Title page

Prognostic value of macrophage phenotypes in resectable non-small cell lung cancer assessed by multiplex immunohistochemistry

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Running title:
TAM phenotypes in NSCLC

Abstract

Macrophages are important inflammatory cells that regulate innate and adaptive immunity in cancer. Tumor-associated macrophages (TAMs) are thought to differentiate into two main phenotypes, pro-inflammatory M1 and pro-tumorigenic M2. Currently, the prognostic impact of TAMs and their M1 and M2 phenotypes is unclear in non-small cell cancer (NSCLC). The present study was set up to evaluate an approach for identifying common M1 and M2 macrophage markers and explore their clinical significance in NSCLC. Using multiplex chromogenic immunohistochemistry, tissue micro-arrays of 553 primary tumors and 143 paired metastatic lymph nodes of NSCLC specimens were stained to detect various putative macrophage phenotypes: M1 (HLA-DR/CD68), M2 (CD163/CD68), M2 (CD204/CD68) and pan-macrophage (CD68/CK). Correlation analyses were performed to examine the relationship between TAMs and adaptive/innate immune infiltrates. Greater frequency of stromal HLA-DR$^+$/CD68$^+$M1 subpopulation was statistically associated with lower T stage and more favorable ECOG performance status in primary tumors. HLA-DR$^+$/CD68$^+$M1 TAM level significantly decreased from pathological stage I to III. In a compartment-specific correlation analysis, moderate to strong correlations were observed between both TAM subsets (M1 and M2) with CD3, CD8, CD4 and CD45RO positive immune cells. Survival analyses, in both stromal and intra-tumoral compartments, revealed that high levels of HLA-DR$^+$/CD68$^+$M1 (stroma, HR=0.73, *P*=0.03; intra-tumor, HR=0.7, *P*=0.04), CD204$^+$M2 (stroma, HR=0.7, *P*=0.02; intra-tumor, HR=0.6, *P*=0.004) and CD68 (stroma, HR=0.69, *P*=0.02; intra-tumor, HR=0.73, *P*=0.04) infiltration were independently associated with improved NSCLC-specific survival. In lymph nodes, the intra-tumoral level of HLA-DR$^+$/CD68$^+$M1 was an independent positive prognostic indicator (Cox model, HR=0.38, *P*=0.001). In conclusion, high levels of M1, CD204$^+$M2 and CD68 macrophages are independent prognosticators of prolonged survival in NSCLC.

Introduction

In addition to intrinsic mechanisms within neoplastic cancer cells, cancer development depends on complex crosstalk between the tumor and the host's innate and adaptive immune systems [1]. Assessment of the tumor-immune contexture may provide information on the prognostic and predictive value of immune-related biomarkers, and improve understanding of tumor behavior [2,3]. Current knowledge suggests that the composition of the immune response influences the development and prognosis of non-small cell lung cancer (NSCLC) [4]. More recently, immune profiling of NSCLCs has provided prognostic data able to supplement the current TNM classification, producing a TNM-Immune-cell score (TNM-I) model [5]. In search for other immunological markers which could potentially contribute to a NSCLC TNM-I, *in situ* macrophages, known as tumor-associated macrophages (TAMs), are of great interest.

Macrophages constitute a heterogeneous and ubiquitous population of innate myeloid-derived cells, with pivotal roles in phagocytosis, inflammation and tissue repair in both normal homeostasis and disease [6]. In malignancy, TAMs interact with tumor cells to produce a rich source of cytokines, growth factors and proteases that shape the tumor microenvironment [7]. TAMs mainly originate from bone marrow (monocytic precursors), and differentiate according to tumor-derived signals[8]. It is proposed that TAMs polarize into one of two major lineages: M1 (classically activated) and M2 (alternatively activated) [9]. M1 macrophages secrete pro-inflammatory cytokines, largely express MHC class II (such as HLA-DR), and are thought to exhibit anti-tumoral functions through stimulation of T-cell mediated anti-tumor immunity [10]. M2 macrophages are often identified by the expression of CD163 (hemoglobin-scavenger receptor) or CD204 (macrophage-scavenger receptor-1), and are thought to contribute in tumor progression through increased metastatic ability, angiogenesis, immunosuppression via inhibition of the anti-tumoral immunity of both M1 and T-helper (Th1) cells, and by attracting activating regulatory T-cells and Th2 cells [9,11].

The prognostic impact of TAMs is inconsistent for different types of cancer. In a meta-analysis of different solid tumors, the presence of TAMs was associated with unfavorable outcomes in breast, head and neck, ovarian, gastric and bladder carcinomas, and with favorable outcomes in colorectal carcinoma (CRC) [12]. In NSCLC, the prognostic relevance of TAMs is still under debate [13]. Contradictory reports in NSCLC may relate to choice of

marker, low statistical power, homogeneous cohorts (using a particular tumor stage), and wide variation in the used method to assess patterns of macrophage infiltration [14].
The most common marker used to identify TAMs is the pan-macrophage CD68 antibody. However, CD68 is not exclusively expressed by TAMs; and other tumor tissue components (such as malignant epithelial and stromal cells) may express CD68 on their surface to some extent [15]. Moreover, single labeling of macrophages based on CD68 does not distinguish between M1 and M2 subsets. Recent studies attempt to use two or three different macrophage-associated markers to phenotype M1 and M2 and assess their effector functions [16]. Measuring TAMs using multiplex chromogenic immunohistochemistry (IHC) provide subset detail and may have higher detection accuracy, but this is limited to the use of appropriate chromogens for visualizing co-localized markers. The use of translucent chromogens produces color changes at sites of co-localization, allowing easy and reliable identification within the boundaries set by the sensitivity and specificity of the primary antibodies [17].

Due to previous contradictory findings and their wide methodological variation in NSCLC [13], the current study was conducted to profile tissue-based macrophages according to widely accepted M1 (HLA-DR) and M2 (CD163 and CD204) markers in combination with the pan-macrophage marker CD68. TAMs infiltration and association to prognosis was evaluated, in tissues from 553 resected NSCLC specimens and 143 matched lymph nodes, both in cancer cell islets and associated-stroma.

Materials and methods

Study cohort

The study population (previously described in [18,19]) is a consecutive series of 633 stage I-III NSCLC patients operated at University Hospital of North Norway and Nordland Hospital between 1990 and 2010. Of 633 potential cases, 553 were eligible for inclusion and 80 were excluded due to: neoadjuvant therapy before surgical resection (n=15), inadequate tissue in FFPE blocks (n=26), and presence of other malignancies before NSCLC diagnosis (n=39). Of the 553 eligible cases, 172 were diagnosed as LN+, of which 143 (N1, n=97; N2, n=47) had available tissue for assessment. Clinicopathological data were retrieved from clinical records and histopathology reports. The records included follow-up data until October 2013. The median follow-up was 86 months (34-267 months). All tumor specimens were restaged and reclassified by two lung pathologists according to the latest UICC and WHO guidelines [20,21]. The collection and reporting of clinicopathological variables, survival information and marker expression data was conducted according to the REMARK (Reporting Recommendations for Tumor Marker Prognostic Studies) guidelines [22]. The study was approved by the Norwegian data protection authority and regional committee for health research ethics (Reference no.2016/714).

Tissue microarray

The tissue microarray (TMA) methodology has been described in detail [23]. Briefly, full-faced tissue section slides were evaluated and the most representative areas were marked on H&E slides. From each patient's FFPE block, four or five representative core punches of 0.6 mm in diameter were transferred from donor to TMA recipient blocks, including two cores from tumor epithelium, two cores from tumor stroma, and one core from the normal alveolar area. TMAs were constructed using a manual MTA-1 tissue arrayer (Estigen, Estonia).

Multiplexed-IHC

The TMA blocks were sectioned at a thickness of 4 μm and baked overnight at 37 °C. The slides were processed using the Ventana Discovery-Ultra platform (Roche, Tucson, USA). The following mouse primary monoclonal antibodies were used for immunostaining: CD68 (clone: KP-1, #790-2931; Ventana), CD163 (clone: MRQ-26, #760-4437; Ventana), CD204

(clone: SRA-E5, #KT022; Transgenic), HLA-DR (clone: TAL.1B5, #M074601-2; Dako) and pan-cytokeratin (CK, clone: AE1/AE3/PCK26, #760-2135; Ventana). CD68, CD163, HLA-DR and pan-CK have clinical applications for *in vitro* diagnostic (IVD) assays. The staining protocol steps are detailed in Table S1. According to applied enzymatic reaction for each staining sequence, the corresponding secondary antibody was loaded: UltraMap anti-mouse (#760-4312, Ventana) and OmniMap anti-mouse (#760-4310, Ventana) for AP and HRP reactions respectively. All the detection kits were from Ventana (#760-124: DAB; #760-247: teal; #760-239: yellow; #760-229: purple). To inactivate the first primary antibody before loading the second primary antibody, enzymatic inhibition, using discovery inhibitor (#760-4840, 12 min,) as well as temperature-induced denaturation (8 min at 90 °C), was applied. All double-stained slides were compared with their corresponding single-stained slide. Three different methods were used for staining quality control: no primary antibody control for each sequence; isotype-matched control; and multi-tumor and normal TMAs.

Evaluation of immunostaining

All slides were digitized using a Pannoramic 250 Flash II scanner (3DHistech, Budapest, Hungary) with a maximum resolution of x40, and viewed using Pannoramic viewer 1.15.4 (3DHistech) and QuPath v.0.12 (Queen's University Belfast, Northern Ireland) software. The CD68 antibody was co-stained with HLA-DR to label M1, and with CD163 or CD204 to label M2. For pan-macrophage assessment, CD68 was co-stained with pan-CK.

The digitized slides were scored independently by two observers (M.R and S.J) for macrophage infiltration in different compartments: (a) tumor stroma (in the primary tumor); and (b) the intra-tumoral area (in both primary tumors and metastatic lymph nodes). Necrotic areas were ignored. In tumor stroma, the percentage of macrophages in the total number of nucleated cells was scored using the following scale: 0 (0–5%), 1 (6–25%), 2 (26–50%) and 3 (>50%). In the intra-tumoral area of both the primary tumor and metastatic lymph nodes, the total number of infiltrating macrophages was scored as follows: 0 (no positive cells), 1 (1–5 positive cells), 2 (≥6 positive cells). If there were more than two disagreements on scores, slides were reassessed to reach a consensus. A mean value of the marker scores was obtained for each patient.

Finally, the stromal M1, $CD204^{+}$M2 and $CD163^{+}$M2 scores were dichotomized into high and low groups using mean as cut-off values. For intra-tumoral infiltration and stromal CD68,

optimal cut-offs (minimal *P*-value) were used for dichotomization. The applied cut-off values are listed in Table S2.B.

Statistical analysis

The statistical analyses were performed using SPSS (Mac OS, version 25) and R (version 3.5.1). Interobserver reliability was calculated using a two-way random-effects model with an absolute agreement definition and Cohen's kappa coefficient with equal weighting. Mann–Whitney U tests were used to examine the association between distribution of different macrophage phenotypes across pathological stages. Correlations were explored between macrophage infiltration and clinicopathological variables (Chi-squared test) and between variables (Spearman's rho coefficient). Survival analysis was estimated by the Kaplan–Meier method, and the log-rank test was used to compare survival between the groups. Disease-specific survival (DSS) was calculated from the date of surgery to the date of NSCLC death. Multivariable Cox regression analyses were performed to identify independent predictors of survival. Stepwise backward conditional selection using 0.10 and 0.05 as entry-and exit-points was used to select variables for the final models. *P* values of < 0.05 were considered statistically significant.

Results

Reliable assessment of macrophage phenotypes

The study evaluated the presence and expression patterns of macrophage subpopulations co-expressing HLA-DR$^+$/CD68$^+$ (M1), CD163$^+$/CD68$^+$ (M2) and CD204$^+$/CD68$^+$ (M2). To find the most appropriate chromogen for cellular colocalization, different dye combinations (DAB, purple, red, yellow, and teal) were tested. HLA-DR, CD163 and CD204 in teal (HRP) and CD68 in yellow chromogen (AP), were the best for manual double-antigen visualizing. In this assay, two over-lapping signals on macrophages appear with a tertiary (green) color, making spatial assessment of the two markers considerably easier (Figure 1A-F). In order to improve differentiation of CD68$^+$ TAMs in tumor islets, pan-CK as an epithelial landmark marker, was co-stained with CD68 (Figure 1G-H).

Figure 2A-B represents the correlation matrix between TAM subsets and immune-related markers previously studied in this cohort. There was a strong correlation between stromal CD163$^+$M2 and CD204$^+$M2 (r=0.92), and moderate correlations between stromal M1 and CD204$^+$M2 or CD163$^+$M2 (r=0.46 and r=0.42, respectively). In the tumoral areas, strong correlation was also observed between CD163$^+$M2 and CD204$^+$M2 (r=0.91), and moderate correlations between M1 and CD204$^+$M2 (r=0.51) or CD163$^+$M2 (r=0.50).

To validate the specificity of TAM subset staining, a single TMA slide consisting of tumor samples from 54 patients were stained in multiplexed-IHC and compared in the combinations of HLA-DR/CD204/CD68 and HLA-DR/CD163/CD68, and the proportion of macrophages coexpressing both M1 and M2 markers were evaluated. By an absolute count of shared-phenotypic positive cells, the majority of TAMs showed a unique phenotypic expression, either M1 or M2, with few macrophages positive for both differentiating markers: HLA-DR$^+$/CD204$^+$/CD68$^+$: median (range) 3.1% (0–10.26%); HLA-DR$^+$/CD163$^{+/}$CD68$^+$: 2.7% (0–11.42%) (Figure 3A-B).
The intraclass correlation coefficients and Kappa values for the macrophage scores are listed in Table S2.A. There was substantial interobserver agreement between the two scorers, with greater consensus for the stroma compartment than the tumor compartment.

To further validate the TMA results, full-faced section slides of 20 (SCC, n=10; ADC, n=10; random selection) patients were evaluated. Heterogeneity between paired sections (full-face

tissue versus TMA cores) from the same patient was very low, and a significant concordance was observed for different macrophage subsets (Table S2. C).

Expression pattern of macrophage markers

The expression patterns of the used markers were fully evaluated in different tumor tissue cell types by two expert pulmonary pathologists (Table S3). As previously reported [15], and confirmed in this assessment, none of the applied antibodies were exclusively expressed on macrophages and can be expressed to some extend by other inflammatory and immune cells. Among these markers, CD68 and HLA-DR had broad immune cell and tissue expression, while CD204 and CD163 were restricted to particular macrophages. In addition, CD68 and HLA-DR were expressed on cancer cells in 23% (n=125) and 51% (n=281) of patients in the cohort, respectively (as illustrated in Figure. 1B). In positive cases, the intensity of CD68 protein expression in the cancer cells was homogenous while varied highly for HLA-DR. The M2-like phenotype was the dominant subset of TAMs in almost all necrotic areas (Figure 3C-D). All the explored antibodies displayed membranous and diffuse cytoplasmic localization on macrophages. CD163 and CD204 antigens had slightly higher cell membrane expression than HLA-DR or CD68.

Macrophage distribution and correlation

High stromal M1 was statistically associated with lower T stage and more favorable ECOG performance status in primary tumors. $CD204^{+}$M2 were closely correlated with patients age. (Table S4). No consistent associations (except between M1 and ECOG) were found between the level of macrophage subsets and clinicopathological variables in the intra-tumoral compartment of primary tumors or metastatic lymph nodes (Table S5).

In the stromal areas, moderate to strong correlations were observed between TAM subsets with CD3 (M1 r=0.47; $CD163^{+}$M2 r=0.39; $CD204^{+}$M2 r=0.38), CD8 (M1 r=0.38; $CD163^{+}$M2 r=0.31; $CD204^{+}$M2 r=0.30), CD4 (M1 r=0.48; $CD163^{+}$M2 r=0.41; $CD204^{+}$M2 r=0.43), and CD45RO (M1 r=0.29; $CD163^{+}$M2 r=0.31; $CD204^{+}$M2 r=0.3) positive immune cells (Figure 2.A). In the tumor area, similar correlations were observed between TAM subsets and T-cell markers (Figure 2.B).

Macrophage distribution was evaluated across TNM stages I, II and III. For pathological stages I to III, levels of stromal CD204$^+$M2, CD163$^+$M2 and pan-CD68 infiltration did not differ significantly, but notably decreased for M1 macrophages (Figure 2.C).

Macrophage and survival: univariate analysis

In the overall cohort, high levels of both intra-tumoral and stromal M1 (P = 0.021 and P = 0.003), CD204$^+$M2 (P = 0.004 and P = 0.013) and pan-CD68 (P = 0.01 and P = 0.006) macrophages were significantly associated with longer DSS (Figure 4; Table 1). For CD163$^+$M2 TAMs, a positive trend was seen for high infiltration in the stromal and intra-tumoral compartments.

In the SCC subgroup (n=307), high levels of stromal CD163$^+$M2 (P <0.001) and CD204$^+$M2 (P= 0.005) and both stromal and intra-tumoral M1 (P <0.001, P=0.016) macrophage infiltration, were associated with improved DSS (Figure S1, Table.S6).

In the ADC subgroup (n=239), high levels of stromal CD68-positive macrophages was associated with longer DSS (P= 0.039) (Figure S2, Table.S6).

In the metastatic lymph nodes, the presence of intra-tumoral M1 macrophages was a significant positive prognostic factor (P= 0.002) (Table 1).

Multivariate survival analysis

To test the prognostic significance of macrophage infiltration when adjusted for known prognostic factors, Cox proportional hazard models were used. In the overall cohort, stromal M1 (HR 0.73; CI 0.5-0.97; P=0.03), CD204$^+$M2 (HR 0.7; CI 0.5-0.94; P=0.02) and CD68 (HR 0.69; CI 0.5-0.94; P=0.02) were associated with significantly longer DSS independent of pStage, vascular invasion, ECOG performance status, and gender. Consistent with findings in stroma, intra-tumoral M1 (HR 0.7; CI 0.5-0.99; P=0.04), CD204$^+$M2 (HR 0.6; CI 0.4-0.8; P=0.004) and CD68 (HR 0.73; CI 0.5-0.99; P=0.04) were independent positive prognostic factors for DSS (Table 2). In metastatic lymph nodes, high intra-tumoral M1 infiltration was an independent positive predictor of DSS (HR 0.38; CI 0.2-0.7; P=0.001).

Discussion

The study describes a multiplex IHC assay for simultaneous identification of co-localized markers in macrophage phenotyping. To our knowledge, this is the first large study to investigate the clinical significance of *in situ* TAMs in stage I-III NSCLC using a chromogen-based IHC approach. The study reveals independent positive associations between the levels of HLA-DR$^+$M1, CD204$^+$M2 and pan-CD68$^+$ TAMs with disease-specific survival in both stromal and intra-tumoral compartments. Our findings also indicate that the presence of intra-tumoral HLA-DR$^+$M1 macrophages in metastatic lymph nodes is a predictor of improved survival.

The traditional approach of TAM analysis is based solely on CD68 expression [24]. Our previous study, involving 335 patients, showed a positive trend between high numbers of CD68$^+$TAMs and clinical outcome in both stromal and intra-tumoral compartments by single-color IHC [25]. In the current study, using a larger sample size and co-staining with pan-CK, CD68$^+$ TAMs showed statistical significance with multivariable analyses. Table S7 summarizes previous studies assessing the prognostic impact of TAMs in NSCLC. In line with the present study, Kim and Eerola *et al* [26,27] showed superior outcome with high intra-tumoral CD68$^+$TAMs. In contrast, other investigators found negative [28–30], none [31–33] or diverging [34,35] associations of CD68$^+$TAM density with patient outcome. These inconsistencies may partly be explained by two major issues, namely CD68 antibody specificity and methodological variation. Evidently, the subjectivity of IHC-stain interpretation can remarkably influence the reproducibility of CD68 scoring. Part of the variability in CD68$^+$TAM scoring may be caused by expression of this marker in tumor cells and other infiltrated immune cells [15]; in this study, tumor cells were positive for CD68 in 23% of the cohort. Non-specific staining may overestimate the level of TAMs and consequently affect the results. The use of pan-CK to differentiate between epithelial and non-epithelial cells probably increases the detection accuracy of intra-tumoral CD68 macrophages. Digital pathology has been used to quantify TAMs in some studies [36,37]. Antibody specificity may bias these studies more than visual microscopic evaluation due to the wide range of macrophage size distribution (5-30 μm) in lung tissue [38]. At the very least, detection of macrophages using morphological attributes in digital pathology requires highly specific algorithms relying on huge annotated datasets for the shape of TAMs.

Currently, there is no consensus on the identification and differentiation of tissue-based macrophage subsets in solid tumors. Recent publications advocate the use of multiple antibodies both to identify macrophages and to characterize TAM subpopulations [39]. When co-staining with CD68 (clone:KP1; IVD antibody) or even in single IHC assays, the most commonly used markers for M2 identification have been CD163 (clone: MRQ-26; IVD antibody), CD204 (clone: SRA-E5, widely used) and CD206 (used mainly for flow cytometry)[16]. For M1, there is less agreement about the best choice of antibodies, however several studies have used HLA-DR (clone: TAL.1B5; IVD antibody) for M1 identification [36,40–42]. HLA-DR is expressed on the membrane of antigen-presenting cells such as macrophages, monocytes, dendritic cells, B cells and activated T cells [43]. Tumor cell expression of HLA-DR has also been reported [44]. In NSCLC, only two studies employed double-IHC staining for analyzing different subsets of TAMs, while the majority used single-IHC staining against M2 antigens (CD204 or CD163) (Table S7). Ohri *et al* reported that intra-tumoral subpopulations, including M1- and M2-like TAMs, were predictors of superior outcome in NSCLC [40]. Similarly, we observed a survival advantage related to high M1 or M2 phenotypes in tumor islet as well as in stromal compartments. Ma *et al* found only intra-tumoral M1 (not M2) to be an independent prognostic indicator [36]. However, both Ma and Ohri *et al* were unable to identify any statistically significant associations between stromal TAM subsets and survival [36,40].

Biologically, the M1 and M2 subpopulations of macrophages are expected to associate with inverse anti-tumoral or pro-tumoral functions, respectively. However, we and other researchers (studying NSCLC, CRC and gastric carcinomas) have observed that both M1 and M2 subtype infiltration were positively associated with the patient's clinical outcome [40,45,46]. Different inferences were made in these studies for the survival benefits of M2 TAM infiltration. In NSCLC, Ohri *et al* suggested that further research might reveal mutual interactions between M1 and M2 TAMs [40]. Edin *et al* anticipated that due to co-presence of M1 and M2 in tumor tissue of CRC, the M1 anti-tumoral attribute may dominate over the M2 pro-tumoral functionality, leading to improved outcome. They also suggested that the intestinal environment is unique, comprising various microorganisms whereby macrophages require functional alteration in order to maintain local tissue homeostasis [45]. In gastric cancer, Kim *et al* speculated that the prognostic aspects of TAM may be largely oriented in relation to

lymphocytic infiltration, as concomitantly high levels of tumor-infiltrating lymphocytes (TILs) and $CD163^{+}$M2 were observed in their population [46]. In our study, the moderate to strong correlation between M1 or M2 and lymphocytic infiltration of CD3, CD8 and CD4 cells may imply that both macrophage phenotypes are involved in effective recruitment of lymphocytes and co-operate with T-helper/cytotoxic cells to induce anti-tumoral immune response [47]. Interestingly, in a recent lung cancer study, Peranzoni *et al* indicated a close relationship between the quantity of $CD206^{+}$ M2-like TAMs and "bystander" $CD8^{+}$ TILs in stroma [48]. Further, using a TAM-depleted murine model, they found that TAMs engage in prolonged interaction with $CD8^{+}$ TILs in stroma, limiting their entry into cancer islets and thereby interrupting their antitumor activity [48]. Taken together, macrophage phenotype clearly differs from tissue to tissue or within a single tissue in relation to their steps of polarization, disease stages and environmental signals. It also appears that, due to the high plasticity of macrophages, such a definition of M1 and M2 subpopulations and their involvement in distinct pro-tumoral and anti-tumoral activities of tumor is limiting; and such established nomenclature based on function probably bears no relevance in the complex tumor microenvironment [49,50].

Tumor stroma consists of a higher proportion of immune cells than intratumoral compartment, in which some immune cell subsets are positive for the markers studied here, together with TAMs (Table S3). Consequently, IHC-based analysis of TAM subsets in tumor stroma requires a reliable technical method that accounts for macrophage markers being co-localized in this context. With this understanding, a set of experiments to characterize macrophage subsets were conducted. In multiplexed chromogenic-IHC, the choice of chromogen or substrate is not important when protein biomarkers are expressed in different cell types. However, evaluating target proteins is more challenging when these are expressed in a single cellular compartment. In this situation, there is a risk of misinterpretation due to the overlap of chromogens and obstruction of one dye with another. By using translucent chromogens, we were able to reliably label co-localized antigens of interest on TAMs. When they are mixed, they can create a unique color, making it relatively easy to identify cells co-expressing the markers. The common dual-chromogen set used by researchers is conventional DAB/red, but in our experiment, this failed to be reliable because the dominant brown color significantly obstructed the red.

A novel finding in this study was the significant prognostic relevance of the M1 phenotype in resected metastatic lymph nodes—the level of intra-tumoral M1 infiltration was a very strong positive predictor of DSS in multivariable analysis, which is in line with its prognostic contribution in primary tumors. We did not find a significant correlation between TAM subsets in lymph nodes compared with primary tumor tissue (data not shown), which may relate to the heterogeneity of macrophages in these tissues [51]. Moreover, in pathological subgroups, stromal infiltration of M1 significantly dropped from stage I to stage III, which supports the previous concept about transition of macrophage phenotypes from proinflammatory to immunosuppressive states during the course of disease [52]. In further support, an animal study on hepatocellular carcinoma showed a shift from a high M1-like phenotype in the early stage to a low M1-like phenotype in the advanced stage [53]. Part of the complexity of macrophage expression can be linked to this temporal plasticity during tumor development.

In conclusion, this study demonstrates that high levels of either stromal or intra-tumoral pan-CD68, HLA-DR$^+$M1 and CD204$^+$M2 macrophages infiltration are independent determinants of favorable clinical outcome in stage I-III NSCLC patients. In addition, high levels of HLA-DR$^+$M1 macrophages in locoregional nodal metastases is an independent positive prognostic marker. From a technical aspect, the current observations support the use of translucent chromogens as a more practical choice for assessing co-localized TAM biomarkers in brightfield multiplex IHC.

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References

1. Gajewski TF, Schreiber H, Fu Y-X. Innate and adaptive immune cells in the tumor microenvironment. Nat Immunol. 2013 Oct;14(10):1014–22.
2. Fridman WH, Zitvogel L, Sautès–Fridman C, Kroemer G. The immune contexture in cancer prognosis and treatment. Nat Rev Clin Oncol. 2017 Jul 25;14(12):717–34.
3. Remark R, Becker C, Gomez JE, Damotte D, Dieu-Nosjean M-C, Sautès-Fridman C, et al. The Non–Small Cell Lung Cancer Immune Contexture. A Major Determinant of Tumor Characteristics and Patient Outcome. Am J Respir Crit Care Med. 2015;191:377–90.
4. Bremnes RM, Busund L-T, Kilvær TL, Andersen S, Richardsen E, Paulsen EE, et al. The Role of Tumor-Infiltrating Lymphocytes in Development, Progression, and Prognosis of Non-Small Cell Lung Cancer. J Thorac Oncol. 2016;11(6):789–800.
5. Donnem T, Kilvaer TK, Andersen S, Richardsen E, Paulsen EE, Hald SM, et al. Strategies for clinical implementation of TNM-Immunoscore in resected nonsmall-cell lung cancer. Ann Oncol. 2016 Feb;27(2):225–32.
6. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. Nature. 2013 Apr 25;496(7446):445–55.
7. Sica A, Larghi P, Mancino A, Rubino L, Porta C, Totaro MG, et al. Macrophage polarization in tumour progression. Semin Cancer Biol. 2008 Oct 1;18(5):349–55.
8. Franklin RA, Liao W, Sarkar A, Kim M V, Bivona MR, Liu K, et al. The cellular and molecular origin of tumor-associated macrophages. Science (80-). 2014 May 23;344(6186):921–5.
9. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol. 2002 Nov 1;23(11):549–55.
10. Gordon S. Alternative activation of macrophages. Nat Rev Immunol. 2003 Jan 1;3(1):23–35.
11. Qian B-ZZ, Pollard JW. Macrophage Diversity Enhances Tumor Progression and Metastasis. Cell. 2010 Apr 2;141(1):39–51.
12. Zhang Q, Liu L, Gong C, Shi H, Zeng Y, Wang X, et al. Prognostic Significance of Tumor-Associated Macrophages in Solid Tumor: A Meta-Analysis of the Literature. Hoque MO, editor. PLoS One. 2012 Dec 28;7(12):e50946.
13. Mei J, Xiao Z, Guo C, Pu Q, Ma L, Liu C, et al. Prognostic impact of tumor-associated

macrophage infiltration in non-small cell lung cancer: A systemic review and meta-analysis. Oncotarget. 2016 Apr 28;2(23).

14. O'Callaghan DS, O'Donnell D, O'Connell F, O'Byrne KJ. The Role of Inflammation in the Pathogenesis of Non-small Cell Lung Cancer. J Thorac Oncol. 2010 Dec 1;5(12):2024–36.
15. Ruffell B, Coussens LM. Macrophages and therapeutic resistance in cancer. Cancer Cell. 2015 Apr 13;27(4):462–72.
16. Heusinkveld M, van der Burg SH. Identification and manipulation of tumor associated macrophages in human cancers. J Transl Med. 2011 Dec 16;9(December 2011):216.
17. van der Loos CM. Chromogens in Multiple Immunohistochemical Staining Used for Visual Assessment and Spectral Imaging: The Colorful Future. J Histotechnol. 2010;33(1):31–40.
18. Hald SM, Rakaee M, Martinez I, Richardsen E, Al-Saad S, Paulsen E-E, et al. LAG-3 in Non-Small-cell Lung Cancer: Expression in Primary Tumors and Metastatic Lymph Nodes Is Associated With Improved Survival. Clin Lung Cancer. 2018 May;19(3):249–259.e2.
19. Rakaee M, Kilvaer TK, Dalen SM, Richardsen E, Paulsen E-E, Hald SM, et al. Evaluation of tumor-infiltrating lymphocytes using routine H&E slides predicts patient survival in resected non-small cell lung cancer. Hum Pathol. 2018 Jun 6;79:188–98.
20. Goldstraw P, Chansky K, Crowley J, Rami-Porta R, Asamura H, Eberhardt WEE, et al. The IASLC lung cancer staging project: Proposals for revision of the TNM stage groupings in the forthcoming (eighth) edition of the TNM Classification for lung cancer. J Thorac Oncol. 2016;11(1):39–51.
21. Travis WD, Brambilla E, Nicholson AG, Yatabe Y, Austin JHM, Beasley MB, et al. The 2015 World Health Organization Classification of Lung Tumors. J Thorac Oncol. 2015;10(9):1243–60.
22. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. REporting recommendations for tumor MARKer prognostic studies (REMARK). Breast Cancer Res Treat. 2006;100(2):229–35.
23. Bremnes RM. High-Throughput Tissue Microarray Analysis Used to Evaluate Biology and Prognostic Significance of the E-Cadherin Pathway in Non-Small-Cell Lung Cancer. J Clin Oncol. 2002;20(10):2417–28.
24. Bingle L, Brown NJ, Lewis CE. The role of tumour-associated macrophages in tumour

progression: implications for new anticancer therapies. J Pathol. 2002 Mar 1;196(3):254–65.

25. Al-Shibli K, Al-Saad S, Donnem T, Persson M, Bremnes RM, Busund LT. The prognostic value of intraepithelial and stromal innate immune system cells in non-small cell lung carcinoma. Histopathology. 2009;55(3):301–12.
26. Kim D-W, Min HS, Lee K-H, Kim YJ, Oh D-Y, Jeon YK, et al. High tumour islet macrophage infiltration correlates with improved patient survival but not with EGFR mutations, gene copy number or protein expression in resected non-small cell lung cancer. Br J Cancer. 2008 Mar 25;98(6):1118–24.
27. Eerola a K, Soini Y, Pääkkö P. Tumour infiltrating lymphocytes in relation to tumour angiogenesis, apoptosis and prognosis in patients with large cell lung carcinoma. Lung Cancer. 1999;26(2):73–83.
28. Li Z, Maeda D, Yoshida M, Umakoshi M, Nanjo H, Shiraishi K, et al. The intratumoral distribution influences the prognostic impact of CD68- and CD204-positive macrophages in non-small cell lung cancer. Lung Cancer. 2018;123(July):127–35.
29. Chen JJW, Yao P-L, Yuan A, Hong T-M, Shun C-T, Kuo M-L, et al. Up-regulation of tumor interleukin-8 expression by infiltrating macrophages: its correlation with tumor angiogenesis and patient survival in non-small cell lung cancer. Clin Cancer Res. 2003;9(2):729–37.
30. Kawai O, Ishii G, Kubota K, Murata Y, Naito Y, Mizuno T, et al. Predominant infiltration of macrophages and CD8 $^{+}$ T Cells in cancer nests is a significant predictor of survival in stage IV nonsmall cell lung cancer. Cancer. 2008 Sep 15;113(6):1387–95.
31. Pei B, Sun B, Zhang Z, Wang A, Ren P. Interstitial tumor-associated macrophages combined with tumor-derived colony-stimulating factor-1 and interleukin-6, a novel prognostic biomarker in non–small cell lung cancer. J Thorac Cardiovasc Surg. 2014 Oct 1;148(4):1208–1216.e2.
32. Ohtaki Y, Ishii G, Nagai K, Ashimine S, Kuwata T, Hishida T, et al. Stromal Macrophage Expressing CD204 is Associated with Tumor Aggressiveness in Lung Adenocarcinoma. J Thorac Oncol. 2010 Oct 1;5(10):1507–15.
33. Kojima H, Shijubo N, Yamada G, Ichimiya S, Abe S, Satoh M, et al. Clinical significance of vascular endothelial growth factor-C and vascular endothelial growth factor receptor 3 in patients with T1 lung adenocarcinoma. Cancer. 2005 Oct

15;104(8):1668–77.

34. Dai F, Liu L, Che G, Yu N, Pu Q, Zhang S, et al. The number and microlocalization of tumor-associated immune cells are associated with patient's survival time in non-small cell lung cancer. BMC Cancer. 2010 May 20;10:220.
35. Welsh TJ, Green RH, Richardson D, Waller DA, O'Byrne KJ, Bradding P. Macrophage and mast-cell invasion of tumor cell islets confers a marked survival advantage in non-small-cell lung cancer. J Clin Oncol. 2005 Dec 10;23(35):8959–67.
36. Ma J, Liu L, Che G, Yu N, Dai F, You Z. The M1 form of tumor-associated macrophages in non-small cell lung cancer is positively associated with survival time. BMC Cancer. 2010 Mar 25;10:112.
37. Carus A, Ladekarl M, Hager H, Pilegaard H, Nielsen PS, Donskov F. Tumor-associated neutrophils and macrophages in non-small cell lung cancer: No immediate impact on patient outcome. Lung Cancer. 2013 Jul 1;81(1):130–7.
38. Dewhurst JA, Lea S, Hardaker E, Dungwa J V., Ravi AK, Singh D. Characterisation of lung macrophage subpopulations in COPD patients and controls. Sci Rep. 2017 Dec 2;7(1):7143.
39. Mantovani A, Marchesi F, Malesci A, Laghi L, Allavena P. Tumour-associated macrophages as treatment targets in oncology. Nat Rev Clin Oncol. 2017 Jul;14(7):399–416.
40. Ohri CM, Shikotra a., Green RH, Waller D a., Bradding P. Macrophages within NSCLC tumour islets are predominantly of a cytotoxic M1 phenotype associated with extended survival. Eur Respir J. 2009;33(1):118–26.
41. van Dongen M, Savage NDL, Jordanova ES, Briaire-de Bruijn IH, Walburg K V., Ottenhoff THM, et al. Anti-inflammatory M2 type macrophages characterize metastasized and tyrosine kinase inhibitor-treated gastrointestinal stromal tumors. Int J Cancer. 2010 Aug 15;127(4):NA-NA.
42. Ino Y, Yamazaki-Itoh R, Shimada K, Iwasaki M, Kosuge T, Kanai Y, et al. Immune cell infiltration as an indicator of the immune microenvironment of pancreatic cancer. Br J Cancer. 2013 Mar 5;108(4):914–23.
43. Nuchtern JG, Biddison WE, Klausner RD. Class II MHC molecules can use the endogenous pathway of antigen presentation. Nature. 1990 Jan 4;343(6253):74–6.
44. He Y, Rozeboom L, Rivard CJ, Ellison K, Dziadziuszko R, Yu H, et al. MHC class II expression in lung cancer. Lung Cancer. 2017;112(December 2016):75–80.

45. Edin S, Wikberg ML, Oldenborg PA, Palmqvist R. Macrophages: Good guys in colorectal cancer. Vol. 2, OncoImmunology. Taylor & Francis; 2013. p. e23038.
46. Kim KJ, Wen XY, Yang HK, Kim WH, Kang GH. Prognostic implication of M2 macrophages are determined by the proportional balance of tumor associated macrophages and tumor infiltrating lymphocytes in microsatellite-unstable gastric carcinoma. Singh PK, editor. PLoS One. 2015 Dec 29;10(12):e0144192.
47. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol. 2010 Oct 20;11(10):889–96.
48. Peranzoni E, Lemoine J, Vimeux L, Feuillet V, Barrin S, Kantari-Mimoun C, et al. Macrophages impede CD8 T cells from reaching tumor cells and limit the efficacy of anti–PD-1 treatment. Proc Natl Acad Sci. 2018;115(17):201720948.
49. Noy R, Pollard JW. Tumor-associated macrophages: from mechanisms to therapy. Immunity. 2014 Jul 17;41(1):49–61.
50. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol. 2008 Dec 1;8(12):958–69.
51. Klein CA. Parallel progression of primary tumours and metastases. Nat Rev Cancer. 2009 Apr 1;9(4):302–12.
52. Biswas SK, Sica A, Lewis CE. Plasticity of macrophage function during tumor progression: regulation by distinct molecular mechanisms. J Immunol. 2008 Feb 15;180(4):2011–7.
53. Wang B, Li Q, Qin L, Zhao S, Wang J, Chen X. Transition of tumor-associated macrophages from MHC class II(hi) to MHC class II(low) mediates tumor progression in mice. BMC Immunol. 2011 Aug 4;12:43.

Figure 1: Multiplexed protein detection using translucent IHC-chromogens for TAM phenotyping in NSCLC. Compartment-specific infiltration of different TAM phenotypes in primary tumor: [A, B] HLA-DR^{+}(teal)/CD68^{+} (yellow) M1subset, [B] an example of HLA-DR tumor epithelial positive case in which the labelled M1 macrophages are easily distinguishable; [C, D] CD163^{+} (Teal)/CD68^{+} (yellow) M2 subset; [E, F] CD204^{+}(Teal)/CD68^{+} (yellow) M2 subset, all the co-localized markers appeared in a tertiary green color. [G, H] CD68^{+} (brown)/pan-CK (yellow). (magnification 15x)

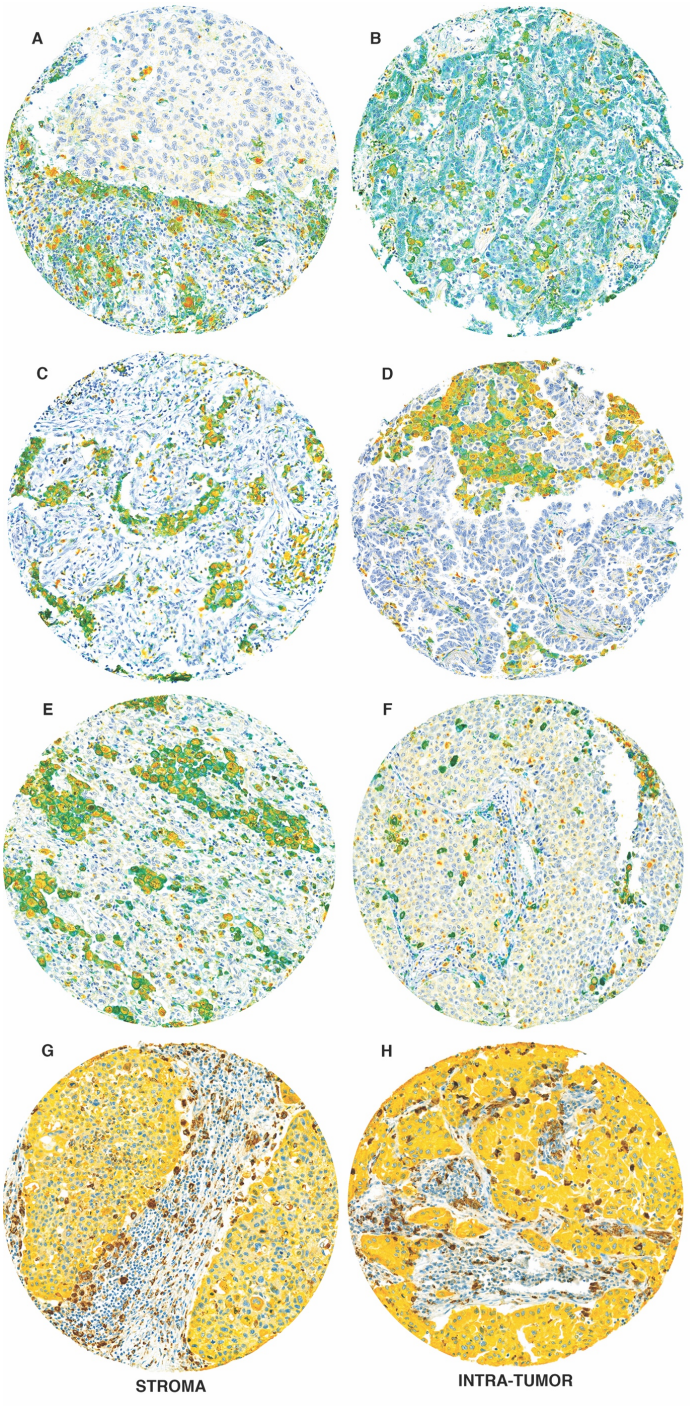

Figure 2: Spearman's rank correlation and Mann-Whitney U test on TAM phenotypes. [A, B] Correlation matrix between different stromal [A] and tumoral [B] TAM subsets and immune-related markers. [C] Dot- and Box-plots of various stromal (S, left column) and tumoral (T, right column) TAM subset distributions across pathological stages I-III in NSCLC. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

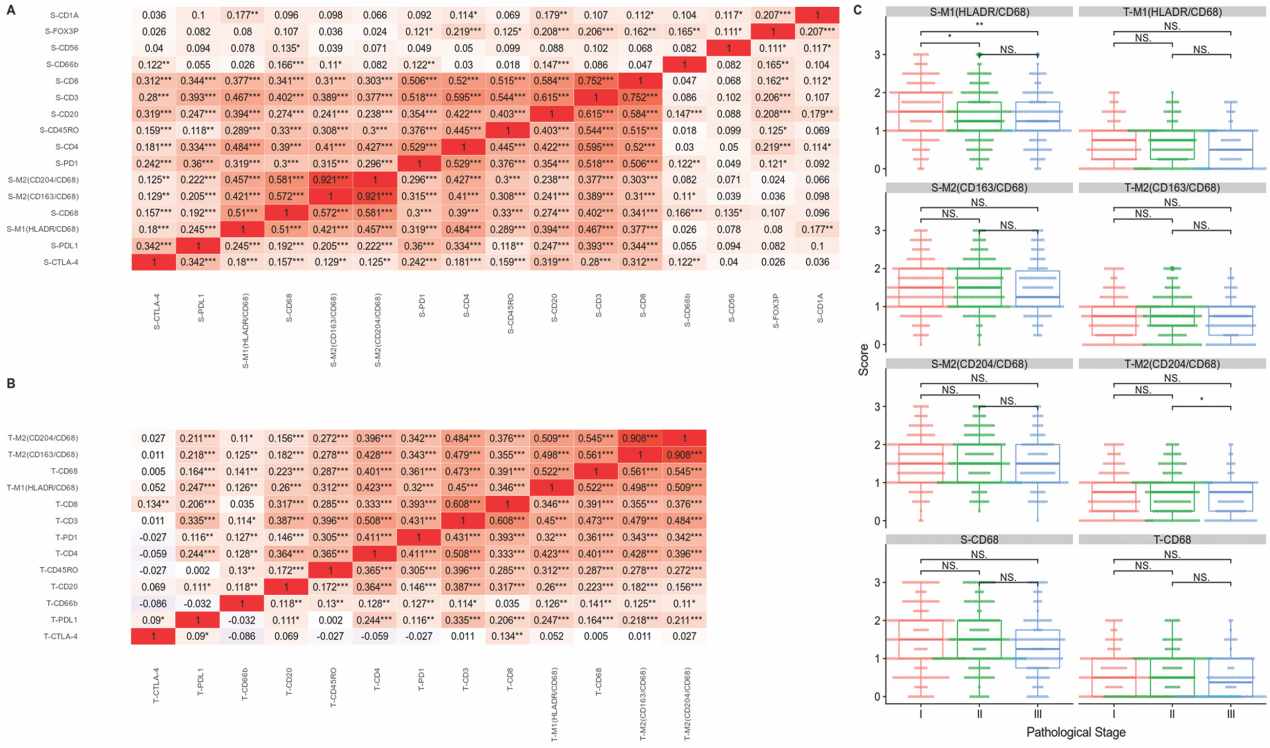

Figure 3: Multiplex IHC for validation of TAM subset (M1 *vs* M2) staining specificity. [A] 3-plexed IHC of M1 and CD163^{+}M2 marker: HLA-DR^{+} (teal)/CD163^{+} (purple)/CD68^{+} (yellow). Distinct phenotypic expression of the markers, M1 (green arrow), CD163^{+}M2 (red arrow), shared M1^{+}/ CD163^{+}M2 (black arrow) phenotype.
[B] 3-plexed IHC of M1 and CD204^{+}M2 marker: HLA-DR^{+} (teal)/CD204^{+} (purple)/CD68^{+} (yellow). Distinct phenotypic expression of the markers, M1 (green arrow), CD204^{+}M2 (red arrow), shared M1^{+}/ CD204^{+}M2 (black arrow) phenotype. [C, D] TAM phenotyping on consecutive TMA sections, demonstrating the dominant level of CD163^{+}M2 over M1 in necrotic areas of same core. [C] HLA-DR^{+} (Teal)/CD68^{+} (yellow) M2, [D] CD163^{+} (teal)/CD68^{+} (yellow) M1, the co-localized markers appeared in a tertiary green color

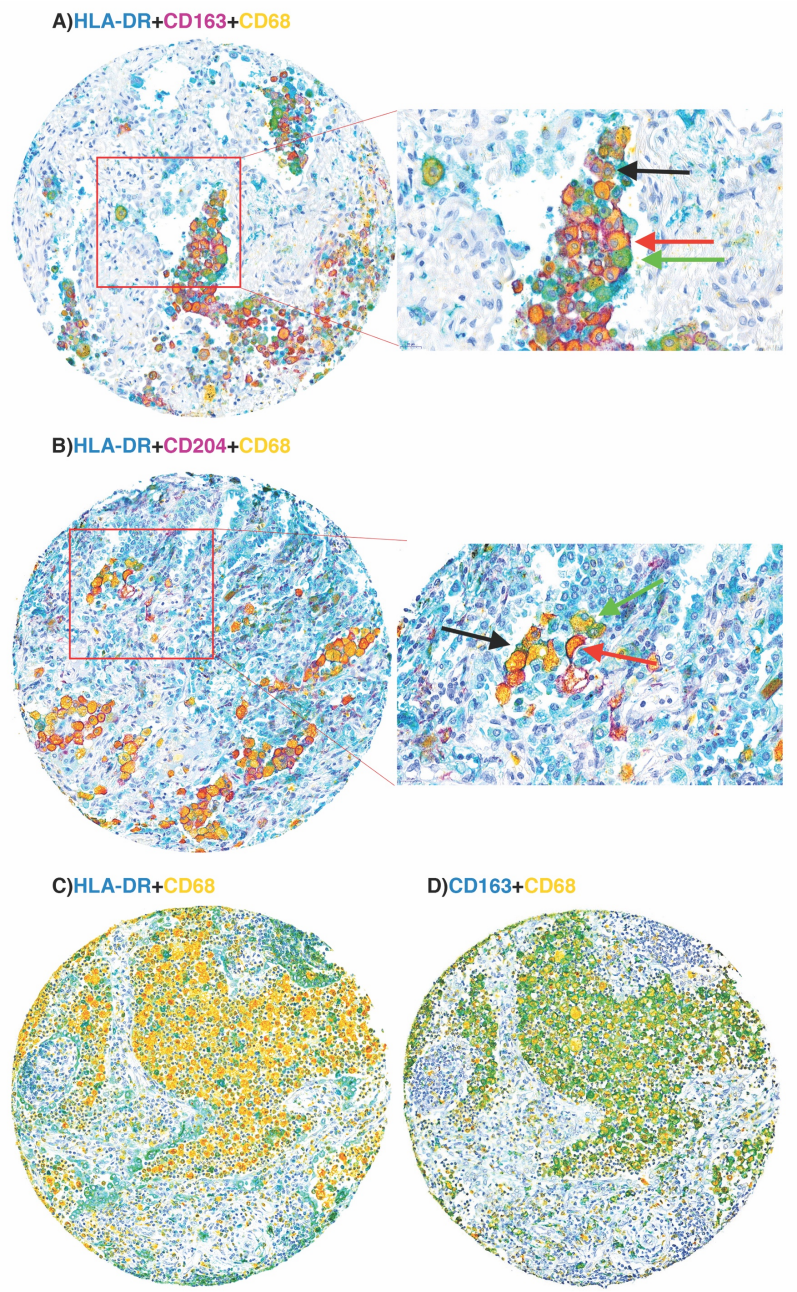

Figure 4: Disease-specific survival curves according to stromal and intratumoral TAM subset levels in primary tumor of NSCLC.

Intratumoral [A] HLA-DR^{+}M1; [B] CD163^{+}M2; [C] CD204^{+}M2; [D] pan-CD68.

Stromal [E] HLA-DR^{+}M1; [F] CD163^{+}M2; [G] CD204^{+}M2; [H] pan-CD68.

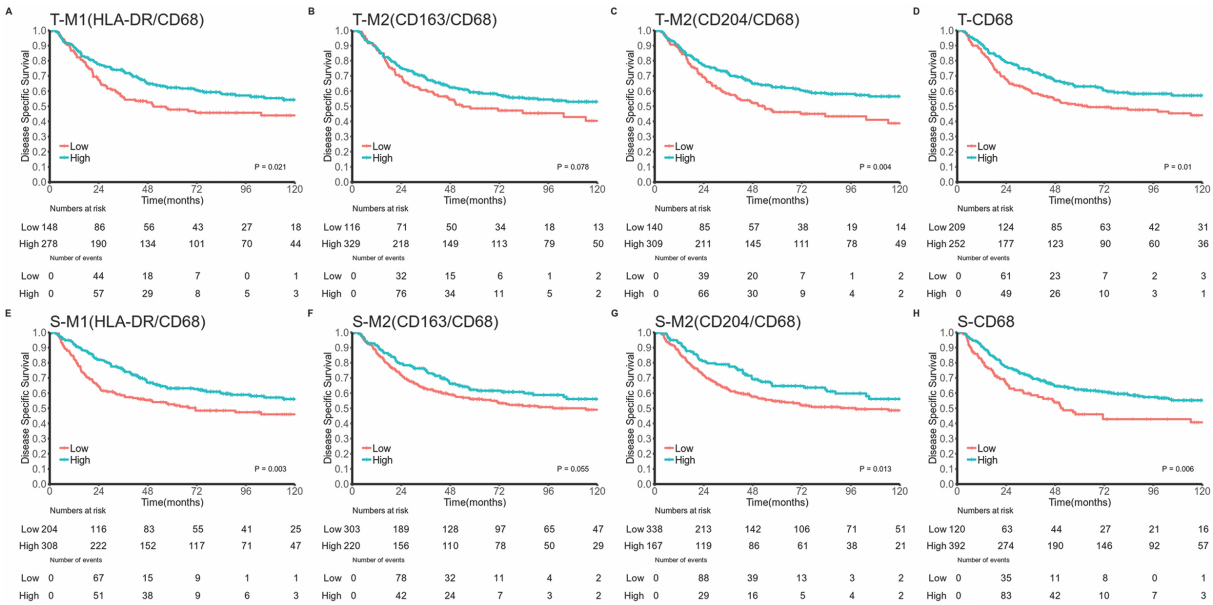

Table 1: Prognostic impact of stromal and intra-tumoral macrophage phenotypes in primary and metastatic lymph nodes of NSCLC patients

	Stroma					**Intra-tumor**				
	N(%)	DSS (%)	Median (month)	HR (95% CI)	*P*	N (%)	DSS (%)	Median (month)	HR(95% CI)	*P*
Primary Tumor										
M1 (HLA-DR$^+$/CD68$^+$)					**0.003**					**0.021**
Low	204(37)	52	71	1		148(27)	48	51	1	
High	308(56)	63	189	0.65(0.5-0.87)		278(50)	62	189	0.7(0.51-0.94)	
Missing	41(7)					127(23)				
M2 (CD163$^+$/CD68$^+$)					0.055					0.078
Low	303(55)	56	98	1		116(21)	48	54	1	
High	220(40)	62	189	0.76(0.57-1.01)		329(59)	59	235	0.75(0.5-1.03)	
Missing	30(5)					108(20)				
M2 (CD204$^+$/CD68$^+$)					**0.013**					**0.004**
Low	338(61)	55	98	1		140(25)	46	54	1	
High	167(30)	65	N.A	0.68(0.41-0.92)		309(56)	62	235	0.65(0.48-0.87)	
Missing	48(9)					104(19)				
CD68$^+$					**0.006**					**0.01**
Low	120(22)	46	51	1		209(38)	51	64	1	
High	392(71)	62	189	0.6(0.47-0.88)		252(46)	63	235	0.68(0.52-0.91)	
Missing	41(7)					92(16)				
LN+										
M1 (HLA-DR$^+$/CD68$^+$)										**0.002**
Low						28(19)	10	15	1	
High						62(43)	35	56	0.41(0.23-0.72)	
Missing						54(38)				

Abbreviations: NSCLC: non-small cell lung cancer, DSS: disease-specific survival, HR: hazard ratio, CI: confidence interval.

Table 2: Multivariable Cox models for disease specific survival of A) various stromal and intra-tumoral macrophage phenotypes in primary tumor and B) metastatic lymph nodes

Parameter	Stroma		Intra-tumor	
	HR (95% CI)	*P*	HR (95% CI)	*P*
A				
Model 1 M1(HLA-DR$^+$/CD68$^+$) Low vs high	0.73(0.5-0.97)	**0.03**	0.7(0.5-0.99)	**0.04**
Pstage				
I	1		1	
II	1.6(1.1-2.3)	**0.01**	0.2(0.16-0.35)	**<0.001**
III	4.1(2.8-5.7)	**<0.001**	0.3(0.2-0.5)	**<0.001**
Vascular invasion No vs yes	1.8(1.3-2.5)	**0.001**	0.5(0.3-0.8)	**0.002**
ECOG				
0	1		1	
1	1.4(1.05-1.9)	**0.02**	0.5(0.3-1.1)	0.09
2	1.4(0.8-2.5)	0.28	0.9(0.5-1.7)	0.8
Gender Female vs male	1.4(1.03-1.9)	**0.03**	0.7(0.5-1.01)	0.06
Model 2 M2 (CD163$^+$/CD68$^+$) Low vs high	0.76(0.57-1.1)	0.053	0.7(0.5-1.03)	0.08
Pstage				
I	1		1	
II	1.6(1.1-2.3)	**0.007**	0.25(0.17-0.36)	**<0.001**
III	3.8(2.7-5.4)	**<0.001**	0.4(0.2-0.5)	**<0.001**
Vascular invasion No vs yes	1.9(1.3-2.6)	**<0.001**	0.6(0.4-0.8)	**0.002**
ECOG				
0	1		1	
1	1.5(1.09-1.9)	**0.009**	0.6(0.3-1.1)	0.1
2	1.5(0.8-2.6)	0.17	0.9(0.4-1.6)	0.7
Gender Female vs male	1.4(1.04-1.9)	**0.03**	0.7(0-4-0.9)	**0.02**
Model 3 M2 (CD204$^+$/CD68$^+$) Low vs high	0.7(0.5-0.94)	**0.02**	0.6(0.4-0.8)	**0.004**
Pstage				
I	1		1	
II	1.6(1.2-2.3)	**0.005**	1.4(1.03-2.1)	**0.03**
III	3.7(2.6-5.3)	**<0.001**	3.6(2.5-5.2)	**<0.001**
Vascular invasion No vs yes	1.8(1.3-2.5)	**0.001**	1.7(1.2-2.5)	**0.002**
ECOG				
0	1		1	
1	1.4(1.04-1.8)	**0.02**	1.4(1.03-1.9)	**0.03**

2	1.4(0.8-2.5)	0.25	1.5(0.8-2.7)	0.1
Gender Female vs male	1.4(1.04-1.9)	**0.02**	1.3(0.9-1.9)	0.058
Model 4 $CD68^+$ Low vs high	0.69(0.5-0.94)	**0.02**	0.73(0.5-0.99)	**0.04**
Pstage				
I	1		1	
II	1.6(1.1-2.26)	**0.01**	1.5(1.05-2.2)	**0.02**
III	3.7(2.6-5.3)	**<0.001**	3.6(2.5-5.2)	**<0.001**
Vascular invasion No vs yes	1.8(1.2-2.5)	**0.001**	1.8(1.3-2.6)	**<0.001**
ECOG				
0	1		1	
1	1.4(1.05-1.8)	**0.02**	1.4(1.03-1.9)	**0.03**
2	1.4(0.8-2.6)	0.2	1.5(0.8-2.8)	0.1
Gender Female vs male	1.3(0.9-1.8)	**0.053**	1.4(1.06-2.02)	**0.01**
B				
M1 ($HLA\text{-}DR^+/CD68^+$) Low vs high			0.38(0.2-0.7)	**0.001**
T stage				
1			1	
2			1.7(0.7-3.9)	0.18
3			1.7(0.7-4.2)	0.2
4			2.6(0.9-7.1)	0.06
N stage (N1 vs N2)			1.7(0.9-3.1)	0.07

Abbreviations: HR: hazard ratio, CI: confidence interval, ECOG: Eastern Cooperative Oncology Group

Figure S1: Disease-specific survival curves of TAM subset levels in squamous cell carcinoma (SCC) subgroup. Intratumoral [A] HLA-DR^{+}M1; [B] CD163^{+}M2; [C] CD204^{+}M2; [D] pan-CD68. Stromal [E] HLA-DR^{+}M1; [F] CD163^{+}M2; [G] CD204^{+}M2; [H] pan-CD68.

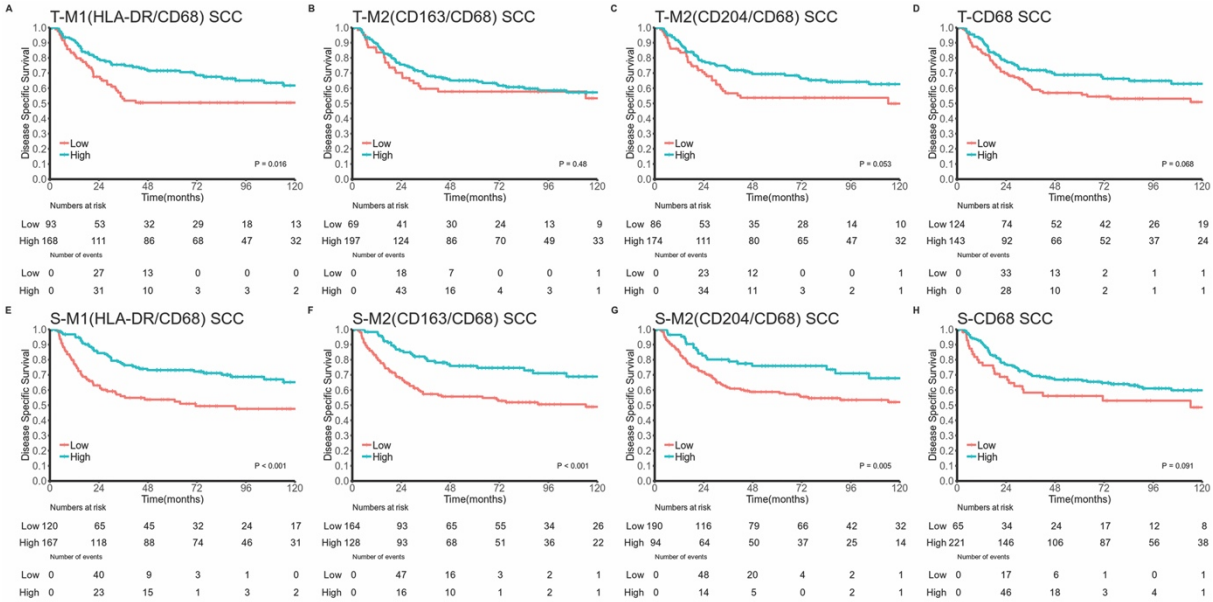

Figure S2: Disease-specific survival curves of TAM subset levels in adenocarcinoma (ADC) subgroup. Intratumoral [A] HLA-DR^{+}M1; [B] CD163^{+}M2; [C] CD204^{+}M2; [D] pan-CD68. Stromal [E] HLA-DR^{+}M1; [F] CD163^{+}M2; [G] CD204^{+}M2; [H] pan-CD68.

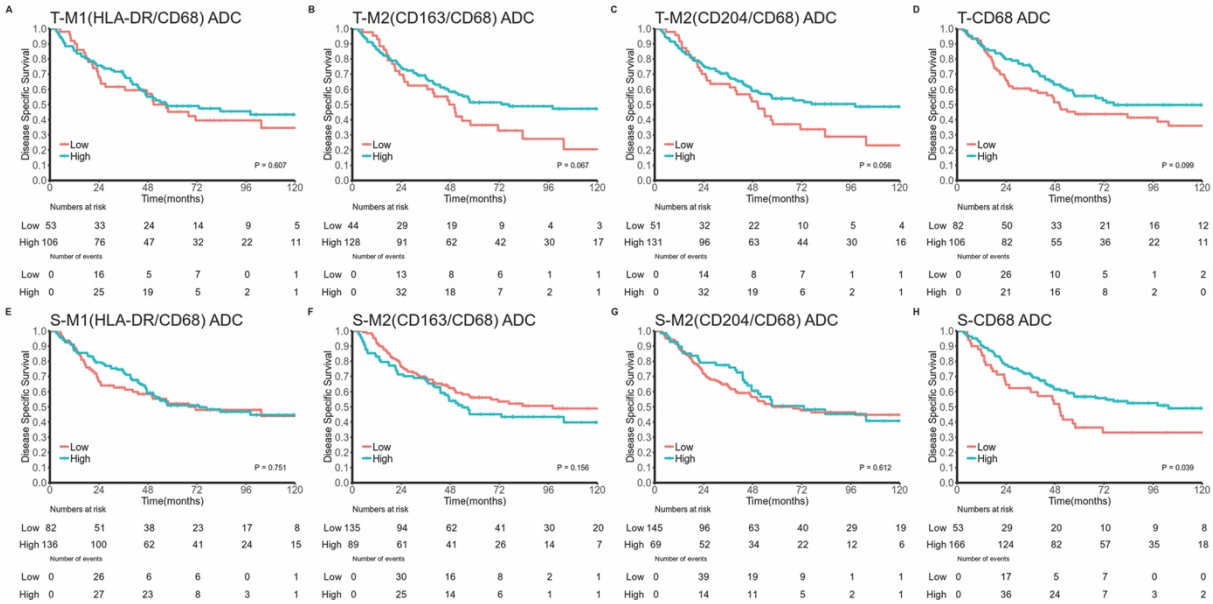

Table S1: The detailed protocol of the sequential multiplexed-IHC for profiling TAMs.

	1st sequence					**2nd sequence**				
	Deparaffinization Cycle (Time)	Pretreatment (Time)	Antibody Dilution (Time)	Chromogen (Time)	Substrate	Antibody Dilution (Time)	Chromogen (Time)	Substrate	Counterstain (Hematoxylin)	Post Counterstain (bluing reagent)
HLADR/CD68	3 (12 min)	CC1 (32 min)	1/150 (32 min)	Teal (H2O2:32 min) (Act:16 min)	HRP	Prediluted (32 min)	Yellow (44 min)	AP	4 min	NA
CD163/CD68	3 (12 min)	CC1 (32 min)	Prediluted (28 min)	Teal (H2O2:32 min) (Act:16 min)	HRP	Prediluted (16 min)	Yellow (44 min)	AP	4 min	NA
CD204/CD68	3 (12 min)	CC1 (32 min)	1/50 (32 min)	Teal (H2O2:32 min) (Act:16 min)	HRP	Prediluted (16 min)	Yellow (44 min)	AP	4 min	NA
CD68/CK	3 (12 min)	CC1 (32 min)	Prediluted (24 min)	DAB (default)	HRP	Prediluted (16 min)	Yellow (60 min)	AP	28 min	4 min

Abbreviations: CD: cluster of differentiation, HLA-DR: human leucocyte antigen-DR isotype, CK: cytokeratin, CC1: cell conditioning 1, Act: activator, HRP: horseradish peroxidase, AP: alkaline phosphatase, NA: not applied.

Table S2: A) Intraclass correlation and Cohen's kappa analysis between scorers for assessed markers in both intratumoral and stromal compartments of primary tumor and metastatic lymph nodes**, B)** the used cut points in the statistical analysis for dichotomization, **C)** paired sample correlation between full-faced tissue section and TMA core scores for 20 cases (*t*-test). For intratumoral compartment of the full-faced section slides, an average number of five fields were used.

	A	Marker localization	ICC	*P*-value	Kappa	*P*-value	**B**	Cut point	**C**	*r*	*P*-value
Primary tumor											
M1(HLA-DR$^+$/CD68$^+$)		intra-tumor	0.86	< 0.001	0.48	< 0.001		0.25		0.84	0.004
M1(HLA-DR$^+$/CD68$^+$)		stroma	0.93	< 0.001	0.53	< 0.001		1		0.86	0.003
M2(CD163$^+$/CD68$^+$)		intra-tumor	0.81	< 0.001	0.33	< 0.001		0.25		0.82	0.01
M2(CD163$^+$/CD68$^+$)		stroma	0.89	< 0.001	0.32	< 0.001		1.5		0.91	< 0.001
M2(CD204$^+$/CD68$^+$)		intra-tumor	0.81	< 0.001	0.35	< 0.001		0.25		0.78	0.02
M2(CD204$^+$/CD68$^+$)		stroma	0.88	< 0.001	0.49	< 0.001		1.75		0.86	0.01
CD68		intra-tumor	0.95	< 0.001	0.8	< 0.001		0.25		0.82	0.01
CD68		stroma	0.97	< 0.001	0.74	< 0.001		0.75		0.84	0.004
Metastatic Lymph nodes											
M1(HLA-DR$^+$/CD68$^+$)		intra-tumor	0.86	< 0.001	0.44	< 0.001		0.25		N.E	N.E

Abbreviation: ICC: intraclass correlation coefficient, N.E: not evaluated

Table S3: Expression pattern of macrophages markers among various tissue cellular compositions assessed in random 50 NSCLC patients (single-IHC staining)

	CD68 (pan)	HLA-DR (M1)	CD163(M2)	CD204 (M2)
TAMs	+++	+++	+++	++
TILs	-/+	++	-/+	-
TANs	-/+	-	-	-
Dendritic Cells*	+	++	-/+	-/+
Plasma Cells	-	-	-/+	-
Fibrocyte/ fibroblast	-/+	-/+	-	-
Endothelial Cells	-	-	-	-
Type 1 Pneumocyte	-	-/+	-	-
Type 2 Pneumocyte	-/+	-/+	-	-
Cancer cells	-/+	++	-/+	-

*Dendritic cell expression assessed in lymphoid aggregates area.
Rarely (-/+), lowly (+), moderately (++), highly (+++) expressed.

Abbreviations: TAMs: tumor-associated macrophages, TILs: tumor-infiltrated lymphocytes, TANs: tumor-associated neutrophils

Table S4: Associations between stromal macrophage phenotypes and clinic-pathological variables in overall cohort of resected primary NSCLC

	M1			CD163 M2			CD204 M2			CD68		
	low	high	*P*	low	high	*P*	low	high	*P*	low	high	*P*
Total	204	308		303	220		338	167		120	392	
Age			0.6			0.2			**0.02**			0.1
≤ 65	91	131		136	87		158	60		44	176	
>65	113	177		167	133		180	107		76	216	
Sex			0.6			0.3			0.4			0.2
Female	65	105		94	78		107	59		35	138	
Male	139	203		209	142		231	108		85	254	
Weight loss			0.07			0.7			0.5			0.8
<10%	177	282		270	199		301	152		107	351	
≥10%	27	25		32	21		36	15		13	40	
Missing		1		1			1				1	
Smoker			0.4			0.4			0.9			0.1
Never	4	12		8	9		11	6		2	15	
Current	129	198		198	133		214	106		73	255	
Former	71	98		97	78		113	55		45	122	
ECOG			**0.02**			0.8			0.2			0.6
0	107	194		177	130		190	104		66	233	
1	77	99		102	76		119	55		44	133	
2	20	15		24	14		29	8		10	26	
Histology			0.52			0.6			0.4			0.8
SCC	120	167		164	128		190	94		65	221	
ADC	82	136		135	89		145	69		53	166	
Other	2	5		4	3		3	4		2	5	
Tstage			**0.004**			0.2			0.5			0.5
T1	63	128		124	73		131	59		34	135	
T2	68	111		103	80		115	61		45	145	
T3	49	40		45	44		54	33		26	70	
T4	24	29		31	23		38	14		15	42	
Nstage			0.4			0.5			0.8			0.7
N0	135	215		205	154		232	118		170	184	
N1	44	66		64	48		72	35		49	59	
N2	25	27		34	18		34	14		26	24	
Pstage			0.08			0.1			0.2			0.1
I	75	135		131	84		141	68		50	163	
II	67	105		92	86		107	64		34	140	
III	62	68		80	50		90	35		36	89	
Vascular invasion			0.5			0.6			0.09			0.6
No	165	255		251	177		285	131		96	322	
Yes	38	51		51	41		51	35		23	68	
Missing	1	2		1	2		2	1		1	2	

Abbreviations: ECOG:Eastern Cooperative Oncology Group performance status, ADC: adenocarcinoma, SCC: squamous cell carcinoma, LCC: large-cell carcinoma, ASC: adenosquamous carcinoma, NOS: not otherwise specified, Nstage: nodal stage, Pstage: pathological stage, Tstage: tumor stage.

Table S5: Associations between intratumoral macrophage phenotypes and clinic-pathological variables in overall cohort of resected primary NSCLC tumor and paired metastatic lymph node

	Primary Tumor												LN+		
	M1			CD163 M2			CD204 M2			CD68			M1		
	low	high	*P*	low	high	*P*	low	high	*P*	low	high	*P*	Low	High	*P*
Total	148	278		116	329		140	309		209	252		28	62	
Age			0.8			0.9			0.8			0.9			0.6
≤ 65	63	121		49	140		61	132		89	109		15	30	
>65	85	157		67	189		79	177		120	143		13	32	
Sex			0.1			0.1			0.3			0.6			0.2
Female	40	93		42	94		50	95		66	85		6	20	
Male	108	185		74	235		90	214		143	167		22	42	
Weight loss			0.9			0.3			0.1			0.2			0.3
<10%	132	248		107	291		130	272		192	221		23	56	
≥10%	16	29		9	37		10	36		17	30		5	6	
Missing		1			1			1			1				
Smoker			0.3			0.6			0.4			0.3			0.5
Never	2	10		3	9		2	11		9	5		0	2	
Current	96	172		68	209		87	194		129	163		19	37	
Former	50	96		45	111		51	104		71	84		9	23	
ECOG			0.1			**0.03**			0.1			0.09			0.7
0	73	167		55	198		71	187		110	158		17	33	
1	63	92		48	111		56	102		82	77		9	23	
2	12	19		13	20		13	20		17	17		2	6	
Histology			0.8			0.5			0.4			0.8			0.2
SCC	93	168		69	197		86	174		124	143		15	30	
ADC	53	106		44	128		51	131		82	106		12	32	
Other	2	4		3	4		3	4		3	3		1	0	
Tstage			0.1			0.4			0.6			0.053			0.3
T1	38	98		39	100		51	98		71	82		3	14	
T2	60	96		38	129		46	119		65	104		11	25	
T3	33	49		27	58		27	55		48	37		7	15	
T4	17	35		12	42		16	37		25	29		7	8	
Nstage			0.2			0.9			0.1			0.3			0.5
N0	95	199		81	227		97	216		144	171				
N1	35	58		25	74		26	72		41	61		21	42	
N2	18	21		10	28		17	21		24	20		7	20	
Pstage			0.2			0.8			0.3			0.5			0.3
I	55	116		46	131		59	127		82	106				
II	49	102		40	121		43	115		70	89		9	27	
III	44	60		30	77		38	67		57	57		19	35	
Vascular invasion			0.6			0.6			0.9			0.3			0.1
No	119	219		94	264		113	250		173	198		17	49	
Yes	28	58		20	64		26	58		35	52		10	13	
Missing	1	1		2	1		1	1		1	2		1		

Abbreviations: ECOG: Eastern Cooperative Oncology Group performance status, ADC: adenocarcinoma, SCC: squamous cell carcinoma, LCC: large-cell carcinoma, ASC: adenosquamous carcinoma, NOS: not otherwise specified, Nstage: nodal stage, Pstage: pathological stage, Tstage: tumor stage.

Table S6: Subgroup analysis on prognostic value of stromal and intra-tumoral macrophage phenotypes in NSCLC patients

Variable	**Stroma**										**Intra-tumor**									
	Squamous cell carcinoma					Adenocarcinoma					Squamous cell carcinoma					Adenocarcinoma				
	N (%)	DSS (%)	Median (month)	HR(95% CI)	*P*	N(%)	DSS(%)	Median (month)	HR(95% CI)	*P*	N (%)	DSS (%)	Median (month)	HR(95% CI)	*P*	N(%)	DSS(%)	Median (month)	HR(95% CI)	*P*
M1 (HLA-DR$^+$/CD68$^+$)					**<0.001**					0.75					**0.016**					0.6
Low	120(39)	54	71	1		82(34)	52	71	1		93(30)	50	N.A	1		53(22)	45	51	1	
High	167(54)	73	235	0.48(0.32-0.71)		136(57)	51	73	0.94(0.63-1.4)		168(55)	71	235	0.6(0.4-0.91)		106(44)	49	57	0.8(0.56-1.4)	
Missing	20(7)					21(9)					46(15)					80(34)				
M2 (CD163$^+$/CD68$^+$)					**<0.001**					0.15					0.5					0.06
Low	164(53)	56	114	1		135(57)	56	98	1		69(23)	58	N.A	1		44(18)	36	50	1	
High	128(42)	76	N.A	0.44(0.28-0.68)		89(37)	45	54	1.3(0.9-1.9)		197(64)	65	235	0.8(0.54-1.3)		128(54)	51	76	0.6(0.42-1.03)	
Missing	15(6)					15(6)					41(13)					67(28)				
M2 (CD204$^+$/CD68$^+$)					**0.005**					0.61					0.053					0.056
Low	190(62)	59	235	1		145(61)	50	68	1		86(28)	54	114	1		51(21)	37	50	1	
High	94(31)	76	N.A	0.51(0.3-0.8)		69(29)	51	73	0.9(0.59-1.3)		174(57)	70	235	0.6(0.43-1.01)		131(55)	54	98	0.65(0.42-1.01)	
Missing	23(7)					25(10)					47(15)					57(24)				
CD68$^+$					0.09					**0.039**					0.06					0.09
Low	65(21)	56	114	1		53(22)	36	50	1		124(40)	57	N.A	1		82(34)	44	50	1	
High	221(72)	67	235	0.68(0.43-1.07)		166(70)	57	103	0.63(0.4-0.98)		143(47)	69	235	0.68(0.46-1.03)		106(45)	56	77	0.7(0.47-1.06)	
Missing	21(7)					20(8)					40(13)					51(21)				

Abbreviations: DSS: disease-specific survival, HR: hazard ratio, CI: confidence interval.

Table S7: Published data on prognostic effect of tumor-associated macrophages by chromogenic-IHC in NSCLC

(Author/ year)	Cohort size	Sample type	Tumor histology	Tumor stage	IHC	Marker(s)	Subset	Antibody clone	Cut-off	Assessed area	Prognostic impact (PI) for high infiltration
(Li *et al*, 2018)	297	WT	ADC Other	I-IV	SS	1-CD68	Pan	PG-M1	median	stroma	CD68 and CD204 independent negative PI (Overall cohort and ADC subgroup)
						2-CD204	M2	SRA-E5			
										tumor	NS
(Pei *et al*, 2014)	417	TMA	ADC, SCC	I-IIIA	SS	CD68	Pan	KP-1	zero	ND	NS
(Carus *et al*, 2013)	335	WT	ADC, SCC	I-IIIA	SS	CD163	M2	EDHu-1	median	ND	NS
(Ito *et al*, 2012)	304	WT	ADC	I	SS	CD204	M2	SRA-E5	median	stroma	Negative PI for PFS NS in multivariable analysis
(Hirayama *et al*, 2012)	208	WT	SCC	I-III	SS	CD204	M2	SRA-E5	median	stroma	Independent negative PI
(Ma *et al*, 2010)	100	WT	ADC, SCC	I-IV	DS	1-CD68/ HLA-DR	M1	KP1/ LN3	median	tumor	M1: independent positive PI M2: NS
						2-CD68/ CD163	M2	KP1/ 10D6			
										stroma	NS
	170	WT	ADC	I-III	SS	1-CD204	M2	SRA-E5	median	stroma	CD204: negative PI

(Ohtaki *et al*, 2010)						2-CD68	Pan	KP1			CD68: NS
(Dai *et al*, 2010)	99	WT	SCC, ADC	I-IV	SS	CD68	Pan	KP1	median	tumor	Independent positive PI
										stroma	Independent negative PI
(Al-Shibli *et al*, 2009)	335	TMA	SCC, ADC	I-IIIA	SS	CD68	Pan	KP1	optimal	tumor	NS
										stroma	NS
(Ohri *et al*, 2009)	40	WT	SCC, ADC	I-IV	DS	1-CD68/ HLA-DR	M1	PGM1/ TAL.1B5	median	tumor	All M1 and M2 markers: positive PI CD68/MRP8-14: positive independent PI
						2-CD68/ iNOS	M1	PGM1/ 2D2-B2			
						3-CD68/ CD163	M2	PGM1/ 10D6			
						4-CD68/ MRP8-14	M1	PGM1/ 27E10			
						5-CD68/ VEGF	M2	PGM1/ 14-124			
										stroma	NS
(Kim *et al*, 2008)	144	TMA	SCC, ADC	I-IV	SS	CD68	Pan	M0876	median	tumor	Independent positive PI

										stroma	NS
(Kawai *et al*, 2008)	199	WT	SCC, ADC, other	IV	SS	CD68	Pan	KP1	median	tumor	NS
										stroma	Negative PI
(Kojima *et al*, 2005)	129	WT	ADC	I-III	SS	CD68	Pan	PGM1	mean	stroma	NS
(Welsh *et al*, 2005)	162	WT	SCC, ADC, other	I-IV	SS	CD68	Pan	PGM1	median	tumor	Independent positive PI
										stroma	Independent negative PI
(Chen *et al*, 2003)	35	WT	SCC, ADC	I-III	SS	CD68	Pan	KP1	median	ND	Negative PI
(Takanami *et al*, 1999)	113	WT	ADC	I-IV	SS	CD68	Pan	KP1	optimal	ND	Independent negative PI
(Eerola *et al*, 1999)	38	WT	LCC	I-III	SS	CD68	Pan	PGM-1	median	tumor	Positive PI

Abbreviations: TMA: tissue microarray, WT: whole tissue, SCC: squamous cell carcinoma, ADC: Adenocarcinoma, LCC: large cell carcinoma; IHC: immunohistochemistry, SS: single staining, DS: double staining, ND: not differentiated, NS: not significance, CD: cluster of differentiation, HLA-DR: human leukocyte antigen- DR isotype, iNOS: Inducible nitric oxide synthase, MRP8-14: myeloid-related proteins 8-14, VEGF: Vascular endothelial growth factor

Table S7 references:

Al-Shibli K, Al-Saad S, Donnem T, Persson M, Bremnes RM, Busund LT (2009) The prognostic value of intraepithelial and stromal innate immune system cells in non-small cell lung carcinoma. *Histopathology* **55**: 301–312, doi:10.1111/j.1365-2559.2009.03379.x.

Carus A, Ladekarl M, Hager H, Pilegaard H, Nielsen PS, Donskov F (2013) Tumor-associated neutrophils and macrophages in non-small cell lung cancer: No immediate impact on patient outcome. *Lung Cancer* **81**: 130–137, doi:10.1016/J.LUNGCAN.2013.03.003.

Chen JJW, Yao P-L, Yuan A, Hong T-M, Shun C-T, Kuo M-L, Lee Y-C, Yang P-C (2003) Up-regulation of tumor interleukin-8 expression by infiltrating macrophages: its correlation with tumor angiogenesis and patient survival in non-small cell lung cancer. *Clin Cancer Res* **9**: 729–737.

Dai F, Liu L, Che G, Yu N, Pu Q, Zhang S, Ma J, Ma L, You Z (2010) The number and microlocalization of tumor-associated immune cells are associated with patient's survival time in non-small cell lung cancer. *BMC Cancer* **10**: 220, doi:10.1186/1471-2407-10-220.

Eerola a K, Soini Y, Pääkkö P (1999) Tumour infiltrating lymphocytes in relation to tumour angiogenesis, apoptosis and prognosis in patients with large cell lung carcinoma. *Lung Cancer* **26**: 73–83.

Hirayama S, Ishii G, Nagai K, Ono S, Kojima M, Yamauchi C, Aokage K, Hishida T, Yoshida J, Suzuki K, Ochiai A (2012) Prognostic Impact of CD204-Positive Macrophages in Lung Squamous Cell Carcinoma: Possible Contribution of Cd204-Positive Macrophages to the Tumor-Promoting Microenvironment. *J Thorac Oncol* **7**: 1790–1797, doi:10.1097/JTO.0B013E3182745968.

Ito M, Ishii G, Nagai K, Maeda R, Nakano Y, Ochiai A (2012) Prognostic Impact of Cancer-Associated Stromal Cells in Patients With Stage I Lung Adenocarcinoma. *Chest* **142**: 151–158, doi:10.1378/CHEST.11-2458.

Kawai O, Ishii G, Kubota K, Murata Y, Naito Y, Mizuno T, Aokage K, Saijo N, Nishiwaki Y, Gemma A, Kudoh S, Ochiai A (2008) Predominant infiltration of macrophages and $CD8^+$ T Cells in cancer nests is a significant predictor of survival in stage IV nonsmall cell lung cancer. *Cancer* **113**: 1387–1395, doi:10.1002/cncr.23712.

Kim D-W, Min HS, Lee K-H, Kim YJ, Oh D-Y, Jeon YK, Lee S-H, Im S-A, Chung DH, Kim YT, Kim T-Y, Bang Y-J, Sung SW, Kim JH, Heo DS (2008) High tumour islet macrophage infiltration correlates with improved patient survival but not with EGFR mutations, gene copy number or protein expression in resected non-small cell lung cancer. *Br J Cancer* **98**: 1118–1124, doi:10.1038/sj.bjc.6604256.

Kojima H, Shijubo N, Yamada G, Ichimiya S, Abe S, Satoh M, Sato N (2005) Clinical significance of vascular endothelial growth factor-C and vascular endothelial growth factor receptor 3 in patients with T1 lung adenocarcinoma. *Cancer* **104**: 1668–1677, doi:10.1002/cncr.21366.

Li Z, Maeda D, Yoshida M, Umakoshi M, Nanjo H, Shiraishi K, Saito M, Kohno T, Konno H, Saito H, Minamiya Y, Goto A (2018) The intratumoral distribution influences the prognostic impact of CD68- and CD204-positive macrophages in non-small cell lung cancer. *Lung Cancer* **123**: 127–135, doi:10.1016/j.lungcan.2018.07.015.

Ma J, Liu L, Che G, Yu N, Dai F, You Z (2010) The M1 form of tumor-associated macrophages in non-small cell lung cancer is positively associated with survival time. *BMC Cancer* **10**: 112, doi:10.1186/1471-2407-10-112.

Ohri CM, Shikotra a., Green RH, Waller D a., Bradding P (2009) Macrophages within NSCLC tumour islets are predominantly of a cytotoxic M1 phenotype associated with extended survival. *Eur Respir J* **33**: 118–126, doi:10.1183/09031936.00065708.

Ohtaki Y, Ishii G, Nagai K, Ashimine S, Kuwata T, Hishida T, Nishimura M, Yoshida J, Takeyoshi I, Ochiai A (2010) Stromal Macrophage Expressing CD204 is Associated with Tumor Aggressiveness in Lung Adenocarcinoma. *J Thorac Oncol* **5**: 1507–1515, doi:10.1097/JTO.0B013E3181EBA692.

Pei B, Sun B, Zhang Z, Wang A, Ren P (2014) Interstitial tumor-associated macrophages combined with tumor-derived colony-stimulating factor-1 and interleukin-6, a novel prognostic biomarker in non–small cell lung cancer. *J Thorac Cardiovasc Surg* **148**: 1208–1216.e2, doi:10.1016/J.JTCVS.2014.05.003.

Takanami I, Takeuchi K, Kodaira S (1999) Tumor-Associated Macrophage Infiltration in Pulmonary Adenocarcinoma : Association with Angogenesis and Poor Prognosis. *Oncology* **57**: 138–142.

Welsh TJ, Green RH, Richardson D, Waller DA, O'Byrne KJ, Bradding P (2005) Macrophage and mast-cell invasion of tumor cell islets confers a marked survival advantage in non-small-cell lung cancer. *J Clin Oncol* **23**: 8959–8967, doi:10.1200/JCO.2005.01.4910.