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CD47 overexpression is associated with decreased neutrophil apoptosis/ phagocytosis and poor prognosis in non-small-cell lung cancer patients

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Background: Non-small-cell lung cancer (NSCLC) patients often exhibit neutrophilia, which has been associated with poor clinical outcomes. However, the mechanisms that lead to neutrophilia have not been fully established. CD47 is an antiphagocytic molecule that promotes neutrophil recruitment.

Methods: Blood was collected from 50 treatment-naive patients with advanced NSCLC and from 25 healthy subjects. The frequency of CD66b$^+$ cells and the expression of CD47 were determined by flow cytometry. Neutrophil apoptosis was determined by 7-amino-actinomycin D/Annexin V-APC staining. Phagocytosis was assessed by flow cytometry. Reactive oxygen species production after phorbol 12-myristate 13-acetate treatment was quantified by 2′,7′-dichlorofluorescein fluorescence. Pro-inflammatory plasma cytokines were quantified using a cytometric bead array assay.

Results: The percentage of circulating neutrophils was significantly higher in patients than in controls ($P<0.001$). Patient-derived neutrophils had a higher oxidative potential than those of controls ($P=0.0286$). The number of neutrophils in late apoptosis/ necrosis was lower in patients than in controls ($P=0.0317$). Caspase 3/7 activation was also lower in patients than in controls ($P=0.0079$). CD47 expression in whole-blood samples and in the neutrophil fraction was higher in NSCLC patients than in controls ($P=0.0408$ and $P<0.001$). Patient-derived neutrophils were phagocytosed at a lower rate than those of controls ($P=0.0445$). CD47 expression in neutrophils negatively correlated with their ingestion by macrophages ($P=0.0039$). High CD47 expression was associated with a lower overall survival.

Conclusions: Increased CD47 expression on the surface of neutrophils was associated with a delay in neutrophil apoptosis and with an impairment in their phagocytic clearance by macrophages, suggesting that CD47 overexpression may be one of the underlying mechanisms leading to neutrophilia in NSCLC patients.

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Lung cancer remains the leading cause of cancer death worldwide (Arrieta *et al*, 2013) and the second most common cancer among both men and women (Global Burden of Disease Cancer *et al*, 2017). The majority of patients are diagnosed with advanced-stage disease, and despite technological advancements on all fronts of clinical practice, the prognosis for patients with lung cancer remains dismal (Arrieta *et al*, 2013). Haematologic abnormalities, including anaemia, thrombocytosis and leukocytosis are frequently observed in lung cancer patients. Although neutrophilic leukocytosis (also known as neutrophilia) has long been reported in patients with lung cancer, the underlying mechanisms leading to this condition have not been fully elucidated.

Acute inflammation is a physiological process through which the innate immune system responds to irritation, injury and infection. If unresolved, acute lung inflammation can progress to chronic inflammation, which has been directly implicated in a wide range of lung diseases such as acute respiratory distress syndrome, asthma, cystic fibrosis, chronic obstructive pulmonary disease, emphysema and lung cancer (Robb *et al*, 2016). Neutrophils are undoubtedly major effectors of acute and chronic inflammation. Consequently, a common feature between these diseases is chronic neutrophilic inflammation, as evidenced by an exacerbated and sustained infiltration of neutrophils into the lungs with concurrent neutrophilic leukocytosis (Gernez *et al*, 2010, Anand and Anand, 2012).

In patients with non-small-cell lung cancer (NSCLC), there is a strong correlation between poor clinical outcomes and high neutrophil content, both locally (Ilie *et al*, 2012) and systemic (Arrieta *et al*, 2010; Sanchez-Lara *et al*, 2012; Carus *et al*, 2013; Gu *et al*, 2015; Peng *et al*, 2015; Yin *et al*, 2015), suggesting that neutrophils and inflammation have an important role during carcinogenesis.

Owing to their pro-inflammatory functions and potential toxicity against host tissue, neutrophil numbers are tightly regulated through a fine balance between their production, retention, mobilisation, margination and clearance from the system (Silvestre-Roig *et al*, 2016). Therefore, the successful resolution of inflammation involves the cessation of further neutrophil recruitment, the induction of neutrophils to undergo programmed cell death as well as the phagocytic clearance of dying cells (Serhan and Savill, 2005; Mora-Jensen *et al*, 2011).

Healthy cells are protected from phagocytosis by displaying anti-phagocytosis molecules such as CD31, CD200, plasminogen activator inhibitor 1 and the integrin-associated protein/CD47 (Gitik *et al*, 2014). CD47 is expressed on the surface of many cells types but it is particularly abundant on red blood cells (RBCs) and leukocytes (Oldenborg *et al*, 2000). Binding of CD47 to its macrophage receptor, signal regulatory protein alpha (SIRP-α), initiates a signal transduction cascade that results in the inhibition of phagocytosis (Oldenborg *et al*, 2001). Conversely, in cells undergoing apoptosis, CD47 expression is reduced and phagocytosis increased. Under this setting, CD47 can also be redistributed into patches distant from the phagocytic synapse.

It has been shown that various types of human tumours upregulate the expression of CD47 as a mechanism to avoid immune-mediated elimination (Goto *et al*, 2014). Recently, it has been show that CD47 expression in NSCLC specimens and cell lines correlates with clinical staging, lymph node metastasis and distant metastasis (Zhao *et al*, 2016).

CD47 supports the migration of neutrophils and activated T cells to the sites of infection via its interactions with SIRP-α, SIRP-γ and a subset of integrins (Liu *et al*, 2001, 2002; Azcutia *et al*, 2012), which indicates that CD47 expression on neutrophils, leukocytes and endothelium is critical for these immune cells to localise to sites of inflammation. Furthermore, neutrophils can be eliminated by the blockade of the anti-phagocytic signal CD47 (Majeti *et al*, 2009; Chao *et al*, 2011).

It is thus possible that changes in the expression of CD47 (which reportedly affect the accumulation and clearance of neutrophils from the system) could underlie, at least in part, the neutrophilia observed in patients with NSCLC. The aim of this study was to evaluate the expression of CD47 in circulating neutrophils, to associate this expression with clinical characteristics and prognosis in patients with advanced NSCLC and finally, to evaluate the phenotype, apoptosis, activation state, reactive oxygen species (ROS) production and inflammatory cytokines between patients with NSCLC and healthy subjects.

MATERIALS AND METHODS

Study design. A total of 50 patients with NSCLC in stage IIIB and IV were enrolled from January to December 2013 at the National Institute of Cancer in Mexico. All patients signed an informed consent document that was approved by the Institutional Review Board and Ethics Committee (INCAN (013/020/ICI) (CV773/13)) from the National Institute of Cancer. The inclusion criteria were as follows: adult patients (>18 years); newly diagnosed, disease stage IIIB or IV; histopathological confirmation of NSCLC; Eastern Cooperative Oncology Group (ECOG) status of 0–2; free of any treatment (radiation, chemotherapy or immunotherapy) and eligible to receive chemotherapy (carboplatin–taxol) at the time of diagnosis; and no history of autoimmune disease or of recent steroid therapy. Standard clinicopathological characteristics (age, sex, smoking history, wood-smoke exposure, BMI, metastases, oligometastases, central nervous system (CNS) metastases, pleural effusion, history of diabetes mellitus and hypertension) were recorded from patients at the time of diagnosis. Blood samples of healthy subjects ($N=25$), paired by age and gender, were obtained from the Blood Transfusion Center bank, and information concerning smoking history, wood-smoke exposure and comorbidities was recorded. Detailed characteristics of the patients can be found in Supplementary Table 1.

Sample collection. A volume of 16 ml of blood was drawn into two 8 ml EDTA tubes (BD Biosciences, San Jose, CA, USA) from patients and healthy subjects. One milliliter of blood was used for fresh whole-blood immunofluorescence staining and analysed by flow cytometry. Seven milliliter were centrifuged, the plasma was collected and stored at $-80\,^{\circ}$C for subsequent cytokine analysis. Eight milliliter were used for neutrophil isolation and functional assays.

Phenotypic characterisation by flow cytometry. Phenotypic characterisation and activation state assessment of neutrophils were performed by flow cytometry analyses according to the standard protocols. In brief, antibodies were added to 100 μl of fresh whole blood and incubated in the dark for 15 min at room temperature. Afterwards, red blood cells were lysed using RBC lysis buffer 1$\times$ and cells were washed twice with cell staining buffer. Cells were then acquired and analysed. The following combination of monoclonal antibodies was used: anti-CD66b-PerCp Cy5.5 (G10F5); and anti-CD47-FITC (CC2C6) (Biolegend, San Diego, CA, USA). Fluorescence Minus One (FMO) negative controls correspond to samples stained with all the fluorescent conjugates, minus the one being tested. Gates were set using fluorescence minus one. All antibodies were used according to the manufacturers' instructions. Finally, samples were acquired in a FACS Aria II Flow Cytometer (BD Biosciences) and analysed with FlowJo software 10.1 (Tree Star, Ashland, OR, USA). The leukocyte population was gated based on morphological parameters on a forward *vs* side scatter (FSC/SSC) plot. Neutrophils were defined as CD66b^{+}. The mean fluorescence intensity (MFI) was calculated by FACS Aria II and was used to present the expression levels of CD47 molecules on the membrane of neutrophils in this study.

Cytokine quantification. Plasma levels of the cytokines and chemokines IL-8, IL-2, IL-17A, IFN-γ, IL-4, IL-1β, IL-6, IL-10, IL-12p70 and TNF were quantified using a Pro-Inflammatory and Th1/Th2/Th17cytometric bead array assay kit (BD Biosciences, Mexico City, Mexico) according to the manufacturer's instructions. Samples were acquired using a FACS Aria II Flow Cytometer (BD Biosciences) and analysed with FCAP Array Software V. 3.0 (Soft Flow, Pecs, Hungary).

Neutrophil isolation. Eight millilitre blood samples of healthy subjects and patients were diluted in PBS at a ratio of 1 : 1 v/v before separation by density gradient centrifugation (Polymorphprep, AXIS-SHIELD PoC AS, Oslo, Norway). The interphase contains mononuclear cells and polymorphonuclear leukocytes (PMNs). The purity and viability of PMNs isolated using this method were within acceptable parameters (higher than 90%). Recovered neutrophils were gown in propylene tubes or 24-well microplates in RPMI 1640 medium supplemented with 10% FBS at 37 °C, 5% CO_2 for 24 h after which functional assays (apoptosis and phagocytosis) were performed.

Caspase activation and apoptosis. Polymorphonuclear leukocytes (2×10^6 cells) were grown in RPMI for 24 h, after which caspase activation and spontaneous apoptosis was evaluated. An anti-CD66b monoclonal antibody was used to identify neutrophil-specific populations. The activity of caspases 3 and 7 was evaluated with the CellEvent Kit Caspase-3/7 Green Detection Reagent (Life Technologies, Eugene, OR, USA). Polymorphonuclear leukocytes (2×10^6 cells) were grown in RPMI for 24 h, after which cells were incubated for 30 min at 37 °C with 5 μM ml^{-1} of Green Detection Reagent. Samples were then observed under a microscope FLoid Cell Imaging Station (Life Technologies) with a $\times 20$ objective. Micrographs from five randomly selected fields were taken for each sample. Positive cells were counted and the data were analysed with Image J Software 1.49 (NIH, Bethesda, MD, USA).

Apoptosis of PMN was determined using the Annexin V-APC apoptosis detection kit (BD Biosciences) according to the manufacturer's protocol. Briefly, 24 h after plating, cells were incubated with an anti-CD66b mAb for 15 min and washed (2 ×) with cold cell staining buffer. Cells were then re-suspended in Annexin V Binding Buffer. Samples were then stained with 5 μl of Annexin V-APC and 2.5 μl of 7-amino-actinomycin D (7-AAD) solution for 15 min at room temperature (25 °C) in the dark. Additional Annexin V Binding Buffer was added to each tube. Cells were analysed on a FACS Aria II Flow Cytometer (BD Biosciences) and analysed with FlowJo software 10.1 (Tree Star). Excitation was set at 488 nm, and 7-AAD fluorescence was detected at 650 nm long-pass filter.

Cell culture and THP-1 differentiation to macrophages. The THP-1 cell line was obtained from ATCC (TIB-202), and maintained at 2×10^5 cells per ml in RPMI 1640 medium supplemented with 10% FCS and 2 μM l^{-1} L-glutamine. A total of 2×10^6 THP-1 cells per ml were differentiated using 200 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St Louis, MO, USA) for 72 h. Differentiation of PMA-treated cells was enhanced by changing the PMA-containing media 72 h after the initial treatment with fresh RPMI 1640 (10% FCS and 1% L-glutamine) for 5 additional days.

Phagocytosis. Blood samples from five patients and four healthy subjects were used for neutrophil isolation. One fraction of PMNs was used to determine CD47 expression and another fraction was resuspended in PBS and labelled with 10 μM CellTracker Green (5-chloromethyfluorescein; CMFDA, Invitrogen, Waktham, MA, USA.) for 30 min, 37 °C. The CMFDA-labelled PMNs were incubated in RPMI in round-bottom polypropylene tubes at 37 °C, 5% CO_2 for 24 h, to induce spontaneous apoptosis. Differentiated THP-1 macrophages were co-cultured with CMFDA-labelled PMNs at a ratio of 5 : 1 (PMN to MDM) for 4 h at 37 °C, 5% CO_2. For each condition, cells were stained with anti-CD66b to exclude eosinophils from the analysis. Macrophages were detached and analysed by flow cytometry as follows: macrophages were distinguished by their typical high FSC characteristics. A minimum of 10 000 CD66b-negative events were acquired. Macrophages that had ingested CMFDA-labelled apoptotic neutrophils became BL1-positive. To determine the phagocytic index, the total number of the THP-1 events acquired was divided by the number of CMFDA-positive THP-1 within the CD66b-negative gate. Flow cytometric analysis was performed with FlowJo software, version 10.1 (Tree Star).

Reactive oxygen species production. The production of ROS was measured by membrane permeable 2′-7′-dichlorofluorescin diacetate (H_2DCFDA; Life Technologies Inc., Catalogue No. D-399), which is oxidised to fluorescent 2′,7′-dichlorofluorescein (DCF) by ROS following cleavage of its acetate groups by intracellular esterases. Immediately after isolation, neutrophils (1×10^6 cells) from patients and controls were incubated in sterile round-bottom polypropylene tubes with RPMI medium alone, or RPMI supplemented with 30 nM PMA for 25 min at 37 °C, 5% CO_2. Samples were then incubated with 10 μM H_2DCFDA dissolved in DMSO for 30 min at 37 °C, after which DCF MFI was measured by flow cytometry (excitation wavelength, 488 nm). After gating away cellular debris, the geometric mean fluorescence values of spontaneous and stimulated samples were recorded from CD66b$^+$ cells.

Statistical analyses. Continuous data were summarised as arithmetic means with s.d.'s or medians with ranges according to data distribution. Baseline characteristics of patients, CD47, neutrophils and cytokines expression levels were compared using Student's *t*-tests or Mann–Whitney *U*-tests (according to data distribution determined by the Kolmogorov Smirnov test), and χ^2-tests. The correlation among the percentage of phagocytosis and MFI of CD47 of CD66b$^+$ was computed by linear regression and analysed by Spearman test. Overall survival (OS) curves were estimated by the Kaplan–Meier method while comparisons among groups were analysed with log-rank or Breslow tests. Statistically significant and borderline variables (*P*-values $\leqslant 0.1$) were included in the Cox multivariate analysis. Statistical significance was determined as $P \leqslant 0.05$ with a two-sided test. All data were analysed using the SPSS software package version 20 (SPSS, Inc., Chicago, IL, USA).

Study approval. This study was approved by the Institutional Review Board and Ethics Committee (INCAN (013/020/ICI) (CV773/13)) from the National Institute of Cancer.

All subjects signed a written informed consent form before inclusion in the study.

RESULTS

Clinical characteristics of NSCLC patients and healthy subjects. Supplementary Table 1 shows the baseline clinical characteristics of treatment-naive NSCLC patients ($N = 50$) and healthy subjects ($N = 25$) included in this study. Both groups had an even distribution, with no significant differences found with regards to age or gender. Significant differences were found with regards to smoking history (46% *vs* 12%; $P = 0.004$) and wood-smoke exposure (40% *vs* 4%; $P = 0.001$), which were more commonly reported among NSCLC patients. Approximately 84% of patients had adenocarcinoma and 90% presented stage IV disease. Central nervous system metastases at the time of diagnosis were found in 14% of the patients and pleural effusion in 50%. Finally, 86% of patients presented a functional performance status (ECOG) of 1. The haematologic characteristics of treatment-naive patients are

summarised in Supplementary Table 1. Approximately 64% of the patients exhibited a white blood cell count within normal parameters ($1.5–7.7 \times 10^9 l^{-1}$). Leukocytosis (leukocytes $>10 \times 10^9 l^{-1}$) was present in 28% of the patients (14 out of50). Neutrophilic leukocytosis (leukocytes $>10 \times 10^9 l^{-1}$ and an absolute neutrophil count (ANC) $>7.7 \times 10^9 l^{-1}$) was present in 20% of patients. Only 2% of the patients (1 out of 50) exhibited isolated neutrophilia (ANC$>7.7 \times 10^9 l^{-1}$ with a leukocyte count within normal parameters). Neutropenia (ANC$<1.5 \times 10^9 l^{-1}$) was present only in 4% of the patients (2 out of 50).

Neutrophil frequency. As shown in Figure 1A, the granulocytic and lymphocytic populations (leukocytes) were selected from the FSC-A *vs* SSC-C dot plot and neutrophils were identified based on CD66b expression. The percentage of neutrophils (CD66b$^+$ cells) was significantly elevated in patients as compared to healthy donors (56.5 ± 17.5 *vs* 37.5 ± 7.6; $P<0.001$; Figure 1B and Supplementary Table 2). Similarly, the expression level of CD66b (measured as MFI) was also significantly higher in neutrophils obtained from NSCLC patients as compared to those from controls (6938 ± 2333 *vs* 2994 ± 2449; $P<0.0178$; Figure 1C and Supplementary Table 2).

Expression of CD47 in peripheral blood cells and neutrophils. Whole-blood expression of CD47 in patients and controls was evaluated by flow cytometry following the gating strategy shown in (Figure 1D). The percentage of CD47$^+$ cells in whole blood was similar in patients and controls (median 82.1% (67.8–99.3) *vs* 89.2% (66.5–91.5); $P=0.142$; Figure 1E and Supplementary Table 2). However, the abundance of CD47 in a per cell basis, quantified as MFI, was significantly higher in NSCLC patients (22 464 (15 204–28 814) *vs* 16 455 (14 542–17 061); $P=0.0408$; Figure 1F and Supplementary Table 2). Similarly, when the expression of CD47 was evaluated in the neutrophil population (CD66b$^+$), the levels of CD47 were markedly higher in patients than in controls (600.7 ± 198 *vs* 255.9 ± 106.6; $P<0.001$; Figure 1G and Supplementary Table 2).

Neutrophil oxidative state. No significant differences were found between patients and healthy subjects with regards to spontaneous ROS production. In contrast, the levels of ROS produced after PMA stimulation were significantly ($P=0.0286$) higher in neutrophils from patients (34 405 (28 664–47 540)) than in neutrophils from healthy subjects (19 061 (14 307–27 499); Figure 2).

Caspase activation and spontaneous apoptosis in neutrophils. Spontaneous apoptosis, assessed as Annexin V binding and membrane permeability to 7-AAD, was significantly lower in neutrophils from patients with NSCLC than in those from healthy controls after 24 h of *ex vivo* culture (10.59 *vs* 18.28; $P=0.0317$; Figure 3C and D). In line with this, samples from healthy subjects exhibited a higher percentage of fluorescent cells, indicative of caspase-3/7 activation, than samples from NSCLC patients (506.8 *vs* 145.4; $P=0.0079$; Figure 3A and B).

***In vitro* macrophage phagocytosis of control and patient-derived neutrophils.** To assess the rate at which macrophages engulf either patient- or control-derived neutrophils, the fluorescence of CMFDA$^+$CD66b$^-$ cells was quantified by flow cytometry according to the gating strategy shown in Figure 4A. This population represents macrophages that incorporated the dye through the engulfment of neutrophils. The percentage of CMFDA$^+$CD66b$^-$ macrophages was lower in NSCLC samples as compared to controls (65.02 (52.0–78.8) *vs* 79.10 (70.0–88.0); $P=0.0445$; Figure 4B). To determine whether increased neutrophil expression of CD47 was associated with the observed reduction in the phagocytic index, we sought a correlation between expression of CD47 and the phagocytic index (Figure 4D). To this end, the expression of CD47 was evaluated in parallel blood samples (i.e., from the same patients/controls) as the ones used to determine the phagocytic index. In accordance with the results from the complete cohort previously shown (Figure 1G), in this subset of patients the expression of CD47 in CD66b$^+$ cells was higher than in controls (22 464 (15 204–28 814) *vs* 16 455 (14 542–17 061); $P=0.0408$; Figure 4C). The results from the correlation analysis are shown in Figure 4D where a significant negative association between CD47 expression and macrophage phagocytosis can be clearly observed ($r^2=-0.7179$; $P=0.0039$).

Factors associated with OS. The median follow-up of patients was 16.9 months, with a range of 9.2–24.6 months. The mean OS was 17.5 months (95% CI 14.015–21.067; Supplementary Table 6). Patients with NSCLC were divided into two groups according to the median expression of CD47 in whole blood and OS in each group was compared. The group of NSCLC patients whose CD47 expression in whole blood was $\leqslant 1635.5$ did not reach the median survival at the time of the last follow-up. In contrast, the group of patients whose CD47 MFI exceeded 1635.5 had a significantly lower OS (13.6 months (4.3–22.8); $P=0.007$; Figure 5A). In a multivariate analysis, high expression of CD47 in whole blood was an independent predictive factor of poor OS (RR 3.46 (95% CI 1.2–9.7); $P=0.018$; Figure 5C).

When groups were dichotomised according to the median expression of CD47 in neutrophils (CD66b$^+$ cells) a similar trend was observed, albeit without statistical significance. Whereas the majority of patients in the low CD47 group were alive at the last follow-up (OS: NR), those in the high CD47 group exhibited a lower OS (13.6 months (1.4–25.9); $P=0.140$; Figure 5B). The expression of CD47 in whole blood was significantly higher in patients with stage IV disease than in patients with stage IIIB (1783 MFI *vs* 975 MFI; $P=0.020$; Supplementary Table 3). There was a tendency, which did not reach statistical significance, for high CD47 expression to correlate with the presence of oligometastases ($P=0.064$; Supplementary Table 3). The expression of CD47 in whole blood was significantly higher among patients with low albumin levels ($\leqslant 3.4$ mg dl^{-1}) than in those with higher albumin levels (2501.5 *vs* 1316; $P=0.021$). Low albumin levels (<3.4) were associated with lower median OS ($P<0.001$; Supplementary Table 7). In agreement with this, in a multivariate analysis, the level of serum albumin was an independent predictive factor of OS (0.49 (0.25–0.95); $P=0.037$; Figure 5C). Other factors such as clinical and demographic characteristics (gender, age >60, smoking history, tumour histology, oligometastases and CNS metastases at the time of diagnosis) did not impact median OS (Supplementary Table 6). There was a tendency for patients with ECOG 1 and clinical stage IIIB to have a better OS (Supplementary Table 6).

Association between plasma cytokines and CD66b/CD47 percentage and expression. The plasma levels of various pro-inflammatory cytokines were evaluated. Compared to healthy donors, patients had significantly higher levels of IL-8, IL-12p70, IL-29, IL-31 and IL-32 in plasma (Supplementary Table 4). Furthermore, there was a significant correlation between the expression of CD47 in whole blood and the levels of both IL-8 ($r=0.45$; $P=0.0051$) and IL-4 ($r=0.44$; $P=0.0126$). These correlation coefficients indicate that the expression of CD47 increases together with that of IL-8 and IL-4 (Figure 6A and B and Supplementary Table 5). Similarly, there was a strong correlation between the percentage of CD47-positive cells in whole blood and the plasma levels of both IL-4 ($r=0.39$; $P=0.0297$) and IL-12 ($r=0.54$; $P=0.0016$; Figure 6C and D and Supplementary Table 5). There was also a significant association between the percentage of CD66b$^+$ cells and the plasma levels of IL-4 ($r=0.35$; $P=0.0479$), IL-8 ($r=0.51$; $P=0.0014$) and IL-31 ($r=0.35$; $P=0.0494$; Figure 6E–G and Supplementary Table 5). Finally, the expression of CD47 in neutrophils positively correlated with

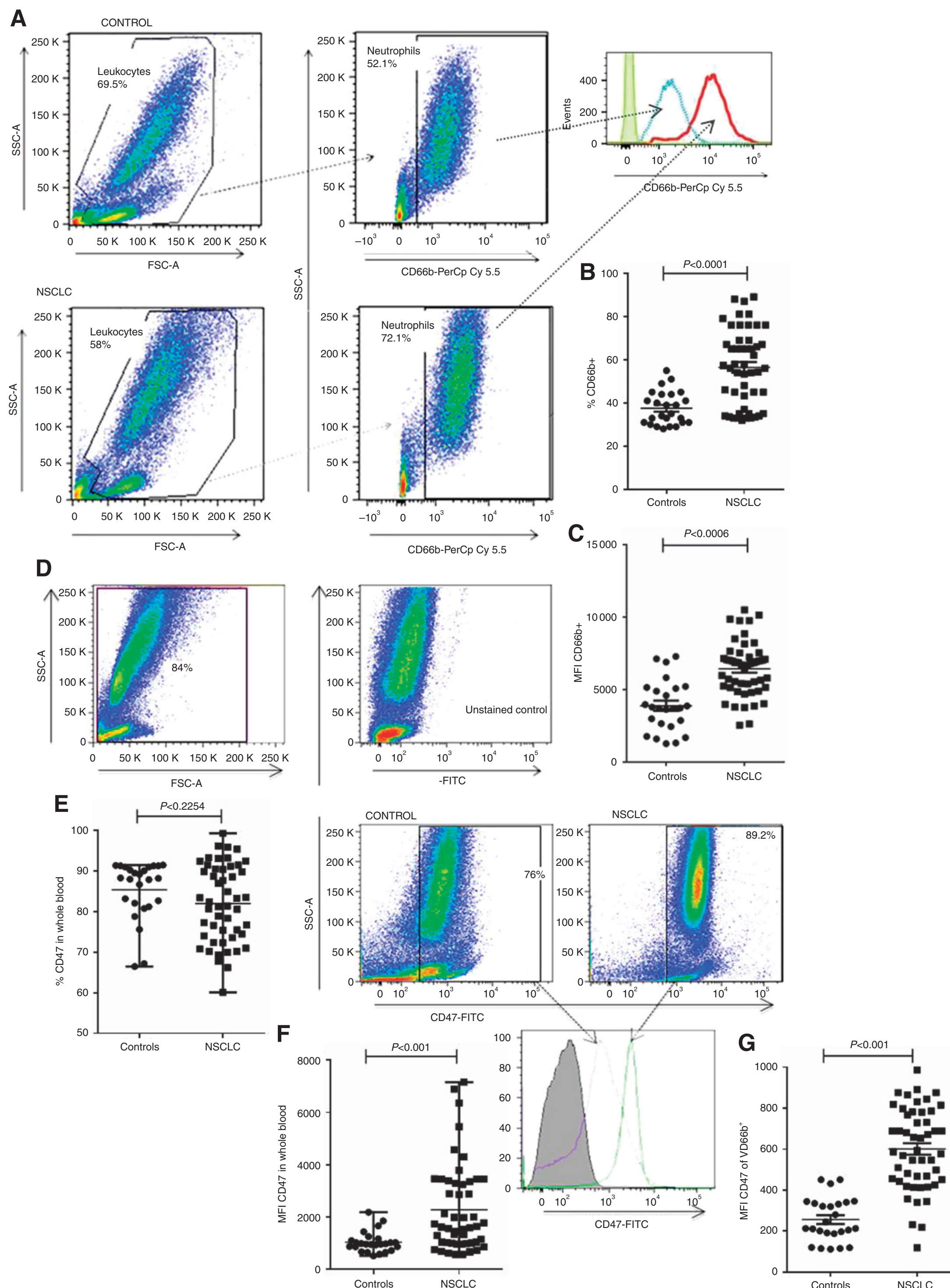


Figure 1. Neutrophil abundance and expression of CD47 in NSCLC patients and controls. Gating strategy for the flow cytometry analysis performed in whole-blood samples from patients and healthy donors. From the FSC-A *vs* SSC-C dot plot, the granulocytic and lymphocytic populations (leukocytes) were analysed. (**A**) Neutrophils were identified based on CD66b expression and physical characteristics (SSC). Positive cells for CD66b correspond to neutrophils. The histogram represents the MFI of the CD66b marker. The green line is the fluorescence minus one control (FMO), the blue line corresponds to the control subject and red line to a patient. (**B**) Scatter plot showing the frequency of neutrophils ($CD66b^+$ cells) and CD66b MFI (**C**) in peripheral blood samples from NSCLC patients and controls. (**D**) CD47 expression was quantified in leukocytes identified based on FSC and SSC (left dot plot). The gate used to define $CD47^+$ cells was set using the FMO control ($FITC^-$). Shown are representative dot plots from a control and a patient, and a histogram to illustrate the differences in the expression of CD47 (grey shade indicates FMO, purple line corresponds to a control subject and green line to a patient). (**E**) Scatter plot showing the frequency of $CD47^+$ cells in whole blood of controls and NSCLC patients. (**F**) Scatter plot showing the MFI of CD47 in peripheral blood cells. (**G**) MFI of CD47 in $CD66b^+$ cells (neutrophils) from NSCLC patients and controls. Each symbol represents one individual (controls, $N = 25$; patients, $N = 50$). Lines indicate mean ± s.e.m. Means were compared using unpaired *t*-tests (**B**, **C**, **G**) or unpaired Mann–Whitney tests (**E**, **F**).

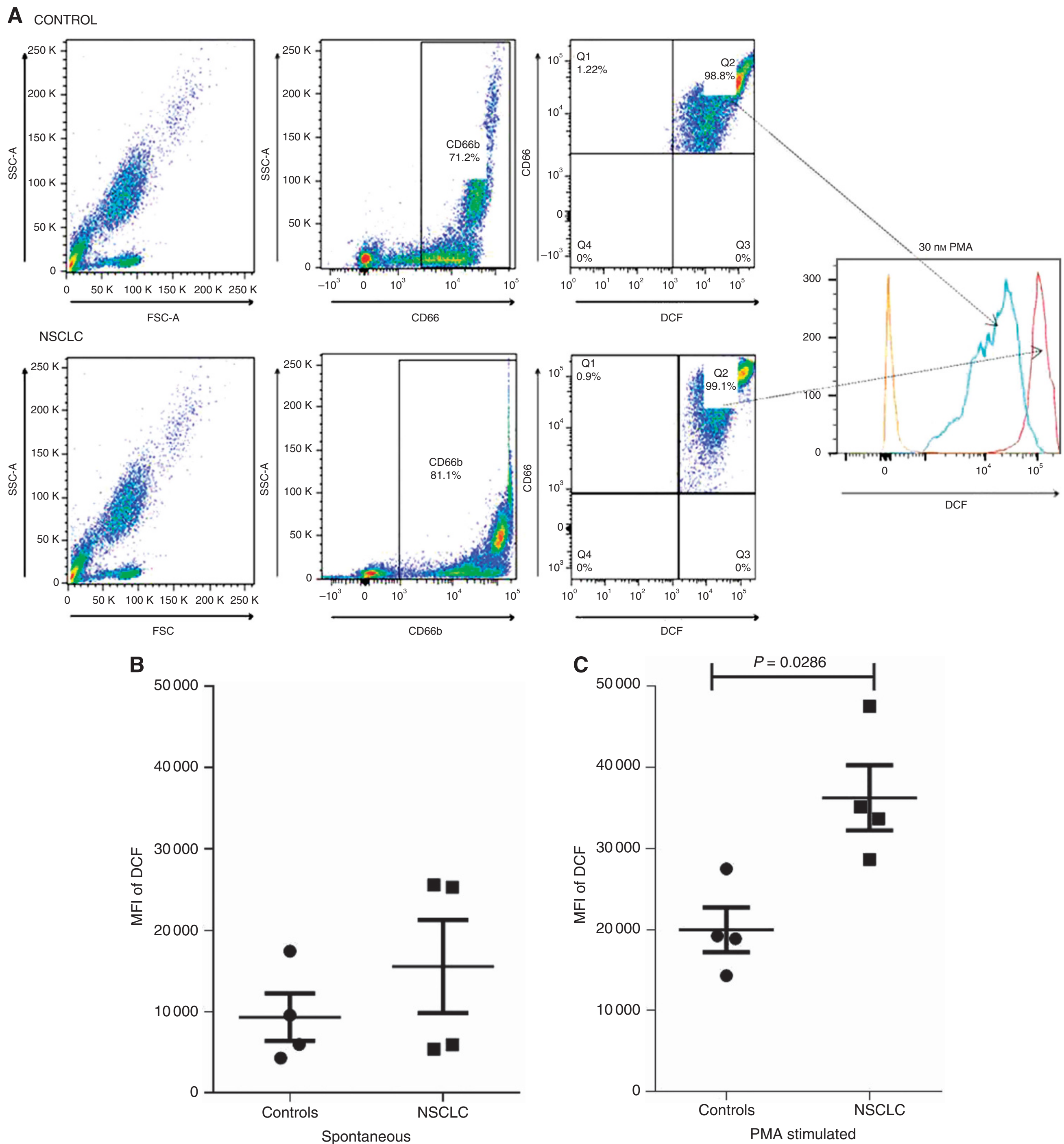


Figure 2. Spontaneous and PMA-induced ROS production in circulating neutrophils. (A) 2′,7′-dichlorodihydrofluorescein diacetate (DCF) fluorescence was used as an indicator of ROS production in neutrophils identified based on CD66b expression and physical characteristics. Shown are representative dot plots from a patient, and a control and a histogram comparing ROS production in neutrophils from a patient (red line) and a control (blue line). The yellow line indicates the FMO control. (B) Scatter plot showing spontaneous ROS (DCF MFI) production by neutrophils from patients and controls. (C) Scatter plot showing PMA-stimulated ROS production. Each symbol represents one individual (controls, $n=4$; patients, $n=4$). Lines indicate mean ± s.e.m. Unpaired *t*-test analyses were performed.

the plasma levels of IL-4 ($r=0.39$; $P=0.0297$) and IL-31 ($r=0.39$; $P=0.0297$; Figure 6H and I and Supplementary Table 5).

DISCUSSION

Patients with NSCLC often exhibit neutrophilic leukocytosis (neutrophilia) at the time of diagnosis or during the course of the disease. Several studies have confirmed that neutrophil content, both local and systemic, inversely correlates with positive outcomes. In particular, the neutrophil–lymphocyte ratio has emerged as an independent and reliable prognostic factor for patients with lung cancer, in both early (Tomita *et al*, 2012; Yao *et al*, 2013; Tanoglu *et al*, 2014; Zhang *et al*, 2015) and advanced stages (Arrieta *et al*, 2010; Sanchez-Lara *et al*, 2012; Gu *et al*, 2015; Peng *et al*, 2015; Yin *et al*, 2015). However, much uncertainty still exists about the underlying mechanisms that lead to neutrophilia in the context of cancer.

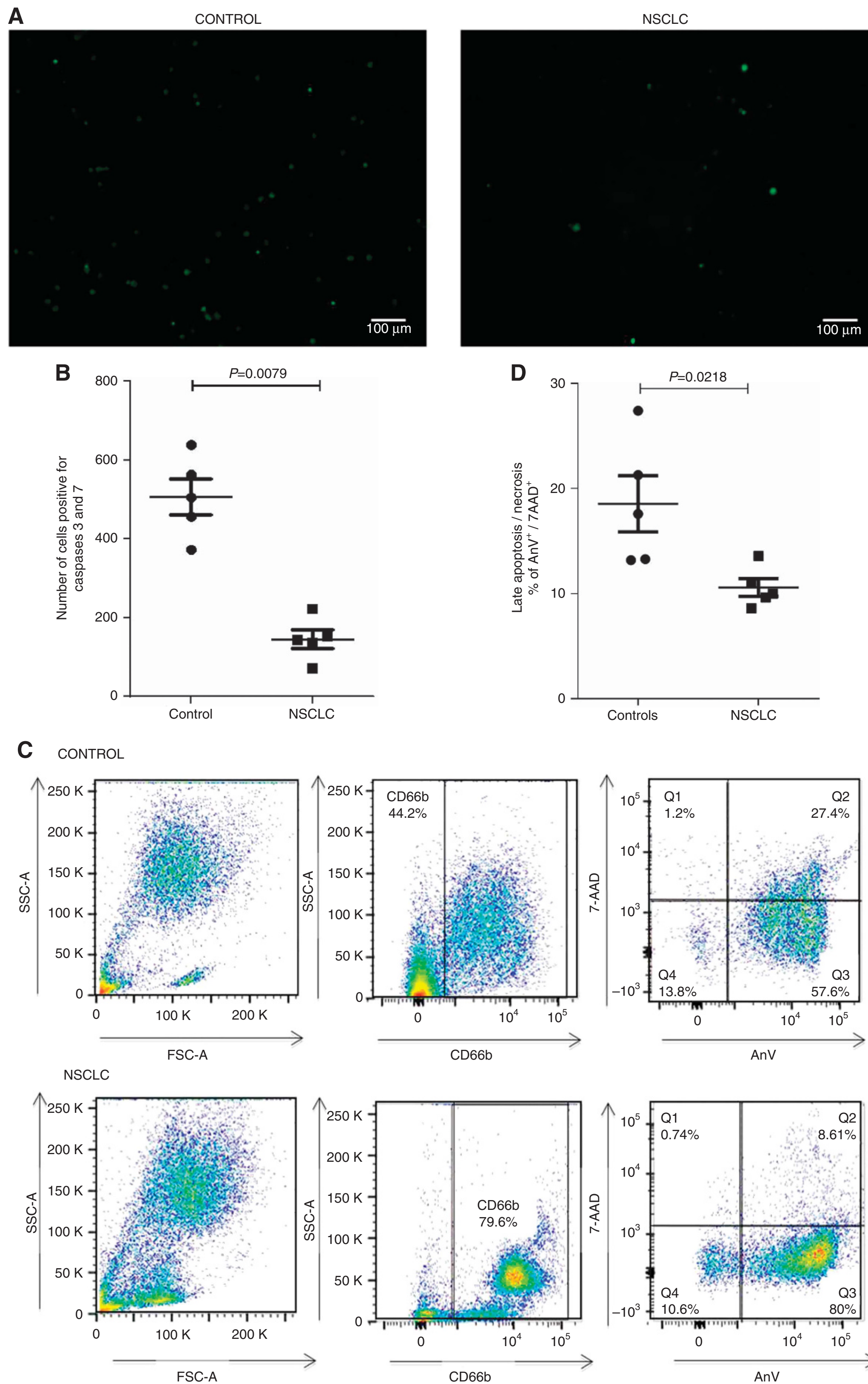


Figure 3. Spontaneous apoptosis in neutrophils from patients and controls. The PMNs from patients with NSCLC and healthy donors were isolated from peripheral blood and incubated during 24 h. Spontaneous apoptosis was quantified by counting cells with activated caspases 3 and 7, and by quantifying Annexin V- and 7-AAD-positive cells. (**A**) Representative micrographs showing caspase 3 and 7 activation (green fluorescence). (**B**) Number of PMN cells positive for caspases 3 and 7 ($n=5$). (**C**) Neutrophils were gated as detailed previously, and Annexin V-APC and 7-AAD fluorescence was measured. (**D**) Scatter plot showing the percentage of cells positive for Annexin V and 7-AAD (late apoptosis/necrosis). Each symbol represents one individual (controls, $n=5$; patients, $n=5$). Lines indicate mean ± s.e.m. Unpaired t-test analyses were performed.

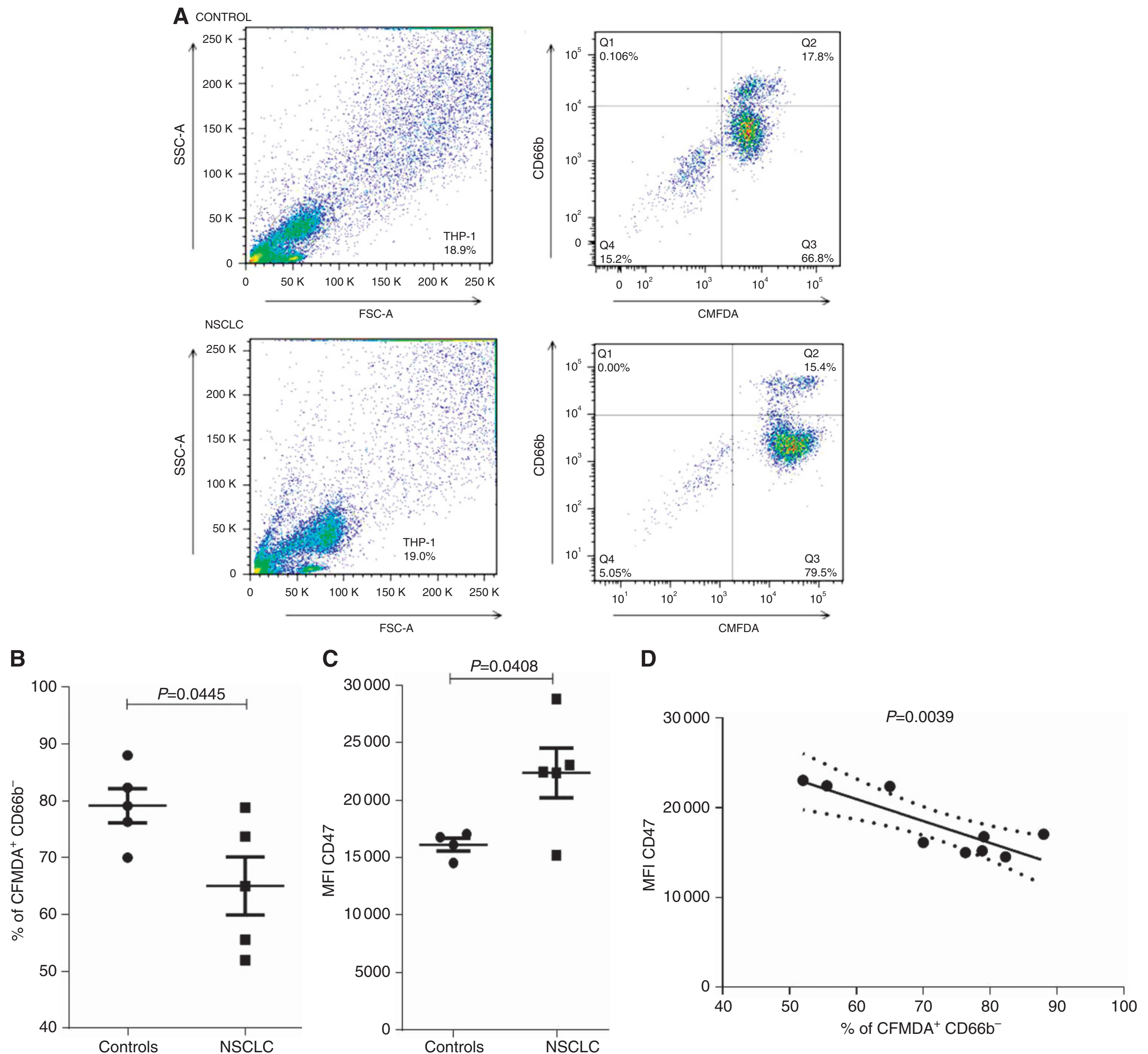
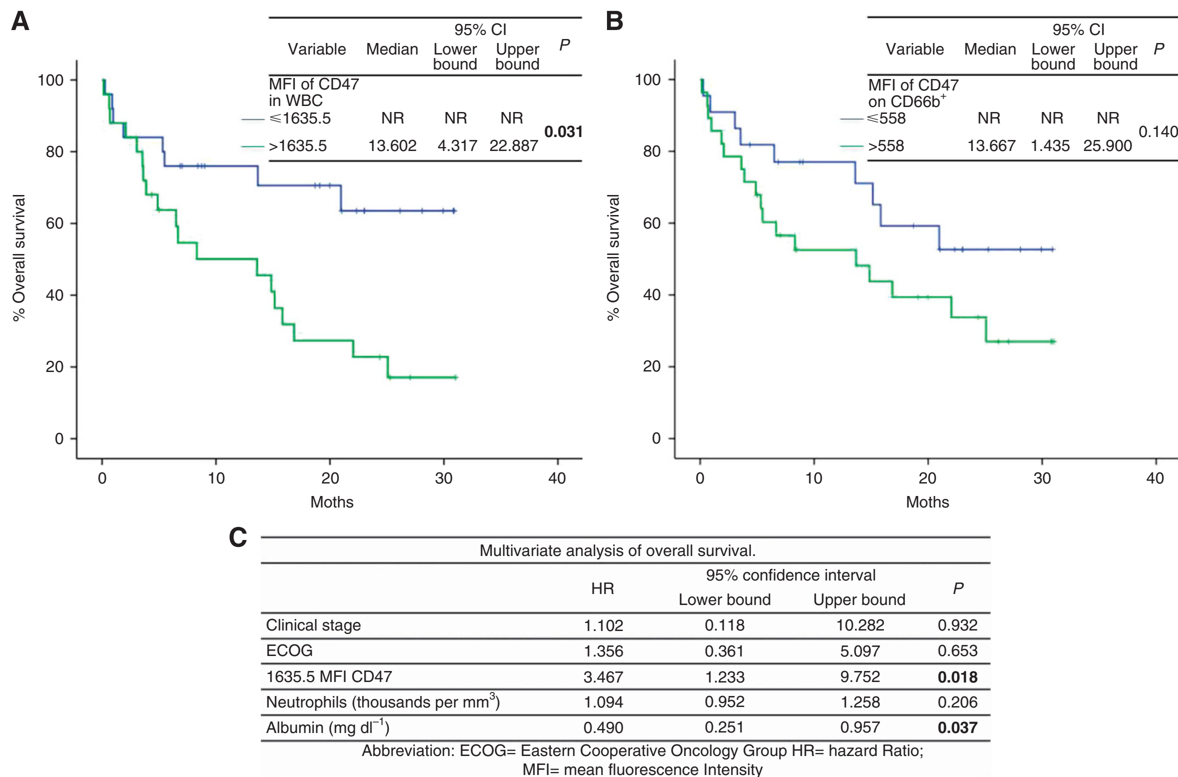


Figure 4. Phagocytosis of neutrophils from NSCLC patients and controls. (**A**) Neutrophils from patients and controls were labelled with CMFDA and incubated with macrophages. Neutrophil engulfment was quantified by counting the fraction of CD66b$^-$ cells positive for CMFDA. (**B**) Scatter plot showing the percentage of macrophages that engulfed neutrophils (CMFDA$^+$CD66b$^-$) from patients and controls. (**C**) CD47 expression in neutrophils from patients and controls. (**D**) Spearman correlation of CD47 expression in neutrophils *vs* % of CFMDA$^+$CD66b$^-$ cells ($r^2 = -0.7179$). Each symbol represents one individual (controls, $n=5$; patients, $n=5$). Lines indicate mean ± s.e.m. Unpaired *t*-test analyses were used to compare means.

Neutrophil homeostasis is maintained by a fine balance between granulopoiesis, bone marrow retention and mobilisation, intravascular margination and clearance from the system (Tak *et al*, 2013; Silvestre-Roig *et al*, 2016). Several parameters of the neutrophil life cycle were evaluated in the current study, with special emphasis on the activation state of neutrophils and the mechanisms by which neutrophils are removed in patients with NSCLC. Our results show that the number of neutrophils (percentage of CD66b-positive cells in whole blood) and the level of expression of CD66b (measured on a per cell basis) is increased in NSCLC patients as compared to controls. CD66b is a GPI-anchored glycoprotein of the carcinoembryonic antigen family that is located in the specific granules (Ducker and Skubitz, 1992). Increased expression of CD66b is caused by degranulation of specific granules and represents a surrogate marker of neutrophil activation (Faurschou and Borregaard, 2003).

Neutrophils utilise a wide array of mechanisms to exert their effector functions during infection and wound healing. In addition to releasing granules, activated neutrophils have been shown to produce high amounts of ROS. Therefore, to further assess the activation state of neutrophils, ROS production was evaluated using a broad spectrum fluorescent probe. Although no significant differences were noted in the relative oxidative activity of neutrophils between patients and controls, neutrophils isolated from patients exhibited a higher oxidative potential than those isolated from controls, as evidenced by the significantly higher production of ROS in patient-derived neutrophils stimulated with PMA. Various functions of mature neutrophils are activated by pro-inflammatory cytokines, which can lead to the secretion of superoxide anion ($O2^-$) and other ROS with activation of NADPH-oxidase through the activation of MAPK/extracellular signal-regulated kinase (Suzuki *et al*, 1999, 2001).



Multivariate analysis of overall survival.

	HR	95% confidence interval		P
		Lower bound	Upper bound	
Clinical stage	1.102	0.118	10.282	0.932
ECOG	1.356	0.361	5.097	0.653
1635.5 MFI CD47	3.467	1.233	9.752	**0.018**
Neutrophils (thousands per mm^3)	1.094	0.952	1.258	0.206
Albumin (mg dl^{-1})	0.490	0.251	0.957	**0.037**

Abbreviation: ECOG= Eastern Cooperative Oncology Group HR= hazard Ratio; MFI= mean fluorescence Intensity

Figure 5. Kaplan–Meier overall survival (OS) curve according to CD47 expression. (**A**) Survival analysis of patients dichotomised according to CD47 expression in whole-blood cells. The cut-off point (1635.5) of CD47 corresponds to the median MFI value in whole-blood cell samples. (**B**) Survival analysis of patients dichotomised according to CD47 expression in CD66b$^+$ cells. The cut-off point (558) of CD47 was determined using ROC curves. (**C**) Multivariate analysis of factors involved in OS.

Interleukin-8, a member of the ELR$^+$ CXC chemokine family, induces neutrophils to leave the bloodstream and promotes neutrophil recruitment into the airways during lung cancer (Carpagnano *et al*, 2011). Furthermore, previous studies have shown that neutrophils can release IL-8 under various circumstances (Marchi *et al*, 2014). Indeed, it has been suggested that neutrophil-derived IL-8 can promote angiogenesis (Tecchio *et al*, 2013). In agreement, our results indicate that the plasma levels of IL-8 are higher in patients than in controls. Furthermore, there was a correlation between the levels of IL-8 and the percentage of circulating neutrophils. It is thus likely that IL-8 increases the number of neutrophils in the peripheral blood of NSCLC patients, perhaps through the inhibition of apoptosis and in an autocrine manner as it has been previously reported (Dumas *et al*, 2012).

Although IL-4 has been largely regarded as an anti-inflammatory cytokine (Woytschak *et al*, 2016), there is evidence indicating that it also has important pro-inflammatory functions. It has been previously shown that IL-4 promotes the recruitment of eosinophils, macrophages, fibroblasts, B cells and more recently neutrophils (Ratthe *et al*, 2009). Furthermore, a previous report by the same group demonstrated that IL-4 delays neutrophil apoptosis and increases neutrophil IL-8 production (Girard *et al*, 1997). Indeed, previous reports have shown that IL-4 protects B cells from spontaneous and induced apoptosis (Lemaire *et al*, 1999), and, in lung cancer, IL-4 functions as an autocrine growth factor that prevents the induction of apoptosis in neoplastic cells (Todaro *et al*, 2008). In addition, we found a significant correlation between the levels of IL-4 and the expression of CD47 in neutrophils, which may be another mechanism involved in the anti-apoptotic effect of IL-4 on neutrophils. Our results, which show a correlation between the frequency of circulating neutrophils and the levels of IL-4 and IL-8, indicate that a similar pro-inflammatory anti-apoptotic mechanism may underlie neutrophilia in patients with NSCLC.

In vivo studies have shown that under physiological conditions neutrophils undergo spontaneous apoptosis (Filep and El Kebir, 2009). Similarly, it has been shown that neutrophils in culture spontaneously produce ROS, which in turn induces apoptosis (Aoshiba *et al*, 1999; Scheel-Toellner *et al*, 2004). Given that NSCLC patients exhibited an increase in the number of neutrophils without signs of an increase in the number of immature neutrophils, we hypothesised that the rate at which neutrophils undergo cell death could be decreased in NSCLC patients. To explore this possibility, neutrophils isolated from both patients and controls were kept *in vitro* for 24 h, after which the rate at which neutrophils undergo spontaneous cell death was evaluated. Unlike neutrophils obtained from controls, cultured neutrophils from NSCLC patients had lower levels of extracellular Annexin V and 7-AAD as well as lower levels of activated caspase 3 and 7, indicating a delay in apoptosis.

Although phagocytosis is generally viewed as the culminating step of programmed cell death, there is evidence suggesting that non-apoptotic neutrophils can be phagocytosed by macrophages at the same rate as apoptotic neutrophils (Lagasse and Weissman, 1994). Our results show that patient-derived neutrophils are phagocytosed at a lower rate than those obtained from healthy donors. Patient-derived neutrophils showed an increase in the expression of CD47, which negatively correlated with the rate at which they were phagocytosed.

CD47 is ubiquitously expressed in human cells where it acts as a 'marker of self'. CD47 inhibits phagocytosis through its binding to SIRP-α, a receptor found on the surface of phagocytic cells, including macrophages and dendritic cells (Zhao *et al*, 2011; Feng *et al*, 2015). Binding of CD47 to SIRP-α causes the phosphorylation

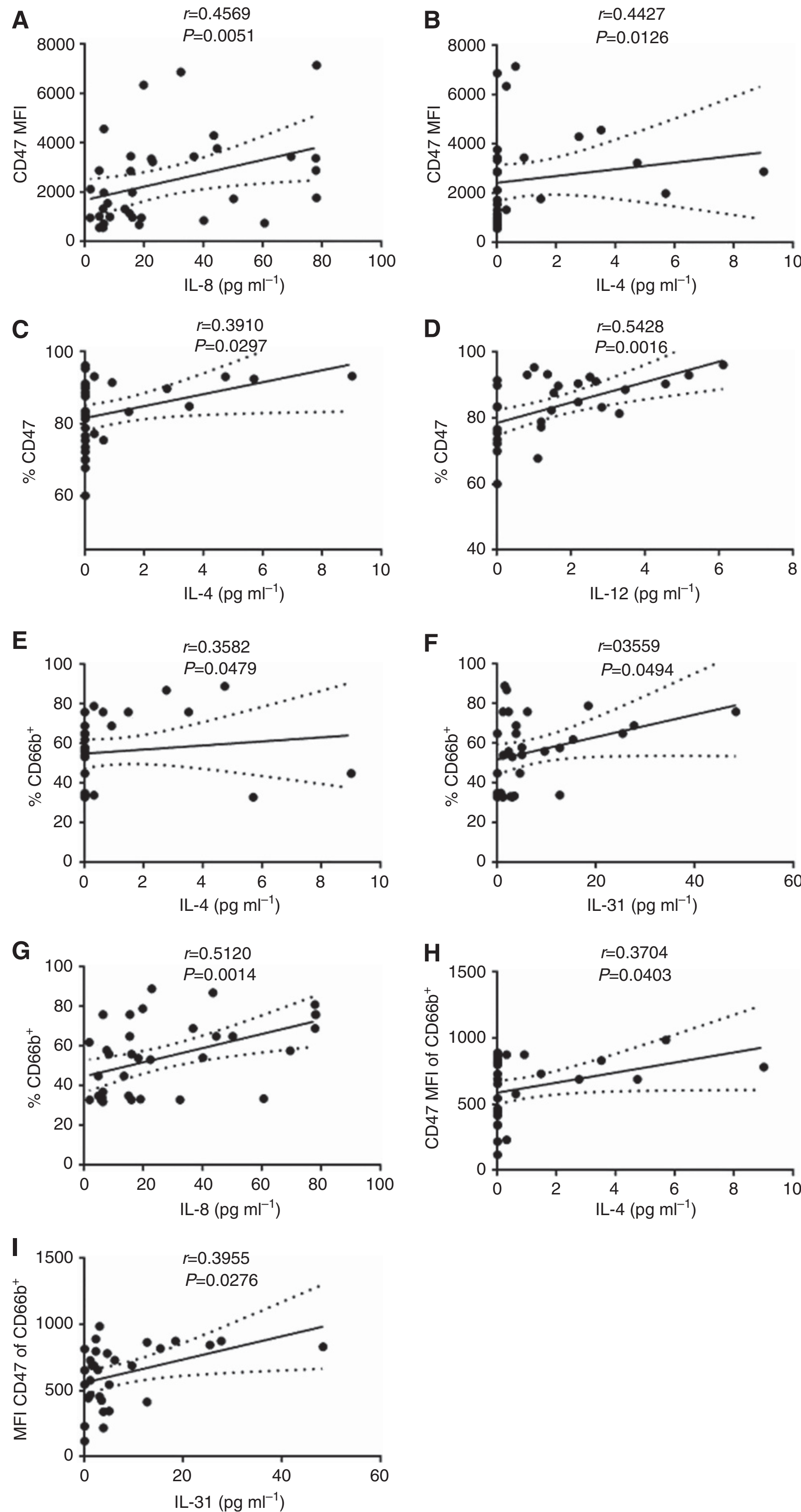


Figure 6. Linear regression and correlation between plasma cytokine levels and CD66b/CD47 percentage/expression. Correlation between CD47 expression (in whole blood) and plasma levels of IL-8 (**A**) and IL-4 (**B**). Correlation between the percentage of CD47^{+} cells (in whole blood) and plasma levels of IL-4 (**C**) and IL-12 (**D**). Correlation between the percentage of CD66b^{+} (neutrophils) and the plasma levels of IL-4 (**E**), IL-31 (**F**) and IL-8 (**G**). Correlation between CD47 expression (in CD66b^{+} cells) and plasma levels of IL-4 (**H**) and IL-31 (**I**). Statistical analyses were performed using the Spearman correlation test. Each symbol represents one patient ($n = 31$).

of the immunoreceptor tyrosine-based inhibitory motif on SIRP-α, the recruitment of Src homology region 2 domain-containing phosphatase (SHP)-1 and SHP-2 to the membrane, which inhibits myosin-IIA accumulation at the phagocytic synapse, ultimately resulting in the inhibition of phagocytosis (Fujioka *et al*, 1996).

Conversely, CD47-deficient circulating cells are rapidly cleared by splenic macrophages (Brown and Frazier, 2001). Thus, it is possible that in patients with NSCLC, CD47 overexpression may cause an increase in the number of circulating neutrophils by reducing their clearance from the system. It has been reported that cancer cells and circulating tumour cells may express CD47 as a mechanism to avoid immune system attack (Chao *et al*, 2012; Eruslanov *et al*, 2014; McCracken *et al*, 2015). Indeed, CD47 overexpression has been found in haematologic malignancies (Jaiswal *et al*, 2009; Majeti *et al*, 2009), gastric cancer (Yoshida *et al*, 2015) and NSCLC (Zhao *et al*, 2016), where its expression correlates with a bad prognosis.

Several studies have shown that high CD47 expression in tumours correlates with poor prognosis. However, to the best of our knowledge, this is the first study in NSCLC patients in which the level of expression of CD47, evaluated in whole-blood samples, negatively correlates with OS. Indeed, the survival analysis of groups according to the level of expression of CD47 in peripheral whole blood shows that patients with low expression of CD47 have a better OS than patients with high CD47 expression. Although the survival analysis of patients according to the expression of CD47 in neutrophils did not reach statistical significance, there was a trend suggesting that high CD47 expression in neutrophils leads to poorer survival outcomes. It is possible that the relatively small size of this cohort precluded the detection of any true differences that might exist. Alternatively, it is possible that the stark differences in survival observed from the whole-blood analysis are caused by the summed effect of other immune cell subsets in which CD47 expression is also increased. Although further mechanistic studies will likely determine the specific contribution of each immune cell subset, the translational relevance of the current study is supported by a previous report showing that CD47 overexpression in peripheral blood samples predicts recurrence in breast cancer patients (Nagahara *et al*, 2010). Thus, evaluating the expression of CD47 in whole blood could be used as a non-invasive method to identify patients with worse prognosis.

Recent studies have advanced CD47 as a novel molecular target for the use of therapeutic antibodies. Promising results have been obtained using anti-CD47 antibodies for the treatment of non-Hodgkin lymphoma, breast, bladder, lung and ovarian carcinomas (Chao *et al*, 2010; Weiner *et al*, 2010; Pardoll, 2012). Our results suggest that an additional advantage of CD47-blocking therapies could be the control neutrophil accumulation. However, future research is warranted in order to determine the clinical implications of CD47-blocking strategies. Whereas it is likely that CD47 antibodies could have an overall positive effect on tumour control (Ilie *et al*, 2012), it remains unclear whether other antitumour immune cell subsets could be negatively affected.

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CONFLICT OF INTEREST

José Rafael Borbolla-Escoboza is a full time AstraZeneca employee (Oncology Medical Affairs); Lourdes Barrera is a full time AstraZeneca employee (Oncology GPPS). However, the authors declare no conflict of interests since no AstraZeneca or competitor molecules were tested. The remaining authors declare no conflict of interests.

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