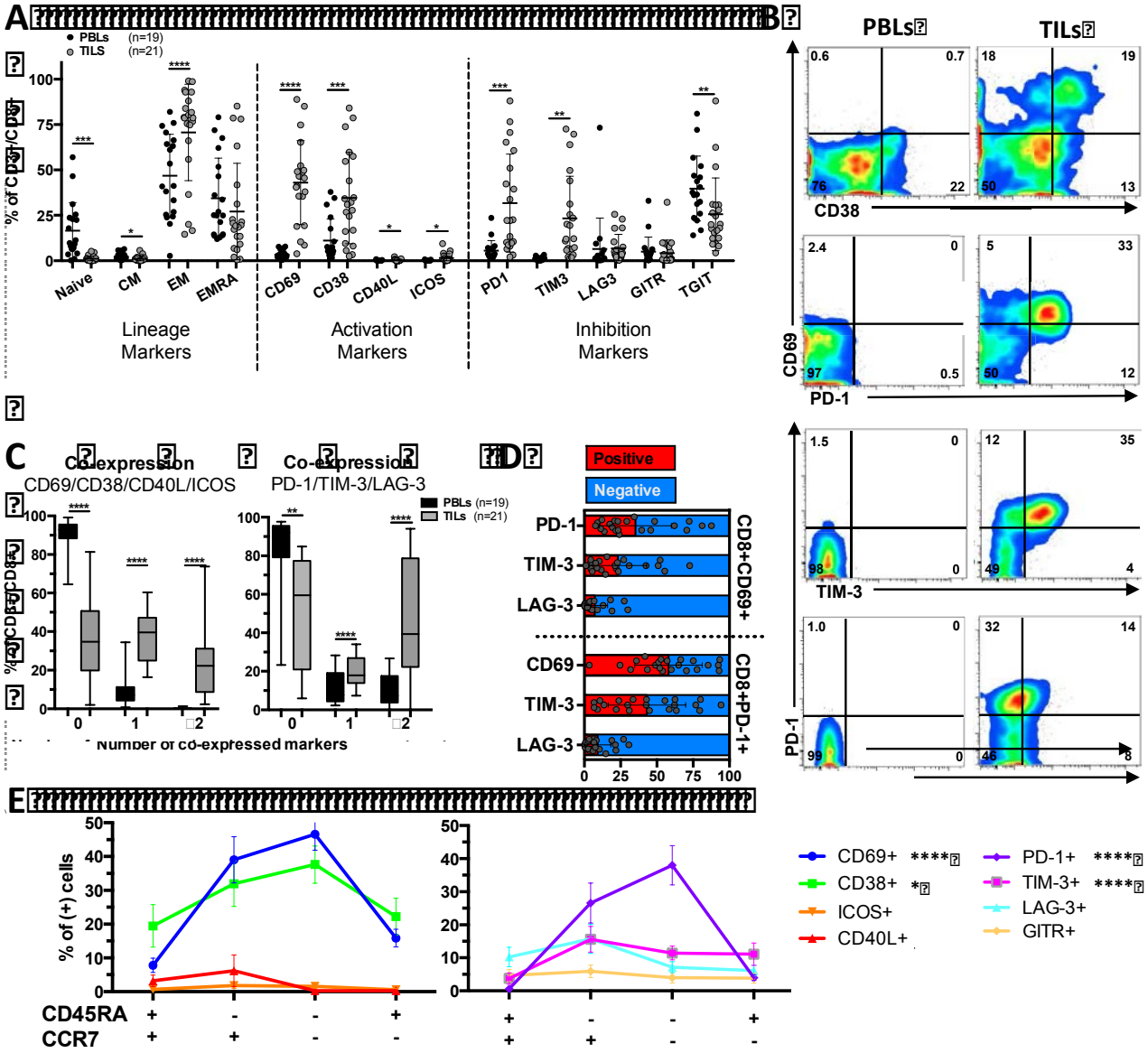

Figure 3. CD8+ ccRCC TIL exhibit particular phenotypic characteristics compared to PBL. (**A**) Percentages of CD8+ PBL (black dots) and TIL (grey dots) expressing lineage, activation or inhibitory receptors (mean ± SD) in ccRCC-bearing patients (n=21). (**B**) Density plots on the co-expression of CD69, CD38 and PD-1 (upper panel), in addition to PD-1, TIM-3 and LAG-3 (lower panel) on the and TIL from 1 representative ccRCC-bearing patient. The cell percentage in each quadrant is displayed. (**C**) Percentage of PBL (black box) and TIL (grey box) expressing 0, 1 or ≥ 2 activation (up to 4, left panel) or inhibitory (right panel) markers. (**D**) Frequencies (mean and SD) of PD-1+, TIM-3+ and LAG-3+ cells among the CD8+CD69+ TIL (top); frequency of CD69+, TIM-3+ and LAG-3+ cells among the CD8+PD-1+ TIL (bottom). (**E**) Mean values (and SEM) of activation and inhibitory receptor expression in relation to the differentiation status of CD8+ TIL; colour code is displayed. *P < 0.05, **P < 0.01, ***P < 0.001, ***P < 0.0001, Mann-Whitney or Kruskal-Wallis test.

A sub-group of ccRCC TIL exhibits an enhanced expression of inhibitory receptors

To further characterize the heterogeneous phenotype of the TIL in ccRCC we used an unsupervised approach including the fractions CD4+ and CD8+ TIL expressing the analyzed markers to sub-classify the tumours. We found two clusters of TIL, named 'Cluster A' (n=13, 61%) and 'Cluster B' (n=8, 39%) (Figure 4A). Principal component analysis revealed that while Cluster A shared major characteristics with KL, the TIL from Cluster B exhibited different traits compared to KL and PBL (Figure 4A). CD4+ or the CD8+ TIL from Cluster A did not displayed differences on the expression of the AM or InR when compared to KL, and thus were named *KL-like* (Figure 4B). When compared to autologous PBL, KL-like TIL displayed an enhanced expression of AM (CD69 and ICOS), a down-regulation of CD40L, and no significant changes in the expression of InR (data not shown).

In contrast, CD4+ TIL from Cluster B exhibited an enhanced expression of both AM (CD69 and CD38) and InR (PD-1, TIM-3, CTLA-4, GITR and TIGIT) compared to KL-like tumours and KLs (Figure 4B and 5C). Likewise, CD8+ TIL from the same cluster displayed increased fraction of cell expressing AM (CD69, CD38 and ICOS) and InR (PD-1 and TIM-3) (Figure 4B and 4C). The analysis of the co-expression of AM and InR across the two clusters revealed that the fraction of cells co-expressing AM and InR, in addition to those expressing InR and not AM, were augmented in Cluster B as compared to KL-like tumours; therefore, the former was named *Inhibited-like* (Figure 4D).

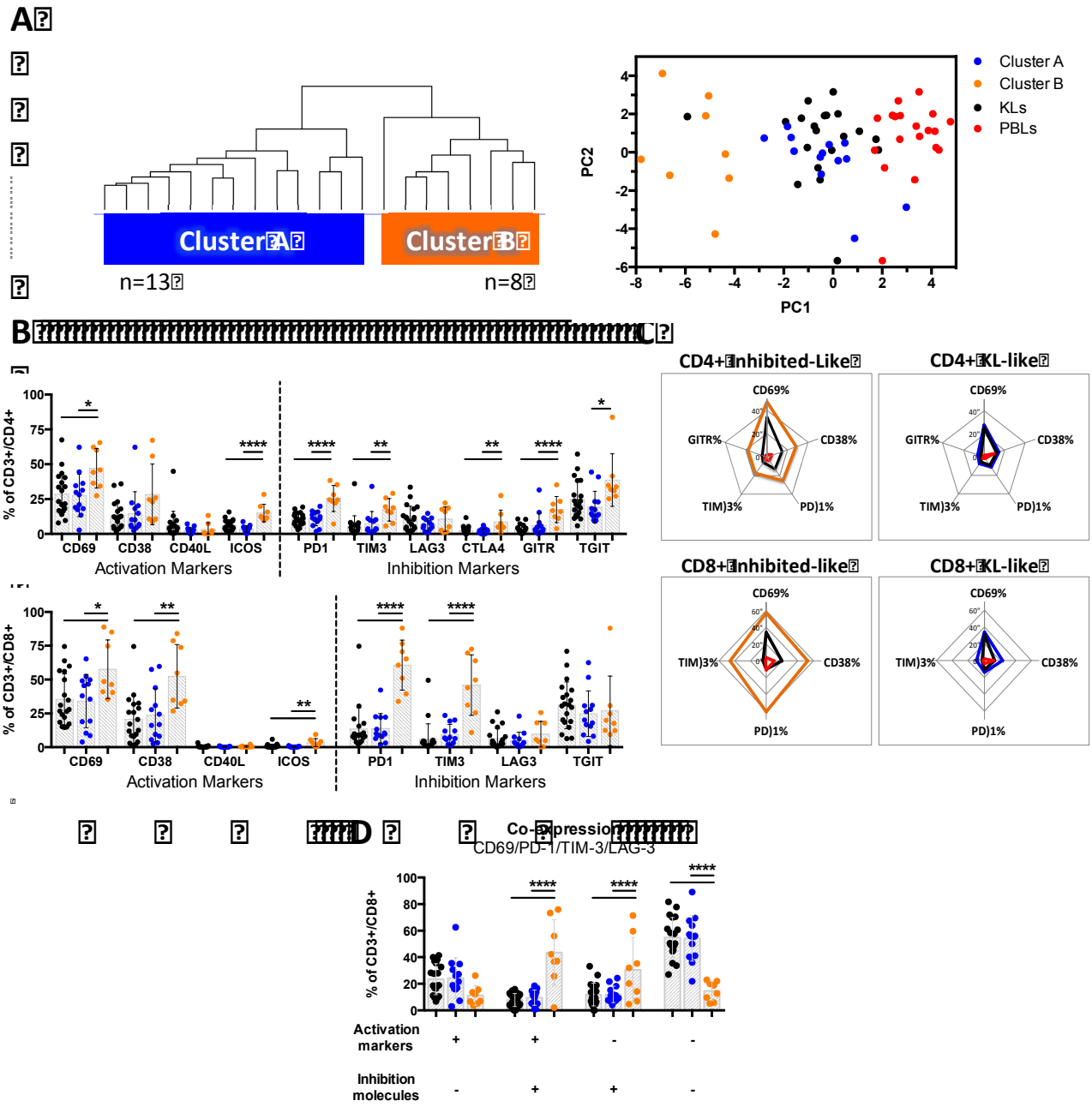

Figure 4. Two groups of TIL in ccRCC with distinct phenotypic traits (**A**) Unsupervised hierarchical clustering of the ccRCCs (right) and principal component analysis (left) based on the T cell phenotypes; TIL clusters (A blue and B orange squares and dots), KLs (black dots) and PBL (red dots). (**B**) Percentages of CD4+ and CD8+ T cells expressing activation or inhibitory receptors (mean ± SD) in KLs (black dots), Cluster A TIL (blue dots) and Cluster B TIL (orange dots) (n=21). (**C**) Radar chart displaying the percentages of CD4+ (upper panels) and CD8+ (lower panels) T cells expressing activation or inhibitory receptors among TIL (coloured lines), matched-KL (black lines) and PBL (red lines) in Cluster A (blue) and Cluster B (orange). (**D**) Dotplot displaying the fractions of CD8+ TIL co-expressing activation and/or inhibitory receptors in Cluster A and B, and KLs. *P < 0.05, **P < 0.01, ***P < 0.001, ***P < 0.0001, Mann-Whitney or Kruskal-Wallis test.

ccRCC shapes the phenotype of PBL

To investigate whether the phenotype of circulating T lymphocytes are different in ccRCC patients and healthy individuals, we compared the expression on AM and InR on PBL from 19 ccRCC-bearing patients and 5 healthy controls (HC). PCA analysis demonstrated differences between PBL from HC and ccRCC-patients (Figure 5A). The detailed analysis of the CD4+ T cells phenotype demonstrated that PBL from ccRCC-patients display increased fractions of cells expressing AM (CD69) and InR (PD-1, TIM-3, CTLA-4 and GITR) (Figure 5B). Similarly, in comparison to HC, CD8+ PBL from ccRCC-patients displayed an expansion of the subpopulations expressing AM (CD69 and ICOS) and InR (PD-1, TIM-3 and LAG-3) (Figure 5C). Interestingly, the expression of CD38 did not follow the same pattern, as it was enhanced in CD4+ (Figure 5B) and CD8+ (Figure 5C) PBL from HC compared to ccRCC-patients.

To assess if these phenotypic traits were related to particular features of the ccRCC tumour immune microenvironment rather than an unspecific systemic inflammation induced by cancer, we compared the PBL phenotype between patients with ccRCC and other non-clear cell RCC (nccRCC n=8, 5 oncocytomas, 2 papillary and 1 chromophobe tumour). Interestingly, CD4+ and CD8+ PBL from ccRCC-patients displayed enhanced expressions of PD-1 compared to nccRCC-patients (data not shown).

Finally, to assess how these phenotypic traits were modulated by the TME, we compared the phenotype of autologous PBL and TIL. We found that the percentages of TIL expressing AM or InR were positively correlated with those in PBL in the CD4+ (CD69, TIGIT, CD38 and PD-1) and CD8+ (PD-1, GITR, TIGIT, CD40L, CD38 and ICOS) compartments (Figure 5D). Figure 5E displays the correlation between the fraction of T cells expressing PD-1 among PBL and TIL.

Taking into account that ccRCC could shape the PBL' phenotype, we next wondered if the patients from each Cluster displayed different PBL phenotype. Interestingly, we found that patients with Inhibited-like tumours exhibited increased fractions of PB CD4+/PD-1+ and CD8+/PD-1+ cells when compared to those with KL-like tumours; this correlation was not found for any other marker (Figure 5F).

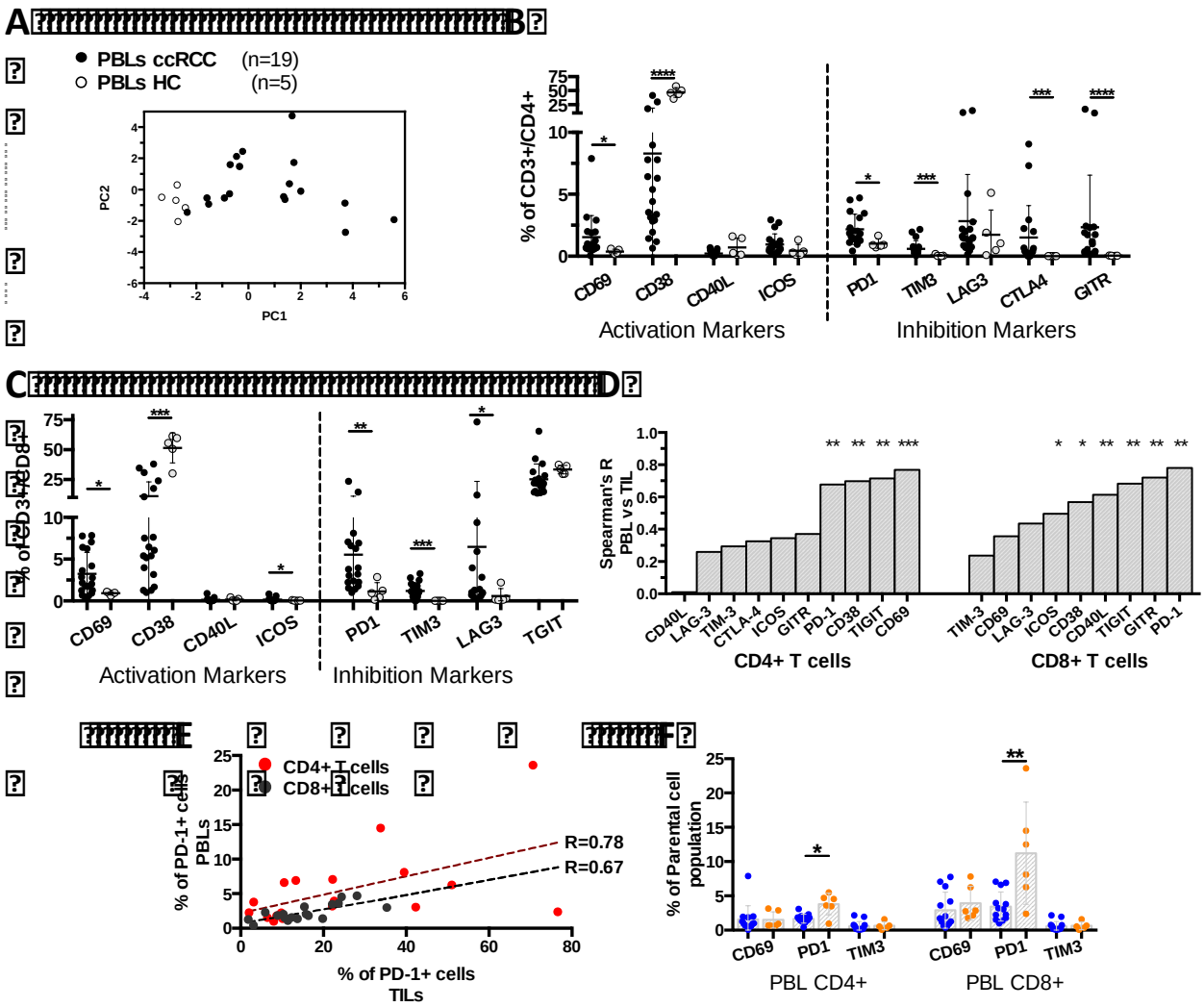

Figure 5. ccRCC shapes the phenotype of PBL. (**A**) Principal component analysis of the T cell phenotypes of PBL in HC (empty dots) and ccRCC-bearing patients (black dots). Percentages of CD4+ (**B**) and CD8+ (**C**) PBL expressing activation or inhibitory receptors (mean ± SD) in HC and ccRCC-bearing patients. (**D**) Spearman's R values for the correlation between the fractions of TIL and PBL expressing activation and inhibitory receptors. **(E)** Dot plot displaying the fractions of CD4+ (red dots) and CD8+ (grey dots) cells expressing PD-1 in the PBL (Y-axis) and TIL (X-axis) compartments; Spearman's R value for each regression is displayed. (**F**) Dotplot displaying the fractions of CD4+ and CD8+ PBL expressing activation or inhibitory receptors in KL-like (blue dots) and Inhibited-like (orange dots) ccRCC-bearing patients. *P < 0.05, **P < 0.01, ***P < 0.001, ***P < 0.0001, Mann-Whitney test.

Discussion Unpublished Results

In this study we found an over-representation of the ccRCC-memory TIL expressing AM and InR as compared to autologous PBL. These results are consistent with previous studies reporting an expansion of the CD69+, HLA-DR+ (324) (200) (325) (326) (328) (104), PD-1+, TIM-3 or LAG-3+ (333) (104) TIL as compared to autologous PBL in RCC-patients. In addition, in this study we found some interesting results that extend our understanding of the T cell behavior in ccRCC.

Regarding the ccRCC CD4+ TIL compartment, we found some unexpected phenotypic traits. First, we found an almost exclusive up-regulation of CD69 (early AM) and not of other AM such as CD38, CD40L and ICOS. The former result questions if CD4+ RCC-TIL have been activated through a TCR-dependent process, since multiples studies have suggested that the antigen-driven activated T cells express CD38 (453), while CD69 can be induced be several cytokines in the absence of TCR-activation (454). We also found a very limited expansion of the CD4+CD40L+ RCC TIL (2.5% of the population), which represents 20-fold less than CRC TIL (unpublished results). It seems possible that the dissociation between the expression of CD69 and CD40L in RCC CD4+ TIL reflects the lack of cytokines (e.g. IL-2 and IL-15) and co-stimulatory molecules (such as ICOSL) necessary for the induction/maintenance of the latter AM in the TME (455) (456) (457) (458). Furthermore, scarce studies have suggested that CD40 can be expressed by RCC tumour cells (459), and that could be a potential mechanism inducing the down-regulation of CD40L on CD4+TIL. This result is relevant since CD40L-CD40 interactions are crucial for DC and B cell differentiation, and T cell priming in cancer (460) (461). In addition to a dysfunctional and possibly unspecific CD4+ TIL activation, we also found a significant expansion of the CD4+ T cells expressing PD-1, GITR, LAG-3 and ICOS, a phenotype compatible with Treg (462). Further studies to confirm if this particular phenotype corresponds to Treg should be followed. Altogether, these preliminary results suggest that RCC CD4+ TIL display features of a poorly coordinated, yet enhanced, activation and a possible skewing towards a Treg phenotype.

In the other side, the CD8+ TIL compartment displayed an enhanced co-expression of CD69, CD38, PD-1 and TIM-3. The simultaneous up-regulation of AM and InR in the CD8+TIL in ccRCC challenges the idea that the expression of PD-1 is an exclusive feature of the "exhausted" T cells. Although in the context of chronic antigenic stimulation PD-1 up-regulation has been indeed related to CD8+ T cell exhaustion (39), current evidence suggests that under other inflammatory conditions PD-1 expression is not necessarily correlated with T-cell functional status (463); instead, it can be expressed at different stages of differentiation, from recently activated and highly cytotoxic (464) (465) (359) to exhausted and inhibited T lymphocytes (466). In this study we explored the co-expression of several InR (including PD-1 and TIM-3) and CD69 (an AM that is rapidly expressed and down-regulated upon T-cell activation) by ccRCC-TIL, to get insights of the contribution of T-cell activation in the expression of

InR in ccRCC. We found that more than half of the EM CD8+/PD-1+ TIL expressed CD69 (similar to TIM-3+ TIL), suggesting that the up-regulation of this InR is probably related to CD8+ TIL activation (463). The functional and cytotoxic status of the CD8+/PD-1+/CD69+ is currently unknown, and further research is needed to address this question. In addition, it is still unclear if the enhanced expression of CD69 and/or PD-1 represents a TCR-dependent activation of TIL, since both molecules can be also induced by an inflammatory microenvironment in the absence of TCR activation (454) (354) (247) (248) (249). Interestingly, we found a sub-group of tumours ('Inhibited-like') characterized by the expansion of CD8+CD69−PD-1+ T cells, more advanced T stages and a trend towards higher Fuhrman grades, suggesting that this population could potentially represent the exhausted TIL (data not shown). The same group of tumours was characterized by enhanced percentages of CD4+GITR+LAG3+ TIL, suggesting a highly inhibitory microenvironment. Interestingly, a second sub-group of tumours was found, characterized this time by an expansion of the EMRA population, an enhanced expression of CD69 and ICOS, a lack of CD40L expression and no changes in the InR in TIL as compared to autologous PBL. This phenotype highly resembled that of KL, suggesting that these cells could represent resident memory T cells that probably were not subjected to an inhibitory microenvironment (467), and therefore could be associated with an efficient anti-tumour immune response.

The virtual absence of CM CD4+ and CD8+ T cells in RCC (which is not found in other cancer such as CRC) and the lack of expression of CD40L in CD4+ (discussed above) and CD8+ TIL support a deficient TLS formation and T-cell priming in these tumours. Furthermore, recent evidence suggests that CD40L expression permits CD8+ T lymphocytes to execute immunologic helper functions (468), and the lack of expression of this AM in RCC as compared to other tumours (unpublished results) suggests this mechanism could be altered. The expansion of the EMRA population, particularly in the KL-like and KL, suggests that the kidney microenvironment could potentially induce a terminally differentiated phenotype on infiltrating T cell, that needs to be studied in detail.

Another interesting finding in our study was that the fractions of TIGIT+ TIL in the tumour were decreased in comparison to autologous CD4+ and CD8+ PBL. This is a striking result since in other neoplasias (e.g. melanoma) this populations is expanded within the tumour, and it has been suggested that they represent the tumour-specific TIL (469). The dissociation between the expression of CD69, PD-1 and TIGIT is striking and highlights the necessity of studying the mechanisms of induction of these molecules during T-cell activation and differentiation, in order to understand the ccRCC TIL biology.

Finally, a question that needs to be further addressed is the positive and strong correlation between the percentages of TIL and PBL expressing CD69, PD-1 and GITR. Although previous studies have reported that RCC patients displayed increased percentages of Treg and PD-1+ PBL (393) (363) (104)

(256) (389), it is still unknown known if these cells represent re-circulating TIL, or if they are induced by the systemic inflammatory response associated with cancer.

In summary, our results suggest that a sub-group of RCC is characterized by the expansion of TIL displaying a poorly coordinated (yet enhanced) activation, a possible skewing towards a CD4+Treg phenotype and more advanced tumour stages. This research should follow to further characterize this group of tumours, and understand their origin and natural history.

Discussion

RCC is characterized by an inflammatory microenvironment that simultaneously promotes the tumour development and hampers the in situ immune response. This project aimed to characterize the immune microenvironment associated with a Th1 and CTL immune response and its relation with patients' clinical outcome in ccRCC. This information was necessary to harmonize the gaps between several findings in RCC suggesting 1) a link between high densities of CD8+TIL, a Th1 gene signature and poor clinical outcome, 2) an altered function of the infiltrating DC and 3) a high sensitivity to anti–PD-1 agents.

Unifying the idea of increased CD8+ TIL densities and poor prognosis in ccRCC

In this study we found that the increased numbers of infiltrating CD8+ TIL were associated with poor clinical outcome in three independent ccRCC cohorts by quantitative IHC or gene expression analyses. To our knowledge, this is the third study describing such an association (175) (128), and supporting a positive correlation between increased densities of CD8+ TIL and higher Fuhrman grades in RCC (330) (329).

Previous studies have demonstrated that immune response in RCC is characterized by an enhanced infiltration CD8+ TIL (324) (200) (325) (326) (327) (328) (104) that, despite exhibiting an activated phenotype, are characterized by diminished proliferative capacities and lack of clonal expansion (337) (338) (326) (331) (332) (333). The current evidence suggests that the highly inflammatory RCC TME can be responsible of the enhanced T-cell infiltration (247) (248) (249), but simultaneously impedes the establishment of an effective anti-tumour immune response. Indeed, several studies have demonstrated that RCC tumour cells can express a wide range of molecules that attract T lymphocytes into the tumour (e.g. CCL4, CCL5, CXCL9-11 and CXCL16), among other immune cells (322) (319) (320) (321) (323). In addition, an inflammatory contexture in RCC has been linked with augmented T cell infiltrations; Beuselinck et al. analyzed the immune contexture of the four molecular sub-groups of ccRCC (ccrcc1-ccrcc4), and found that ccrcc4 tumours were associated with an inflammatory signature composed of genes associated with a CD8 and Th1 response, in addition to copious amounts of inflammatory/chemotactic/immunomodulatory transcripts (e.g. TNF, IRF family, IFNG, IL12, IL10, LAG3, PDCD1, PDL1 and PDL2) as well as genes associated with macrophages and blood vessels, and

the worst patients' clinical outcome (470). These results were confirmed by IHC, since ccrcc4 tumours displayed the highest densities of CD8+ T cells (470). Furthermore, unpublished results form our group showed that the densities and gene-signatures of macrophages, B and T cell are positively correlated in RCC, which supports an inflammatory-driven recruitment of lymphocytes. Contrariwise, in CRC the immune profile characterized by increased infiltration of CD8+ T cells, NK cells and scarce macrophages, are associated with early TNM stages and longer overall survival (116); in this case where the densities of cytotoxic and memory T cells are negatively correlated with macrophage infiltration, a more controlled and orchestrated immune response is likely.

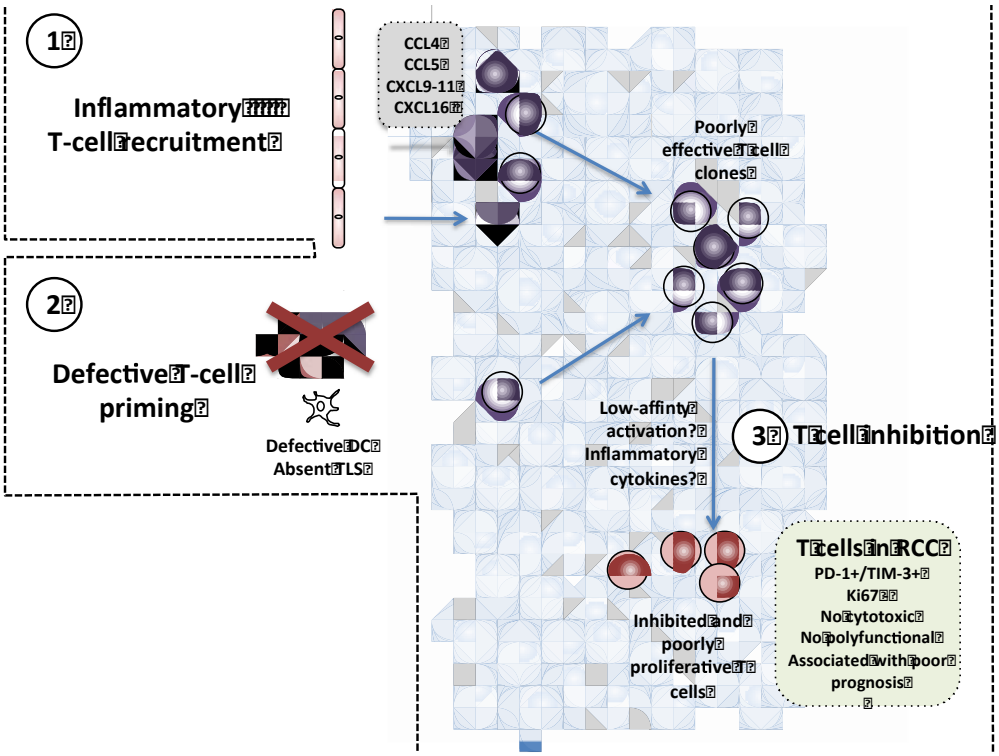

Figure 6. Potential mechanisms inducing overactive but defective T cell responses in RCC. Cartoon depiction of the possible mechanisms inducing an enhanced, yet inhibited, CD8+ TIL infiltration in RCC. In the top, the expression of inflammatory and chemotactic molecules by RCC tumour cells could potentially recruit no tumor-specific CD8+ T cells directly from blood vessels (**No. 1**). In the middle, the lack of TLS and mature DC impedes the T-cell priming and the expansion of tumour-specific high-affinity TIL clones (**No. 2**). Finally, T-cell activation under a highly inflammatory/inhibitory context could potentially induce the development of exhausted T cells (**No. 3**), that are associated with a poor clinical outcome.

Altogether these results suggest that the inflammatory RCC microenvironment can contribute to the enhanced recruitment of potentially no tumor-specific T cells (Figure 6). Nevertheless, once inside the tumour, CD8+ TIL can acquire different phenotypes and functional orientations depending of the TME. Interestingly, Nakano et al. described that while high CD8+ TIL densities were associated with

poor prognosis in RCC, increased infiltrations with CD8+/Ki67+ double-positive cells were correlated with the opposite clinical outcome (175). These results support that increased infiltrations with CD8+ TIL can be associated with multiple contextures, and only when they are actively proliferating, they are associated with good clinical outcome. In the present study, we demonstrated that the prognosis associated with increased CD8+ TIL densities in RCC could be modulated by the phenotype and localization of the DC, as well as the expression of immune checkpoint by the tumour and immune cells.

DC compartmentalization and orchestration of the immune response in ccRCC

As previously reported in all sub-types of RCC (310) (311) (312), in this study we found two different populations of DC infiltrating ccRCC: the first, in the tumour core isolated from other immune cells [accounting for roughly 70% of the DC and named Non-TLS (NTLS-DC)]; and the second, embedded within the peri-tumour TLS (named TLS-DC). Previous reports have suggested that while the first population represents immature and probably tolerogenic DC, the second displays an activated and mature phenotype and is capable of priming T cells (310) (313) (314) (312).

The quantification of NTLS-DC and TLS-DC allowed to identify tumours with different immune contextures but similar numbers of CD8+ TIL. Indeed, increased densities of TLS-DC identified a group of patients with high CD8+ TIL densities and low risk of recurrence and death. This finding suggests that the absence of TLS and mature DC impedes the intra-tumour T-cell priming and the development of an efficient immune response (33) (Figure 6). In favour of this hypothesis, we found that TLS-DC express high quantities of HLA-DR and CD83 and colocalize with PNAd+ vessels (HEV) suggesting they can indeed participate in the in situ T-cell education. Contrary to TLS-DC, NTLS-DC express low quantities of HLA-DR and CD83 and do not colocalize with PNAd+ vessels (HEV), suggesting they cannot participate in T-cell priming. Its seems feasible that NTLS-DC accumulation in RCC could be related to an inflammatory milieu given that previous reports described that the presence of inflammatory cytokines such as TNF, TGF-β, IL-6, IL-8 and VEGF can both recruit and induce tolerogenic DC in RCC (315) (313) (316) (312) (317). In this case, and as for CD8+ TIL, enhanced NTLS-DC infiltrations might denote an inflammatory milieu instead of a tumour-specific immune response. Previous studies have demonstrated that particular populations of RCC-infiltrating DC can express pro-tumour and CTL-inhibiting molecules (312). To get insights in the role of NTLS-DC in RCC development and spreading, it would be interesting to assess the functional and cytokine profile of the NTLS-DC as compared to TLS-DC. A cartoon depicting the phenotype, potential molecules associated with their development and clinical impact of the DC populations in RCC is illustrated in Figure 7.

As previously mentioned, HEV and conventional vessels can help discriminating between TLS-DC and NTLS-DC. Compared to conventional blood vessels, HEV are more appropriate in T-cell recruitment due to the abundant expression of ICAM1, highly glycosylated/sulphated sialomucins and chemokines such as CCL19, CCL21, CXCL12 and CXCL13 (471). In tumours, there seem to be a symbiotic interaction between mature DC and HEV: in the one hand, HEV induce the recruitment and accumulation of mature DC through the secretion of diverse chemokine including CXCL13, CCL19 and CCL21; in the other hand, mature DC help maintaining the HEV phenotype (probably through mechanisms dependent of LTa) (472). Both type of cells efficiently induce the recruitment of naïve lymphocytes into the tumor.

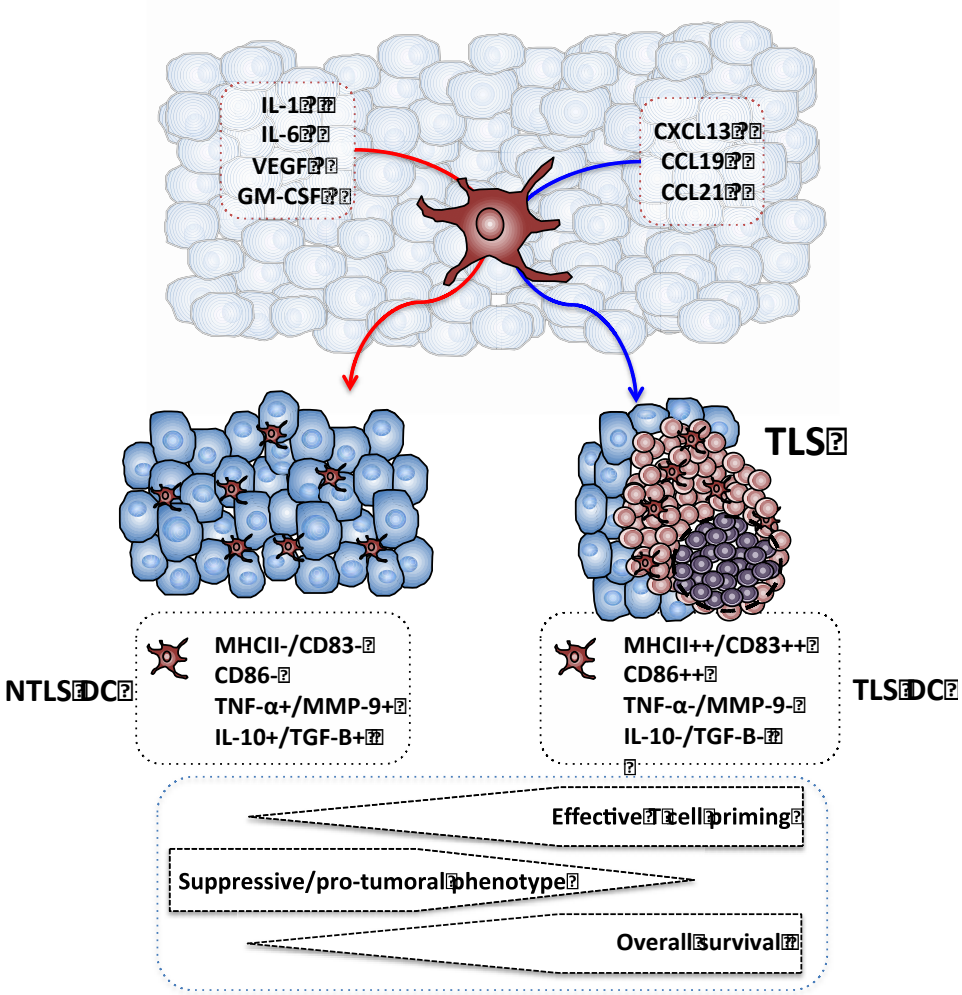

Figure 7. DC compartmentalization, T-cell priming and prognosis in RCC. Cartoon depiction of the two major types of DC in ccRCC. On the left, the expression of inflammatory molecules can induce the stromal accumulation of immature DC, characterized the production of pro-tumoural molecules and poorly effective T-cell priming capacities. On the right, the presence of CXCL13, CCL19 and CCL21 can potentially promote the development of DC within TLS, exhibiting a mature phenotype and capable of inducing T-cell priming.

Immune checkpoints, CD8+ T cells and prognosis in ccRCC

In view of the elevated sensitivity of RCC to PD-1 blocking agents (207) (208), we also wondered how does the expression of immune checkpoints by TIL and tumour cells could modulate the prognosis associated with CD8+ lymphocyte infiltration in RCC. We demonstrated that RCC CD8+ TIL often co-express PD-1 and LAG-3, and that their expression was associated with poor clinical outcome. Interestingly, patients whose tumours exhibited both high densities of PD-1+ lymphocytes and PD-L1+ and/or PD-L2+ tumour cells had the worst prognosis. In addition, we found a dissociation between the infiltration with TLS-DC and the expression immune checkpoints.

Previous studies have already demonstrated an association between the enhanced expressions of PD-1 or its ligands in RCC and patients' poor clinical outcome (366) (367) (368) (361) (362). In addition to supporting these findings, this study increases our understanding of the mechanisms inhibiting the T cell responses in ccRCC in several ways:

1. First, in 3 independent cohorts, we demonstrated through gene expression analysis, IHC and FC that relevant fractions of RCC CD8+ TIL express inhibitory receptors. Nevertheless, our data suggest that the sole expression of PD-1 on T cells is probably not sufficient to inhibit their anti-tumour activity; instead, an inhibitory microenvironment characterized by the expression of PD-1 ligands must be present for this purpose.
2. Second, the densities of TLS-DC are higher in tumours that lack tumour expression of PD-1 ligands. This result suggests that the expression of PD-L1 and L2 could have a common origin with the dysfunctional TLS development.
3. And third, our results suggest a potential role of PD-L2 and LAG-3 expression in the inhibition of CD8+ TIL in ccRCC.

The mechanisms inducing the expression of PD-1 in the RCC TIL are poorly understood. While some works in melanoma suggest PD-1 is expressed in TIL upon TCR activation (204) (357), other studies suggest that its expression could be induced by the interaction of γ-chain cytokines or IFN-α with their receptors on T cells (354) (355). Interestingly, previous studies have demonstrated that the gene expression of PD-1, TGFB1-3, IL10 and TNF (and not IL-2 or IL-4) are highly correlated in ccRCC (470). Whether the expression of PD-1 in RCC TIL represents the activation of high-affinity tumour-specific lymphocytes within an inhibitory milieu, or the indirect action of an inflammatory microenvironment on T cells in the context of low-affinity TCR interactions, remains to be determined (Figure 6).

As for CD8+ TIL, increased densities of PD-1+ TIL can also be accompanied by different immune contextures in RCC. The group of tumours characterized by a high expression of PD-1 ligands in the absence of fully functional mature DC is associated with poor prognosis. A second group, displaying low or nonexistent expression of PD-1 ligands and abundant mature TLS-DC, exhibited a good clinical outcome. In addition to the phenotype and localisation of DC within the TME, the expression of PD-1 ligands in the TME seems to modulate the prognostic significance of PD-1+ and CD8+ cells in RCC.

We did not investigate in detail the mechanisms responsible for the induction of the expression of PD-L1 and PD-L2 in RCC. Nevertheless, the strong correlation between the expression IFNG and PD-L2 genes suggests a potential mechanism of PD-L2 up-regulation in cancer that, to the best of our knowledge, has only be described in immune cells (473). The weaker correlation between IFNG and PD-L1 transcripts confirms that the latter molecule could be induced by other mechanisms, such as an hypoxic microenvironment (320) (372) (373). In addition, we did not investigated the expression of PD-1 ligands in the myeloid cell compartment, and this is a question that need to be addressed in the future.

Finally, our results suggest PD-L2 could play an important role in the inhibition of CTL responses, but this finding needs to be confirmed in larger cohorts. A couple of observations from clinical trials support PD-L2 could be expressed in RCC: on the one hand, the response rates among patients with advanced RCC goes from 10% when using drugs inhibiting just PD-L1 (208) to 25% when inhibiting the PD-1 receptor (207); and, on the other hand, although very few, there are PD-L1 negative tumours that respond to anti-PD-1 treatment (207). To date, only one additional study has assessed the expression of PD-L2 in RCC, and found that its expression was limited to 1 out of 6 tumours (213). In view of these results, efforts should be made in order to precisely characterize the expression of PD-L2 in RCC, and confirm its role in the inhibition of CTL responses.

Towards the identification of the tumours with a suppressive microenvironment in ccRCC

All these results suggest that a sub-group of ccRCC is characterized by an enhanced and probably inflammatory-driven immune response, which is inefficient due to the expression of inhibitory molecules in the TME and/or the deficient orchestration of T cell priming. Our analysis in the retrospective cohort demonstrated that approximately 20-25% of primary and metastatic tumours display these characteristics. Consistently, the molecular sub-group displaying a similar immune profile (ccrcc4) represents 20% of all tumours (470). In addition, preliminary results on the ccRCC TIL phenotype suggest that a subgroup of tumours (representing roughly 35-40%) shares many of these features, including the over-expression of immune checkpoints, higher CD8+ TIL densities by IHC,

more advanced tumour and nuclear grades. Interestingly, a recent study suggested that an increased tumor cell expression of PD-L1 and high counts of CD8-positive T cells were associated with shorter survival in patients with metastatic RCC receiving VEGF-targeted agents (474). The potential three different immune contextures one can find in ccRCC are depicted in Figure 8.

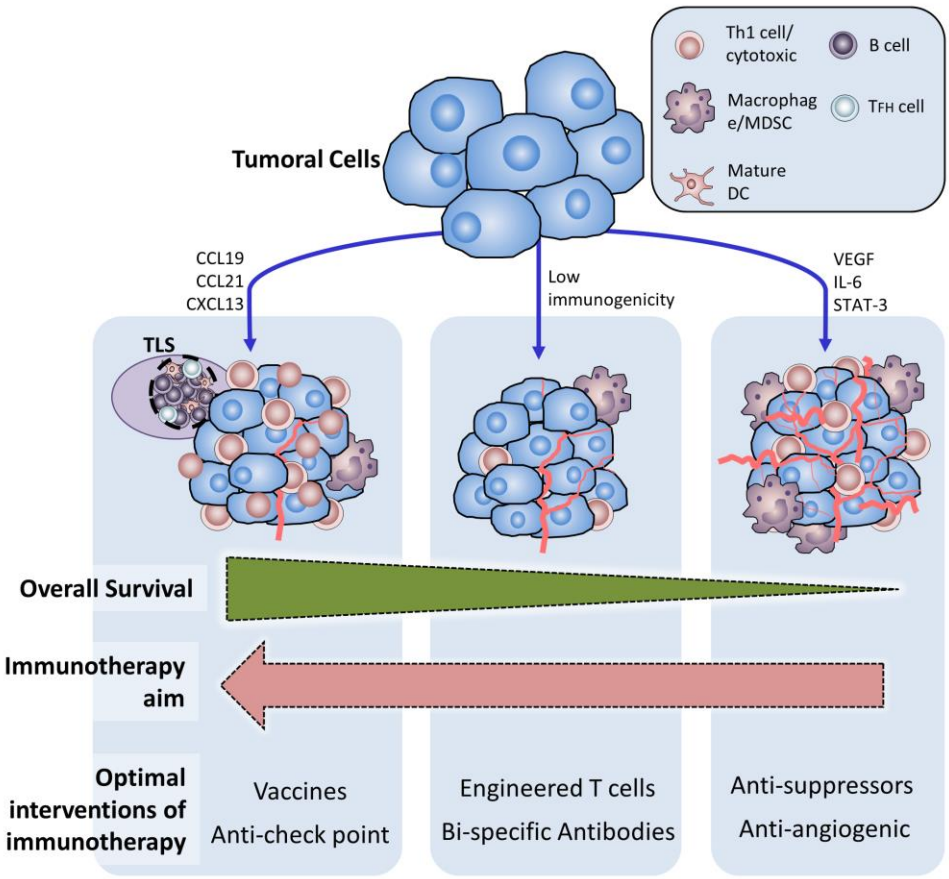

Figure 8. ccRCC immune contexture scenarios and personalized medicine for immunotherapy. Cartoon depiction of the major immune contexture scenarios for ccRCC. A strong immune response (left) can be induced by the presence of CX3CL1, CXCL9 and CXCL10 and is characterized by Th1/Cytotoxic T cells infiltration and the presence of Tertiary Lymphoid Structures (TLS), while associated with a highest overall survival. In the other extreme, those tumours associated with high expression of IL-6, VEGF-A and STAT-3 induce a rather inflammatory and pro-angiogenic environment (right) associated with poor overall survival. Knowing the possible scenarios of the immune contexture allows to use the optimal immunotherapy treatment in each case, to lastly induce an appropriate immune response in the tumour microenvironment (475).

Although preliminary, these results provide important clues to identify the sub-group of ccRCC characterized by an inflammatory and inhibited immune microenvironment, which can potentially help in predicting the patients' clinical outcome and choosing the most suitable therapy. Interestingly, the

phase 1 studies on the effect of Anti-PD-1 treatment (nivolumab) on patients with advanced RCC have shown an objective response of approximately 30% (207). It seems feasible that the group of patients responding to checkpoint blockade is relevantly enriched in tumours displaying this inhibitory immune profile, and further efforts should be made to validate their clinical utility in larger and independent cohorts. In agreement with this hypothesis, preliminary data from clinical trials of PD-1 blockade in other cancers suggest that the presence of: 1) infiltrating CD8+ or PD-1+ T cells (212) and/or 2) PD-L1+ tumour (213) (207) (210) or immune cells (211) (214), are the more sensitive parameters to predict the patients' response to treatment (215).

Interestingly, the patients whose tumours exhibited an inhibitory immune profile also carried higher percentages of PD-1+ T cell in their PB. These preliminary results suggest that the immunophenotypic profile of PBL could be a potential tool to identify the patients with an inhibited tumour immune microenvironment. Consistently with our results, one study previously showed that the fractions of PD-1+ T cells are expanded in the PB of patients with RCC compared to HC, difference that is lost after tumourectomy (363). The analysis of the phenotype of other circulating immune cells in RCC-bearing patients has suggested that the tumour influences the circulating monocytes (293), and contribute to the expansion of CD4+ PB T cells with a Treg (104) (256) (389) or Th2 orientation (383). The expansion of all the aforementioned immune populations has been related with advanced tumours stages and poor prognosis.

In addition, the origin of these circulating immune cells is poorly understood. In our study, although there is an expansion PBL expressing PD-1 in patients whose tumours present an inhibitory profile, the PD-1+ lymphocytes from PB and tumour TI display a different phenotype. It is likely that circulating PD-1+ cells could represent recirculating TIL, that under the influence of a different microenvironment, change their phenotype. These findings need further research.

Revisiting the immunoscore

The 'Immunoscore' (technique to quantify the in situ CD8+ and CD3+ cell infiltrate in cancer) has shown promising results in the prediction of the patients' clinical outcome in several cancers (180). Nevertheless, our results invites to revise the general dogma that CD8+ T-cell infiltration is associated with a diminished risk of progression and death across all tumours (26). In this study we found that increased CD8+ TIL densities can be accompanied by strikingly different immune contextures, notably a bona fide or an inhibitory immune profile. We propose that the latter is overrepresented in RCC as compared to other tumours, biasing the prognostic significance of CD8+ TIL when they are analyzed out of their context. Interestingly, a similar picture has been described in NSCLC, where high CD8+ T cell infiltration accompanied with low density of TLS-DC are associated with patients' poor prognosis

(167). In addition, other studies have also suggested that CTL or Th1-signatures could be associated with poor outcome in Hodgkin lymphoma (476) (174), also a pathology highly sensitive to anti-PD-1 treatments (210).

These data provide evidence supporting the complexity of the immune contextures in RCC, and the difficulties behind its one-dimensional analysis. The inclusion of more adapted parameters should be considered in the current routine analysis of the tumour immune contexture, comprising DC infiltration, the characterization of TLS and the expression of immune checkpoints, to correctly predict patients' clinical outcome and response to immunotherapies.

Conclusions

This work has allowed us to conclude that:

I. Increased densities of CD8+ TIL are associated with patients' poor clinical outcome in primary and metastatic ccRCC.

II. The phenotype and localization of DC, as well as the expression of immune checkpoints (PD-1, LAG-3, PD-L1 and PD-L2) in the tumour microenvironment, modulate the prognosis associated with increased infiltrations with CD8+ TIL in ccRCC:
    a. While intra-tumour immature DC (NTLS-DC) were associated with poor clinical outcome, high densities of mature DC within lymphoid aggregates (TLS-DC) identify a group of patients with prolonged survival and high CD8+ TIL densities.
    b. Patients whose tumours exhibited both high densities of PD-1+ TIL and PD-L1+ and/or PD-L2+ tumour cells have the worst prognosis.

III. Three main immune contextures with different clinical outcomes were found in primary ccRCC:
    a. The first, characterized by low densities of TIL and moderated expression of inhibition or activation molecules, was associated with early tumour stages and good clinical outcome.
    b. The second, characterized by increased numbers of CD8+ TIL, high densities of TLS-DC and no expression of PD-1 ligands on tumour cells, was associated with early tumour stages and good clinical outcome.
    c. The third, characterized by increased numbers of CD8+ TIL, but low quantities of TLS-DC and enhanced expression of immune checkpoints in TIL and PD-1 ligands on tumour cells, was associated with advanced tumour stages and poor clinical outcome.

Perspectives and Limitations of the Study

This study is descriptive and provides modest insights into the mechanisms behind the inhibition of CTL response in ccRCC. Thus, several questions remind open and need to be further addressed.

First, regarding the impact of TLS-DC infiltration in CD8+ T cell education in RCC:

1. Is the functional and proliferative capacity of CD8+ TIL associated with the presence and phenotype of DC in RCC? Furthermore, does the clonality of T cells change in function of the infiltration with TLS-DC and an orchestrated immune response?

Second, although we proposed diverse mechanisms triggering the compartmentalization of DC in RCC, there is a necessity to confirm:

2. Which are the main mediators inducing an orchestrated immune response and the differentiation of DC into TLS-DC or NTLS-DC in RCC?
3. And, which is the phenotype of NTLS-DC and its potential pro-tumour or inhibitory role in RCC.

Third, recent evidence suggests that the expression of PD-1 in TIL can identify the tumour-specific lymphocytes in melanoma. This concept is consistent with the fact that the block of PD-1 receptor induces strong anti-tumour immune response in a sub-set of patients with RCC. Nevertheless, it is difficult to understand how PD-1+ cell could be associated with poor clinical outcome in RCC. This study makes evident the need to know:

4. Which are the signals inducing an enhanced expression of PD-1 in TIL and PD-1 ligands in tumour cells, particularly PD-L2, and
5. Whether the expression of PD-1 in RCC TIL represents the activation of high-affinity tumour-specific lymphocytes within an inhibitory milieu, or the direct action of an inflammatory microenvironment on T cells in the context of low-affinity TCR interactions.

Recent evidence also suggests that the expression of PD-1 ligands by myeloid-derived cells could play a central role in the inhibition of the CTL response in tumours. Therefore, it is relevant to determine:

6. Which is the role of the expression of PD-1 ligands by immune cells in the inhibition of the CTL responses in RCC?

Finally, we characterized a group of ccRCC with a highly inhibited immune profile. We consider highly relevant to determine:

7. The potential use of this immune profile as a potential biomarker for predicting the response to checkpoint blockade, particularly to anti-PD-1 treatments.

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