HUMAN IMMUNOLOGY

Antibody combinations for optimized staining of macrophages in human lung tumours

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Abstract
The analysis of tumour-associated macrophages (TAMs) has a high potential to predict cancer recurrence and response to immunotherapy. However, the heterogeneity of TAMs poses a challenge for quantitative and qualitative measurements. Here, we critically evaluated by immunohistochemistry and flow cytometry two commonly used pan-macrophage markers (CD14 and CD68) as well as some suggested markers for tumour-promoting M2 macrophages (CD163, CD204, CD206 and CD209) in human non–small cell lung cancer (NSCLC). Tumour, non-cancerous lung tissue and blood were investigated. For immunohistochemistry, CD68 was confirmed to be a useful pan-macrophage marker although careful selection of antibody was found to be critical. The widely used anti-CD68 antibody clone KP-1 stains both macrophages and neutrophils, which is problematic for TAM quantification because lung tumours contain many neutrophils. For TAM counting in tumour sections, we recommend combined labelling of CD68 with a cell membrane marker such as CD14, CD163 or CD206. In flow cytometry, the commonly used combination of CD14 and HLA-DR was found to not be optimal because some TAMs do not express CD14. Instead, combined staining of CD68 and HLA-DR is preferable to gate all TAMs. Concerning macrophage phenotypic markers, the scavenger receptor CD163 was found to be expressed by a substantial fraction (50%-86%) of TAMs with a large patient-to-patient variation. Approximately 50% of TAMs were positive for CD206. Surprisingly, there was no clear overlap between CD163 and CD206.
1 INTRODUCTION

Tumour-associated macrophages (TAMs) are heterogeneous in phenotypes and functions and may exert either tumour-promoting or tumour-suppressive activity.\(^1\)\(^-\)\(^4\) Therefore, immunoprofiling of TAMs has a great potential as a prognostic tool and as a predictor of immunotherapy efficacy for individuals with cancer. Lung cancer is the most frequent cause of cancer-related death worldwide, and non–small cell lung cancer (NSCLC) accounts for about 85% of all lung cancer cases.\(^5\) Several immunohistochemistry (IHC)-based analyses of NSCLC tumour sections suggested that the density of TAMs may potentially be used to predict patient survival after the operation. Six studies reported that the survival of NSCLC patients was prolonged when more macrophages had infiltrated the tumour tissue.\(^6\)\(^-\)\(^11\) In contrast, seven studies showed that high numbers of TAMs were correlated with shorter survival.\(^7\)\(^,\)\(^9\)\(^,\)\(^12\)\(^-\)\(^16\) Five other studies reported no association between TAM density and prognosis.\(^17\)\(^-\)\(^21\) Thus, although these reports collectively support a key role of TAMs for tumour development, the available data are contradictory and it remains unclear why high numbers of TAMs were associated with better or worse prognosis in the different studies.

The heterogeneity of macrophages poses a challenge for quantitative and qualitative analyses. In fact, the use of different molecular targets and antibody clones to stain TAMs may explain some of the discrepancies in the literature concerning the role of TAMs in human cancer. Most published IHC studies in NSCLC have used CD68 as a pan-macrophage marker. CD68 is a transmembrane glycoprotein that is mainly located in the endosomal/lysosomal compartment of macrophages.\(^22\)\(^,\)\(^23\) However, CD68 has been reported to be expressed by several other cell types such as neutrophils, dendritic cells, fibroblasts and endothelial cells.\(^24\)\(^-\)\(^27\) Therefore, the use of CD68 can potentially lead to an overestimation of the number of macrophages, in particular in lung cancer tumours, which may be highly infiltrated by neutrophils.\(^28\) Accordingly, the validity of CD68 as a pan-macrophage marker in lung tumours needs to be clarified.

According to a popular but controversial model proposed separately by Mills and Mantovani, macrophages with a tumour-suppressive phenotype are called M1, whereas tumour-promoting macrophages are called M2.\(^29\)\(^-\)\(^31\) Most TAMs are considered to have an M2 or M2-like phenotype.\(^30\)\(^-\)\(^32\) Several molecular markers have been suggested to be specific for M2 TAMs, including the haemoglobin scavenger receptor CD163,\(^33\)\(^-\)\(^35\) the scavenger receptor-A CD204,\(^15\)\(^,\)\(^36\)\(^-\)\(^38\) macrophage mannose receptor receptor CD206,\(^16\) CD209 (also known as dendritic cell–specific intercellular adhesion molecule-1-3 grabbing non-integrin, DC-SIGN)\(^39\) and TREM-1.\(^40\) In accordance with the Mills & Mantovani model, specific quantification of M1 or M2 TAMs should provide more precise prognostic information compared to total TAM numbers. The validity of this strategy has received support from several reports indicating that high densities of M1 TAMs were associated with prolonged survival,\(^34\)\(^,\)\(^35\)\(^,\)\(^41\) whereas high numbers of M2 TAMs were associated with shorter survival.\(^36\)\(^-\)\(^38\)\(^,\)\(^40\)\(^-\)\(^42\) Unfortunately, a major limitation for clinical implementation of the approach is that there is currently no consensus concerning which markers should be used to identify tumour-suppressive M1 TAMs and tumour-promoting M2 TAMs, respectively. For example and to illustrate the problem, two studies came to the surprising conclusion that high numbers of TAMs with an M2 phenotype were associated with better prognosis.\(^11\)\(^,\)\(^34\)

Macrophages reside in different histological locations in lung tumours. Inside the tumour itself, some TAMs infiltrate the islets of cancer cells, but most TAMs are present in the tumour stroma which consists of normal, non-cancerous cells.\(^7\) When the tumour contains areas with preserved lung tissue, tissue-resident alveolar macrophages may be found. Macrophages are also present in the tertiary lymphoid structures (TLS) that are frequently observed at the periphery of lung tumours.\(^43\)\(^,\)\(^44\) Macrophages in the germinal centres of TLS are called tingible body macrophages and their function is to engulf apoptotic cells.\(^45\)\(^,\)\(^46\) Several reports indicated that macrophages in different histological areas of a tumour, such as tumour islets versus stroma, may play opposite roles in tumour development.\(^7\)\(^,\)\(^9\) Therefore, the different compartments of a tumour should be taken into consideration when analysing TAMs.

In the present study, we critically evaluated by IHC and flow cytometry two commonly used pan-macrophage markers (CD14 and CD68) as well as several suggested markers for M2 macrophages (CD163, CD204, CD206 and CD209). The data presented here should represent a useful resource for scientists aiming at staining macrophages in human tumours and normal lung tissue.
2 | MATERIAL AND METHODS

2.1 | Patients and lung tissue samples

Tissue samples from lung tumour and from a non-cancerous part of the lung were collected from eleven NSCLC patients following surgical resection of lung lobes at the Department of Cardiothoracic Surgery at Oslo University Hospital, Norway. The diagnosis of lung cancer was based on histopathology criteria, and TNM stage of the cancers varied from I to IIIIB. Five patients were diagnosed with squamous cell carcinoma and six patients with adenocarcinoma. Non-cancerous lung tissue was sampled from a part of the lung specimen located as far away from the tumour tissue as possible. The patients were smokers or former smokers, and they had not received neoadjuvant chemotherapy, preoperative radiotherapy or immunosuppressive therapy. Written informed consent was obtained from all patients before inclusion. The study has been approved by The Norwegian Regional Ethical Committee (ref: S-05307).

2.2 | Immunohistochemistry (IHC)

Tissue samples from primary lung tumour and non-cancerous lung tissue were fixed in 10% formalin for 24 hours and embedded in paraffin before 4-µm-thick serial sections were made. Staining of the sections was performed using a Ventana Discovery Ultra automated slide stainer (Ventana Medical System, Roche, Cat. No. 750-601). After deparaffinization of the sections, heat-induced antigen retrieval was performed by using cell conditioning 1 buffer (CC1, Ventana Medical System, Cat. No. 950-500) for 32 minutes, before staining with the primary monoclonal antibodies (mAbs). Next, the tissue sections were incubated with secondary antibody conjugated with peroxidase. The primary and secondary antibodies used are listed in Tables S1 and S2. Bound antibodies were visualized using the kit ChromoMap DAB Kit (Ventana Medical System, Cat. No. 760-159).

For paired immunostaining, we established the procedure shown in Table S3. The primary and secondary antibodies used are listed in Tables S4 and S5. Briefly, the primary antibodies against CD14, CD163 or CD206 were applied on the tissue sections, before incubation with the secondary antibodies. Bound antibodies were visualized using Discovery Teal-HRP detection kit (Ventana Medical System, Cat. No. 760-247). Next, to block for potential binding of subsequently added antibodies to the already applied primary and secondary antibodies, the tissue sections were treated with cell conditioning 2 buffer (CC2, Ventana Medical System, Cat. No. 950-123) at 100°C for 24 minutes. Then, the primary antibodies against CD68 were added and visualized using Discovery Yellow AP detection kit (Ventana Medical System, Cat. No. 760-239). Finally, the tissue sections were counterstained with haematoxylin (Thermo Fisher Scientific, Waltham, MA, USA, Cat. No. 6765015). Isotype- and concentration-matched irrelevant mouse IgG1 (Dako, Agilent Technologies, Cat. No. X0931), mouse IgG3 (R&D Systems, Cat. No. MAB007) and rabbit antibodies (Vector Laboratories, Cat. No. I-1000) were used as negative controls.

2.3 | Immunofluorescence

Formalin-fixed, paraffin-embedded, 4-µm-thick tissue sections were deparaffinized and rehydrated, followed by antigen retrieval by boiling for 20 minutes in Tris-EDTA buffer with pH 9 (Dako, Cat. No. K8004). Double immunostaining of macrophages was performed by applying a mixture of mouse anti-CD68 mAb clone PG-M1 (diluted 1:100) (Dako, Cat. No. M0876) and mouse anti-CD163 mAb clone 10D6 (diluted 1:100) (Novocastra, Leica Biosystems, Cat. No. NCL-CD163) diluted in phosphate-buffered saline (PBS) with bovine albumin (Bio-Rad, Cat. No. 805090). The sections were incubated with the primary mAb overnight at 4°C. After washing in PBS with 1% Tween-20 (Sigma-Aldrich, Cat. No. P2287) for 5 minutes, the sections were incubated for 2 hours at room temperature with a mixture of Alexa Fluor 488-labelled goat anti-mouse IgG1 (diluted 1:500) (Molecular Probes, Cat. No. A-21121) and Alexa Fluor 594-labelled goat anti-mouse IgG3 (diluted 1:500) (Molecular Probes, Cat. No. A-21155). Hoechst was added to the final washing buffer to visualize the nuclei. Concentration- and isotype-matched irrelevant mouse IgG1 (Dako, Cat. No. X0931) and mouse IgG3 (R&D Systems, Cat. No. MAB007) were used as negative controls.

2.4 | Flow cytometry

Fresh NSCLC tumour samples from resected lung were mechanically dissociated using scissors and digested in DMEM (Gibco, Thermo Fisher Scientific, Cat. No. 12491-015) supplemented with 2 mg/mL collagenase A and 500 units/mL DNase I (both from Roche, Cat. No. 10103586001 and Cat. No. 11284932001, respectively) by incubation with a stirring magnet at 37°C for 1 hour. Single-cell suspension was obtained by filtering the dissolved tissue through a cell strainer (BD Falcon, Cat. No. 734-0004). The supernatants containing enzymes were removed after centrifugation at 410 g at 4°C for 6 minutes, before the cell pellet was dissolved in cold PBS supplemented with 10% foetal bovine serum (Sigma-Aldrich, Cat. No. F7524). Blood samples were obtained from the same patients, and the peripheral blood mononuclear cells (PBMCs) were isolated using a density gradient medium (Lymphoprep, Axis-Shield, Cat. No. 1114544). For cell surface staining, cells were stained in a 96-well V-bottom plate kept on ice. First, to block non-specific binding of mAb, cells were incubated for 15 minutes with 12.5 µg/mL IgG purified from mouse serum (Sigma-Aldrich, Cat. No. I8765). Next,
| Marker used to identify TAMs | mAb clone | Producer | Patient number | Main conclusion | Reference |
|-----------------------------|-----------|----------|----------------|----------------|-----------|
| CD68                        | KP-1      | Dako     | 113            | High number of CD68+ TAMs was correlated with worse survival. | Takanami et al 1999 |
| CD68                        | KP-1      | Dako     | 95             | No correlation between CD68+ TAM density and survival. | Johnson et al 2000 |
| CD68                        | KP-1      | Roche    | 129            | No correlation between CD68+ TAM density and survival. | Kojima et al 2005 |
| CD68                        | KP-1      | Roche    | 335            | No association between CD68+ TAM number and survival. | Al-Shibli et al 2009 |
| CD68                        | KP-1      | Abcam    | 417            | Presence of CD68+ TAM was not associated with survival. | Pei et al 2014 |
| CD68                        |          | Zhongshan Golden Bridge Biotechnology | 99          | High CD68+ TAM density in the tumour islets was associated with longer survival. High CD68+ TAM density in tumour stroma was associated with shorter survival. | Dai et al 2010 |
| CD68                        | KP-1      | Unknown  | 75             | High number of CD68+ TAM correlated with improved overall survival. | Remark et al 2016 |
| CD68/HLA-DR                  | KP-1/LN3  | Invitrogen/Invitrogen | 100          | High density of M1 (HLA-DR+CD68+) TAMs was associated with longer survival. | Ma et al 2010 |
| CD68/CD163                  | KP-1/1D6  |Invitrogen/Invitrogen | 100          | High density of M1 (HLA-DR+CD68+) TAMs was associated with longer survival. | Ma et al 2010 |
| CD68/HLA-DR                  | KP-1      | Roche    | 553            | High HLA-DR+CD68+, CD204+CD68+ and CD68+ TAM densities in stromal and intratumoral compartments were associated with prolonged survival | Rakaee et al 2019 |
| CD68/iNOS                   | KP-1/SP126 | Roche/Spring | 80          | High density of CD68+CD163+ M1 TAMs in tumour islets was associated with longer survival. High density of CD68+CD163+ M2 TAMs in stroma and tumour islets was associated with shorter survival. | Jackute et al 2018 |
| CD68/D163                   | KP-1/SP126 | Roche/Roche | 80          | High density of CD68+CD163+ M1 TAMs in tumour islets was associated with longer survival. High density of CD68+CD163+ M2 TAMs in stroma and tumour islets was associated with shorter survival. | Jackute et al 2018 |
| CD68                        | PG-M1 SRA-E5 | Dako TransGenic Inc. | 297          | A high number of CD68+ TAMs or CD204+ TAMs in tumour stroma was a worse prognostic factor. | Li et al 2018 |
| CD68                        | PG-M1     | Dako     | 38             | High number of CD68+ TAMs was associated with improved survival | Eerola et al 1999 |

(Continues)
| Marker used to identify TAMs | mAb clone | Producer | Patient number | Main conclusion | Reference |
|-----------------------------|-----------|----------|----------------|----------------|-----------|
| CD68                        | PG-M1     | Dako     | 162            | High CD68+ TAM density in tumour islets was associated with improved survival. High stromal CD68+ TAM density was associated with worse prognosis. | Welsh et al 2005 |
| CD68                        | PG-M1     | Dako     | 144            | High CD68+ TAM density in tumour islets was associated with better prognosis. | Kim et al 2008 |
| CD68/CD163                  | PG-M1/10D6| Dako/Novocastra | 40           | High M1 TAM density in tumour islets was associated with prolonged survival. | Ohri et al 2009 |
| CD68/HLA-DR                 | PG-M1/TAL.1B5| Dako/Hycult |              |                |           |
| CD68/iNOS                   | PG-M1/2D2-B2 | Dako/R&D Systems |              |                |           |
| CD68                        | Unknown   | Dako     | 117            | CD68+ TAM density was not associated with survival. | Toomey et al 2003 |
| CD68                        | Unknown   | Dako     | 35             | High CD68+ TAM density correlated with shorter survival. | Chen et al 2003 |
| CD68                        | Unknown   | Dako     | 199            | High CD68+ TAM density in cancer stroma was associated with poor survival. | Kawai et al 2008 |
| CD68/iNOS                   | Unknown   | Zhongshan, Santa Cruz Biot., BioLegend | 65           | High density of CD68+ TAMs and CD68+CD206+ M2 TAMs were associated with shorter survival | Zhang et al 2011 |
| CD163                       | EDHu-1    | AbD Serotec | 335          | CD163+ TAM density was not associated with survival. | Carus et al 2013 |
| CD204                       | SRA-E5    | TransGenic Inc | 170         | High CD204+ TAM density in tumour stroma was associated with poor prognosis. | Ohtaki et al 2010 |
| CD204                       | SRA-E5    | TransGenic Inc | 208         | High CD204+ TAM density in tumour stroma was associated with poor prognosis. | Hirayama et al 2012 |
| CD204                       | SRA-E5    | TransGenic Inc | 304         | High CD204+ TAM density was associated with higher cancer recurrence. | Ito et al 2012 |
| TREM-1                      | AF1278    | R&D Systems | 68           | High number of TREM-1+ TAMs was correlated with shorter survival. | Ho et al 2008 |
the cells were incubated with fluorochrome-labelled mAbs for 20 minutes on ice in the dark. The mAbs used for flow cytometry are listed in Tables S6-S8. Propidium iodide (PI) (Bio-Rad, Cat. No. 1351101) was used to exclude dead cells and was added immediately before flow cytometry acquisition of the samples. Staining of cell surface molecules was done prior to intracellular staining of CD68. For intracellular immunostaining of CD68, the single-cell suspension was incubated for 20 minutes in 0.5 mL fixation buffer (BioLegend Cat. No. 420801). After centrifugation (350 g, 7 minutes) fixed cells were washed with 1 mL permeabilization wash buffer (BioLegend, Cat. No. 421002) and centrifuged at 350 g for 7 minutes twice. To block non-specific mAb binding to intracellular epitopes, the cells were incubated with 12.5 µg/mL mouse IgG for 15 minutes. Fluorochrome-labelled anti-CD68 mAb was added and incubated for 30 minutes in the dark at room temperature. Stained cells were analysed with a BD LSRFortessa™ Cell Analyzer instrument (BD Biosciences, model no. 647794E6) and FlowJo software version 10.

2.5 | Image analyses of tissue sections

Images of the tissue sections stained by IHC were captured by an Olympus BX51 microscope, model BX51TF using a Colorview digital camera (Olympus); or by a Nikon Eclipse microscope, model N i-U using an Infinity 2 digital camera (Lumenera Corporation). Images of the tissue sections labelled with immunofluorescence were captured by a Nikon Eclipse model 80i microscope (Nikon) using an Axiocam 506 mono digital camera (Zeiss). For quantitative analysis, we selected five representative areas in total with both tumour cell areas and tumour stroma. Images of the representative areas were captured with an Olympus BX51 microscope at a 400x magnification. If a tissue section had areas with both low abundance and high abundance of stained cells, both areas were selected as representative areas and included in the analysis. Individual cells were identified by strong brown stain and manually counted by using AnalySIS Pro software (Olympus). The cell counting was repeated three times for each area by two scientists. All images were analysed in a blinded fashion.

2.6 | Statistical analysis

For quantitative analysis, the number of counted cells is presented as mean ± standard deviation for each marker. Groups were compared and potential differences were identified using the t test with Bonferroni-Dunn to correct for multiple comparisons. All statistical analysis was carried out using GraphPad Prism, version 6. P < .05 was considered statistically significant.

3 | RESULTS

3.1 | TAMs in lung tumour stroma express CD68, CD14 and CD163

We first performed a literature search to determine which mAb have previously been used for IHC of TAMs in NSCLC prognostic studies. We found in total 25 studies and most investigators (20 reports) had used an anti-CD68 mAb to identify macrophages (Table 1). The anti-CD68 mAb clone KP-1 was utilized in 11 studies whereas the anti-CD68 clone PG-M1 was used in 5 studies (Table 1). In the four remaining reports, the mAb clone name was not specified. Most studies used CD68 alone, but, in a few reports, CD68 was used in combination with another marker in order to identify M1 or M2 macrophages (Table 1). Taken together, the anti-CD68 mAb clone KP-1 has so far been the most commonly used mAb to label TAMs in prognostic NSCLC studies.

Three potential pan-macrophage markers (CD68, CD163 and CD14) were evaluated for IHC labelling of lung tumour sections. We tested the anti-CD14 mAb clone EPR3653,47 the anti-CD163 mAb clone 10D648 and two anti-CD68 mAbs (clones PG-M1 and KP-1).49,50 Tumour samples were obtained from 6 patients with NSCLC, and a series of adjacent tumour sections were made in order to compare the different mAbs. In all 6 patients examined, TAMs were mainly observed in the tumour stroma and were positive for CD14, CD163 and CD68 (Figure 1). Staining with the anti-CD14 mAb clone EPR3653, anti-CD163 clone 10D6 or anti-CD68 clone PG-M1 resulted in similar numbers of positive cells (Figure 1A,C,E). In contrast, the anti-CD68 mAb clone KP-1 gave a high background in tumour cell areas and throughout the whole section, as well as a strong stain in neutrophil-rich areas, suggesting that KP-1 may stain both TAMs and neutrophils as previously reported24,25 (Figure 1G,H). Notably, since CD14 and CD163 are membrane-associated molecules, the anti-CD14 and anti-CD163 mAbs labelled cell membranes, whereas the anti-CD68 mAbs resulted in granular stains in the cytoplasm (Figure 1). In summary, a high signal-to-noise ratio for immunostaining of TAMs was obtained with the anti-CD14 mAb EPR3653, the anti-CD163 mAb 10D6 and the anti-CD68 mAb PG-M1, whereas the widely used anti-CD68 mAb KP-1 gave high background and seemed to stain both TAMs and neutrophils.

3.2 | Alveolar macrophages are strongly positive for CD68 and CD163 but show variable expression of CD14

Alveolar macrophages constitute a substantial portion of macrophages in normal lungs and can also be present in lung
tumours. Therefore, we examined the expression of CD14, CD68 and CD163 by alveolar macrophages using the same mAbs as against TAMs. For this purpose, we used non-cancerous lung tissue from patients with lung cancer, because there are more alveoli and thereby alveolar macrophages

in non-cancerous lung tissue than in lung tumours. Non-cancerous lung from 7 patients was examined. Alveolar macrophages were identified by their distinct morphology and intra-alveolar localization. Immunostaining of CD14 revealed heterogeneous signal intensity between patients (Figure 2A-C). In patient #1, nearly all alveolar macrophages were labelled by the anti-CD14 mAb (Figure 2A), whereas the signal for CD14 was minimal or absent in the 6 other patients investigated (Figure 2C and data not shown). In all 7 patients, alveolar macrophages were labelled by the anti-CD163 mAb.
(Figure 2D), the anti-CD68 mAb clone PG-M1 (Figure 2E) and the anti-CD68 mAb clone KP-1 (Figure 2F). As previously observed in tumour tissue (Figure 1G), the anti-CD68 KP-1 mAb gave a high background in normal lung tissue (Figure 2F). Thus, alveolar macrophages are strongly positive for CD68 and CD163, whereas CD14 expression varies from patient to patient.

### 3.3 Macrophages in the TLS around lung tumours express CD68 but not CD163

Macrophage-containing TLS are commonly formed at the periphery of lung tumours.43,44 We tested whether the different mAbs could detect macrophages in TLS from the same 6 patients examined in Figure 1. Inside TLS, a weak CD14 stain was found in macrophages present in areas resembling germinal centres (white arrowheads in Figure 3A). These cells were presumably tingible body macrophages because such macrophages are typically found in germinal centres.45,46,51 The other macrophages in the TLS, hereafter termed TLS-associated macrophages, identified by morphology and haematoxylin and eosin (H&E) stain were not labelled by the anti-CD14 mAb (Figure 3A and data not shown). In contrast, TAMs in the areas surrounding the TLS were labelled by the anti-CD14 mAb (black arrowheads in Figure 3A). The anti-CD163 mAb did not stain any cell in TLS, including in germinal centres, whereas the TAMs in the areas surrounding

### Table 2 Detection and signal intensity of stained lung macrophage populations in lung sections

| Macrophage population       | CD14 (clone EPR3653) | CD163 (clone 10D6) | CD68 (clone PG-M1) | CD68 (clone KP-1) |
|-----------------------------|-----------------------|--------------------|--------------------|-------------------|
| TAMs                        | +/+++                 | ++                 | ++                 | ++                |
| Alveolar macrophage         | −/+                   | ++                 | +                  | +                 |
| TLS-associated macrophage   | −                     | −                  | +                  | +                 |
| Tingible body macrophage    | +                     | −                  | ++                 | ++                |

*Note: +++: strong signal; +: weak signal; −: no signal.*
the TLS were labelled (black arrowheads in Figure 3C). When using anti-CD68 mAbs (clones PG-M1 or KP-1), the signal was strong for tingible body macrophages and for TAMs surrounding the TLS, but weaker for TLS-associated macrophages (Figure 3E,G). Thus, TLS-associated macrophages are weakly positive for CD68 and negative for CD14 and CD163. Tingible body macrophages in the germinal centres of TLS are positive for CD68, weakly positive for CD14 and negative for CD163. All data presented so far are summarized in Table 2.

### 3.4 The anti-CD68 mAb clone KP-1 labels neutrophils

Because CD68 has been reported to be expressed by neutrophils,24,25 we investigated whether the anti-CD68 mAbs PG-M1 and KP-1 labelled neutrophils in lung cancer tissues, and performed IHC on tissue sections from the same 6 patients as above. Representative data from one patient are shown in Figure 4. Using H&E stain, we first identified tumour areas containing numerous neutrophils characterized by their multilobular nuclei (Figure 4A,B). Adjacent lung cancer tissue sections showed that neither the anti-CD14 mAb (Figure 4C), the anti-CD163 mAb (Figure 4D) nor the anti-CD68 mAb clone PG-M1 (Figure 4E) stained neutrophils. In contrast, use of the anti-CD68 mAb clone KP-1 resulted in a relatively strong stain of neutrophils (Figure 4F). Thus, the anti-CD68 mAb clone KP-1 does not only label macrophages (as shown above) but also neutrophils. In contrast, the anti-CD68 mAb clone PG-M1 appears to be more macrophage-specific since it does not stain neutrophils. Therefore, we made use of clone PG-M1 to stain CD68 in subsequent analyses of tumour tissue sections.

### 3.5 TAMs and alveolar macrophages in lung tumours co-express CD68 and CD163

Single-stained tumour tissue sections shown in Figure 1 suggested that TAMs may be positive for both CD68 and CD163. To investigate this further, we used fluorescence microscopy and performed double staining of CD68 (using clone PG-M1) and CD163 of lung tumour tissue from the same 6 patients. Representative data from one patient are shown in Figure 5. Numerous CD68+CD163+ double-positive TAMs were observed (Figure 5A). The signal intensity for CD68 and for CD163 varied within each tissue section. Several TAMs showed a strong signal for CD68 and a weak signal for CD163 (white arrowhead in Figure 5B), while other TAMs exhibited the opposite pattern (white arrow in Figure 5B). Moreover, several CD68+ TAMs without CD163 expression were detected in tumour stroma (white arrows in Figure 5C). The proportion of CD163 positive cells varied among patients. In the tumour tissue from the 6 patients, we found, by manual counting of 8 microscopy fields (magnification x400) per section, that 5%-30% of the CD68+ TAMs were negative for CD163, whereas only 1%-5% of the CD163+ TAMs were negative for CD68 (data not shown). All alveolar macrophages were strongly labelled by both anti-CD68 and anti-CD163 mAbs (Figure S1A). In accordance with the data obtained by single stain (Figure 3), TLS-associated macrophages...
were positive for CD68 but negative for CD163 (white arrows in Figure S1B). CD68\(^{+}\)CD163\(^{+}\) double-positive TAMs were found surrounding the TLS (white arrowheads in Figure S1B). Isotype-matched control mAbs showed no signal in the examined tissue areas (Figure S2A-C). Taken together, these results indicate that the anti-CD68 mAb clone PG-M1 detects most TAMs and label a higher number of TAMs than the anti-CD163 mAb. Alveolar macrophages co-express CD68 and CD163, while TLS-associated macrophages only express CD68.

3.6 Combined staining of CD68 with a plasma membrane marker improves the identification of macrophages

The results presented above provide support for CD68 as the most reliable pan-macrophage marker in lung tumours, because CD68 was shown to be expressed by most TAMs, alveolar macrophages, TLS-associated macrophages and tingible body macrophages (Table 2). In contrast, these four macrophage sub-populations were not all consistently detected by anti-CD14 or anti-CD163 mAbs (Table 2). Unfortunately, CD68 labelling poses a challenge for cell counting because CD68 molecules are present intracellularly and appear as cytoplasmic granules or dots in the cells, a pattern that may be difficult to distinguish from non-specific background stain (Figure 1E). In contrast, cells stained with mAb specific for a molecule present on the plasma membrane, such as CD14 and CD163, are easier to count because of the distinct labelling of the cell contours (Figure 1A,C). Therefore, we hypothesized that combining anti-CD68 mAb with a mAb specific for a macrophage-associated plasma membrane marker (such as CD14, CD163 or CD206) may improve the detection and quantification of macrophages in tissue sections. Indeed, the identification of macrophages was greatly enhanced by such a double stain, both for alveolar macrophages and TAMs (Figure 6).
To evaluate this double stain approach for cell counting, we quantified the number of CD68⁺ macrophages (Figure 7A,D), CD163⁺ macrophages (Figure 7B,E) and double-stained CD68⁻CD163⁺ macrophages (Figure 7C,F) in adjacent sections from lung tumour from 6 patients. Five representative areas were selected and counted manually for each section. The quantification revealed that the mixture of the two mAbs resulted in a higher macrophage number than the single stain approach with anti-CD68 or anti-CD163 mAb in many of the selected areas in the tissue sections from all 6 patients (Figure 7G and data not shown). As CD68 is the most commonly used marker for detection of macrophages, the mean of the number of CD68⁺ cells was set to 100% for all patients (Figure 7H). The average number of macrophages detected by the mAb mixture was significantly increased to 130%-190% for patients #3 and #4 (Figure 7H). For patients #2, #5 and #7, staining with the mAb mixture resulted in a tendency towards more macrophages. Notably, the data also showed that higher numbers of macrophages typically were counted in sections stained with anti-CD163 mAb alone compared to anti-CD68 alone (Figure 7G,H). This may seem surprising since the immunofluorescence data presented in Figure 5 indicated that most macrophages in lung tumours expressed CD68 whereas only a sub-population (70%-95%) expressed CD163. A likely explanation for this apparent paradox is that more macrophages stained with anti-CD68 mAb are missed during manual quantification, as compared to anti-CD163 stain, illustrating the challenge of using an intracellular granule stain such as CD68 to quantify cells. In summary, the data indicate that macrophages are easier to identify and count by combining the cytoplasmic CD68 stain with a membranous surface stain.

3.7 | CD163 expression is essentially restricted to macrophages

Molecular markers for macrophages were further investigated using flow cytometry. First, we examined CD163 expression in tumour tissue from 4 patients (Figure 8). Tumour tissue from the same patients was previously evaluated by immunofluorescence (Figure 5). In this initial experiment, we used the markers CD14 and HLA-DR, which represent a commonly used gating strategy in flow cytometry for identification of macrophages as double-positive CD14⁺HLA-DR⁺ cells. The nucleated cells were first gated to remove debris from all recorded events (Figure 8A). Next, the cells were gated using a single-cell gate to exclude doublets and cell clumps (Figure 8B). Live leucocytes were then gated by use of propidium iodide (PI) and CD45 (Figure 8C), whereas CD19⁺ B cells and CD3⁺ T cells were excluded (Figure 8D). From the live, non–T cell, non–B cell leucocyte gate (Figure 8D), cells were gated into CD163⁻positive and CD163⁻negative cells (Figure 8E,H,K,N).

Among the CD163⁻ cells, there was a small population of CD14⁺HLA-DR⁺ macrophages, confirming that some macrophages do not express CD163 (Figure 8F,I,L,O). In contrast, essentially all CD163⁺ cells were shown to express both CD14 and HLA-DR, revealing that CD163 expression is essentially restricted to macrophages (Figure 8G,J,M,P). Thus, CD163 is a macrophage-specific marker, but it is not expressed by all macrophages.
Flow cytometry confirms that CD163 is expressed by most but not all TAMs

The data presented in Figure 8 were further analysed to elucidate in more detail the association between CD163, CD14 and HLA-DR expression. After exclusion of CD3+ T cells and CD19+ B cells (Figure 9A-D) the live leucocytes were separated into three populations based on HLA-DR and CD14 expression (Figure 9E,I,M,Q). The expression of CD163 was investigated in all three populations, that is the HLA-DR+CD14− population (Figure 9F,J,N,R), the HLA-DR+CD14+ bona fide macrophage population (Figure 9G,K,O,S) and the HLA-DR CD14− population (Figure 9H,L,P,T). A clear difference in CD163 expression was observed, as a substantial proportion of HLA-DR+CD14+ cells were positive for CD163 whereas the two CD14− populations were negative or contained very few CD163+ cells, confirming that CD163 expression is essentially restricted to macrophages. The analysis also verified our observation made by immunofluorescence that not all macrophages express CD163 since the proportion of CD163+ cells among CD14+HLA-DR+ macrophages varied from 48.2% to 84.9% (mean: 64%) for the 4 patients investigated (Figure 9G,K,O,S and Figure S4). Notably, the CD163 stain did not separate the macrophages into two distinct populations (Figure 9G,K,O,S). Instead, the intensity for CD163 varied from low to relatively high, which is consistent with the results obtained by immunofluorescence (Figure 5). We also considered the possibility that CD163 may be expressed intracellularly by all TAMs. To test this hypothesis, we performed
intracellular flow cytometry staining in fixed and permeabilized single cells from a lung tumour sample from 1 patient and found that 68% of the HLA-DR⁺CD14⁺ TAMs were positive for CD163 (Figure S5). Thus, there is a significant fraction of TAMs, approximately one-third, that do not express CD163, neither on the cell surface nor intracellularly.

3.9 | Flow cytometry shows that CD68 expression is not restricted to macrophages

Immunostaining of tissue sections (Figure 5) and previous reports suggested that CD68 may be expressed by other cells than macrophages.²⁴,⁵⁴ To clarify this using flow cytometry, we examined tumour tissue from 3 NSCLC patients with the anti-CD68 mAb clone Y1/82A (Figure 10). Because CD68 is predominantly located in intracellular vesicles, fixation and permeabilization were included in the staining protocol. CD45⁺ leucocytes were first gated (Figure 10A-C) and CD68⁺ cells were identified in the CD45⁺CD3⁻CD19⁻ population (Figure 10D-E). Next, the expression of CD14 and HLA-DR among the CD68⁺ cells was assessed (Figure 10G). This analysis revealed that the majority (58.8%) of CD68⁺ cells expressed both HLA-DR and CD14 (indicated in yellow in Figure 10G). However, a substantial fraction (25.8%) of the CD68⁺ cells was HLA-DR⁺ and CD14⁻ (indicated in green in Figure 10G). In addition to the two HLA-DR⁺ populations, a population of HLA-DR⁻CD14⁻ cells (indicated in red in Figure 10G) was also observed among
These findings suggest that other immune cells than macrophages are also stained by the anti-CD68 mAb since all macrophages are expected to express HLA-DR. Moreover, the observation that a relatively large fraction of CD68+ cells were HLA-DR−CD14− strongly suggests that there is a population of TAMs which does not express CD14 and that is excluded when using the classical flow cytometry definition of macrophages (HLA-DR+CD14+ gate).
In addition to macrophages, the CD68⁺HLA-DR⁺CD14⁻ population may potentially contain some CD11c⁺ myeloid dendritic cells (Figure 10F). Staining of CD11c revealed that 14.9% of CD11c⁺HLA-DR⁺CD14⁻ cells, presumably representing dendritic cells, expressed CD68 (Figure S6). However, it should be noted that CD11c is not sufficient by itself to identify dendritic cells because most lung macrophages are also positive for CD11c.28,54,55 Notably, the CD68⁺HLA-DR⁺CD14⁻CD11c⁻ population contained smaller and less granulated cells (Figure 10I) compared to the CD68⁺HLA-DR⁺CD14⁺ population, which showed size and granularity typical to that of macrophages (Figure 10H). We also analysed the small population of cells (14.8%) that lacked both CD14 and HLA-DR (indicated in red in Figure 10G). Because immunostaining revealed that one of the evaluated mAbs towards CD68 detected neutrophils (Figure 4), we included a mAb against CD11b to be able to identify CD11b⁺ granulocytes and natural killer cells in the CD68⁺CD14⁻HLA-DR⁻ population. However, only 0.4% of the CD68⁺CD14⁺HLA-DR⁻ cells could be gated by this strategy (Figure 10J). The remaining fraction of CD11b⁺ cells contained both large cells with a high granularity (SSC-H) and smaller and less granulated cells (Figure 10K). Further analysis showed that no CD68 expression could be found on CD56⁺ natural killer cells (Figure S7), and virtually all neutrophils (99.3%) and most eosinophils (91.7%) were found to be negative for CD68 (Figure S8). In contrast, all monocytes in blood were shown to express CD68 (Figure S9). Taken together, the data strongly suggest that CD68 expression in lung tumour is not strictly restricted to macrophages, in particular because CD14⁺HLA-DR⁻ cells and probably some dendritic cells also express CD68.

3.10 | CD68 is a sensitive marker for detection of most macrophages

In the next analysis, we wanted to examine whether CD68 is a suitable marker for detection of all macrophages in lung tumour tissue. CD68 expression was evaluated in tumour tissue from the same patients as presented in Figure 10, and three cell populations were examined: HLA-DR⁺CD14⁺, HLA-DR⁺CD14⁻ and HLA-DR⁻CD14⁻ (Figure 11). Before analysis, CD3⁺ T cells and CD19⁺ B cells were excluded from the CD45⁺ leucocyte population (Figure 11A-D). Essentially all CD14⁺HLA-DR⁺ macrophages expressed CD68 (Figure 11E, F), indicating that CD68 can be used to detect all bona fide macrophages in tumour. The HLA-DR⁺CD14⁺ population contained 31.8% CD68⁺ cells, presumably representing macrophages and dendritic cells that did not express CD14 (Figure 11H). Finally, some CD68⁺ cells (11.1%) were found in the CD14⁻HLA-DR⁻ population (Figure 11G), suggesting that CD68 expression is not strictly restricted to macrophages, as mentioned in the previous section. Thus, these data are consistent with CD68 being expressed by virtually all macrophages in lung tumour tissue.

3.11 | A better gating strategy for TAMs in flow cytometry based on CD68 and HLA-DR

The results from the flow cytometry evaluation of CD14, CD163 and CD68 indicated that all three markers had limitations as pan-macrophage markers. CD163 was found to be a rather specific marker for the monocyte/macrophage lineage but CD163 was not detected on all macrophages in lung tumours. CD68 was not strictly specific for the macrophage/monocyte lineage since it was also expressed by HLA-DR⁻ cells. However, CD68 was detected on a larger fraction of macrophages in tumour, as compared to CD163 and CD14. Based on these observations, we hypothesized that CD68 in combination with HLA-DR would specifically detect most macrophages, including macrophages that are negative for CD14. Therefore, such a strategy should identify more macrophages than the commonly used definition of macrophages in flow cytometry being double-positive CD14⁺HLA-DR⁺ cells. To compare the two gating strategies, flow cytometry was performed on adenocarcinoma tumours from 3 patients (Figure 12). For this purpose, the first three gates were set to exclude debris, cell clumps and CD45⁻ non-leucocytes (Figure 12A-C). Next, CD3⁺ T cells and CD19⁺ B cells were excluded from the CD45⁺ leucocytes (Figure 12D). From this population, macrophages were gated by the current definition (HLA-DR⁺CD14⁺) (Figure 12E,G,I) or our suggested new strategy (HLA-DR⁺CD68⁺) (Figure 12F,H,J). The percentage of macrophages was calculated from the total population of leucocytes (single, CD45⁺ leucocytes). The HLA-DR⁺CD68⁺ based strategy identified 25%-50% more macrophages than the current definition of macrophages (Figure 12). Thus, we conclude that the combination of HLA-DR and CD68 is a better strategy to gate most macrophages in tumours as compared to HLA-DR and CD14.

3.12 | Evaluation of commonly used markers for the M2 macrophage phenotype

M2-associated markers including CD163 have been used for the purpose of identifying tumour-promoting macrophages in lung tumours in previous studies. Because we observed that CD163 was expressed by a subset of TAMs, we wanted to compare the CD163 expression to that of other suggested markers for the M2 macrophage phenotype.
M2 markers (CD204, CD206 and CD209) using the classical gating strategy (CD14\(^+\)HLA-DR\(^+\)) for macrophages. Lung cancer tissue from 3 patients was included in the analysis (Figure 13). Live leucocytes were gated and CD19\(^+\) B cells and CD3\(^+\) T cells were excluded from the population (Figure 13A-D). The expression levels of the M2-associated markers CD163 (Figure 13F), CD204 (Figure 13H), CD206 (Figure 13J) and CD209 (Figure 13L) differed within the macrophage population of each patient. Both CD206 and CD163 were expressed by approximately 60% and 85% of CD14\(^+\)HLA-DR\(^+\) macrophages, respectively, whereas no expression of CD204 was found among the same population. Labelling of CD206 separated the macrophages into a CD206\(^+\) and a CD206\(^-\) population (Figure 13J). A small fraction of the HLA-DR\(^+\)CD14\(^+\)CD11c\(^-\) population (I) and HLA-DR\(^-\)CD14\(^+\)CD11b\(^-\) population (K). The percentage of CD68\(^+\) cells was calculated from the total number of CD45\(^+\) leucocytes. All other percentages (blue) were calculated from the number of CD68\(^+\) cells. All per cent values are means of results obtained on tissue from 3 patients with lung adenocarcinoma (patients #9, #10 and #11). DCs, dendritic cells

**FIGURE 10** Intracellular flow cytometry analysis of CD68\(^+\) cells in lung tumour. Intracellular flow cytometry staining of CD68 was performed on fixed and permeabilized cells from NSCLC tumour. (A) SSC-A and FSC-A were used to exclude cellular debris from all recorded events. (B) Single cells were gated using FSC-A and FSC-H, thereby excluding doublets. (C) CD45 was used to define the leucocyte population. (D) The CD68\(^+\) population was identified after (E) CD19\(^+\) and CD3\(^+\) cells had been excluded from the leucocyte population. (F) Dendritic cells were defined as CD11c\(^+\) and CD14\(^-\), gated from (G) the HLA-DR\(^+\)CD14\(^+\) population of CD68\(^+\) leucocytes. (H) The HLA-DR\(^+\)CD14\(^-\) population was further separated based on CD11b expression. Cellular size and granularity were evaluated in the HLA-DR\(^+\)CD14\(^-\) population (H), HLA-DR\(^+\)CD14\(^+\)CD11c\(^-\) population (I) and HLA-DR\(^-\)CD14\(^+\)CD11b\(^-\) population (K). The percentage of CD68\(^+\) cells was calculated from the total number of CD45\(^+\) leucocytes. All other percentages (blue) were calculated from the number of CD68\(^+\) cells. All per cent values are means of results obtained on tissue from 3 patients with lung adenocarcinoma (patients #9, #10 and #11). DCs, dendritic cells
3.13 | CD163 and CD206 expression identifies three macrophage populations in lung tumours

The observation that the two M2 markers CD163 and CD206 were expressed by approximately 60%-85% of macrophages in tumour (Figure 13) suggested that the two molecules may be co-expressed by the same population of TAMs. To test this hypothesis, CD3+ T cells and CD19+ B cells were excluded from CD45+ leucocytes. CD45+ cells were defined as leucocytes. CD3+ T cells and CD19+ B cells were excluded. The markers HLA-DR and CD14 were used to gate CD14+ macrophages, a HLA-DR-CD14− population and a HLA-DR+CD14+ population. The expression of CD68 in these populations was further assessed in (F), (G) and (H), respectively. The percentage of CD14+ macrophages was calculated from the total number of CD45+ leucocytes. The percentages of CD68 expression (blue) was calculated from the population of which the expression was assessed. All per cent values are means of results obtained on tissue from 2 patients diagnosed with lung adenocarcinoma (patients #9 and #10).

were detected on a significant fraction of macrophages in lung tumours.
Gating of macrophages based on CD68^+HLA-DR^+ identified more cells than the commonly used gating strategy based on CD14^+HLA-DR^+. Intracellular flow cytometry staining of CD68 was performed on fixed and permeabilized tumour tissue-derived single cells. (A) SSC-A and FSC-A were used as parameters to exclude cellular debris from all recorded events. (B) Single cells were gated using FSC-A and FSC-H, thereby excluding doublets. (C) CD45^+ was used to identify leucocytes; thereafter, (D) CD3^+ and CD19^+ leucocytes were excluded from the CD45^+ population before markers of macrophages were assessed. Macrophages defined as HLA-DR^+ and CD14^+ (E)(G)(I) were compared to a population of macrophages defined as HLA-DR^+ and CD68^+ (F)(H)(J). Tissue was obtained from 3 patients, and the first four gates are representative for all 3 patients. The case number and histological subtype for each patient are indicated. The per cent values were calculated from the total number of gated CD45^+ leucocytes.
Flow cytometry analysis of expression of M2-associated markers on CD14+ macrophages in lung tumour. (A) Nucleated cells were gated to exclude cellular debris from all recorded events. (B) Single cells were gated using FSC-A and FSC-H, thereby excluding doublets. (C) Dead cells stained with propidium iodide (PI) were excluded, and the remaining cells expressing CD45 were defined as live leucocytes (CD45+PI-). (D) CD3+ T cells and CD19+ B cells were excluded. (E) Macrophages were defined as HLA-DR+ and CD14+. Isotype controls were used to determine the level of background caused by non-specific binding of the mAb. From the macrophage gate, M2-associated markers were assessed: (F) CD163, detected by clone RM3/1; (G) isotype-matched control mAb for CD163; (H) CD204, clone 7C9C2; (I) isotype-matched control mAb for CD204; (J) CD206, clone 15-2; (K) isotype-matched control mAb for CD206; (L) CD209, clone 9E9A8; and (M) isotype-matched isotype control mAb for CD209. The percentage of HLA-DR+CD14+ macrophages was calculated from the total number of live leucocytes (CD45+PI-). All other percentages (blue) were calculated from the population of HLA-DR+CD14+ macrophages. All per cent values are means of data obtained on lung squamous cell carcinoma and lung adenocarcinoma from 3 patients (patients #4, #5 and #8).
In this study, we critically evaluated antibodies that may be used for quantitative and qualitative analysis of macrophages in human lung tumours by IHC and flow cytometry. CD68 was confirmed to represent a pan-macrophage marker that is expressed by all four macrophage populations that are present in lung tumours, namely TAMs, alveolar macrophages, TLS-associated macrophages and tingeble body macrophages. In contrast, two other potential pan-macrophage markers evaluated, CD14 and CD163, were found not to be optimal because they were not expressed by all macrophages. Although CD68 was confirmed to represent a pan-macrophage marker in human lung tumours, several important limitations of this marker were observed. First, careful selection of antibody was found to be critical. Several anti-CD68 mAbs including clones KP-1 and PG-M1 which recognize different epitopes of the CD68 antigen are available. The widely used anti-CD68 clone KP-1 appears to stain both macrophages and neutrophils. In contrast, the anti-CD68 clone PG-M1 was found to stain TAMs but not neutrophils. Labelling of neutrophils and other granulocytes by KP-1 has been previously reported by several laboratories. In fact, it is well established that CD68 is expressed by both macrophages and neutrophils. It is unclear why the anti-CD68 mAb clone KP-1 stains neutrophils, whereas the anti-CD68 clone PG-M1 does not, but it could be due to differences in CD68 glycosylation in the two cell types as previously suggested. For IHC of TAMs in lung tumours, we recommend the use of anti-CD68 clone PG-M1 rather than KP-1 because lung tumours may contain many neutrophils. The difference in specificity between clone KP-1 and PG-M1 may in part explain the inconsistencies of the results from studies concerning the prognostic significance of TAMs in NSCLC.

Another limitation of CD68 as a marker to identify macrophages on tissue sections is that CD68 molecules are present intracellularly and appear as cytoplasmic granules or dots, a pattern that may be difficult to distinguish from non-specific background stain. Our data showed that combining anti-CD68 stain with another mAb specific for a macrophage-associated plasma membrane marker (such as CD14, CD163 or CD206) could greatly improve the identification of macrophages on tumour sections. In particular, analysis of tumour sections stained with a mixture of anti-CD163 and anti-CD68 mAbs resulted in a significant increase in the number of quantified macrophages compared to staining with each mAb alone. However, it should be kept in mind that none of the macrophage-associated plasma membrane marker tested (CD14, CD163 and CD206) was expressed by all TAMs. Therefore, although double staining of, for example, CD68 and CD163 makes it easier to count TAMs, it is likely that some TAMs (e.g. CD163− TAMs) will be missed by this method. Thus, identification of all macrophages in lung tumours may require additional mAbs or different antibody combinations.

A commonly used strategy for identification of macrophages by flow cytometry is based on double expression of HLA-DR and CD14, but CD68 has also been used. Here, we compared several gating strategies and found that all HLA-DR+CD14+ cells were positive for CD68 whereas only 64% were positive for CD163. CD163 was therefore excluded as a potential pan-macrophage marker in flow cytometry. Importantly, our data suggest that a significant fraction of macrophages in human lung tumours do not express CD14. Among all CD68+, non−T cell/non−B cell leucocytes, we found that approximately 30% of the HLA-DR~ cells did not express CD14. Our IHC data also revealed that TLS-associated macrophages and alveolar macrophages in most patients were negative for CD14. Macrophages with low or no CD14 expression have been previously reported in human non-diseased lung tissue. Interestingly, the cytokine IL-4 has been shown to down-regulate CD14 expression on macrophages in vitro. It should also be noted that there exists a soluble form of CD14 and that proteases mediate the shedding of CD14 from the macrophage cell surface. Because many macrophages in tumour lack CD14, we suggest an improved strategy for identification of macrophages in flow cytometry by use of CD68 and HLA-DR, with the purpose of including CD14-negative macrophages.

Each method has strengths and limitations and this certainly applies to IHC and flow cytometry. In order to label most TAMs, we established two different, yet related protocols adapted for each method. Both strategies take advantage of CD68 as a decent, although not perfect, pan-macrophage marker. In IHC, CD68 labelling poses a challenge for cell counting because CD68 molecules are present intracellularly and appear as cytoplasmic granules or dots in the cells. We could show that combining anti-CD68 stain with another mAb specific for a macrophage-associated plasma membrane marker such as CD163 could greatly improve the identification of macrophages on tumour sections. In flow cytometry, the intracellular localization of CD68 does not pose a problem for cell counting, but cell fixation and permeabilization are required before CD68 staining. Because CD68 is not strictly macrophage-specific, we suggest an improved strategy for identification of macrophages in flow cytometry as CD68+HLA-DR+ double-positive cells.

CD163, CD204, CD206 and CD209 have previously been suggested as markers for human M2 macrophages. We failed to detect significant CD204 and CD209 expression on macrophages in lung tumours from 3 patients investigated, whereas previous IHC studies found both CD209+ cells and CD204+ cells in NSCLC tumours. This discrepancy may be explained by patient-to-patient variation, by use of different mAb clones or...
different methods. In contrast, we detected both CD163 and CD206 on subsets of macrophages. Notably, there was no perfect correlation between CD163 and CD206 expression on TAMs. Instead, the markers divided HLA-DR⁺CD14⁺ lung tumour macrophages into three sub-populations: CD163⁺CD206⁺, CD163⁺CD206⁻ and CD163⁻CD206⁻ TAMs. If CD163 and CD206 were indeed markers for M2 macrophages, and this remains to be clarified, it would...
imply that there are at least two distinct M2 macrophage sub-populations in human lung tumours: CD163+CD206+ TAMs and CD163+CD206- TAMs. Moreover, CD163 was detected on essentially all circulating blood monocytes as previously reported,56,57 but the monocytes were negative for CD206. Most TAMs are likely to derive from circulating monocytes. Therefore, the CD163+CD206- population, which constituted 21%-34% of the HLA-DR+CD14+ cells, may in fact represent monocytes that have recently infiltrated the tumour.

For the flow cytometry analysis, we included some additional macrophage markers that were not used in IHC, such as HLA-DR, CD204 and CD209, because it is much easier to combine antibodies for multistaining in flow cytometry than in IHC. However, these markers, in particular HLA-DR and CD204, are certainly of interest for staining TAMs on tissue sections. It should be noted that IHC-based labelling of HLA-DR on macrophages can be challenging because i) HLA-DR levels vary on the surface of TAMs and ii) other tumour-infiltrating immune cell types such as B cells also express HLA-DR.68

Although it is well accepted that macrophages may either suppress or promote tumour development depending on their activation phenotype, most investigators currently consider that the M1-M2 macrophage nomenclature represents an oversimplification. For example, a recent study based on single-cell RNA sequencing defined as many as 14 distinct transcriptional states of monocytes and macrophages in human lung tumours.69 Macrophages are highly responsive to their surroundings. Therefore, it is likely that the phenotype of TAMs is influenced by the molecular composition of the tumour microenvironment. In vitro studies have shown that IL-4 may induce CD206 expression by macrophages.64,65 Therefore, it is possible that IL-4 in the tumour induces CD206 on CD163+ monocytes, thereby generating CD163+CD206+ TAMs. Furthermore, the presence of CD163 on blood monocytes, but its absence on many TAMs, suggests that CD163 expression is lost by some monocyte-derived TAMs. Anti-inflammatory signals such as glucocorticoids and IL-10 have been reported to induce the surface expression of CD163 on monocytes and macrophages.56,57,64,65,70 whereas inflammatory mediators such as IFN-γ, lipopolysaccharide and tumour necrosis factor-α were shown to suppress CD163 expression.70 Therefore, IFN-γ-producing immune cells in the tumour, such as natural killer cells and Th1 cells, may potentially suppress the expression of CD163 on monocyte-derived macrophages. Accordingly, the CD163+CD206- sub-population, which constituted 9%-45% of HLA-DR+CD14+ cells, may potentially contain tumour-suppressive ‘M1’ macrophages.2

CONFLICT OF INTEREST
No conflict of interest was declared.

AUTHORS’ CONTRIBUTIONS
AF performed IHC-based experiments and wrote the manuscript. RS performed flow cytometry work, analysed the results and contributed to writing the manuscript. CH analysed and evaluated IHC-based results and contributed to writing the manuscript. LT performed cell counting. BS performed flow cytometry work. HA, PW, ÅH and OB provided material from lung cancer patients. IØ discussed the results and contributed to writing the manuscript. AC designed, supervised and evaluated the experiments and wrote the manuscript. All authors revised the manuscript and approved the final version of the manuscript.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.