Characteristics and clinical impacts of the immune environments in colorectal and renal cell carcinoma lung metastases: influence of tumor origin

Romain Remark1,2,3, Marco Alifano3,5, Isabelle Cremer1,2,3, Audrey Lupo1,2,3,4,5, Marie-Caroline Dieu-Nojean1,2,3, Marc Riquet3,6, Lucile Crozet1,2,3, Hanane Ouakrim1,2,3, Jeremy Goe1,2,3, Aurélie Cazes3,6, Jean-François Fléjou2,7, Laure Gibault3,8, Virginie Verkarre3,9, Jean-François Régnard3,5, Olivier-Nicolas Pagès5, Stéphane Oudard3,6, Bernhard Mlecnik1,2,3, Catherine Sautès-Fridman1,2,3, Wolf-Herman Fridman1,2,3,10,§, and Diane Damotte1,2,3,5,§.

1Institut National de la Santé et de la Recherche Médicale (INSERM), U872, Centre de Recherche des Cordeliers, Paris, France; 2Université Pierre et Marie Curie-Paris 6, UMRS 872; 3Université Paris Descartes-Paris 5, UMRS 872; 4Université Denis Diderot-Paris 7; 5Services d'anatomie-pathologique et de chirurgie thoracique, Hôpital Hôtel Dieu, AP-HP, Paris, France; 6Services d'anatomie-pathologique, oncologie et de chirurgie thoracique, Hôpital Européen Georges Pompidou, AP-HP, Paris, France; 7Service d'anatomie-pathologique, Hôpital Saint-Antoine, AP-HP, Paris, France; 8Service d'anatomie-pathologique, Hôpital Cochin, AP-HP, Paris, France; 9Service d'anatomie-pathologique, Hôpital Necker-Enfants Malades, AP-HP, Paris, France and 10Service d'Immunologie Biologique, Hôpital Européen Georges Pompidou, AP-HP, Paris, France.

§These authors contributed equally to this work.

Corresponding author:
Diane Damotte, M.D.-Ph.D., INSERM U872, Centre de Recherche des Cordeliers, 15 rue de l’Ecole de Médecine, 75006 Paris, France
Phone: +33 1 42 34 87 12
Fax: +33 1 42 34 86 41
E-mail: diane.damotte@htd.aphp.fr

Conflicts of interest: There are no competing interest, no conflict to disclose.

Running title: In situ immune reaction in lung metastases

Key words: lung metastases, colorectal carcinoma, renal cell carcinoma, lymphocytes, prognosis.
Abbreviations:

CEA: carcinoembryonic antigen
CRC: colorectal cancer
CT: center of the tumor
DC: dendritic cell
DFI: disease-free interval
IM: invasive margin
LLN: lower limit of normal
LM: lung metastasis
NK: natural killer
OS: overall survival
PT: primary tumor
RCC: renal cell carcinoma
TLS: tertiary lymphoid structure
ULN: upper limit of normal


Statement of translational relevance:

Demonstration for the first time, in a large cohort of patients with lung metastasis from two different primary tumors, i.e. colorectal and renal cell carcinoma, that densities of CD8\(^+\) T cells and DC-LAMP\(^+\) mature dendritic cells (“immune pattern”), evaluated in paraffin sections, were independent prognostic factors of patients’ survival, and stronger prognosticators than currently evaluated clinical and pathological parameters. Furthermore, tumor immune environment is reproduced throughout cancer disease, from primary tumor to relapsing metastasis. This finding is the first important step for further extensive studies on the role of the tumor cells in shaping their own immune environment and the patients’ outcome.
Abstract

Purpose:

If immune cells are involved in tumor surveillance and have a prognostic impact in most primary tumors, little is known about their significance in metastases. Since patient’s survival is heterogeneous, even at metastatic stages, we hypothesized that immune cells may be involved in the control of metastases. We therefore characterized the tumor immune microenvironment and its prognostic value in colorectal (CRC) and renal cell carcinoma (RCC) metastases, and compared it to primary tumors.

Experimental Design:

We analyzed by immunohistochemistry (n=192) and qPCR (n=32) the immune environments of CRC and RCC lung metastases.

Results:

Metastases from CRC and RCC have different immune infiltrates. Higher densities of DC-LAMP+ mature dendritic cells (P<0.0001) and lower densities of NKp46+ NK cells (P<0.0001) were observed in CRC as compared to RCC metastases, whereas densities of T cells were similar. High densities of CD8+ and DC-LAMP+ cells correlated with longer overall survival (OS) in CRC (P=0.008) and shorter OS in RCC (P<0.0001). High NK cell densities were associated with improved survival in RCC (P=0.002) but not in CRC. Densities of immune cells correlated significantly from primary to relapsing metastases for the same patient. A Th1 orientation was found in CRC metastases, whereas a heterogeneous immune gene expression was found in RCC metastases.
Conclusions:

Our results demonstrate a major prognostic value of the immune pattern (CD8+/DC-LAMP+ cell densities) in CRC and RCC, reproducible from primary to metastatic tumors, although with opposite clinical impacts, and highlight the role of the tumor cell in shaping its immune environment.
Introduction

Immune cells are found in human solid tumors, and the immune pattern of the tumor microenvironment is a major predictor of patient survival in a large array of primary tumors (1). Thus, a high density of T cells with a Th1 and CD8+ T cells cytotoxic orientation or of mature dendritic cells (DC) are beneficial in most cancers, especially in colorectal (2-4), lung (5), breast (6), gastric (7), pancreatic (8), urothelial (9), hepatocellular (10), esophageal (11), ovarian cancer (12) and melanoma (13), with the exception of RCC in which high densities of CD8+ and CD45RO+ cells are associated with poor prognosis (14, 15).

Even if metastatic spreading is the main cause of death by cancer (16), metastatic patients have heterogeneous survival (17). A classical view of cancer progression is that genetic modifications (18) may allow malignant cells to escape local and systemic immune control (19) and consequently invade and metastasize in distant organs. This hypothesis would predict that the immune microenvironment in metastatic sites should be poor and have no impact on clinical outcome. Only a limited number of studies have reported the presence of immune cells in metastatic lesions. They showed that high densities of CD8+ T cells were associated with longer survival in CRC (20) and ovarian cancer (21), and potential response to chemotherapy in liver metastases from CRC (22). Another question, which remains largely unanswered, concerns the respective roles of the malignant cells and the seeding organ in shaping the immune microenvironment.

We therefore analyzed the immune environment of CRC and RCC metastases seeded in a same organ, the lung, compared coincident and relapsing metastases in the lung and the primary tumor from the same patients, and determined their clinical impacts.
We report that tumor cells induce a characteristic and reproducible immune pattern in the primary and metastatic tumors, supporting the hypothesis that the malignant cells, rather than the host organ, shape their microenvironment. We found that a high infiltration by DC-LAMP⁺ mature DC and CD8⁺ T cells is a major predictor of good survival in lung metastases from CRC, whereas it is associated with poor survival in lung metastases from RCC. This demonstrates that the immune microenvironment pattern remains a major prognostic factor even in advanced cancer stages, but with different consequences depending on the origin of the primary tumor. Altogether our results suggest a strong influence of the tumor origin on the immune environment characteristics and clinical impact.
Patients and methods

Patients

We constituted a retrospective and unselected cohort of 140 patients with CRC lung metastasis operated at Hôtel-Dieu hospital between 2000 and 2010 and 52 patients with RCC lung metastasis, operated at Hôtel-Dieu or Laennec/Hôpital Européen Georges Pompidou hospitals between 1992 and 2010. In the RCC series, 51 out of 52 patients were treated with radical nephrectomy and one with partial nephrectomy. None of the patients had signs of local recurrence of primary tumor. We also analyzed 25 CRC and 24 RCC primary tumors from the same patients, operated at Saint-Antoine, Cochin or Necker-Enfants Malades hospitals between 1987 and 2008. In addition, 14 coincident and 12 recurrent CRC lung metastases from the same patients were studied. Altogether, 218 lung metastases from 192 patients were analyzed.

Among these 192 patients, 32 frozen samples of lung metastases were available for patients with CRC (n=19) or RCC (n=13).

Baseline characteristics of these patients are summarized in supplementary tables S1 and S2.

All experiments were performed with the agreement of the Ile de France II ethics committee (n° 2012-0612).

Immunohistochemistry

For each tumor two observers (R.R. and D.D., A.L., A.C, L.G. or V.V., expert pathologists) selected the tumor section containing the highest density of immune cells on hematoxylin and eosin-stained slides. Serial 5-µm formalin-fixed and paraffin-embedded tissue sections were...
stained with autostainer Link 48 (Dako). Tissue sections were incubated with primary antibodies (CD3 polyclonal antibody (Dako), CD8 (SP16, Springbioscience), CD20 (L26, Dako), DC-LAMP (1010.01, Dendritics), granzyme B (11F1, Novocastra), NKp46 (195314, R&D Systems) or PNAd (MECA-79, BD Pharmingen)) followed by secondary antibodies coupled with biotin or alkaline phosphatase. Biotinylated antibodies were coupled with streptavidin-peroxidase and peroxidase activity was revealed using 3-amino-9-ethylcarbazole substrate (Vector Laboratories). Alkaline phosphatase activity was revealed using alkaline phosphatase substrate III (Vector Laboratories).

The density of DC-LAMP+ cells was manually counted on the entire section as previously described (23). CD3+, CD8+, granzyme B+ and NKp46+ cells were counted in the center of the tumor (CT) and in the invasive margin (IM) of the tumor with the convergence to the mean method (24). For each slide, 40 to 100 high power fields (1.37 to 3.43 mm²) were examined on each tumor zone. Both immunostaining and scoring were evaluated by three independent observers blinded to clinical data (R.R., L.C. and A.L., expert pathologist).

Gene expression analyses

RNA from the frozen tissues of 32 lung metastases was extracted with the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions and controlled for quantity and quality on an Agilent 2100 Bioanalyser (Agilent Technologies). Then, RT-PCR was performed with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystem). Finally, the quantitative gene expression analysis of selected targets was performed in duplicates with the TaqMan Human Immune Array on an Applied Biosystems 7900HT Fast Real-Time PCR
Expression levels of genes were determined using threshold cycle (Ct) values normalized to actin B (ΔCt) and were represented using the Genesis program.

Statistical analyses

We used the Mann-Whitney test to compare the densities of infiltrating immune cells in the different tumors and ΔCt, and the Wilcoxon matched pairs test to compare the density of infiltrating immune cells in different tumors from the same patient. Since all gene expression comparisons were pre-planned and the 51 genes clustered according to their immune functions before analysis, the P values were not corrected by Bonferroni or similar methods. Correlations were evaluated by the Spearman test. OS curves were estimated by Kaplan-Meier method and differences between the groups of patients were calculated using the log-rank test. The start of follow-up for OS was the time of lung surgery. In addition to mature DC, CD8+ T cells and NK cells densities, the following available clinical parameters were tested: initial stage (CRC), completeness of resection at pulmonary level, number of lung metastases, presence of extra-thoracic metastases at time of lung surgery, thoracic lymph node invasion, CEA level (CRC), initial Fuhrman nuclear grade (RCC), presence of metastases at presentation (RCC), time from lung metastasis diagnosis to surgery (RCC), disease-free interval (RCC), alkaline phosphatase, hemoglobin, neutrophils and platelets levels (RCC). The lower limit of normal (LLN) was used for hemoglobin (cutoff values: men= 13g/dL and women= 12g/dL) and the upper limit (ULN) was used for alkaline phosphatase (cutoff value: 80U/L), neutrophils (cutoff value: 7500/mm³) and platelets (cutoff value: 400,000/mm³). With respect to immune cell densities and number of metastases, the “minimum P value” approach was used to determine the cutoff for the best separation of patients referring to their OS outcome (outcome-oriented approach). Because the P values
obtained might be overestimated, OS log-rank P values were corrected using the formula proposed by Altman et al. (25) and using 10-fold cross-validations as recommended by Faraggi et al. (26). The confidence interval was important around the optimal P value (Table S3). We have also ensured that the significance established at the optimal cutoff remained valid at the quartiles (data-oriented approach). A P value less than 0.05 was considered statistically significant. Independent parameters identified at univariate analysis as possibly influencing outcome (P<0.1) were introduced in a multivariate Cox-proportional hazards regression model. All analyses were performed with Prism 5 (GraphPad), Statview (Abacus Systems) and the R (http://www.r-project.org/).


Results

The densities of immune cells correlate with OS in lung metastases from CRC

Since densities of CD8\(^+\) T cells and DC-LAMP\(^+\) mature DC in primary tumors correlate with survival (1), we counted these cells in lung metastases from 140 CRC patients. We also quantified NKp46\(^+\) NK cells as a marker of innate immune response. High densities of infiltrating CD8\(^+\) T cells (Fig. 1a) and mature DC (Fig. 1b) were associated with prolonged OS (P=0.039 and P=0.001, respectively). Combination of these two immune parameters allowed to identify patients with better outcome (CD8\(^{\text{high}}\)/DC-LAMP\(^{\text{high}}\)) (Fig. 1c, P=0.008). NKp46\(^+\) cell density did not predict clinical outcome (P=0.12, Fig. 1d). Significance was established at the optimal cutoff, but remained valid at quartiles including the median (Table S3). The quantification of CD8\(^+\) T cells separately in the CT and the IM regions yielded similar results (Fig. S1). Univariate analysis of other clinical and pathological parameters is reported in Table 1. At multivariate analysis, (Table 1) immune pattern (CD8\(^+\)/DC-LAMP\(^+\) densities) of metastases was the strongest independent predictor of survival.

As reported in CRC primary tumors (3), gene expression analyses revealed that a strong CD8\(^+\) and DC-LAMP\(^+\) cell infiltration was associated with a higher expression of genes linked to Th1 orientation, cytotoxicity and lymphoid chemokines/chemokine receptors in lung metastases (Fig. 1e). Expressions of clusters of genes associated with Th2 orientation, inflammation, angiogenesis or immuno-suppression were not correlated with the CD8\(^+\)/DC-LAMP\(^+\) densities. However, individual gene expression of vascular endothelium growth factor (VEGF) was inversely correlated with CD8\(^+\)/DC-LAMP\(^+\) infiltration, as reported in primary CRC (3, 27), whereas that of IL17 and CTLA4 were positively correlated (Fig. 1e).
**The in situ immune pattern is reproduced from primary tumors to metastases in CRC**

To investigate whether the *in situ* immune pattern varies during the course of the metastatic disease for a given patient, we analyzed coincident CRC lung metastases occurring in the other lung side (n=14) operated 1 to 9 months after the initial metastatic surgery, and/or relapsing metastasis occurring 14 to 52 months after surgical removal of the lung metastasis (n=12) (Fig. 2a). Densities of CD8⁺ (Fig. 2b), DC-LAMP⁺ (Fig. 2c) and NKp46⁺ (Fig. 2d) cells were not significantly different between two coincident metastatic sites or between the first lung metastasis and its relapse. We found correlations in the densities of immune cells between coincident and relapsing metastases (Fig. 2b-d).

To address the question of the relationship between immune cell densities in the primary tumor and metastasis, we compared immune infiltrates of primary tumors and lung metastases from the same individuals (n=25) (Fig. 2e). Primary CRC differed from lung metastases by significantly higher density of CD8⁺ T cells (P<0.05) (Fig. 2f), but the density of each cell type was positively correlated between the primary and the metastatic tumors (Fig. 2f-h for CD8⁺, DC-LAMP⁺ and NKp46⁺ cells, respectively). We had access to a small number (n=5) of matched hepatic metastases and the correlation was also found between primary CRC, lung, and liver metastases (data not shown).

**The densities of immune cells correlate with OS in lung metastases from RCC**

We have also analyzed a cohort of 52 RCC lung metastases. Patients with high densities of infiltrating CD8⁺ T cells (Fig. 3a) or DC-LAMP⁺ cells (Fig. 3b) have reduced survival (P=0.03). These two immune parameters allowed to identify, with strong significance, patients with poorer outcome (CD8⁺/DC-LAMP⁺) (Fig. 3c, P<0.0001). High density of
NKp46+ cells was associated with improved survival (P=0.002, Fig. 3d). Separate analysis of the CD8+ and NKp46+ immune infiltrates in the CT and IM also correlated with OS (Fig. S2). Significance was established at the optimal cutoff but also conserved at the quartiles (Table S3). Univariate proportional hazard Cox analyses revealed that the immune pattern (CD8+/DC-LAMP+ densities), NKp46+ cell density, presence of metastases at presentation and disease-free interval were the only prognostic factors of patients’ survival in our cohort (Table 1). Our data also suggest that hemoglobin and thoracic lymph node invasion tended to be associated with survival (P=0.061 and 0.086, respectively). In the resulting multivariate proportional hazard Cox model, DFI and immune pattern were independent prognostic factors (P=0.0067 and 0.0039, respectively) (Table 1).

A strong CD8+/DC-LAMP+ infiltration was associated with a higher expression of genes linked to Th1 orientation, lymphoid and myeloid chemokine/chemokine receptors. Contrasting with CRC, cytotoxicity-related genes were highly expressed in both groups of tumors (3, 27) (Fig. 3e) and, interestingly, VEGF gene expression was positively correlated with CD8+/DC-LAMP+ infiltration, as well as that of interleukin 6 (IL6) and signal transducer and activator of transcription 3 (STAT3).

As previously shown in CRC, we found a correlation between the density of infiltrating DC-LAMP+, CD8+ and NKp46+ cells in the primary tumor and in the corresponding lung metastasis (n=24) (Fig. 4a-d), indicating that the in situ immune pattern of the primary tumor was reproduced in the metastasis.
The cell composition, organization and polarization of the immune reaction is different in CRC and RCC lung metastases

Since CD8$^+$, DC-LAMP$^+$ and NKp46$^+$ cell densities in lung metastases have different clinical impacts in CRC and RCC, we compared their microenvironments. Histological analyses revealed profound differences between CRC and RCC lung metastases. We found glands, often necrotic, in an abundant and collagenous stroma surrounded by a high density of Tertiary Lymphoid Structures (TLS) in CRC metastases (Fig. 5a). In contrast, in RCC metastases, tumor cell nests were separated by a thin stroma with few and scattered TLS (Fig. 5a). TLS contained a B cell follicle, a T cell zone and PNAd$^+$ high-endothelial venules (Fig. 5b).

We found similar densities of CD3$^+$ and CD8$^+$ T cells in the whole tumor zone (Fig. 5c). Mature DC, located in the T cell area of TLS, were found at higher density in CRC than in RCC (P<0.0001) (Fig. 5b and c), in accordance with the higher number of TLS in CRC lung metastases. The CRC metastases contained significantly lower densities of NK cells as compared to RCC metastases (P<0.0001) (Fig. 5b and c). No significant differences in the densities of CD8$^+$, DC-LAMP$^+$ and NKp46$^+$ cells were observed in tumors from CRC or RCC patients having received or not pre-operative treatment (chemotherapy, IL2/IFN or association of bevacizumab and chemotherapy) (Fig. S3 and tables S1 and S2 for treatment details).

Whereas expression of genes linked to adaptive immune populations was not significantly different between both types of metastatic tumors, we found a lower expression of CD68 gene in CRC lung metastases (Fig. 5d and Fig. S4 for detailed gene level expression). A similar Th1 orientation was found in CRC and RCC metastases, but a stronger expression of genes linked to Th2 was detected in RCC lung metastases. Genes linked to acute
inflammation were up regulated in CRC lung metastases and genes linked to chronic inflammation, angiogenesis or immunosuppression were up-regulated in RCC lung metastases. A higher expression of cytotoxicity-related genes in RCC metastases was observed, in accordance with their higher NK cell content. Chemokines and receptors genes prone to attract Th1, T regulatory and DC were more expressed in CRC metastases, whereas RCC lung metastases were characterized by the expression of inflammatory chemokines and chemokine receptors genes.
Discussion

The objective of our study was to characterize the immune microenvironment of metastatic lesions and its clinical impact. If several clinical parameters have been reported to be associated with survival in metastatic patients, none has obtained general agreement (17, 28), justifying the search of new non-clinical prognostic markers. We report here a major prognostic value of the immune pattern (densities of mature DC and CD8⁻ T cells) in metastases from CRC and RCC, although with opposite impact on OS. In our cohorts of oligometastatic surgically treated patients, the strongest prognosticator was the immune pattern, i.e. CD8⁺ and DC-LAMP⁺ cell density combination, as reported for many primary tumors (1-13, 23, 27, 29-31). NK cells density had also a prognostic value in RCC. It appears that the immune pattern is a powerful prognostic factor and a potentially important parameter for metastatic patients’ management. Because of the incomplete data collection (especially for laboratory values which were difficult to collect in a retrospective analysis), conclusions remain difficult to draw on the prognostic value of the Memorial Sloan-Kettering Cancer Center (32) and Heng et al. (33) prognostic factor models. Our previous studies demonstrated the highly clinical impact of the CD8⁺ cell densities in primary CRC up to stage III, i.e. without distant metastases at the time of diagnosis (2). In the present study, the impact of the immune pattern on OS was lower in primary tumors (P=0.15 and P=0.01 for CD8⁺ and DC-LAMP⁺ cells, respectively; data not shown) than in lung metastases from CRC (P=0.039 and P=0.001 for CD8⁺ and DC-LAMP⁺ cells, respectively; data not shown).

Clinical significance of CD8⁺ T cells density appears to be contrasted, according to primary tumor’s origin. Whereas we found similar densities of CD8⁻ T cells in both CRC and RCC metastases, the prognostic value of these cells was different. Similar conflicting observations about the prognostic role of immune infiltrate have been reported in primary CRC and RCC.
(2, 4, 15, 27) and one could hypothesize that the seeding organ (colon or kidney) may explain this variability in the outcome. Since it remains valid in the lung metastases, our data support the idea that the kind of primary tumor is essential in determining the prognostic value of the host immune infiltrate at metastatic level. This is in accordance with the fact that primary RCC appears as an exception to the well documented general findings that Th1/CD8 immune cell infiltrate and high density of mature DC correlate with favorable prognosis in the majority of solid tumors (1). The differential clinical impacts of the T cells might be due to their site of activation. Indeed we have previously reported that TLS in early stages of non-small cell lung cancer may act as potential structures of anti-tumor T cell generation (23, 34). We found more TLS, reflected by higher densities of mature DC and higher expression of CCL19 gene, a chemokine expressed in TLS (34), in CRC than in RCC metastases. Since TLS are scarce in RCC lung metastases and numerous in lung metastases from CRC, one may postulate that the T cells present in the former have not been educated in tumor-adjacent TLS (35) and reflect rather a chronic inflammatory reaction which is known to be deleterious for the host (36). Indeed, gene expression analyses revealed significant differences between lung metastases from CRC and RCC, which share a Th1 profile, but the latter exhibit also a Th2, inflammatory and immunosuppressive pattern. The high expression of VEGF, IL6 and M-CSF genes in RCC may also inhibit the differentiation of DC and induce monocyte differentiation to macrophages (37-39), which could initiate an impaired T cell response in RCC, resulting in poor prognosis. Interestingly, VEGF gene expression was positively correlated with high CD8⁺ and DC-LAMP⁺ infiltration in RCC lung metastases and with low CD8⁺ and DC-LAMP⁺ infiltration in CRC lung metastases. Since it has been suggested that VEGF may induce non-coordinated immune responses (27), affect cytotoxic Th1 adaptive immune responses (39, 40) and contribute to the progression of malignant disease, the correlation between CD8⁺/DC-LAMP⁺ densities and VEGF expression could be one...
explanation among others to explain the negative impact associated with this immune signature. Moreover, up regulation of IL6 and STAT3 genes in the CD8$^{\text{high}}$/DC-LAMP$^{\text{high}}$ group could reflect the inflammatory milieu of the RCC microenvironment (41, 42). It could also explain the reasons that immunotherapies, which modify the acute/chronic inflammatory microenvironment, are often reported to have some efficacy in metastatic renal cell carcinoma (43).

The Von Hippel Lindau phenotype, often found in RCC, may also be involved in the shaping of peculiar tumor microenvironments, through induction of hypoxia, production of VEGF, induction of regulatory immune circuits (44-47) and increased sensitivity of tumor cells to NK cell lysis (48). It may also influence differently the stroma characteristics, the vascularization or the collagen content which could also impact on the migration, organization and functionality of intra-tumor immune cells (49). Together, these data may explain the negative clinical impact of the adaptive immune pattern at the primary and advanced stages of RCC.

We found that CRC and RCC have a correlated pattern of DC-LAMP$^+$, CD8$^+$ and NKp46$^+$ cells, from primary tumor to relapsing metastasis, which could reflect, either a potential “imprinting” of the immune microenvironment by the tumor cells or the possibility that the immune contexture in the primary tumor, results in “educated” immune cells that are recalled in the metastatic sites.

In conclusion, our findings highlight the fact that during all steps of cancer development, reciprocal interactions occur between immune and cancer cell and are critical for patients’ survival. The immune signature appears to be a phenotypic marker for the disease and is remarkably reproduced between primary and metastatic sites in the same patient. The
immune contexture affects OS in lung metastases from CRC and RCC, and the analysis of the immune pattern might be useful to guide therapeutics (50).

Acknowledgements: Authors thank Patricia Bonjour, Véronique Ducruit, Tessa Fredriksen for technical assistance and Martine Bovet for help in clinical data collection; the Hôtel-Dieu hospital tumor bank (n° DC 2009-947), the tumorothèque cancer-est (Tumo0203) and the “Centre d’Imagerie Cellulaire et de Cytométrie” (Cordeliers Research Center, Paris).

Financial support: This work was supported by Institut National de la Santé et de la Recherche Médicale (INSERM), Université Paris-Descartes, Université Pierre et Marie Curie, Institut National du Cancer, Cancéropole Ile de France and Labex Immuno-oncology (2011-1-PLBIO-06-INSERM 6-1, PLBIO09-088-IDF-KROEMER, 11LAXE62_9UMS872 FRIDMAN).
References


Figure Legend

Figure 1

Prognostic value of the densities of CD8^+ T cells, DC-LAMP^+ mature DC and NKp46^+ NK cells in lung metastases from CRC. Kaplan-Meier curves for the duration of OS according to a separated (a, b) and combined (c) analysis of CD8^+ and DC-LAMP^+ densities in CRC lung metastases. (d) Kaplan-Meier curves for the duration of OS according to the densities of NKp46^+ cells in CRC lung metastases (n=84). The numbers of at risk patients according to a separated and combined analysis of CD8^+ and DC-LAMP^+ densities and NKp46^+ cells densities were given. Statistical comparison was performed by the log-rank test and all OS log-rank P values were corrected using the formula proposed by Altman et al. (e) Expression of genes related to immune cell populations, Th1/Th2 orientations, inflammation, angiogenesis, immuno-suppression, cytotoxicity, chemokines/chemokine receptors according to the densities of CD8^+ and DC-LAMP^+ cells (high/high versus low/low) in lung metastases from CRC. Expression levels of genes were determined using threshold cycle (Ct) values normalized to actin B [ACTB] (ΔCt). We used Mann-Whitney test to identify genes with significantly different levels of expression among patient groups (high versus low CD8^+/DC-LAMP^+ densities). *P<0.05 for individual gene expression.

Figure 2

CD8^+ T cells, DC-LAMP^+ mature DC and NKp46^+ NK cell densities in coincident or relapsing metastases and in primary colorectal cancer. (a) Surgical treatment for coincident and relapsing CRC lung metastases. (b-d) Coincident or relapsing metastases have the same densities of CD8^+, DC-LAMP^+ and NKp46^+ cells. (e) Surgical treatment for primary CRC and their lung metastases. (f-h) CRC primary tumors were more infiltrated by CD8^+ cells than lung metastases, but have similar densities of DC-LAMP^+ and NKp46^+ cells.
R values show the positive correlations (0.5<R<0.9 and P<0.05, Spearman test) between coincident metastases, relapsing metastases, primary tumors and associated metastases according to the CD8⁺, DC-LAMP⁺ and NKp46⁺ cell densities.

PT=Primary Tumor, LM=Lung Metastasis. ns, not significant, *P<0.05, (Wilcoxon matched pairs test).

Figure 3

Prognostic value of the densities of CD8⁺ T cells, mature DC (DC-LAMP⁺) and NK cells (NKp46⁺) in lung metastases from RCC. Kaplan-Meier curves for the duration of OS according to a separated (a, b) and combined (c) analysis of CD8⁺ and DC-LAMP⁺ cell densities. (d) Kaplan-Meier curves for the duration of OS according to the densities of NKp46⁺ cells. The numbers of at risk patients according to a separated and combined analysis of CD8⁺ and DC-LAMP⁺ densities and NKp46⁺ cells densities were given. Statistical comparison was performed by the log-rank test and all OS log-rank P values were corrected using the formula proposed by Altman et al.

(e) Expression of genes related to immune cell populations, Th1/Th2 orientations, inflammation, angiogenesis, immuno-suppression, cytotoxicity, chemokines/chemokine receptors according to the CD8⁺ and DC-LAMP⁺ cell densities (high/high versus low/low) in lung metastases from RCC. Expression levels of genes were determined using threshold cycle (Ct) values normalized to actin B [ACTB] (ΔCt). We used Mann-Whitney test to identify genes with significantly different levels of expression among patient groups (high versus low). *P<0.05 for individual gene expression.
Figure 4

CD8⁺ T cells, DC-LAMP⁺ mature DC and NKp46⁺ NK cell densities in metastases and in primary RCC tumors. (a) Surgical treatment for primary RCC and their lung metastases. (b, c and d) RCC primary tumors were less infiltrated by DC-LAMP⁺ cells than lung metastases. R values show the positive correlations (0.5< R<0.9 and P<0.05, Spearman test) between primary tumors and lung metastases according to the CD8⁺, DC-LAMP⁺ and NKp46⁺ cell densities.

PT=Primary Tumor, LM=Lung Metastasis. ns, not significant, *P<0.05, (Wilcoxon matched pairs test).

Figure 5

Comparison of the immune contexts in CRC and RCC lung metastases. (a) Representative pictures of CRC and RCC lung metastases (Hematoxylin-Eosin-Safran (HES) staining) showing the organization of tumors. Original magnification: ×40 and ×200. TLS=Tertiary Lymphoid Structure, T=Tumor, S=Stroma. (b) Location and organization of CD20⁺ B cell follicles (red) surrounded by high-endothelial venules (blue), DC-LAMP expressing mature dendritic cells (red, black arrows), CD3⁺ T cells (red), CD8⁺ T cells (red) and NKp46⁺ NK cells (red) in CRC (left) and RCC (right) lung metastases. Original magnification: ×200 and ×400. (c) Quantification of CD3⁺, CD8⁺, DC-LAMP⁺ and NKp46⁺ cells in lung metastases from colorectal (CRC-LM, n=140) and renal cell carcinoma (RCC-LM, n=52). Whiskers length represents 10-90 percentile. ns, not significant; ***P<0.0001 (Mann-Whitney test). (d) Heat map of the expression levels of genes according to the origin of lung metastases (CRC and RCC) represented using the Genesis program. LM=Lung Metastasis. ns, not significant; *P<0.05 (Mann-Whitney test).
Table 1

Univariate and multivariate Cox proportional hazards analyses for OS according to clinical parameters and immune cell densities in CRC and RCC lung metastases.

To be able to perform regression with a categorical variable, they were coded before entered into the Cox model.

‡The stage was determined by pathological examination at the time of diagnosis. None of the variables violated the proportional hazards assumption.
Figure 1

a. **CD8$^+$ T cells:**

![Graph showing survival of CD8$^+$ T cells with Kaplan-Meier plots for CD8$^{hi}$ (n=71) and CD8$^{lo}$ (n=69). The survival curve for CD8$^{hi}$ shows a more rapid decline compared to CD8$^{lo}$, with a statistically significant difference (P=0.039). At risk patients: Hi (71, 53, 40, 24, 12, 4), Lo (69, 52, 30, 15, 5, 2).]

b. **DC-LAMP$^+$ mature DC:**

![Graph showing survival of DC-LAMP$^+$ mature DC with Kaplan-Meier plots for DC-LAMP$^{hi}$ (n=116) and DC-LAMP$^{lo}$ (n=24). The survival curve for DC-LAMP$^{hi}$ shows a more rapid decline compared to DC-LAMP$^{lo}$, with a statistically significant difference (P=0.001). At risk patients: Hi (116, 91, 62, 33, 16, 5), Lo (24, 14, 8, 6, 2, 1).]
c. CD8$^+$ and DC-LAMP$^+$ cells:

![Graph showing survival analysis for different CD8 and DC-LAMP combinations.]

- CD8$^{hi}$/DC-LAMP$^{hi}$ (n=67)
- CD8$^{lo}$/DC-LAMP$^{hi}$ or CD8$^{hi}$/DC-LAMP$^{lo}$ (n=53)
- CD8$^{lo}$/DC-LAMP$^{lo}$ (n=20)

At risk patients:
- Hi/Hi: 67, 51, 39, 24, 13, 4
- MIX: 20, 12, 7, 6, 2, 1
- Lo/Lo: 53, 42, 24, 9, 3, 1

P=0.008

---

d. NKp46$^+$ NK cells:

![Graph showing survival analysis for different NKp46 expression levels.]

- NKp46$^{hi}$ (n=58)
- NKp46$^{lo}$ (n=26)

At risk patients:
- Hi: 58, 49, 39, 23, 14, 5
- Lo: 26, 17, 8, 3, 0, 0

P=0.12
e.

Immune cell populations

- High expression
- Low expression

Th1 orientation

- mean(ΔCt) P = 0.04

Th2 orientation

- mean(ΔCt) P = 0.42

Inflammation

- mean(ΔCt) P = 0.04

Angiogenesis

- mean(ΔCt) P = 0.23

Immunosuppression

- mean(ΔCt) P = 0.032

Cytotoxicity

- mean(ΔCt) P = 0.03

Chemokines/chemokine receptors

- High CD8+/DC-LAMP+ densities
- Low CD8+/DC-LAMP+ densities
Figure 2

a.

Coincident metastases:

Relapsing metastases:
b.

\[ R = 0.644 \]
\[ R = 0.643 \]ns

\[ R = 0.614 \]
\[ R = 0.580 \]ns

c.

\[ \text{Number of CCE cells/mm}^2 \]

\[ \text{First side} \]
\[ \text{Second side} \]
\[ \text{First metastasis} \]
\[ \text{Metastatic phase} \]
d.

![Graph showing comparison between primary tumor and metastases]

R = 0.696 ns
R = 0.895 ns

Number of NKG46 cells/mm²

First side, Second side, First metastasis, Metastatic relapse

---

e.

**Primary tumor versus metastases:**

![Diagram illustrating progression from primary tumor to metastasis]
f. \( R = 0.693 \)

![Graph showing the number of CD8+ cells/mm²](image)

R = 0.693

---

g. \( R = 0.659 \)

![Graph showing the number of DCLAMP cells/mm²](image)

R = 0.659

---
h.
Figure 3

a. CD8$^+$ T cells:

![Survival Curve for CD8$^+$ T cells](image)

b. DC-LAMP$^+$ mature DC:

![Survival Curve for DC-LAMP$^+$ mature DC](image)
c. CD8$^+$ and DC-LAMP$^+$ cells:

![Graph showing overall survival for different CD8 and DC-LAMP expression levels]

- CD8$^{lo}$ / DC-LAMP$^{lo}$ (n=26)
- CD8$^{lo}$ / DC-LAMP$^{hi}$ or CD8$^{hi}$ / DC-LAMP$^{lo}$ (n=17)
- CD8$^{hi}$ / DC-LAMP$^{hi}$ (n=9)

<table>
<thead>
<tr>
<th>At risk patients</th>
<th>CD8$^{lo}$/DC-LAMP$^{lo}$</th>
<th>CD8$^{lo}$/DC-LAMP$^{hi}$ or CD8$^{hi}$/DC-LAMP$^{lo}$</th>
<th>CD8$^{hi}$/DC-LAMP$^{hi}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hi/Hi</td>
<td>9</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>MIX</td>
<td>18</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>Lo/Lo</td>
<td>25</td>
<td>19</td>
<td>13</td>
</tr>
</tbody>
</table>


d. NKp46$^+$ NK cells:

![Graph showing overall survival for different NKp46 expression levels]

- NKp46$^{hi}$ (n=28)
- NKp46$^{lo}$ (n=24)

<table>
<thead>
<tr>
<th>At risk patients</th>
<th>NKp46$^{hi}$</th>
<th>NKp46$^{lo}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hi</td>
<td>28</td>
<td>20</td>
</tr>
<tr>
<td>Lo</td>
<td>24</td>
<td>12</td>
</tr>
</tbody>
</table>
e.

Immune cell populations

mean(ΔCt) P = 0.0046

Th1 orientation

mean(ΔCt) P = 0.0094

Th2 orientation

mean(ΔCt) P = 0.36

Inflammation

mean(ΔCt) P = 0.16

Angiogenesis

mean(ΔCt) P = 0.130

Immuno-suppression

mean(ΔCt) P = 0.165

Cytotoxicity

mean(ΔCt) P = 0.126

Chemokines/chemokine receptors

mean(ΔCt) P = 0.018

High CD8+/DC-LAMP+ densities

Low CD8+/DC-LAMP+ densities
Figure 4

a.

Primary tumor versus lung metastasis:

b.
c. $R = 0.547$

![Graph showing data with $R = 0.547$.](image)

d. $R = 0.817$

![Graph showing data with $R = 0.817$.](image)
Figure 5

a.

Colorectal cancer - Lung metastasis

Renal cell carcinoma - Lung metastasis
b. 

Colorectal cancer - Lung metastasis

Renal cell carcinoma - Lung metastasis
c.

**CD3^+ T cells:**

**CD8^+ T cells:**

**DC-LAMP^+ mature DC:**

**NKp46^+ NK cells:**
d.

![Heatmap Image]

**Immune cell populations**
- CD68
- CD3E
- CD4
- CD8A

**Th1 orientation**
- IL18
- TBX21
- IFNG
- IL12A
- IL12B
- LTA

**Th2 orientation**
- IL5
- IL4
- IL10
- IL13

**Inflammation and angiogenesis**
- FN1
- C3
- VEGF
- STAT3
- CSF1
- ACE
- CD34
- IL6
- IL7
- TNF
- IL3
- IL8
- IL1A
- IL1B
- PTGS2
- SELE
- CSF3
- IL17

**Immuno-suppression**
- TGFBI
- CSF1
- CTLA4
- IL10

**Cytotoxicity**
- GZMB
- GLNY
- PRF1
- IL15

**Chemokines/chemokines receptors**
- CCR2
- CCL2
- CCR5
- CCL3
- CCL5
- CCR4
- CCL19
- CXCL11
- CXCR3
- CXCL10
- CCR7

*Colorectal carcinoma - LM*  
*Renal cell carcinoma - LM*
### Table 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analyses</th>
<th>Multivariate analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard ratio</td>
<td>95% CI</td>
</tr>
<tr>
<td>Stage‡ (stages 3+4 vs 1+2)</td>
<td>1.68</td>
<td>(0.88 - 3.20)</td>
</tr>
<tr>
<td>Presence of extrathoracic metastases (yes vs no)</td>
<td>1.56</td>
<td>(0.88 - 2.75)</td>
</tr>
<tr>
<td>Completeness of resection (R1 vs R0)</td>
<td>2.49</td>
<td>(0.77 - 8.05)</td>
</tr>
<tr>
<td>CEA level (≥5ng/ml vs &lt;5ng/ml)</td>
<td>1.55</td>
<td>(0.86 - 2.82)</td>
</tr>
<tr>
<td>NK cells (high vs low)</td>
<td>0.58</td>
<td>(0.28 - 1.16)</td>
</tr>
<tr>
<td>Thoracic lymph node invasion (yes vs no)</td>
<td>1.49</td>
<td>(0.60 - 3.66)</td>
</tr>
<tr>
<td>Number of metastases (&gt;2 vs ≤2)</td>
<td>1.84</td>
<td>(1.03 - 3.28)</td>
</tr>
<tr>
<td>Immune pattern (high/mix/low)</td>
<td>0.54</td>
<td>(0.39 - 0.76)</td>
</tr>
<tr>
<td>Initial Fuhrman nuclear grade (3+4 vs 1+2)</td>
<td>1.32</td>
<td>(0.56 - 3.11)</td>
</tr>
<tr>
<td>Time from lung metastasis diagnosis to surgery (&gt;1year vs ≤1year)</td>
<td>1.77</td>
<td>(0.83 - 3.76)</td>
</tr>
<tr>
<td>Number of metastases (multiple vs 1)</td>
<td>1.30</td>
<td>(0.61 - 2.79)</td>
</tr>
<tr>
<td>Presence of extrathoracic metastases (yes vs no)</td>
<td>1.34</td>
<td>(0.51 - 3.55)</td>
</tr>
<tr>
<td>Completeness of resection (R1 vs R0)</td>
<td>1.40</td>
<td>(0.42 - 4.65)</td>
</tr>
<tr>
<td>Alkaline phosphatase (&gt;80U/L vs ≤80U/L)</td>
<td>1.52</td>
<td>(0.75 - 4.33)</td>
</tr>
<tr>
<td>Neutrophils (&gt;7500/mm³ vs ≤7500/mm³)</td>
<td>0.87</td>
<td>(0.29 - 2.62)</td>
</tr>
<tr>
<td>Platelets (&gt;400,000/mm³ vs ≤400,000/mm³)</td>
<td>0.80</td>
<td>(0.23 - 2.82)</td>
</tr>
<tr>
<td>DFI (≥1year vs &lt;1year)</td>
<td>2.23</td>
<td>(1.02 - 4.86)</td>
</tr>
<tr>
<td>Metastases at presentation (synchronous vs metachronous)</td>
<td>2.33</td>
<td>(0.91 - 5.33)</td>
</tr>
<tr>
<td>Thoracic lymph node invasion (yes vs no)</td>
<td>2.26</td>
<td>(0.96 - 5.33)</td>
</tr>
<tr>
<td>Hemoglobin (men: ≤13g/dL vs &gt;13g/dL and women: ≤12g/dL vs &gt;12g/dL)</td>
<td>2.68</td>
<td>(0.22 - 0.95)</td>
</tr>
<tr>
<td>NK cells (high vs low)</td>
<td>0.46</td>
<td>(1.58 - 4.57)</td>
</tr>
<tr>
<td>Immune pattern (high/mix/low)</td>
<td>2.68</td>
<td>(1.58 - 4.57)</td>
</tr>
</tbody>
</table>
Clinical Cancer Research

Characteristics and clinical impacts of the immune environments in colorectal and renal cell carcinoma lung metastases: influence of tumor origin

Romain Remark, Marco Alifano, Isabelle Cremer, et al.

Clin Cancer Res  Published OnlineFirst June 19, 2013.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-12-3847
Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2013/06/19/1078-0432.CCR-12-3847.DC1
Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.