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Prognostic Value of Macrophage Phenotypes in Resectable Non–Small Cell Lung Cancer Assessed by Multiplex Immunohistochemistry[1]

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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: proinflammatory M1 and protumorigenic 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 microarrays 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. 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 intratumoral compartments, revealed that high levels of HLA-DR$^+$/CD68$^+$M1 (stroma, hazard ratio [HR] = 0.73, P = .03; intratumor, HR = 0.7, P = .04), CD204$^+$M2 (stroma, HR = 0.7, P = .02; intratumor, HR = 0.6, P = .004), and CD68 (stroma, HR = 0.69, P = .02; intratumor, HR = 0.73, P = .04) infiltration were independently associated with improved NSCLC-specific survival. In lymph nodes, the intratumoral level of HLA-DR$^+$/CD68$^+$M1 was an independent positive prognostic indicator (Cox model, HR = 0.38, P = .001). In conclusion, high levels of M1, CD204$^+$M2, and CD68 macrophages are independent prognosticators of prolonged survival in NSCLC.

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Introduction

In addition to intrinsic mechanisms within neoplastic cancer cells, cancer development depends on complex cross talk 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

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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 proinflammatory cytokines, largely express MHC class II (such as HLA-DR), and are thought to exhibit antitumoral functions through stimulation of T-cell–mediated antitumor 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 antitumoral immunity of both M1 and T-helper (Th1) cells, and 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) provides subset detail and may have higher detection accuracy, but this is limited to the use of appropriate chromogens for visualizing colocalized markers. The use of translucent chromogens produces color changes at sites of colocalization, 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 were evaluated, in tissues from 553 resected NSCLC specimens and 143 matched lymph nodes, both in cancer cell islets and in associated-stroma.

Materials and Methods

Study Cohort

The study population (previously described in Hald et al[18] and Rakaee et al[19]) is a consecutive series of 633 stage I to 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 formalin-fixed paraffin-embedded 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 Union for International Cancer Control and World Health Organization guidelines.[20,21] The collection and reporting of clinicopathological variables, survival information, and marker expression data were conducted according to the 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 hematoxylin and eosin slides. From each patient's formalin-fixed paraffin-embedded 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, AZ). 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 minutes) as well as temperature-induced denaturation (8 minutes at 90°C), was applied.

All double-stained slides were compared with their corresponding single-stained slide. To assess the cross-reactivity of the two sequence components (chromogens, primary and secondary antibodies), three different strategies were applied: 1) full staining protocol without first sequence primary

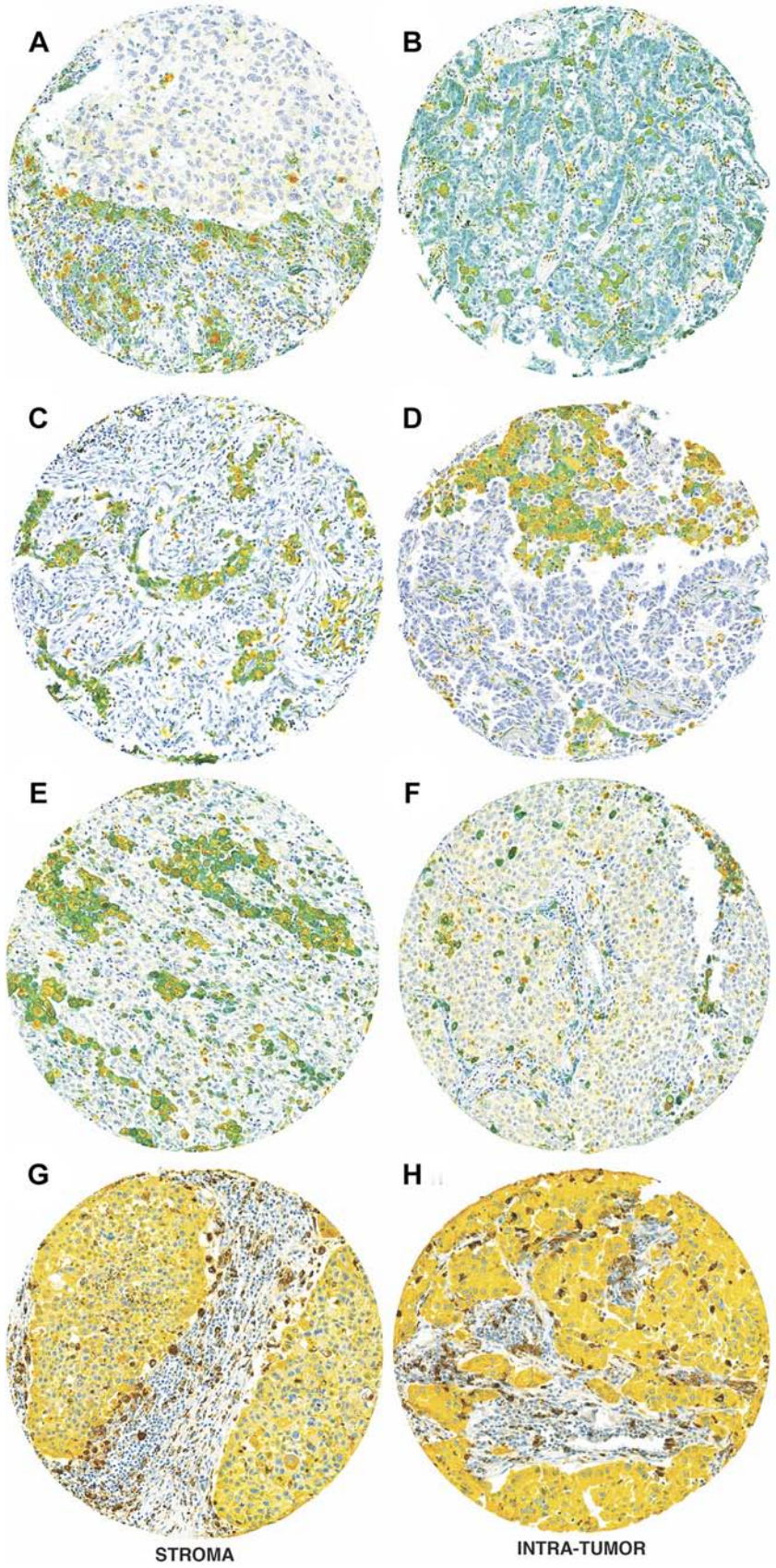


Figure 1. Multiplexed protein detection using translucent IHC chromogens for TAM phenotyping in NSCLC. Compartment-specific infiltration of different TAM phenotypes in primary tumor: (A, B) HLA-DR$^+$(teal)/CD68$^+$ (yellow) M1subset, (B) an example of HLA-DR tumor epithelial positive case in which the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

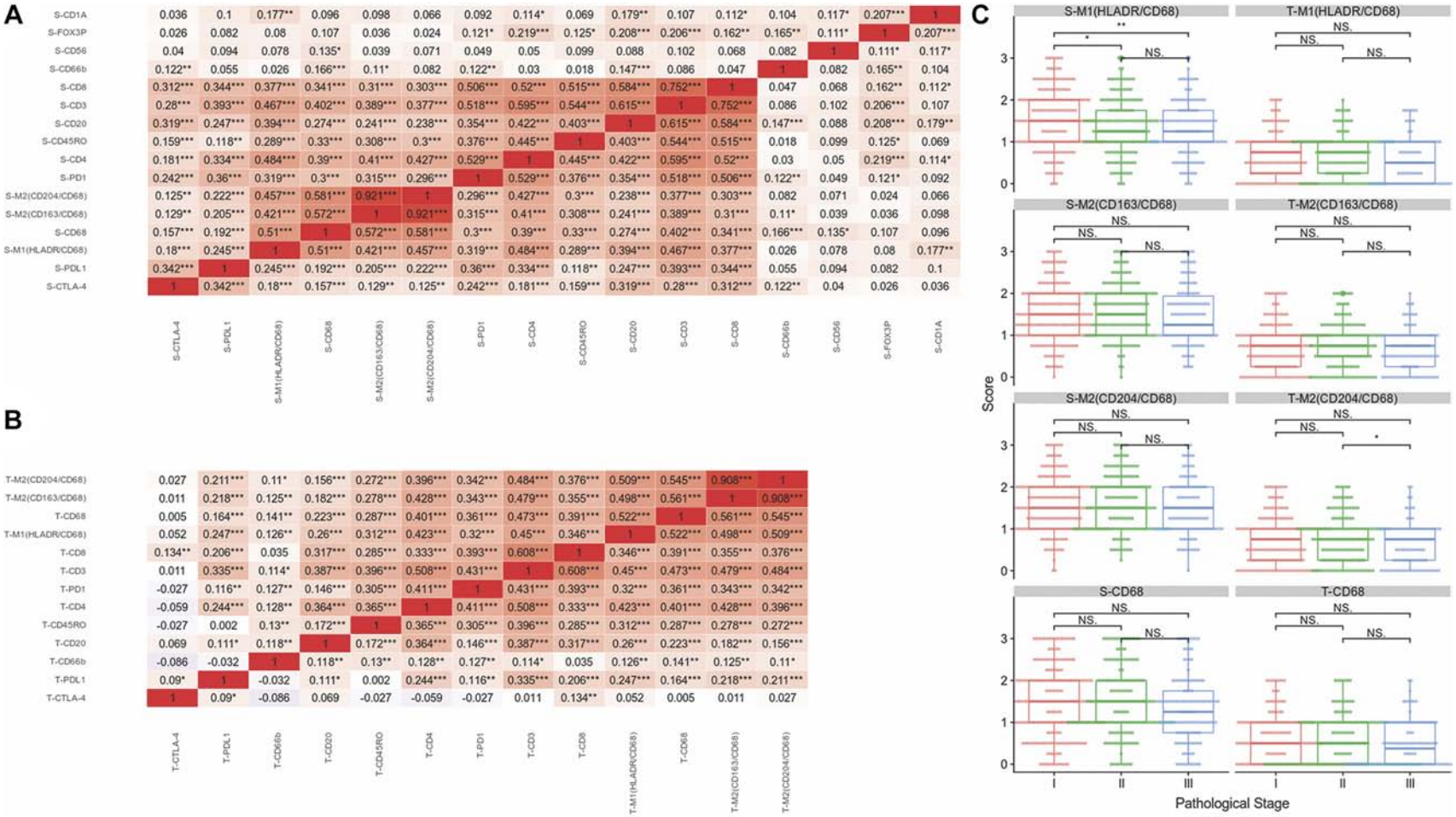
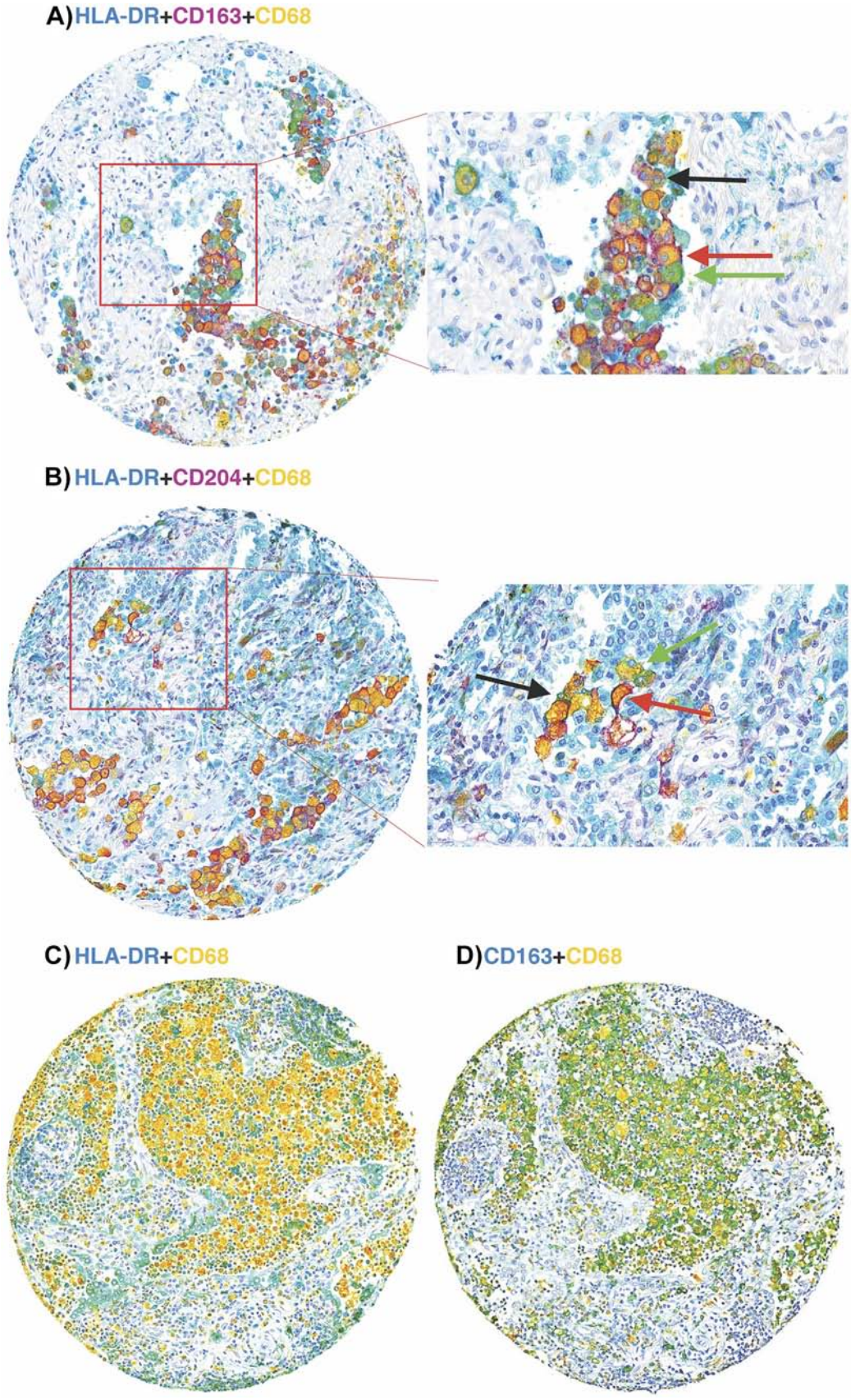


Figure 2. Spearman's rank correlation and Mann-Whitney *U* test on TAM phenotypes. (A, B) Correlation matrix between different stromal (A) and tumoral (B) TAM subsets and immune-related markers. (C) Dot- and box-plots of various stromal (S, left column) and tumoral (T, right column) TAM subset distributions across pathological stages I to III in NSCLC. *$P < .05$, **$P < .01$, ***$P < .001$.


A) HLA-DR+CD163+CD68
B) HLA-DR+CD204+CD68
C) HLA-DR+CD68
D) CD163+CD68

antibody, 2) full staining protocol without second sequence primary antibody, and 3) full staining protocol without first sequence secondary antibody and chromogen and second sequence primary antibody. In addition, isotype-matched control antibody for each sequence and multitumor and normal TMAs were included for staining quality control.

Evaluation of Immunostaining

All slides were digitized using a Pannoramic 250 Flash II scanner (3DHistech, Budapest, Hungary) with a maximum resolution of ×40 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 intratumoral area (in both primary tumors and metastatic lymph nodes). Macrophages in intratumoral areas were defined as macrophages infiltrating into tumor-cell-nests. Macrophages in stroma were defined as macrophages in the area not occupied by tumor-cell-nests. 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 intratumoral 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), and 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 cutoff values. For intratumoral infiltration and stromal CD68, optimal cutoffs (minimal *P* value) were used for dichotomization. The applied cutoff 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 (χ^2 test) and between variables (Spearman's ρ 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 < .05 were considered statistically significant.

Results

Reliable Assessment of Macrophage Phenotypes

The study evaluated the presence and expression patterns of macrophage subpopulations coexpressing $HLA\text{-}DR^+/CD68^+$ (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 overlapping signals on macrophages appear with a tertiary (green) color, making spatial assessment of the two markers considerably easier (Figure 1, *A-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 1, *G-H*).

Figure 2, *A-B* represents the correlation matrix between TAM subsets and immune-related markers previously studied in this cohort. There were 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 were observed 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: $HLA\text{-}DR^+/CD204^+/CD68^+$: median (range) 3.1% (0%-10.26%); $HLA\text{-}DR^+/CD163^{+/}CD68^+$: 2.7% (0%-11.42%) (Figure 3, *A-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 total 20 squamous cell carcinoma (SCC, n = 10; random selection) and adenocarcinoma (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

Figure 3. Multiplex IHC for validation of TAM subset (M1 vs. M2) staining specificity.(A) Three-plexed IHC of M1 and $CD163^+$M2 marker: $HLA\text{-}DR^+$ (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) Three-plexed IHC of M1 and $CD204^+$M2 marker: $HLA\text{-}DR^+$ (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) $HLA\text{-}DR^+$ (teal)/$CD68^+$ (yellow) M2, (D) $CD163^+$ (teal)/$CD68^+$ (yellow) M1; the colocalized markers appeared in a tertiary green color.

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 1*B*). In positive cases, the intensity of CD68 protein expression in the cancer cells was homogenous, while it varied highly for HLA-DR. The M2-like phenotype was the dominant subset of TAMs in almost all necrotic areas (Figure 3, *C-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 Eastern Cooperative Oncology Group (ECOG) performance status in primary tumors. CD204$^+$M2 was 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 intratumoral 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 were notably decreased for M1 macrophages (Figure 2*C*).

Macrophage and Survival: Univariate Analysis

In the overall cohort, high levels of both intratumoral and stromal M1 (P = .021 and P = .003), CD204$^+$M2 (P = .004 and P = .013), and pan-CD68 (P = .01 and P = .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 intratumoral compartments.

In the SCC subgroup (n = 307), high levels of stromal CD163$^+$M2 (P < .001) and CD204$^+$M2 (P = .005) and both stromal and intratumoral M1 (P < .001, P = .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 were associated with longer DSS (P = .039) (Figure S2, Table S6).

In the metastatic lymph nodes, the presence of intratumoral M1 macrophages was a significant positive prognostic factor (P = .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 (hazard ratio [HR] 0.73; confidence interval [CI] 0.5-0.97; P = .03), CD204$^+$M2 (HR 0.7; CI 0.5-0.94; P = .02), and CD68 (HR 0.69; CI 0.5-0.94; P = .02) were associated with significantly longer DSS independent of pStage, vascular invasion, ECOG performance status, and gender. Consistent with findings in stroma, intratumoral M1 (HR 0.7; CI 0.5-0.99; P = .04), CD204$^+$M2 (HR 0.6; CI 0.4-0.8; P = .004), and CD68 (HR 0.73; CI 0.5-0.99; P = .04) were independent positive prognostic factors for DSS (Table 2). In metastatic lymph nodes, high intratumoral M1 infiltration was an independent positive predictor of DSS (HR 0.38; CI 0.2-0.7; P = .001).

Discussion

The study describes a multiplex IHC assay for simultaneous identification of colocalized 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 to 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 DSS in both stromal and intratumoral compartments. Our findings also indicate that the presence of intratumoral 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 intratumoral 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 et al.[26] and Eerola et al.[27] showed superior outcome with high intratumoral 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. Nonspecific staining may overestimate the level of TAMs and consequently affect the results. The use of pan-CK to differentiate between epithelial and nonepithelial cells probably increases the detection accuracy of intratumoral 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

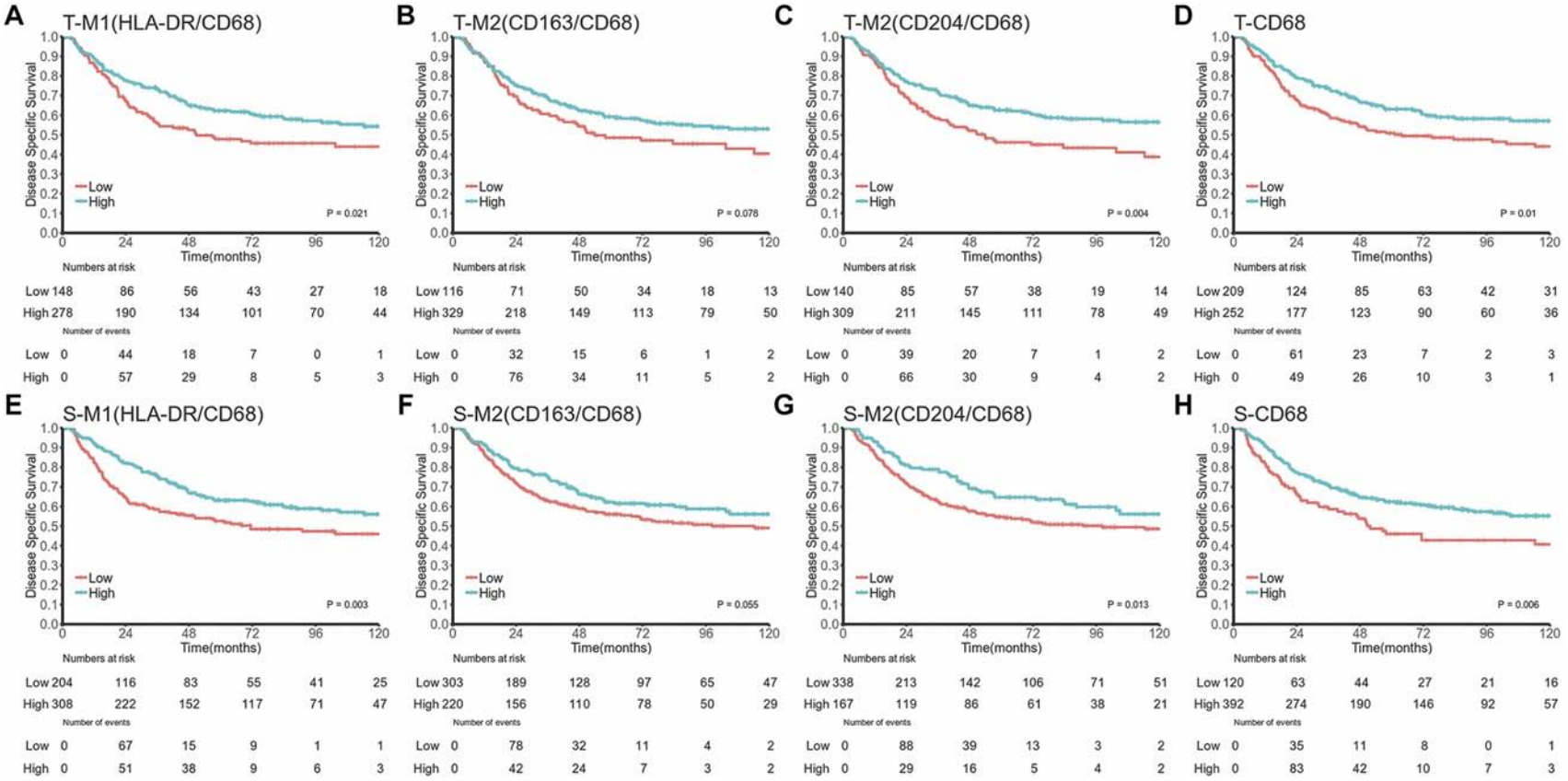


Figure 4. DSS curves according to stromal and intratumoral TAM subset levels in primary tumor of NSCLC.Intratumoral (A) HLA-DR$^+$M1; (B) CD163$^+$M2; (C) CD204$^+$M2; (D) pan-CD68.Stromal (E) HLA-DR$^+$M1; (F) CD163$^+$M2; (G) CD204$^+$M2; (H) pan-CD68.

Table 1. Prognostic Impact of Stromal and Intratumoral Macrophage Phenotypes in Primary and Metastatic Lymph Nodes of NSCLC Patients

	Stroma					Intratumor				
	N (%)	DSS (%)	Median (Months)	HR (95% CI)	*P*	*N* (%)	DSS (%)	Median (Months)	HR(95% CI)	*P*
Primary tumor										
M1					**.003**					**.021**
(HLA-DR$^+$/CD68$^+$)										
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					.055					.078
(CD163$^+$/CD68$^+$)										
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					**.013**					**.004**
(CD204$^+$/CD68$^+$)										
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$^+$					**.006**					**.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										**.002**
(HLA-DR$^+$/CD68$^+$)										
Low						28 (19)	10	15	1	
High						62(43)	35	56	0.41 (0.23-0.72)	
Missing						54 (38)				

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 intratumoral 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 intratumoral M1 (not M2) to be an independent prognostic indicator.[36] However, both Ma et al 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 antitumoral or protumoral functions, respectively. However, we and other researchers (studying NSCLC, CRC, and gastric carcinomas) have observed that both M1 and M2 subtype infiltrations 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 antitumoral attribute may dominate over the M2 protumoral 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 cooperate with T-helper/cytotoxic cells to induce antitumoral 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 protumoral and antitumoral 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 colocalized in this context. With this understanding, a set of experiments to characterize macrophage subsets was 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 colocalized

Table 2. Multivariable Cox Models for DSS of A) Various Stromal and Intratumoral Macrophage Phenotypes in Primary Tumor and B) Metastatic Lymph Nodes

Parameter	Stroma		Intratumor	
	HR (95% CI)	*P*	HR (95% CI)	*P*
A				
Model 1 M1(HLA-DR$^+$/CD68$^+$) Low vs. high	0.73 (0.5-0.97)	**.03**	0.7 (0.5-0.99)	**.04**
Pstage				
I	1		1	
II	1.6 (1.1-2.3)	**.01**	0.2 (0.16-0.35)	**<.001**
III	4.1 (2.8-5.7)	**<.001**	0.3 (0.2-0.5)	**<.001**
Vascular invasion No vs. yes	1.8 (1.3-2.5)	**.001**	0.5 (0.3-0.8)	**.002**
ECOG				
0	1		1	
1	1.4 (1.05-1.9)	**.02**	0.5 (0.3-1.1)	.09
2	1.4 (0.8-2.5)	.28	0.9 (0.5-1.7)	.8
Gender Female vs. male	1.4 (1.03-1.9)	**.03**	0.7 (0.5-1.01)	.06
Model 2 M2 (CD163$^+$/CD68$^+$) Low vs. high	0.76 (0.57-1.1)	.053	0.7 (0.5-1.03)	.08
Pstage				
I	1		1	
II	1.6 (1.1-2.3)	**.007**	0.25 (0.17-0.36)	**<.001**
III	3.8 (2.7-5.4)	**<.001**	0.4 (0.2-0.5)	**<.001**
Vascular invasion No vs. yes	1.9 (1.3-2.6)	**<.001**	0.6 (0.4-0.8)	**.002**
ECOG				
0	1		1	
1	1.5 (1.09-1.9)	**.009**	0.6 (0.3-1.1)	.1
2	1.5 (0.8-2.6)	.17	0.9 (0.4-1.6)	.7
Gender Female vs. male	1.4 (1.04-1.9)	**.03**	0.7 (0-4-0.9)	**.02**
Model 3 M2 (CD204$^+$/CD68$^+$) Low vs. high	0.7 (0.5-0.94)	**.02**	0.6 (0.4-0.8)	**.004**
Pstage				
I	1		1	
II	1.6 (1.2-2.3)	**.005**	1.4 (1.03-2.1)	**.03**
III	3.7 (2.6-5.3)	**<.001**	3.6 (2.5-5.2)	**<.001**
Vascular invasion No vs. yes	1.8 (1.3-2.5)	**.001**	1.7 (1.2-2.5)	**.002**
ECOG				
0	1		1	
1	1.4 (1.04-1.8)	**.02**	1.4 (1.03-1.9)	**.03**
2	1.4 (0.8-2.5)	.25	1.5 (0.8-2.7)	.1
Gender Female vs. male	1.4 (1.04-1.9)	**.02**	1.3 (0.9-1.9)	.058
Model 4 CD68$^+$ Low vs. high	0.69 (0.5-0.94)	**.02**	0.73 (0.5-0.99)	**.04**
Pstage				
I	1		1	
II	1.6 (1.1-2.26)	**.01**	1.5 (1.05-2.2)	**.02**
III	3.7 (2.6-5.3)	**<.001**	3.6 (2.5-5.2)	**<.001**
Vascular invasion No vs. yes	1.8 (1.2-2.5)	**.001**	1.8 (1.3-2.6)	**<.001**
ECOG				
0	1		1	
1	1.4 (1.05-1.8)	**.02**	1.4 (1.03-1.9)	**.03**
2	1.4 (0.8-2.6)	.2	1.5 (0.8-2.8)	.1
Gender Female vs. male	1.3 (0.9-1.8)	**.053**	1.4 (1.06-2.02)	**.01**
B				
M1 (HLA-DR$^+$/CD68$^+$) Low vs. high			0.38 (0.2-0.7)	**.001**
T stage				
1			1	
2			1.7 (0.7-3.9)	.18
3			1.7 (0.7-4.2)	.2
4			2.6 (0.9-7.1)	.06
N stage (N1 vs. N2)			1.7 (0.9-3.1)	.07

antigens of interest on TAMs. When they are mixed, they can create a unique color, making it relatively easy to identify cells coexpressing 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 intratumoral 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 intratumoral pan-CD68, HLA-DR$^+$M1, and CD204$^+$M2 macrophages infiltration are independent determinants of favorable clinical outcome in stage I to III NSCLC patients. In addition, high levels of HLA-DR$^+$M1 macrophages in locoregional nodal metastases are 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 colocalized TAM biomarkers in brightfield multiplex IHC.

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