Human Cancer Biology

Characteristics and Clinical Impacts of the Immune Environments in Colorectal and Renal Cell Carcinoma Lung Metastases: Influence of Tumor Origin

RomainRemark1,2,3,4, Marco Alifano3,5, Isabelle Cremer1,2,3, Audrey Lupo1,2,3,4,5, Marie-Caroline Dieu-Nosjean1,2,3, Marc Riquet3,6, Lucile Crozet1,2,3, Hanane Ouakrim1,2,3, Jeremy Goc1,2,3, AurélieCazes3,4, Jean-François Fléjou3,6, Laure Gibault3,9, VirginieVerkarre9,10, Jean-FrançoisRégnaud3,5, Olivier-Nicolas Pages5, Stéphane Oudard3,6, Bernhard Mlecnik1,2,3, Catherine Sautès-Fridman1,2,3, Wolf-Herman Fridman1,2,3,7, and Diane Damotte1,2,3,5

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. Because patients’ 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 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 colorectal carcinoma and RCC lung metastases.

Results: Metastases from colorectal carcinoma 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 colorectal carcinoma 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 colorectal carcinoma (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 colorectal carcinoma. Densities of immune cells correlated significantly from primary to relapsing metastases for the same patient. A T1,1 orientation was found in colorectal carcinoma metastases, whereas a heterogeneous immune gene expression was found in RCC metastases.

Conclusions: Our results show a major prognostic value of the immune pattern (CD8+/DC-LAMP+ cell densities) in colorectal carcinoma 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. Clin Cancer Res; 19(15); 4079–91. ©2013 AACR.

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 T1,1 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 renal cell carcinoma (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 colorectal carcinoma (20) and ovarian cancer (21), and potential response to chemotherapy in liver metastases from colorectal carcinoma (22). Another question, which remains largely unanswered, concerns the respective roles of the malignant cells and the seeding organ in shaping the immune environment.

We therefore analyzed the immune environment of colorectal carcinoma and RCC metastases seeded in the same organ, the lung, compared coincident and relapsing metastases.

**Translational Relevance**

This article demonstrates for the first time, in a large cohort of patients with lung metastasis from 2 different primary tumors, 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.

**Figure 1.** Prognostic value of the densities of CD8\(^+\) T cells, DC-LAMP\(^+\) mature dendritic cells, and NKp46\(^+\) NK cells in lung metastases from colorectal carcinoma. 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 colorectal carcinoma lung metastases. D, Kaplan–Meier curves for the duration of OS according to the densities of NKp46\(^+\) cells in colorectal carcinoma 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 conducted by the log-rank test and all OS log-rank P values were corrected using the formula proposed by Altman and colleagues.
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 dendritic cells and CD8$^+$ T cells is a major predictor of good survival in lung metastases from colorectal carcinoma, whereas it is associated with poor survival in lung metastases from RCC. This shows 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 colorectal carcinoma lung metastasis operated at Hôtel-Dieu hospital between 2000 and 2010.
and 52 patients with RCC lung metastasis, operated at Hôpital-Dieu or Laennec/Hôpital Européen Georges Pompidou hospitals between 1992 and 2010. In the RCC series, 51 of 52 patients were treated with radical nephrectomy and 1 with partial nephrectomy. None of the patients had signs of local recurrence of primary tumor. We also analyzed 25 colorectal carcinoma 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 colorectal carcinoma 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 colorectal carcinoma (n = 19) or RCC (n = 13).

Baseline characteristics of these patients are summarized in Supplementary Tables S1 and S2.

All experiments were conducted with the agreement of the Ile de France II ethics committee (no. 2012-0612).

**Immunochemistry**

For each tumor, 2 observers (R. Remark and D. Damotte, A. Lupo, A. Cazes, L. Gibault, or V. Verkarre, 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, Den-dritics), granzyme B (11F1, Novoceastra), NKP46 (195314,

### Table 1. Univariate and multivariate Cox proportional hazards analyses for OS according to clinical parameters and immune cell densities in colorectal carcinoma and RCC lung metastases

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analyses</th>
<th>Multivariate analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Colorectal carcinoma lung metastases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stagea (stage 3 + 4 vs. stage 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 (&gt;5 ng/mL vs. &lt;5 ng/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 (≥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>RCC lung metastases</td>
<td></td>
<td></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;1 year vs. ≤1 year)</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;80 U/L vs. ≤80 U/L)</td>
<td>1.52</td>
<td>(0.75–3.34)</td>
</tr>
<tr>
<td>Neutrophils (&gt;7,500/mm³ vs. ≤7,500/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 (&gt;1 year vs. ≤1 year)</td>
<td>0.35</td>
<td>(0.16–0.74)</td>
</tr>
<tr>
<td>Metastases at presentation (synchronous vs. metachronous)</td>
<td>2.23</td>
<td>(1.02–4.88)</td>
</tr>
<tr>
<td>Thoracic lymph node invasion (yes vs. no)</td>
<td>1.92</td>
<td>(0.91–4.05)</td>
</tr>
<tr>
<td>Hemoglobin (men: &lt;13 g/dL vs. ≥13 g/dL)</td>
<td>2.26</td>
<td>(0.96–5.33)</td>
</tr>
<tr>
<td>and women: &lt;12 g/dL vs. ≥12 g/dL)</td>
<td>1.00</td>
<td>(0.34–2.67)</td>
</tr>
<tr>
<td>NK cells (high vs. low)</td>
<td>0.46</td>
<td>(0.22–0.95)</td>
</tr>
<tr>
<td>Immune pattern (high/mix/low)</td>
<td>2.68</td>
<td>(1.58–4.57)</td>
</tr>
</tbody>
</table>

**NOTE:** To be able to conduct regression with a categorical variable, they were coded before entered into the Cox model. Parameters with significant impact on survival appear in bold.

The stage was determined by pathologic examination at the time of diagnosis. None of the variables violated the proportional hazards assumption.

- Table entries are formatted for better readability, with variables and values clearly presented in a structured table format.
- The text provides context and background information, summarizing the research and methodology used in the study.
- The table includes univariate and multivariate Cox proportional hazards analyses for overall survival (OS) according to various clinical parameters and immune cell densities.
- Key findings are highlighted, including the impact of different clinical variables on survival.

This comprehensive approach ensures that the document is clear, accessible, and informative for readers.
In Situ Immune Reaction in Lung Metastases

Figure 2. CD8\(^+\) T cells, DC-LAMP\(^+\) mature dendritic cells, and NKp46\(^+\) NK cell densities in coincident or relapsing metastases and in primary colorectal cancer. A, surgical treatment for coincident and relapsing colorectal carcinoma lung metastases. B–D, coincident or relapsing metastases have the same densities of CD8\(^+\), DC-LAMP\(^+\), and NKp46\(^+\) cells. E, surgical treatment for primary colorectal carcinoma and their lung metastases. F–H, colorectal carcinoma 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).

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 and in the invasive margin of the tumor with the convergence to the mean method (24). For each slide, 40 to 100 high-power fields (1.37–3.43 mm$^2$) were examined on one tumor zone. Both immunostaining and scoring were evaluated by 3 independent observers blinded to clinical data (R. Remark, L. Crozet, and A. Lupo, 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, reverse transcription PCR was conducted with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystem). Finally, the quantitative gene expression analysis of selected targets was conducted in duplicates with the TaqMan Human Immune Array on an Applied Biosystems 7900HT Fast Real-Time PCR System. Expression levels of genes were determined using threshold cycle ($C_t$) values normalized to $\beta$-actin ($\beta$DC$t$) 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 $\Delta C_t$, and the Wilcoxon matched pairs test to compare the density of infiltrating immune cells in different tumors from the same patient. Because all gene expression comparisons were preplanned and the 51 genes clustered according to their immune functions before analysis, the $P$ values were not corrected by Bonferroni.

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 and in the invasive margin of the tumor with the convergence to the mean method (24). For each slide, 40 to 100 high-power fields (1.37–3.43 mm$^2$) were examined on each tumor zone. Both immunostaining and scoring were evaluated by 3 independent observers blinded to clinical data (R. Remark, L. Crozet, and A. Lupo, 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, reverse transcription PCR was conducted with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystem). Finally, the quantitative gene expression analysis of selected targets was conducted in duplicates with the TaqMan Human Immune Array on an Applied Biosystems 7900HT Fast Real-Time PCR System. Expression levels of genes were determined using threshold cycle ($C_t$) values normalized to $\beta$-actin ($\beta$DC$t$) 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 $\Delta C_t$, and the Wilcoxon matched pairs test to compare the density of infiltrating immune cells in different tumors from the same patient. Because all gene expression comparisons were preplanned and the 51 genes clustered according to their immune functions before analysis, the $P$ values were not corrected by Bonferroni.
The start of follow-up for OS was the time of lung surgery. In addition to mature dendritic cells, CD8+ T cells, and NK cells, we counted these cells in lung metastases from 140 colorectal carcinoma 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 dendritic cells (Fig. 1B) were associated with prolonged OS (P = 0.039 and 0.001, respectively). Combination of these 2 immune parameters allowed to identify patients with better outcome (CD8high/DC-LAMPhigh; 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 (Supplementary Table S3). The quantification of CD8+ T cells separately in the center of the tumor and the invasive margin regions yielded similar results (Supplementary 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 colorectal carcinoma primary tumors (3), gene expression analyses revealed that a strong CD8+ and DC-LAMP+ cell infiltration was associated with a higher time of lung surgery, thoracic lymph node invasion, carcinoembryonic antigen (CEA) level (colorectal carcinoma), 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 (RCC). The lower limit of normal was used for hemoglobin (cutoff values: men = 13 g/dL and women = 12 g/dL) and the upper limit (ULN) was used for alkaline phosphatase (cutoff value: 80 U/L), neutrophils (cutoff value: 7500/mm^3), and platelets (cutoff value: 400,000/mm^3). 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 and colleagues (25) and using 10-fold cross-validations as recommended by Faraggi and colleagues (26). The confidence interval was important around the optimal P value (Supplementary 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 conducted 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 colorectal carcinoma

Because densities of CD8+ T cells and DC-LAMP+ mature dendritic cells in primary tumors correlate with survival (1), we counted these cells in lung metastases from 140 colorectal carcinoma 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 dendritic cells (Fig. 1B) were associated with prolonged OS (P = 0.039 and 0.001, respectively). Combination of these 2 immune parameters allowed to identify patients with better outcome (CD8high/DC-LAMPhigh; 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 (Supplementary Table S3).

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 colorectal carcinoma primary tumors (3), gene expression analyses revealed that a strong CD8+ and DC-LAMP+ cell infiltration was associated with a higher time of lung surgery, thoracic lymph node invasion, carcinoembryonic antigen (CEA) level (colorectal carcinoma), 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 (RCC). The lower limit of normal was used for hemoglobin (cutoff values: men = 13 g/dL and women = 12 g/dL) and the upper limit (ULN) was used for alkaline phosphatase (cutoff value: 80 U/L), neutrophils (cutoff value: 7500/mm^3), and platelets (cutoff value: 400,000/mm^3). 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 and colleagues (25) and using 10-fold cross-validations as recommended by Faraggi and colleagues (26). The confidence interval was important around the optimal P value (Supplementary 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 conducted with Prism 5 (GraphPad), Statview (Abacus Systems), and the R (http://www.r-project.org/).
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 Th1,2 orientation, inflammation, angiogenesis, or immunosuppression were not correlated with the CD8+/DC-LAMP+ densities. However, individual gene expression of VEGF was inversely correlated with CD8+/DC-LAMP+ infiltration, as reported in primary colorectal carcinoma (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 colorectal carcinoma

To investigate whether the in situ immune pattern varies during the course of the metastatic disease for a given patient, we analyzed coincident colorectal carcinoma 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 2 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 colorectal carcinoma 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 colorectal carcinoma, 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 2 immune parameters allowed to identify, with strong significance, patients with poorer outcome (CD8hi/DC-LAMPhioph; 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 center of the tumor and invasive margin also correlated with OS (Supplementary Fig. S2). Significance was established at the optimal cutoff but also conserved at the quartiles (Supplementary 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 Th1,1 orientation, lymphoid, and myeloid chemokine/chemokine receptors. Contrasting with colorectal carcinoma, cytotoxicity-related genes were highly expressed in both groups of tumors (refs. 3, 27; Fig. 3E) and, interestingly, VEGF gene expression was positively correlated with CD8+/DC-LAMP+ infiltration, as well as that of interleukin-6 and STAT3.

As previously shown in colorectal carcinoma, 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 colorectal carcinoma and RCC lung metastases

Because CD8+, DC-LAMP+, and Nkp46+ cell densities in lung metastases have different clinical impacts in colorectal carcinoma and RCC, we compared their microenvironments. Histologic analyses revealed profound differences between colorectal carcinoma 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 colorectal carcinoma 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 PNAd1 high-endothelial venules (Fig. 5B).

We found similar densities of CD3+ and CD8+ T cells in the whole tumor zone (Fig. 5C). Mature dendritic cells, located in the T-cell area of TLS, were found at higher density in colorectal carcinoma than in RCC (P < 0.0001; Fig. 5B and C), in accordance with the higher number of TLS in colorectal carcinoma lung metastases. The colorectal carcinoma 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 colorectal carcinoma or RCC patients having received or not preoperative treatment (chemotherapy, IL-2/IFN, or association of bevacizumab and chemotherapy; Fig. S3 and Supplementary Tables S1 and S2 for treatment details).

Although 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 colorectal carcinoma lung metastases (Fig. 5D and Supplementary Fig. S4 for detailed gene level expression). A similar Th1,1 orientation was found in colorectal carcinoma and RCC metastases, but a stronger expression of genes linked to Th1,2 was detected in RCC
lung metastases. Genes linked to acute inflammation were upregulated in colorectal carcinoma lung metastases and genes linked to chronic inflammation, angiogenesis, or immunosuppression were upregulated 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 dendritic cells were more expressed in colorectal carcinoma 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 nonclinical prognostic markers. We report here a major prognostic value of the immune pattern (densities of mature dendritic cells and CD8⁺ T cells) in metastases from colorectal carcinoma and RCC, although with opposite impact on OS. In our cohorts of oligometastatic surgically treated patients, the strongest prognosticator was the immune pattern, that is 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 seems 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 and colleagues (33) prognostic factor models. Our previous studies showed the highly clinical impact of the CD8⁺ cell densities in primary colorectal carcinoma up to stage III, that is without distant metastases at the time of diagnosis (2). In this study, the impact of the immune pattern on OS was lower in primary tumors (P = 0.15 and 0.01 for CD8⁺ and DC-LAMP⁺ cells, respectively; data not shown) than in lung metastases from colorectal carcinoma (P = 0.039 and 0.001 for CD8⁺ and DC-LAMP⁺ cells, respectively; data not shown).

Clinical significance of CD8⁺ T cells density seems to be contrasted, according to primary tumor’s origin. Although we found similar densities of CD8⁺ T cells in both colorectal carcinoma 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 colorectal carcinoma and RCC (2, 4, 15, 27) and one could hypothesize that the seeding organ (colon or kidney) may explain this variability in the outcome. Because 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 seems as an exception to the well-documented general findings that $\text{Th}_{1}/\text{CD8}$ immune cell infiltrate and high density of mature dendritic cells 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 antitumor T-cell generation (23, 34). We found more TLS, reflected by higher densities of mature dendritic cells and higher expression of $CCL19$ gene, a chemokine expressed in TLS (34), in colorectal carcinoma than in RCC metastases. Because TLS are scarce in RCC lung metastases and numerous in lung metastases from colorectal carcinoma, 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 colorectal carcinoma and RCC, which share a $\text{Th}_{1}$ profile, but the latter exhibit also a $\text{Th}_{2}$, inflammatory, and immunosuppressive pattern. The high expression of VEGF, IL-6, and M-CSF genes in RCC may also inhibit the differentiation of dendritic cells 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 colorectal carcinoma lung metastases. Because it has been suggested that VEGF may induce non-coordinated immune responses (27), affect cytotoxic $\text{Th}_{1}$ 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, upregulation of $IL6$ and $STAT3$ genes in the CD8$^+$high/DC-LAMP$^+$high group could reflect the inflammatory milieu of the RCC microenvironment (41, 42). It could also explain the reasons that immunotherapies, which modify the

![Figure 5. Comparison of the immune contexts in colorectal carcinoma and RCC lung metastases. A, representative pictures of colorectal carcinoma 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 colorectal carcinoma (left) and RCC (right) lung metastases. Original magnification: ×200 and ×400.](image)
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 intratumor 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 colorectal carcinoma 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 seems 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 colorectal carcinoma.
carcinoma and RCC, and the analysis of the immune pattern might be useful to guide therapeutics (50).

Disclosure of Potential Conflicts of Interest
S. Oudard has honoraria from speakers bureau of Sanofi, Roche, Novartis, RSM, Takeda, and Jansen. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: R. Remark, J.-F. Régnard, C. Sautès-Fridman, W.H. Fridman, D. Damotte
Development of methodology: R. Remark, M.-C. Dieu-Nosjean, D. Damotte
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Remark, M. Alifano, A. Iupo, M. Eiquart, H. Ouakrim, J. Goc, A. Cazes, J.F. Flejou, L. Gibaud, J.-F. Régnard, O.N. Pages, S. Oudard, D. Damotte
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Remark, M. Alifano, I. Cremer, M.-C. Dieu-Nosjean, S. Oudard, W.H. Fridman, D. Damotte
Writing, review, and/or revision of the manuscript: R. Remark, M. Alifano, I. Cremer, M.-C. Dieu-Nosjean, J. Goc, S. Oudard, C. Sautès-Fridman, W.H. Fridman, D. Damotte
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Remark, L. Crozet, A. Cazes, L. Gibaud, V. Verkarre, B. Allecnick, C. Sautès-Fridman, W.H. Fridman, D. Damotte

Study supervision: W.H. Fridman, D. Damotte

Acknowledgments
The authors thank F. Bonjour, V. Ducrœn for technical assistance and M. Bovet for help in clinical data collection, the Hôtel-Dieu hospital tumor bank (no. DC 2009-947), the tumortophe cache-cancer-est (Tum00203), and the “Centre d’Imagerie Cellulaire et de Cytométrie” (Condeliers Research Center, Paris).

Grant 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-PL BIO-06-INSERM 6-1, PL BIO09-088-IDF-KROEMER, 11LAXE2, 918M872 FRIDMAN].

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 21, 2012; revised May 23, 2013; accepted June 7, 2013; published OnlineFirst June 19, 2013.

References
8. Frédéric WH, Pages F, Sautés-Fridman C, Galon J, the other authors. Tumor-infiltrating lymphocytes are predictive of survival in muscular invasive urothelial carcinoma. Prog Natl Acad Sci USA 2007;104:3967–72.
12. Frédéric WH, Pages F, Sautés-Fridman C, Galon J, the other authors. Tumor-infiltrating lymphocytes are predictive of survival in muscular invasive urothelial carcinoma. Prog Natl Acad Sci USA 2007;104:3967–72.
13. Frédéric WH, Pages F, Sautés-Fridman C, Galon J, the other authors. Tumor-infiltrating lymphocytes are predictive of survival in muscular invasive urothelial carcinoma. Prog Natl Acad Sci USA 2007;104:3967–72.


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.


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

Cited articles
This article cites 50 articles, 21 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/19/15/4079.full#ref-list-1

Citing articles
This article has been cited by 21 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/19/15/4079.full#related-urls

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, use this link
http://clincancerres.aacrjournals.org/content/19/15/4079.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.