Dendritic cells in tumor-associated tertiary lymphoid structures license the positive prognostic value of tumor-infiltrating CD8+ T cells

Jérémy Goc\textsuperscript{1,2,3}, Claire Germain\textsuperscript{1,2,3}, Thi Kim Duy Vo-Bourgais\textsuperscript{1,2,3}, Audrey Lupo\textsuperscript{1,2,3,4}, Christophe Klein\textsuperscript{2}, Samantha Knockaert\textsuperscript{1,2,3}, Luc de Chaisemartin\textsuperscript{1,2,3}, Hanane Ouakrim\textsuperscript{1,2,3}, Etienne Becht\textsuperscript{1,2,3}, Marco Alifano\textsuperscript{5}, Pierre Validire\textsuperscript{1,6}, Romain Remark\textsuperscript{1,2,3}, Scott A. Hammond\textsuperscript{7}, Isabelle Cremer\textsuperscript{1,2,3}, Diane Damotte\textsuperscript{1,2,3,4}, Wolf-Herman Fridman\textsuperscript{1,2,3,8}, Catherine Sautès-Fridman\textsuperscript{1,2,3}, and Marie-Caroline Dieu-Nosjean\textsuperscript{1,2,3}.

1 Laboratory Immune Microenvironment and Tumors, INSERM U872, Cordeliers Research Center, Paris, France
2 University Pierre et Marie Curie, UMRS 872, Paris, France
3 University Paris Descartes, UMRS 872, Paris, France
4 Department of Pathology, Hôtel Dieu Hospital, AP-HP, Paris, France
5 Department of Thoracic Surgery, Hôtel Dieu Hospital, AP-HP, Paris, France
6 Department of Pathology, Institut Mutualiste Montsouris, Paris, France
7 Oncology Research, MedImmune LLC, Gaithersburg, Maryland, USA
8 Department of Immunology, European Georges Pompidou Hospital, AP-HP, Paris, France

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Corresponding author:

Dr. Marie-Caroline Dieu-Nosjean

Laboratory Immune Microenvironment and Tumors

UMRS 872 INSERM
Cordeliers Research Center
15, rue de l’école de Médecine
F-75270 Paris cedex 06
France
Phone: +33-1-44-27-90-86
Fax: +33-1-44-27-81-17
E-mail: mc.dieu-nosjean@crc.jussieu.fr

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Conflicts of interest:
Scott Hammond is an employee of MedImmune LLC
The abbreviations used are:

ADC, Adenocarcinoma ; OS, overall survival ; CD8s, stromal CD8+ T cell ; CD8t, tumor nest CD8+ T cell ; DC, mature dendritic cell ; DC-LampHi, high density of DC-Lamp+ cells ; DC-LampLo, low density of DC-Lamp+ cells ; NSCLC, Non-Small-Cell Lung Cancer ; qRT-PCR, quantitative RT-PCR ; SCC, Squamous-Cell Carcinoma ; TIL, Tumor-infiltrating T cells ; TLS, tertiary lymphoid structure.
Abstract

Tumor-infiltrating T cells (TIL), particularly CD45RO+CD8+ memory T cells, confer a positive prognostic value in human cancers. However, the mechanisms that promote a protective T cell response in the tumor microenvironment remain unclear. In chronic inflammatory settings such as the tumor microenvironment, lymphoid neogenesis can occur to create local lymph node-like structures known as tertiary lymphoid structures (TLS). These structures can exacerbate a local immune response, such that TLS formation in tumors may help promote an efficacious immune contexture. However, the role of TLS in tumors has yet to be investigated carefully. In lung tumors, mature dendritic cells (DC) present in tumor-associated TLS can provide a specific marker of these structures. In this study, we evaluated the influence of TLS on the characteristics of the immune infiltrate in cohorts of prospective and retrospective human primary lung tumors (n=458). We found that a high density of mature DC correlated closely to a strong infiltration of T cells that are predominantly of the effector-memory phenotype. Moreover, mature DC density correlated with expression of genes related to T cell activation, Th1 phenotype and cytotoxic orientation. Lastly, a high density of TLS-associated DC correlated with long-term survival, which also allowed a distinction of patients with high CD8+ T cell infiltration but a high risk of death. Taken together, our results show how tumors infiltrated by TLS-associated mature DC generate a specific immune contexture characterized by a strong Th1 and cytotoxic orientation that confers the lowest risk of death. Further, our findings highlight the pivotal function of TLS in shaping the immune character of the tumor microenvironment, in promoting a protective immune response mediated by T cells against cancer.
Introduction
The tumor microenvironment is a complex network of different cell types comprising tumor, stromal and immune cells, interspersed with blood and lymphatic vessels (1). The immune infiltrate in human tumors is heterogeneous depending on the tumor type and the individual. Several reports have identified TIL directed against tumor-associated antigens, indicative of a spontaneous in situ immune response in cancer patients (2–6). Moreover, a strong association between T cell density with clinical outcome has been reported in many types of human solid cancer (7–13). More precisely, memory T cells with Th1 and cytotoxic orientations seem to represent a predominant T cell population for prediction of favorable clinical outcome (14). In colorectal cancer, a comparison of the T cell infiltrate with standard pathological criteria demonstrated the prognostic power of immune criteria (7,15,16). These findings support the proposition that the T cell infiltrate may serve as a new critical marker to aid in classifying cancers (17). Nevertheless, despite the well recognized prognostic value of TIL in some cancers, mechanisms that govern their recruitment and activation into the tumor remain unclear.
It is generally recognized that secondary lymphoid organs, comprising lymph nodes, spleen, and mucosal-associated lymphoid tissues are the primary site of induction of adaptive immune responses (18). In addition, organized lymphoid aggregates termed tertiary lymphoid structures (TLS) can develop locally, at sites of persistent inflammatory disorders. Lymphoid neogenesis has been described in various human pathologies, comprising infections, auto-immune diseases and organ transplant rejections (19–21). TLS exhibit strong structural analogies with canonical secondary lymphoid organs and present features of an ongoing immune reaction site (22–24).
Moreover, several studies in mouse models have demonstrated that TLS can induce a protective primary and secondary immune response independently of secondary lymphoid organs during respiratory viral infection (25,26). However, the potential contribution of TLS to the promotion of an intratumoral immune reaction and their influence on the tumor immune contexture remain poorly investigated.

We have previously reported the presence of TLS in the tumor stroma of early-stage Non-Small Cell Lung Cancer (NSCLC), composed of clusters of DC-Lamp⁺ mature DC (referred as “TLS mature DC”) and T cells within T-cell area adjacent to B-cell follicle (27). These structures are surrounded by PNAd⁺ High Endothelial Venules (HEV), which are specialized blood vessels mediating lymphocyte extravasation into canonical lymphoid organs (28). Moreover, TLS were associated with a specific set of chemoattractant molecules involved in T-cell homing suggesting their participation for the immigration of peripheral blood T cells into the tumor (29). Additionally, DC-Lamp⁺ mature DC which home selectively in TLS have been associated with long-term survival in early-stage NSCLC patients supporting the involvement of TLS in the promotion of a protective immunity (27). We hypothesized that TLS could represent a privileged site for the recruitment and activation of TIL in human lung tumors and aimed to evaluate the potential influence of these structures on the tumor immune contexture.

Here, we investigated the impact of the TLS on the immune contexture in 458 NSCLC comprising all stages of the disease. Using immunohistochemistry, flow cytometry and quantitative RT-PCR (qRT-PCR), we demonstrated that TLS mature DC are strongly associated with a specific Th1 and cytotoxic immune signature and a long-term survival. In addition, the combination of mature DC and CD8⁺ T cell
densities constitutes a powerful and independent prognostic factor for overall survival. Altogether our data emphasize a major role for TLS in shaping the tumor immune contexture, and support their involvement in the promotion of a protective immune response mediated by T cells.
Materials and Methods

Patients

Fresh (n=54 patients), frozen (n=28 patients), and paraffin-embedded (n=376 patients) primary lung tumor samples were obtained from NSCLC patients who underwent a complete surgical resection of their lung tumors at Institut Mutualiste Montsouris or Hotel Dieu Hospital (Paris, France). 376 NSCLC patients operated between June 15, 2001 and November 26, 2004 were retrieved retrospectively. Patients who received neo-adjuvant chemotherapy or radiotherapy were ineligible. The observation time of the cohort was the interval between the surgery and the last contact (last follow-up or death of the patient). At the completion of the study, the minimal clinical follow-up was 90 months for the last patient included in the cohort. The main clinical and pathological features of the patients enrolled are presented in Table 1 for the retrospective study and in Supplementary Table 1 and Table 2 for the prospective study on fresh and frozen tumors (20 common patients between the 2 prospective cohorts), respectively. The data on long-term outcomes were obtained retrospectively by interrogation of municipality registers or the family of patients. A written informed consent was obtained from the patients prior to inclusion in the prospective study. The protocol was approved by the local ethics committee (n°: 2008-133 and 2012-0612) in application with the article L.1121-1 of French law.

Immunohistochemistry and immunofluorescence

For each paraffin-embedded lung tumor, two observers (one expert pathologist and one investigator trained to identify the pathological features of NSCLC) selected the tumor section containing a representative area of tumor with adjacent lung
parenchyma, and the highest density of immune cells on the hematoxylin and eosin-safran stained tissue section. Briefly, serial 5-μm tissue sections were deparaffinized, rehydrated, and pretreated in appropriate buffer for antigen retrieval. The sections were incubated with 5% human serum for 30 minutes before adding the appropriate primary antibodies followed by secondary antibodies (see Supplementary Table 3). Enzymatic activity was performed as described (27). For single staining, sections were counterstained with hematoxylin. Images were acquired using a Nanozoomer (Hamamatsu) operated with NDPview software.

**Method for cell quantification**

The quantification of DC-Lamp+ DC was determined as previously described (27). CD8+ cells were enumerated in the tumor nests and the stroma of the whole tumor section with Calopix software (Tribvn), and expressed as an absolute number of positive cells/mm² of the areas of interest. Both immunostaining and quantification were reviewed by at least two independent observers (J.G., and R.R., T.K.D.V.B. or M.-C.D.-N.).

**Flow cytometry**

Fresh lung tumor specimens were mechanically (manual) dissociated and digested in presence of Cell Recovery solution (BD Biosciences) instead of enzymes which can remove CCR7 and CD62L at the cell surface. Then, total live mononuclear cells were isolated from the tumors, as previously described (29). Mononuclear cells were stained with multiple panels of antibodies conjugated to fluorescent dyes (see Supplementary Table 3). Briefly, after saturation with 2% human serum,
mononuclear cells were incubated with the primary antibodies or appropriate isotype controls for 30 minutes at 4°C in the dark. Cells were washed and fixed in 0.5% formaldehyde before the analysis on a LSR II or Fortessa cytometer (BD Biosciences). Flow cytometry data were analyzed with the Diva (BD Biosciences) and FlowJo (Tree Star, Inc) softwares. The gating strategies are explained in Supplementary Fig. 1.

**RNA extraction and reverse transcription**

Total RNA from frozen tissues was extracted with the RNeasy Mini Kit (Qiagen) according to the manufacturer instructions, and RNA quantity and quality were determined with 2100 Bioanalyzer (Agilent Technologies). Samples with a RNA Integrity Number (RIN) ≥7 were reverse transcribed into cDNA using the High Capacity cDNA kit (Life Technologies) according to the manufacturer’s instructions.

**Quantitative PCR**

Complementary DNA samples were amplified using Low Density Array system according to the manufacturer’s instructions (Applied Biosystems). The arrays (Human Immune Array, TaqMan) were processed on a TaqMan 2900HD (Life Technologies). Four ng of cDNA per qRT-PCR were used. Expression levels of genes were determined using threshold cycle (Ct) values normalized to beta-actin expression as an endogenous control (ΔCt).

**Statistical analysis**
We used the Mann-Whitney test to compare the density of infiltrating immune cells in the different tumors. Correlations were performed using the Spearman test. Overall Survival (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 surgery. Together with mature DC and CD8+ cell densities, the following available clinical parameters were tested: TNM stage according to the new classification 2009 (30), smoking history, histological type according to the classification of the WHO (31), intervention type and emboli. With respect to immune cell densities, the “minimum P value” approach was used to determine the cut-off (Supplementary Fig. 2) for the best separation of patients referring to their OS outcome (outcome-oriented approach). Optimal cut-off values are 1.964, 383 and 114 cells per mm² for DC-Lamp+, CD8S and CD8T cells respectively. Because the P values obtained might be underestimated, OS log-rank P values were corrected using the formula proposed by Altman et al. (32) and validated using 100 repetitions of 2-fold cross-validations. We have also ensured that the significance established at the optimal cutoff remained valid at the quartiles (Supplementary Table 4). A P value less than 0.05 was considered statistically significant. Parameters identified at univariate analysis as possibly influencing the outcome (P<0.05) were introduced in a multivariate Cox-proportional hazards regression model. All analyses were performed with Prism 5 (GraphPad), Statview (Abacus Systems) and R (http://www.r-project.org/). Correlation matrix was represented with Genesis software (Institute for Genomics and Bioinformatics, Gratz, Austria (33)).
Results

Mature DC density is associated with early-differentiated and intermediate effector-memory CD8\(^+\) T cell infiltration in human lung tumors

We performed large-scale flow cytometry analyses on 54 freshly resected human NSCLC to characterize the immune infiltrate according to the density of DC-Lamp\(^+\) mature DC (Fig. 1). We observed a significant higher percentage of total CD3\(^+\), CD3\(^+\)CD4\(^+\) and CD3\(^+\)CD8\(^+\) T cells, a non-significant trend for CD19\(^+\) B cells and no difference for CD3\(^-\)CD56\(^+\) NK cells (Fig. 1A) among total live mononuclear cells from the tumor between patients with a high density of DC-Lamp\(^+\) DC (DC-Lamp\(^{Hi}\) patients) versus patients with a low density of DC-Lamp\(^+\) DC (DC-Lamp\(^{Lo}\) patients). DC-Lamp\(^{Hi}\) tumors had a significantly greater amount of CD62L\(^-\)CD4\(^+\) and CD62L\(^-\)CD8\(^+\) T cells than DC-Lamp\(^{Lo}\) tumors (Fig. 1B, left panel), in accordance with the selective localization of CD62L\(^-\) T cells inside the TLS (29) (Supplementary Fig. 3). We also observed a significant and concomitant increase of antigen-experienced CD62L\(^-\)CD4\(^+\) and CD62L\(^-\)CD8\(^+\) T cells, which represent the majority of TIL among total mononuclear cells (Supplementary Fig. 4A), between DC-Lamp\(^{Hi}\) versus DC-Lamp\(^{Lo}\) tumors (Fig. 1B, right panel). Interestingly, a positive correlation was observed between the proportion of CD62L\(^+\) and CD62L\(^-\) T cell subsets among total live mononuclear cells in the tumors (Supplementary Fig. 4B).

As compared to DC-Lamp\(^{Lo}\) tumors, DC-Lamp\(^{Hi}\) tumors were more infiltrated by activated CD38\(^+\) or CD69\(^+\) CD8\(^+\) T cells (Fig. 1C) and by the four main subpopulations of effector-memory CD8\(^+\) T cells (CD45RA\(^-\)CCR7\(^-\)CD27\(^+\) or -CD28\(^+\) or -)
(Fig. 1D) whereas no difference was seen for terminally differentiated effector-memory T cells (also called “TEM-RA”) which were detected at a very low frequency. Altogether, these results demonstrate that DC-Lamp$^{Hi}$ tumors have higher numbers of TLS T cells, as well as a higher number of activated and effector-memory non-TLS T cells, than DC-Lamp$^{Lo}$ tumors.

**Density of mature DC signals a coordinated in situ Th1, cytotoxic and activated T-cell immune reaction**

Since the density of mature DC which home selectively in TLS is associated with an increased number of TIL, we next evaluated the impact of TLS on the functional characteristics of the immune cell infiltrate with a focus on T lymphocytes. Gene expression levels related to the main immune populations, TLS, T helper 1 (Th1) and T helper 2 (Th2) orientations, CD8$^+$ T cell cytotoxicity, T-cell activation, immunosuppression, inflammation and angiogenesis were assessed in whole frozen tumors from 14 patients with DC-Lamp$^{Hi}$ tumors, and compared to 14 patients with DC-Lamp$^{Lo}$ tumors (Fig. 2A, Supplementary Fig. 5, and Supplementary Tables 2 and 5). As a control, genes related to the TLS cluster (CCL19, CCR7, CD28, CD62-L, and lymphotxin-Alpha) were significantly overexpressed among DC-Lamp$^{Hi}$ versus DC-Lamp$^{Lo}$ tumors ($P=0.0006$). DC-Lamp$^{Hi}$ tumors and not DC-Lamp$^{Lo}$ tumors were also associated with the overexpression of sets of genes clustered into specific groups: immune cell populations ($P=0.0011$), Th1 polarization ($P=0.0002$), CD8$^+$ T cell cytotoxicity ($P=0.0013$), and T cell activation ($P=0.0005$). Most of these molecules were detected at the protein level by IHC and/or by flow cytometry on T cells: immune cells (Fig. 1 and 3, (1), TLS signature (Fig. 1, (29)), Th1-orientation
(Supplementary Fig. 6, (27), chemokine receptors (29), and T-cell cytotoxicity and activation (Supplementary Fig. 6).

In contrast, genes involved in Th2 polarization, immuno-suppression, inflammation (excepting CSF-2) and angiogenesis were not differentially expressed between the two groups of patients (Fig. 2A). We constructed a correlation matrix and demonstrated that the expression of all genes overexpressed among DC-Lamp<sup>Hi</sup> tumors (immune cell subsets: CD3, CD4, CD8, CD20; TLS: CCL19, CCR7, CD28, CD62L, LTA; Th1 orientation: CXCR3, IFN-γ, IL2, IL12B, IL15, T-bet, TNFα; cytotoxicity: FAS-L, GNLY, GZMB, PRF1; and T-cell activation: CCL5, CCR2, CCR4, CCR5, CD40L, CD86, CTLA-4, HLA-DRα, and ICOS) was also significantly correlated (correlation coefficient in Fig. 2B, and P value in Supplementary Fig. 7A-B).

Altogether, these results demonstrate that mature DC density correlates with a specific intratumoral immune contexture characterized by the overexpression and coordination of genes related to T-cell activation, Th1 orientation and cytotoxic effector functions.

High density of mature DC predicts high levels of CD8<sup>+</sup> T cell infiltration in lung tumors

A correlation between CD8<sup>+</sup> T cell infiltration and a favorable clinical outcome was previously reported in many types of human solid cancer (8,9,13–15). As we observed a close association between mature DC density with cytotoxic-effector function, we further investigated the relationship between mature DC and CD8<sup>+</sup> T cell infiltration. Since CD8<sup>+</sup> T cells are expected to establish a contact with tumor
cells to exert their cytolytic function, we discriminated CD8+ T cells present in the
tumor nests and in the stroma in the following analysis.

In a retrospective series of 376 NSCLC patients (Table 1), we quantified stromal
CD8+ T cells (CD8S), tumor nest CD8+ T cells (CD8T) and mature DC-Lamp+ DC
(Fig. 3A, 3B, 3D). As previously observed in early stage lung tumors (27), we
confirmed that mature DC home selectively in the T-cell rich areas of TLS (Fig. 3C-
D) adjacent to PNAd+ vessels and B cell follicles (Fig. 3E-F) in all stage lung tumors.

In accordance with the results above, we observed a higher density of both CD8T
and CD8S cells (Fig. 3G) among DC-LampHi versus DC-LampLo tumors (mean=254
versus 138 CD8T/mm², \( P = 0.0003 \); mean=843 versus 553 CD8S/mm² \( P < 0.0001 \),
respectively). Consequently, substratification of DC-LampHi and DC-LampLo patients
according to CD8S and CD8T cell densities revealed that 84% of DC-LampHi patients
were CD8Hi in at least one region, and in particular 55% were high in both regions
(Fig. 3H). These proportions were reduced in DC-LampLo patients with 61% of CD8Hi
in at least one region, and only 33% in both regions. Interestingly, patients with
CD8SLo/CD8THi tumors were rare in both DC-Lamp groups, in accordance with the
trafficking of infiltrating T cells from the stroma to the tumor nests. The main
differences between DC-LampHi versus DC-LampLo patients concerned the
percentage of CD8SHiCD8THi and CD8SLoCD8TLo patients while the percentages of
mix groups (CD8SHiCD8THi and CD8SLoCD8THi) were quite unchanged. There were no
distinguishable clinical characteristics (except for gender, histologic type and pTNM
stage) between the patients with DC-LampHi versus DC-LampLo tumors
(Supplementary Table 6). The gender and histological type are also correlated, as
most females were diagnosed with a SCC (ADC, SCC, and other types: 85, 11 and
4% of females, and 59, 34, 7% of males, respectively, P=0.0026). This observation may, in part, explain the differential distribution of gender and histological type among the groups of DC-Lamp Hi versus DC-Lamp Lo patients. Using Cox multivariate regression analyses, we demonstrate that pTNM stage and DC-Lamp density were two independent prognostic factors (Supplementary Table 7).

Altogether, these results demonstrate that a high density of mature DC is closely related to a strong CD8+ T cell infiltration.

Density of TLS DC allows the identification of CD8 Hi and CD8 Lo patients with high risk of death

Since we observed that high densities of CD8+ T cells were detected in both groups of DC-Lamp Hi and DC-Lamp o patients, we next evaluated the prognostic value of each variable alone and in combination (Fig. 4).

The Kaplan-Meier curves indicate that the densities of mature DC (P=9.1x10^-05, Fig. 4A), CD8 S cells (P=0.0019, Fig. 4B), and CD8 T cells (P=0.0228, Fig. 4C) were correlated with longer overall survival (OS).

Since the presence of mature DC and CD8+ cells in the tumors positively influence the outcome of lung cancer patients, we stratified the patients into 4 groups according to the high or low density of each marker (DC-Lamp Hi/CD8 Hi, DC-Lamp Hi/CD8 Lo, DC-Lamp Lo/CD8 Hi, and DC-Lamp Lo/CD8 Lo) (Fig. 4D). We observed that the group of patients with DC-Lamp Hi tumors regardless of the density of CD8 S cells had the lowest risk of death (P=3.4 x10^-07, median OS were 92 months for DC-Lamp Hi/CD8 S Hi patients and 100 months for DC-Lamp Hi/CD8 S Lo patients), as was
observed for DC-Lamp\textsuperscript{Hi} patients (Fig. 4A). Interestingly, only the DC-Lamp\textsuperscript{Hi} patients present an improved survival as compared to the whole cohort. In contrast, patients with a low density of both dendritic and CD8\textsubscript{S} cells were at highest risk of death (median OS was 22 months) as compared to each immune marker alone (median OS DC-Lamp\textsuperscript{Lo}=36 months, CD8\textsubscript{S}\textsuperscript{Lo}=40 months). Patients with DC-Lamp\textsuperscript{Lo}/CD8\textsubscript{S}\textsuperscript{Hi} tumors were at an intermediate risk of death (median OS=41 months). Same results were obtained when the analysis was performed on the combination of DC-Lamp with CD8\textsubscript{T} cells (data not shown). Additional analyses with 100 repetitions of two-fold cross-validations confirmed the high and significant prognostic value of DC-Lamp/CD8\textsubscript{S} score (cross-validated 99/100 tests, median \(P\) value= \(4.7\times10^{-04}\)). Using Cox multivariate regression analyses (Table 2), the pTNM stage and DC-Lamp/CD8\textsubscript{S} score were the only criteria significantly and independently associated with OS (HR=1.70 and 0.71, and \(P=2.83\times10^{-07}\) and \(4.50\times10^{-07}\), respectively).

All together, these data demonstrate that DC-Lamp alone is a good marker for the identification of patients with a favorable outcome whereas the combination of CD8 with DC-Lamp allows the identification of patients with the highest risk of death. Finally, the DC-Lamp/CD8\textsubscript{S} score and pTNM stage constitute two independent and powerful prognostic factors.
Discussion
The major clinical impact of TIL has emphasized the need to better identify the mechanisms underlying their recruitment and activation (14). Based on the structural analogy with lymph nodes, we hypothesized that lung tumor TLS could play a key role in the promotion of a local immune reaction. The ability of TLS to promote an efficient and protective T cell response in many chronic inflammatory contexts (19–21) provided the rationale for this study.

By combining immunohistochemical and bio-molecular analyses, we demonstrated that a high density of TLS mature DC is associated with a specific immune orientation characterized by the over-expression and coordination of genes related to T-cell activation, Th1 polarization, and cytotoxic-effector functions. By contrast, immune genes related to Th2 polarization, immuno-suppression, inflammation and angiogenesis were not differentially expressed between groups of patients with high or low densities of TLS mature DC. In agreement with these results, previous reports have also demonstrated that TLS correlated with an increased number of T-bet+ lymphocytes or high levels of cytotoxic gene expression in NSCLC and colorectal cancers, respectively (27,34). This specific Th1 and cytotoxic orientation related to mature DC density suggests a major influence of TLS mature DC in the shaping of the tumor immune contexture.

The significant increase of T cell proportion, comprising CD62L+ T cells among the group of DC-LampHi versus DC-LampLo tumors, is in agreement with the selective presence of CD62L+ T cells located close to PNAd+ HEV (and vice versa) in TLS (29). Interestingly, a crucial role for DC in the maturation of HEV (35) and maintenance of TLS (22,23) has been demonstrated in mice. In addition, close
correlations between the density of HEV and lymphocyte infiltrate have been reported in melanoma (36,37), human breast cancer (38) and in the MCA-induced fibrosarcoma mouse model (39), which argue for functional features of HEV in tumor microenvironment. Altogether, these data strongly support that intratumoral HEV mediate recruitment of CD62L⁺ T cells from the blood into TLS, which could represent a major gateway for T cells into the tumor.

The density of TLS mature DC is also associated with a strong infiltration of experienced CD62L⁻ T cells that are predominantly of the effector-memory phenotype. These experienced T cells may derive, in part, from TLS CD62L⁺ T cells which have undergone a local activation and differentiation. Studies in mouse models indicated that TLS could play a key role in the induction of a local immune response. Development of TLS was associated with the generation of specific CD8⁺ T cells and viral clearance in mice lacking secondary lymphoid organs (19,20). A recent study also demonstrated that the activation, expansion and differentiation of naïve CD8⁺ T cells into effector cells could occur directly in the tumors of mice with B16 melanoma and devoid of secondary lymphoid organs (40). These data highlight that extranodal activation of TIL is possible and can generate an immune response against the tumor independently of a response initiated in canonical lymphoid organs. Furthermore, de novo priming of tumor-infiltrating CD8⁺ naïve T cells has been described in TLS induced by modified vaccine virus Ankara demonstrating that these structures represent a major site for T-cell priming (23). Strikingly, induction of lymphoid neogenesis in tumors manipulated to express LIGHT or lymphotoxin-α generates a massive infiltration of naive T cells, followed by T cell activation, expansion and tumors rejection (41,42). Altogether, these data underline that TLS
may serve as an important site for priming TIL during the generation of a local immune response.

Three major studies have previously reported strong associations between CD8+ T cell infiltrate with the outcome of lung cancer patients (9,43,44). In agreement with these data and similar results in many other types of cancers (8,13–15), we found a strong correlation between CD8+ cell density and improved clinical outcome in NSCLC patients. Compared to our first study performed in early-stage NSCLC patients (27), we confirmed that mature DC density is still a favorable prognostic biomarker for patient survival (cohort of patients with stages I to IV NSCLC).

Interestingly, patients with DC-Lamp^Hi/CD8^Lo tumors were scarce arguing for a causal link between TLS and CD8+ cells density where TLS would be an active site for CD8+ T cell proliferation. More importantly among CD8^Hi patients, those with a concomitant DC-Lamp^Hi density had a significant clinical benefit as compared with patients with no or few TLS. Thus, DC-Lamp represents a new marker allowing the identification of CD8^Hi patients with elevated risk of death, and the combination of both variables allows the identification of a worst-risk group (DC-Lamp^Lo/CD8^Lo patients).

TLS could potentiate an anti-tumor CD8+ T cell response in multiple ways. We previously reported that TLS T cells consisted primarily of CD4+ T cells (27,29). Interestingly, a major role of CD4+ T cell help for the recruitment, activation and effector functions of naïve CD8+ T cells was demonstrated in a model of pancreatic tumors (45). This is consistent with the strong coordination between mature DC infiltration, Th1 polarization, T cell activation and cytotoxic orientation that we report in this study.
We speculate that TLS mature DC could present tumor-associated antigens that will promote continuous generation of specific T cells directly in the tumor. As a result, TLS could induce a local immune reaction that would be more adaptable to the shifting expression of tumor-associated antigens during tumor progression. A major challenge will now be to characterize and to compare the specificity of experienced T cells according to DC density in order to evaluate a potential association between TLS and clonal diversity of TIL.

Altogether, our results suggest that TLS represent a privileged area for T-cell recruitment and activation in the primary site of lung tumor, which could play a major role in the shaping of a tumor immune contexture with survival benefit. Lymphoid neogenesis could represent a major phenomenon to promote a protective immune response in lung or other types of cancer, which may provide new opportunities to improve immunotherapy strategies.

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References


Figures and tables

Figure 1: Phenotypic analysis of the immune cell infiltrate according to the high and low density of mature DC.

Large-scale flow cytometry analysis of immune cell populations stratified by the density of mature DC in 54 fresh lung tumors (34 DC-Lamp^{Lo} tumors versus 20 DC-Lamp^{Hi} tumors). Density of mature DC was evaluated by immunohistochemistry. The percentage of the different cell types among total live mononuclear cells from the tumors, of DC-Lamp^{Lo} (white circles) and DC-Lamp^{Hi} (black circles) tumors is shown. (A) Percentage of total CD3^{+} T cells, CD4^{+} and CD8^{+} T cell subsets, CD19^{+} B cells and CD56^{+}CD3^{-} NK cells in groups of DC-Lamp^{Hi} tumors versus DC-Lamp^{Lo} tumors. (B) Proportion of CD3^{+} CD4^{+} and CD3^{+} CD8^{+} T cell subsets expressing either CD62L^{+} or CD62L^{-} T cells in DC-Lamp^{Hi} versus DC-Lamp^{Lo} tumors. Proportion of CD3^{+} CD8^{+} T cells expressing the activation markers CD38 and CD69 (C) with effector-memory phenotype (D) among groups of DC-Lamp^{Hi} versus DC-Lamp^{Lo} tumors. *, P < 0.05 ; **, P < 0.01 ; Mann-Whitney U test. Abbreviation: NA, not applicable.
Figure 2: Gene expression levels related to immune populations, TLS, Th-orientation, cytotoxicity, T-cell activation, immuno-suppression, inflammation and angiogenesis according to the high and low density of mature DC.

Gene expression levels were assessed by qRT-PCR and determined using threshold cycle (Ct) values normalized (delta Ct) to the actin housekeeping gene (n=28 frozen lung tumor samples). Heat map representation of clusters of genes related to immune populations, TLS, Th1 and Th2 orientation, cytotoxicity, T-cell activation, immuno-suppression, inflammation and angiogenesis is shown in (A). Genes are plotted from the minimal level of expression (blue) to the maximal level (red). Density of mature DC was evaluated by immunohistochemistry. Comparison of gene expression levels between DC-Lamp$^{Hi}$ versus DC-Lamp$^{Lo}$ patient groups were performed using Mann-Whitney test. A correlation matrix was constructed with the same genes. Each square represents in (B) a Spearman correlation coefficient (blue for negative correlation and red for positive correlation).
Figure 3: Analysis of the distribution of CD8\textsubscript{T} and CD8\textsubscript{S} positive cells in DC-Lamp\textsuperscript{Hi} versus DC-Lamp\textsuperscript{Lo} patients.

Immunostainings were performed on 376 paraffin-embedded tumor samples. Notice that CD8\textsuperscript{+} cells (red cells surrounded by a green line) were counted selectively in the stroma (A) and pan-cytokeratins\textsuperscript{+} tumor nests (B, blue cells stained with AE1/AE3 antibodies). TLS are detected by immunohistochemistry in NSCLC counterstained with hematoxylin and eosin (C). TLS are composed by a T-cell rich areas which comprised (D) DC-Lamp\textsuperscript{+} DC (red) in contact with CD3\textsuperscript{+} T cells (blue). (E) TLS are surrounded by PNAd\textsuperscript{+} HEV (red). (F) The CD3\textsuperscript{+} T-cell zone (blue) is adjacent to CD20\textsuperscript{+} B cell follicles (red). Lower magnification of IHC stainings is shown in Supplementary Fig. 8. Quantification of positive cells was performed on the whole section. (G) Distribution of the density of CD8\textsubscript{T} (left) and CD8\textsubscript{S} (right) T cells among groups of DC-Lamp\textsuperscript{Lo} (white bars) versus DC-Lamp\textsuperscript{Hi} tumors (grey bars). Statistical significance was calculated by Mann–Whitney test. (H) DC-Lamp\textsuperscript{Hi} and DC-Lamp\textsuperscript{Lo} patients stratified by high or low density of CD8\textsubscript{S} and CD8\textsubscript{T} cells. Abbreviations: He, hematoxylin; HES, hematoxylin and eosin.
Table 1: Baseline characteristics of the NSCLC patients enrolled in the retrospective study.

All parameters were evaluated among 376 NSCLC patients. Abbreviations: ADC, adenocarcinoma; SCC, squamous cell carcinoma; ND, not done; pT, pathologic T stage; pN, pathologic N stage; pTNM, pathologic TNM stage.
Figure 4: Overall survival for NSCLC patients according to the presence of a high or low density of DC-Lamp⁺ mature DC (A), CD8₆ cells (B) and CD8⁺ cells (C), or both DC-Lamp⁺ DC and CD8₆ cells (D).

OS curves for NSCLC patients were estimated by the Kaplan-Meier method, and differences between groups of patients were evaluated using the Log-rank test. Tables show the number of events, censored and at risk patients according to the cell density group. Tables show the 24-months, 60-months and 120-months OS rates (%) according to the group of patients, respectively.
Table 2: Multivariate Cox proportional hazards analysis for overall survival in NSCLC patients.

All parameters were evaluated among 325 NSCLC patients. Patients were stratified into 4 groups according to the high/low densities of mature DC and stromal CD8+ cells (DC-Lamp<sup>Hi</sup>/CD8<sup>Hi</sup>, DC-Lamp<sup>Lo</sup>/CD8<sup>Hi</sup>, DC-Lamp<sup>Hi</sup>/CD8<sup>Lo</sup>, and DC-Lamp<sup>Lo</sup>/CD8<sup>Lo</sup>). All categorical variables were transformed into discrete numeric variables before they were added into the Cox model. PHA test $P<0.05$ violates the proportional hazards assumption. Abbreviations: OS, overall survival; HR, hazard ratio; PHA, proportional hazards assumption.
Table 1

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Table 2

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<td>0.71</td>
<td>(0.62 - 0.81)</td>
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Dendritic cells in tumor-associated tertiary lymphoid structures license the positive prognostic value of tumor-infiltrating CD8+ T cells


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