

Immunoglobulin-like transcript 3 is expressed by myeloid-derived suppressor cells and correlates with survival in patients with non-small cell lung cancer

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Keywords: CD85k, immunoglobulin-like transcript 3, immune suppression, LILRB4, LIR-5, myeloid-derived suppressor cells, non-small cell lung cancer, overall survival

Abbreviations: APC, antigen-presenting cell; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; HC, healthy control; ILT3, immunoglobulin-like transcript 3; MDSC, myeloid-derived suppressor cell; MFI, mean fluorescence intensity; MO-MDSC, monocytic MDSC; NFκB, nuclear factor κB; NSCLC, non-small cell lung carcinoma; PBMC, peripheral blood mononuclear cell; PMN-MDSC, polymorphonuclear MDSC; sILT3, soluble ILT3; Treg, regulatory T cell; Ts, T suppressor cell.

Myeloid-derived suppressor cells (MDSCs) play an important role in immune suppression and accumulate under pathologic conditions such as cancer and chronic inflammation. They comprise a heterogeneous population of immature myeloid cells that exert their immunosuppressive function via a variety of mechanisms. Immunoglobulin-like transcript 3 (ILT3) is a receptor containing immunoreceptor tyrosine-based inhibition motifs (ITIMs) that can be expressed on antigen-presenting cells and is an important regulator of dendritic cell tolerance. ILT3 exists in a membrane-bound and a soluble form and can interact with a yet unidentified ligand on T cells and thereby induce T-cell anergy, regulatory T cells, or T suppressor cells. In this study, we analyzed freshly isolated peripheral blood mononuclear cells (PBMCs) of 105 patients with non-small cell lung cancer and 20 healthy controls and demonstrated for the first time that ILT3 is expressed on MDSCs. We show that increased levels of circulating MDSCs correlate with reduced survival. On the basis of ILT3 cell surface expression, an ILT3^{low} and ILT3^{high} population of polymorphonuclear (PMN)-MDSCs could be distinguished. Interestingly, in line with the immunosuppressive function of ILT3 on dendritic cells, patients with an increased proportion of PMN-MDSCs and an increased fraction of the ILT3^{high} subset had a shorter median survival than patients with elevated PMN-MDSC and a smaller ILT3^{high} fraction. No correlation between the ILT3^{high} subset and other immune variables was found. ILT3 expressed on MDSCs might reflect a previously unknown mechanism by which this cell population induces immune suppression and could therefore be an attractive target for immune intervention.

Introduction

The immune system influences lung cancer pathogenesis, progression, and response to therapy and thereby strongly contributes to the prognosis of the disease.¹ The influence of the immune system is, however, paradoxical, as the different components can either inhibit tumor growth or promote immune evasion and cancer progression.² Myeloid-derived suppressor cells (MDSCs) are an important contributor to the latter process.³

MDSCs are a heterogeneous population of immature myeloid cells that accumulate in blood, lymphoid organs, and tumor tissue under several pathologic conditions, including cancer.^{3,4} MDSCs are generally characterized by being positive for CD33 and CD11b and low or negative for HLA-DR.⁵ This population can be divided in 2 subgroups with different morphology. Monocytic (MO)-MDSCs are mononuclear and CD14 positive, whereas granulocytic or polymorphonuclear (PMN)-MDSCs are CD14 negative.^{3,5} Both populations contribute to tumor

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Submitted: 12/07/2014; Revised: 01/26/2015; Accepted: 01/27/2015

<http://dx.doi.org/10.1080/2162402X.2015.1014242>

immune escape by inducing immune suppression and tolerance through a variety of mechanisms, such as production of nitric oxide and reactive oxygen species, depletion of arginine and cysteine, and induction of regulatory T cells (Tregs).⁶⁻⁹ However, their regulation and dynamics are poorly understood, especially in humans.¹⁰

An important mediator of the induction of immune tolerance is immunoglobulin-like transcript (ILT) 3 (also known as LILRB4, CD85k, or LIR-5), which is expressed on monocytes and antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs).^{11,12} ILT3 expression marks tolerogenic DCs and has been reported to be elevated in APCs of cancer patients^{13,14} and decreased in autoimmune diseases.^{15,16} ILT3 is believed to signal via its immunoreceptor tyrosine-based inhibitory motifs (ITIMs). These motifs are known to inhibit NF- κ B activation and transcription of costimulatory molecules and thereby render the cell tolerogenic.¹⁷ The 2 extracellular Ig-like domains of ILT3 most likely contain ligand-binding sites; however, the nature of the ligand is still unknown.^{12,17-19} Membrane-bound ILT3 interacts with T cells in a cell-to-cell contact-dependent manner and induces immune suppression or tolerance by inducing anergy in CD4⁺ T cells, suppressing the differentiation of IFN- γ -producing CD8⁺ T cells and inhibiting T-cell proliferation and the induction of Tregs and alloantigen-specific CD8⁺ T suppressor (Ts) cells.^{17,20} Moreover, a soluble form of ILT3 (sILT3) resulting from alternative splicing has been described to interact with T cells and induce anergy.²¹ CD68⁺ tumor-associated macrophages are the major source of sILT3.^{21,22}

We have previously have shown increased levels of MDSCs in patients with treatment-naïve Stage IV non-small cell lung carcinoma (NSCLC).²³ Because MDSCs have the ability to induce immune suppression, we hypothesized that ILT3 expression could be part of a yet unidentified mechanism by which MDSCs mediate immune escape. Therefore, we aimed to assess the expression of ILT3 on MDSCs of lung cancer patients with advanced disease before the start of chemotherapy and evaluate its effect on clinical outcome.

Results

Characteristics of study subjects

This study included 118 patients with Stage IV NSCLC who were participating in the NVALT12 study. Of these, 13 patients were excluded from further analysis because blood samples were not available for processing within 6 hours after the samples were taken. **Table 1** shows the characteristics of the 105 NSCLC study participants and 20 healthy controls (HC) that were included in our analyses. The patient characteristics of this study cohort were similar to those of the total NVALT12 population,²³ therefore no selection bias was introduced.

Levels of MO-MDSCs and PMN-MDSCs are elevated in Stage IV NSCLC patients

The two MDSC subsets, monocytic MDSCs (MO-MDSCs) and peripheral blood mononuclear MDSCs (PMN-MDSCs),

Table 1. Characteristics of study subjects

	Healthy controls	NSCLC patients
Number of subjects	20	105
Age (years) (mean \pm SD)	54 \pm 7.5	61 \pm 8.3
Gender (%)		
Male	4 (20)	53 (50.5)
Female	16 (80)	52 (49.5)
WHO performance score (%)		
0	20 (100)	51 (49)
1		50 (48)
2		3 (3)
Histologic subtype (%)		
Adenocarcinoma		87 (84)
Large cell carcinoma		17 (16)

were assessed in the peripheral blood of patients with Stage IV NSCLC and healthy controls by flow cytometric analysis of freshly obtained PBMCs. The gating strategy for the 2 populations is presented in **Figure 1A** and was performed as previously described.²³ First, mature granulocytes were excluded based on their CD16⁺ expression.^{5,24} Next, MO-MDSCs were characterized as CD11b⁺CD14⁺HLA-DR⁻CD33⁺CD15⁺, whereas PMN-MDSCs were characterized as CD11b⁺CD14⁻HLA-DR⁻CD33⁺CD15⁺.

As reported earlier by our group, levels of MDSCs are elevated in NSCLC patients.²³ Accordingly, in the cohort used in this study the frequencies of both MO-MDSCs and PMN-MDSCs in peripheral blood were significantly higher in patients than in healthy controls, as shown in **Figure 1B**.

ILT3 is expressed by subpopulations of MDSCs

ILT3 expression could be detected on both PMN-MDSCs and MO-MDSCs (**Fig. 2A**). In contrast to MO-MDSCs, which showed homogeneous high expression of ILT3 (right panel), PMN-MDSCs contained 2 subsets of high and low ILT3 expression (left panel). **Figure 2B** shows ILT3 expression on PMN-MDSCs of 4 different patients, compared to CD11b⁻CD14⁻ cells (mainly lymphocytes) and CD11b⁺CD14⁺ cells (mainly monocytes). Whereas the lymphocytes were consistently negative for ILT3 (mean fluorescence intensity [MFI] = 53), monocytes showed high and homogeneous ILT3 expression (median MFI = 7,399). The expression of ILT3 on PMN-MDSCs was intermediate and showed 2 peaks in most patients, although the distribution over ILT3^{high} (MFI > 10³) and ILT3^{low} (MFI < 10³) fractions varied extensively between patients (the percentage of ILT3^{high} cells ranged between 0.2% and 92.9%). In contrast to PMN-MDSCs, virtually all MO-MDSCs were positive for ILT3 with homogeneous expression of the marker, which was slightly, but significantly, lower than expression in the monocyte population (MFI = 6,275, $P < 0.001$).

The ILT3^{high} fraction of PMN-MDSCs is increased in lung cancer patients and is not correlated with frequency of T and B cells or monocytes

The proportions of ILT3^{high} PMN-MDSCs within the total PMN-MDSC population varied considerably between patients.

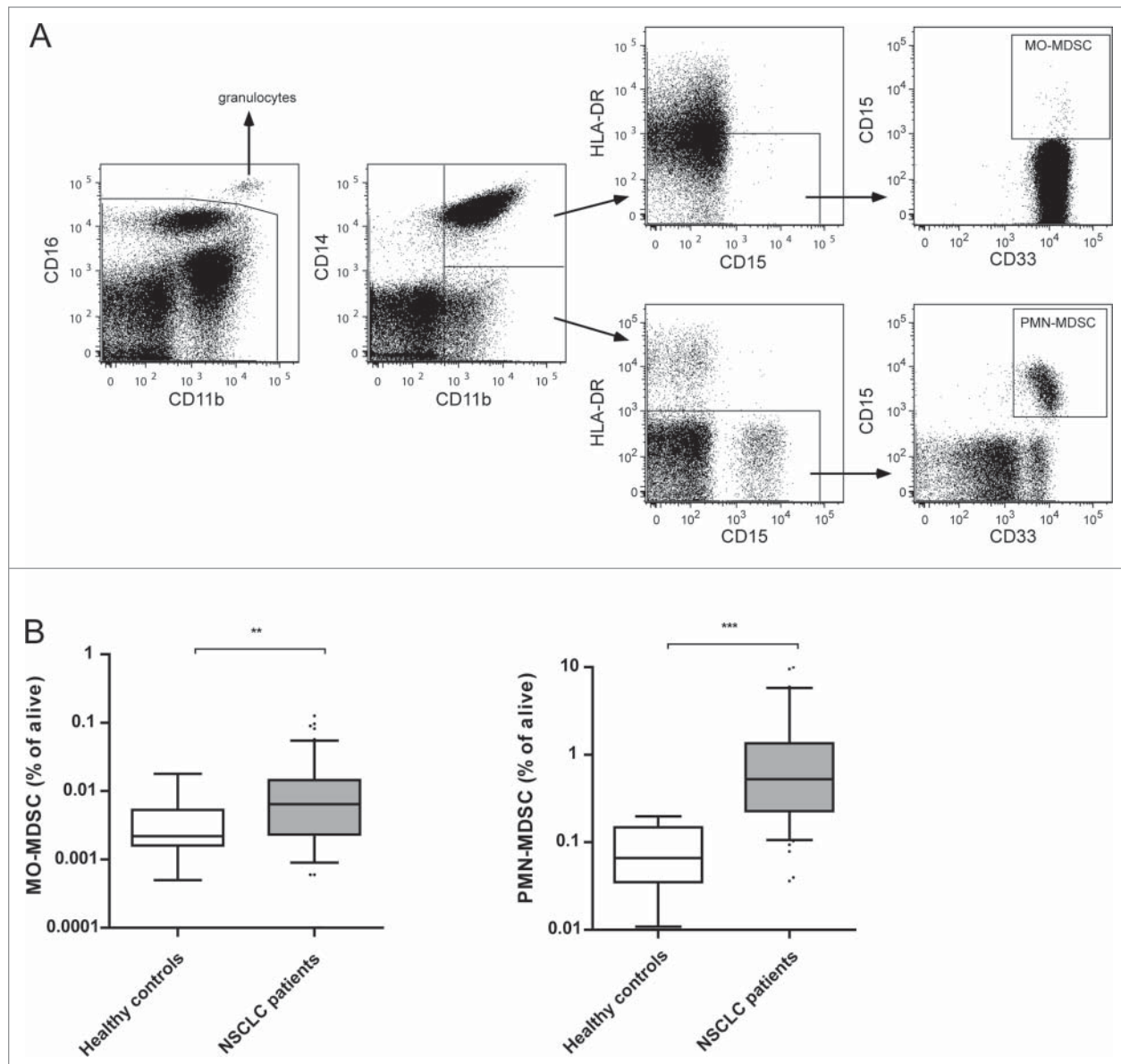


Figure 1. Levels of circulating MO-MDSCs and PMN-MDSCs are increased in patients with non-small cell lung cancer. **(A)** Flow cytometry was performed on freshly isolated PBMCs from NSCLC patients and healthy controls. The gating strategy for determination of circulating MO-MDSCs and PMN-MDSCs is depicted. After gating the live cells, CD16^{high} cells were excluded. MO-MDSCs were characterized as CD14⁺CD11b⁺HLA-DR^{low}CD15⁺ and PMN-MDSCs were characterized as CD14⁻CD11b⁺HLA-DR^{low}CD15⁺. MDSC levels were determined as a proportion of live cells. **(B)** Frequency of MO-MDSCs and PMN-MDSCs in PBMCs was significantly higher in NSCLC patients than in healthy controls. ***P* < 0.01; ****P* < 0.001; Mann-Whitney U test. MDSC, myeloid-derived suppressor cell; MO-MDSC, monocytic MDSC; NSCLC, non-small cell lung carcinoma; PBMC, peripheral blood mononuclear cell; PMN-MDSC, polymorphonuclear MDSC.

As shown in **Figure 3A**, the ILT3^{high} fraction of PMN-MDSCs was significantly higher in NSCLC patients (39 ± 24% [mean ± SD]) compared to healthy controls (12 ± 10%; *P* < 0.0001). The proportion of ILT3^{high} PMN-MDSCs did not correlate with the proportion of ILT3^{high} PMN-MDSCs (**Fig. 3B**). To investigate whether the ILT3^{high} fraction of PMN-MDSCs had an effect on, or was affected by, other immunologic cell populations, we analyzed T cells, the CD4⁺/CD8⁺ T-cell ratio, B cells, and monocytes. No statistically significant correlations were found between the ILT3^{high} fraction of PMN-MDSC and the

proportions of B cells, T cells, the CD4⁺/CD8⁺ ratio and levels of monocytes in NSCLC patients. Furthermore, no correlation with MO-MDSCs existed (**Fig. 3B**). Analyses of absolute numbers of these cell populations gave similar results (data not shown).

Soluble ILT3 is elevated in serum of NSCLC patients and does not correlate with immunologic cell populations

It has been described that, in addition to membrane-bound ILT3, soluble ILT3 (sILT3) can also have immunosuppressive

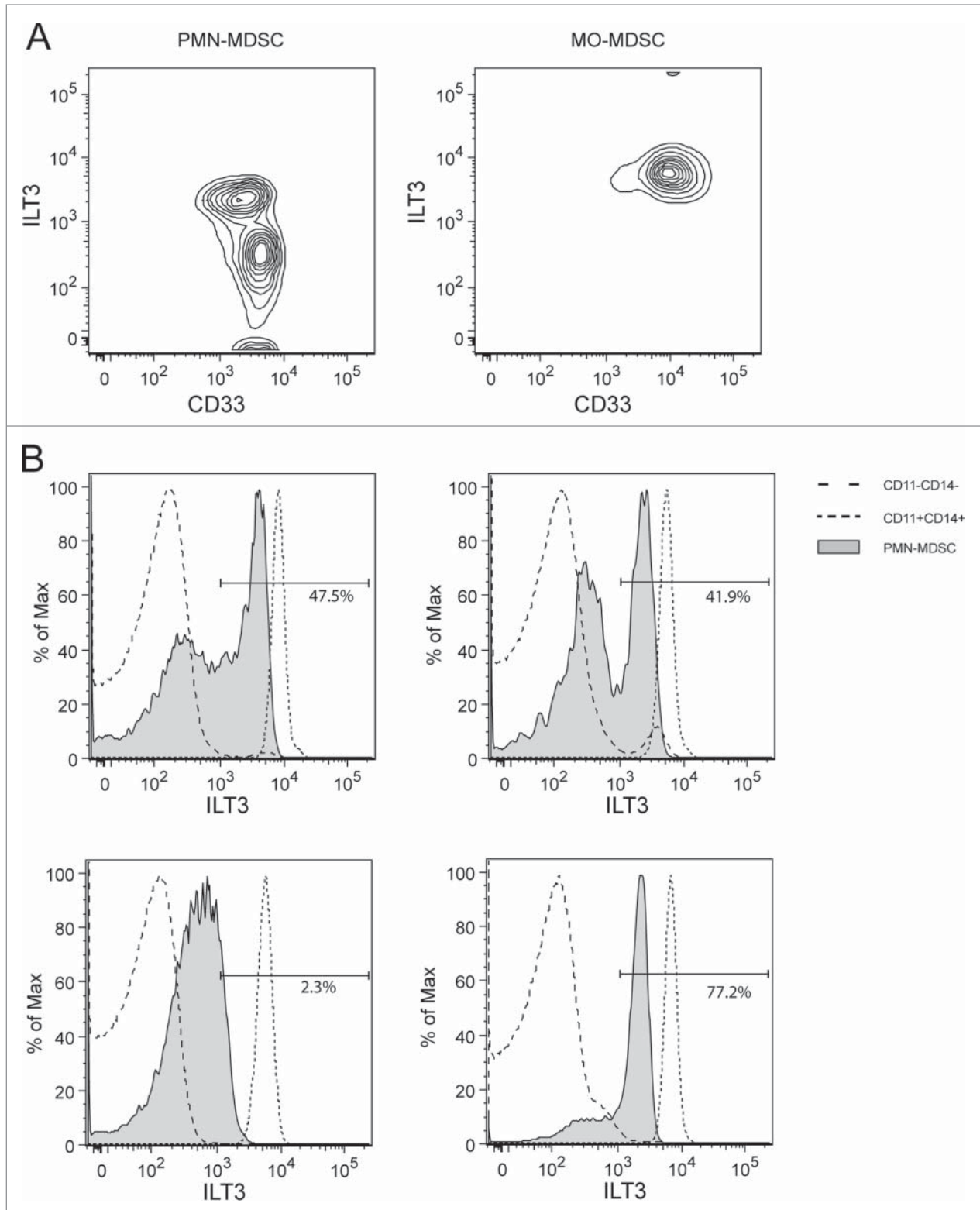


Figure 2. ILT3 expression on myeloid-derived suppressor cells. **(A)** Flow cytometric data of a representative patient, displayed as density plot based on ILT3 and CD33 expression. Left panel: PMN-MDSCs, right panel: MO-MDSCs. **(B)** Histograms of 4 different patients with ILT3 expression of PMN-MDSCs (shaded) compared to the expression within the CD11b⁻CD14⁻ population (dashed line, mainly lymphocytes) and CD11b⁺CD14⁺ population (dotted line, mainly monocytes). Proportions of the ILT3^{high} fraction are displayed as percentage of PMN-MDSCs. ILT3, immunoglobulin-like transcript 3; MDSC, myeloid-derived suppressor cell; MO-MDSC, monocytic MDSC; PMN-MDSC, polymorphonuclear MDSC.

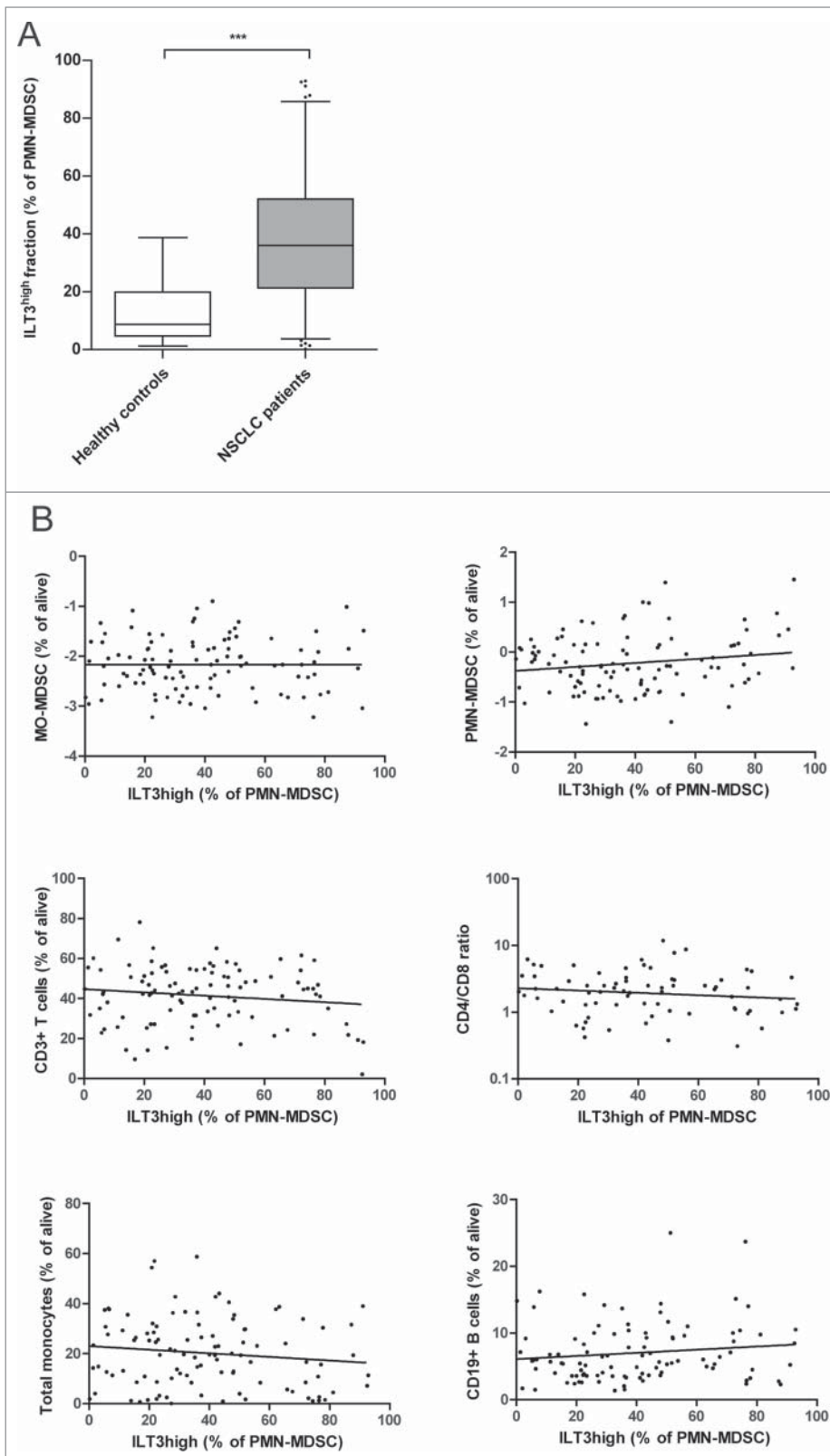
Figure 3. ILT3^{high} proportion of PMN-MDSCs in patients with non-small cell lung cancer. **(A)** ILT3^{high} proportions of PMN-MDSCs were significantly higher in NSCLC patients than in healthy controls. *** $P < 0.001$, Student t test. **(B)** Correlations between the proportion of ILT3^{high} PMN-MDSC and various immune subsets in NSCLC patients were analyzed with the Spearman rho test. None of the tests revealed a significant correlation ($P > 0.05$ for all analyses). ILT3, immunoglobulin-like transcript 3; MDSC, myeloid-derived suppressor cell; MO-MDSC, monocytic MDSC; PMN-MDSC, polymorphonuclear MDSC.

effects.²¹ In multiple types of cancer, sILT3 is present in the serum of patients and is able to strongly abolish T-cell responses against tumor antigens.^{21,22} To test whether sILT3 was present in the serum of the NSCLC patients, sILT3 levels were quantified by enzyme-linked immunosorbent assay (ELISA) in a pilot study of 30 randomly chosen NSCLC patients and 8 healthy controls. As shown in **Figure 4A**, sILT3 was present in the serum of NSCLC patients at significantly higher levels ($P = 0.03$) than in healthy controls. We hypothesized that soluble ILT3 might be produced by ILT3-expressing MDSCs; however, no correlation was found between the serum levels of sILT3 and the proportions of ILT3^{high} cells in the PMN-MDSC population (**Fig. 4B**). Furthermore, sILT3 was not correlated with MFI values of surface ILT3 on monocytes or MDSC populations (data not shown). To check whether sILT3 levels were related to the peripheral immune profile of the patients, we assessed the correlation between sILT3 serum levels and peripheral immune cell proportions in the patient cohort. No significant correlations were found between the levels of sILT3 and the frequency of PMN-MDSCs and MO-MDSCs, T cells, the CD4⁺/CD8⁺ ratio, B cells, and monocytes (**Fig. 4C**).

Increased proportions of circulating MDSCs correlate with a poorer outcome in NSCLC patients

For various types of cancer it has been shown that higher levels of MDSCs correlate with reduced survival of patients.^{25,26} To validate this effect in our patient cohort, patients were divided

into 2 groups based on the proportion of MDSCs. Values that were higher than the mean + 2SD of healthy controls were considered to be elevated. In this way, we identified patients with



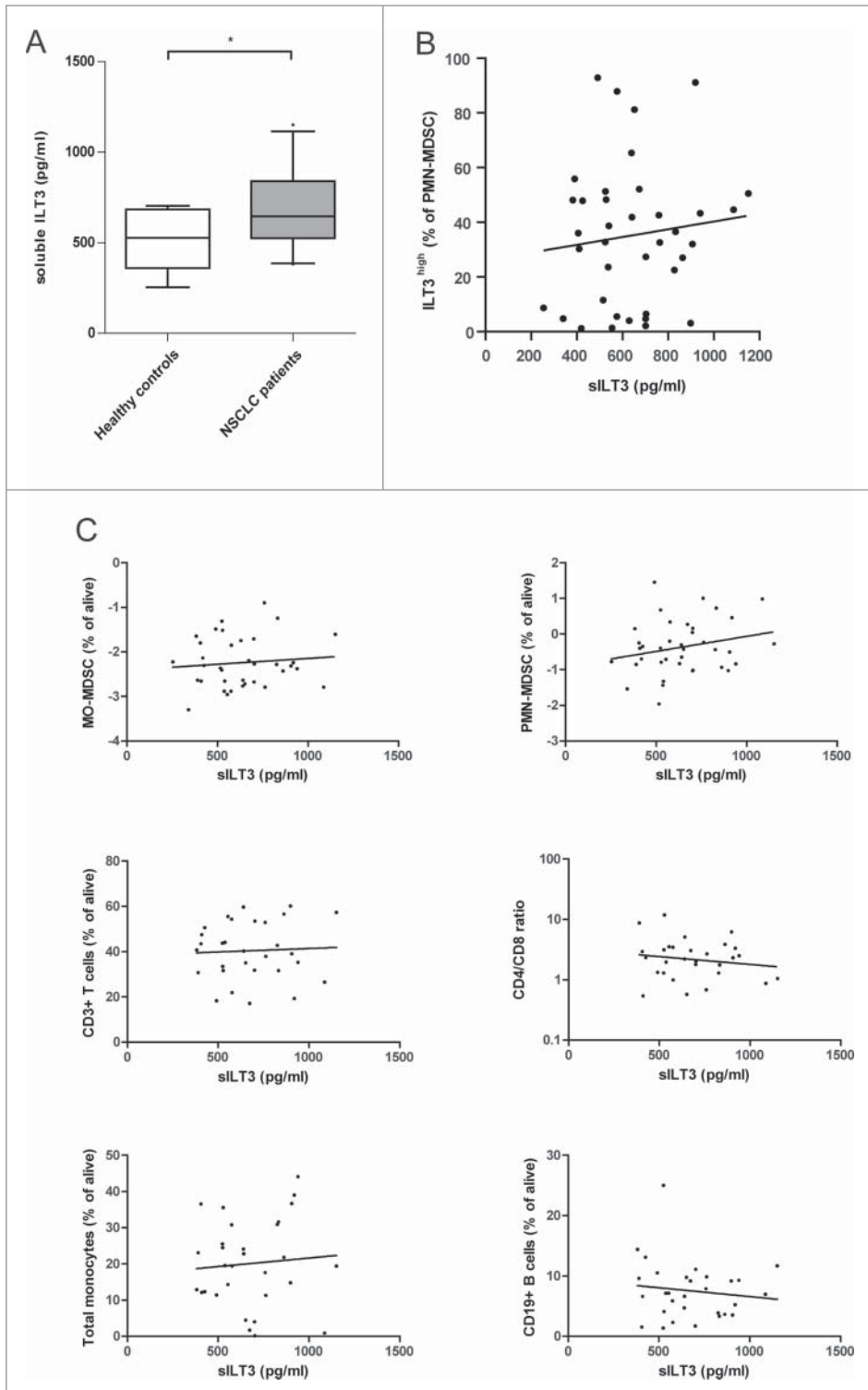


Figure 4. Serum sILT3 in patients with non-small cell lung cancer. **(A)** Soluble ILT3 was measured by ELISA in serum samples of healthy controls (n=8) and patients with Stage IV NSCLC (n=30). Levels of sILT3 were significantly higher in NSCLC patients compared to healthy controls. * $P < 0.05$, Student t test. **(B)** sILT3 levels of NSCLC patients did not correlate with the fraction of ILT3^{high} cells of PMN-MDSC (Spearman rho test). **(C)** Correlations between level of sILT3 in serum and various immune subsets in NSCLC patients were analyzed with the Spearman rho test. None of the tests revealed a significant correlation ($P > 0.05$ in all analyses). ELISA, enzyme-linked immunosorbent assay; ILT3, immunoglobulin-like transcript 3; MDSC, myeloid-derived suppressor cell; MO-MDSC, monocytic MDSC; NSCLC, non-small cell lung carcinoma; PMN-MDSC, polymorphonuclear MDSC; sILT3, soluble ILT3.

are shown in **Figure 5A and B**. Of note, proportions of PMN-MDSC and MO-MDSC were significantly correlated ($P < 0.001$; not shown).

The ILT3^{high} fraction of PMN-MDSC correlates with a poorer outcome in NSCLC patients

To assess whether ILT3 expression on PMN-MDSCs influenced clinical outcome, NSCLC patients were divided into 2 groups based on the percentage of ILT3^{high} cells among PMN-MDSCs, as for the proportions of MDSCs. **Figure 6A** shows a slightly shorter overall survival for patients with a higher percentage of ILT3^{high} PMN-MDSCs, although this did not reach statistical significance ($P = 0.15$). However, it is conceivable that the effect of high ILT3 expression on PMN-MDSCs is limited in patients with low proportions of these cells. Therefore, we performed sub-analysis on the group of patients with the highest levels of PMN-MDSC (above median; **Fig. 6B** and found a significant negative correlation with overall survival ($P = 0.023$, **Fig. 6C**). In contrast, in patients with low proportions of MDSC, the percentage of ILT3^{high} cells did not influence overall survival (**Fig. 6D**). Analysis of progression-free survival gave similar results, but only the effect of MO-MDSC level reached statistical significance (data not shown). Serum levels of sILT3 were not correlated with survival (data not shown).

elevated PMN-MDSC and patients with elevated MO-MDSC. In accordance with reported findings,^{25,26} patients with elevated proportions of PMN-MDSCs had a significantly shorter survival than patients with low proportions of PMN-MDSC ($P = 0.017$). Likewise, patients with elevated proportions of MO-MDSC had a significantly shorter survival than patients with low MO-MDSC values ($P = 0.007$). The survival curves

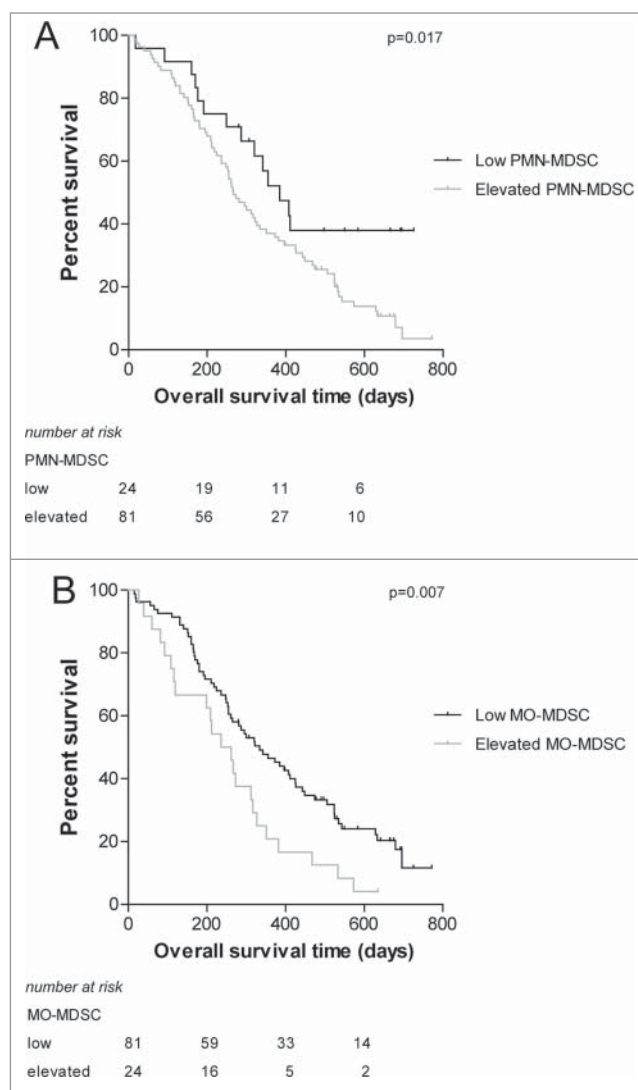


Figure 5. Survival curves of NSCLC patient groups according to the proportion of MDSCs. NSCLC patients were divided into 2 groups based on the mean value + 2SD of healthy controls. (A) Survival of patients with elevated versus low PMN-MDSC levels (B) Survival of patients with elevated versus low MO-MDSC levels. MDSC, myeloid-derived suppressor cell; MO-MDSC, monocytic MDSC; NSCLC, non-small cell lung carcinoma; PMN-MDSC, polymorphonuclear MDSC.

Discussion

This is the first study showing that the immune suppressive molecule ILT3 is expressed by MDSCs. In recent years, MDSCs have received great attention for their immunosuppressive role in cancer, which is executed through a diversity of mechanisms including arginase-1 and iNOS expression and oxidative stress.²⁷ Given that MDSCs are a heterogeneous population of immature cells, these mechanisms are likely to be differentially employed by the different subsets of MDSC and dependent on the context of the microenvironment. With the demonstration of ILT3 expression on the cell membrane of MDSCs, we describe a pathway by which MDSCs might execute their immunosuppressive

function that was previously unknown for these cells. The heterogeneity of MDSCs is further demonstrated by our finding that ILT3 is not expressed on all circulating MDSC of lung cancer patients, but only on MO-MDSCs and a subset of PMN-MDSCs.

Little is known about MDSCs under physiological conditions. In healthy individuals, immature myeloid cells with the same phenotype as MDSCs are continuously generated in the bone marrow, where they differentiate into mature myeloid cells before entering the circulation. Under pathologic conditions they can be released from the bone marrow before maturation. However, in mice, MDSCs are also present in the liver during physiological conditions and are thought to play a pivotal role in maintaining homeostasis.²⁸ Therefore, the function of MDSCs is probably different in healthy controls and cancer patients. It has been reported that MDSCs of diseased mice have an increased capacity to suppress T-cell proliferation compared with MDSCs of normal mice.²⁸ This is supported by the finding that MDSCs from healthy controls show decreased expression of immunosuppressive molecules compared with MDSCs from cancer patients.²⁹ Likewise, in a previous study we showed that arginase-1 is expressed in PBMCs in much larger amounts in lung cancer patients than in healthy controls.^{23,30} Our finding that ILT3 is upregulated on MDSCs of NSCLC patients is in agreement with functional differences with regard to immunosuppression by MDSCs in NSCLC patients and healthy controls.

ILT3 expression on DCs is of critical importance in the induction of tolerance.^{31,32} ILT3 can be induced on APCs by cytokines such as IL-10, interferon (IFN)- α , and IFN- β and by interaction with CD8⁺ T cells.^{33,34} Other inducers of ILT3 on APCs are vitamin D3 analogs, COX-1/2 inhibitors, and tryptophan depletion in the environment, resulting in T-cell non-responsiveness and tolerance.^{18,33,35} Intracellular signaling via the ITIMs of ILT3 induces tolerance in DCs via downregulation of the NF κ B pathway and plays an inhibitory role in antigen presentation.³⁶ However, these signaling pathways are not likely to play an important role in ILT3-expressing MDSCs, since MDSCs are not classic APCs and are defined by low HLA-DR expression. Therefore, ILT3-induced immune suppression by MDSCs is not likely to exert its effectiveness via co-stimulation or antigen presentation, as shown for DCs. However, extracellular signaling by membrane-bound ILT3 and sILT3 has been shown to induce immunosuppressive CD8⁺ T cells and CD4⁺ Tregs.^{18,19} MDSCs might therefore use this extracellular signaling pathway to exert their immunosuppressive function, as this cell population is known to induce Tregs and T cell anergy.

In this study we did not find a correlation between ILT3 expression on MDSCs and the proportion of T cells in PBMCs. However, the functionality of these T cells was not assessed in this study. Given that MDSCs might induce anergy in T cells or induce Tregs from naïve T cells, the functionality rather than the number of T cells could be decreased by ILT3⁺ MDSCs and should be investigated in further studies. Unfortunately, the amount of blood collected from each patient, in combination with the low levels and phenotypic instability of the MDSC populations, did not allow for functional studies to compare the

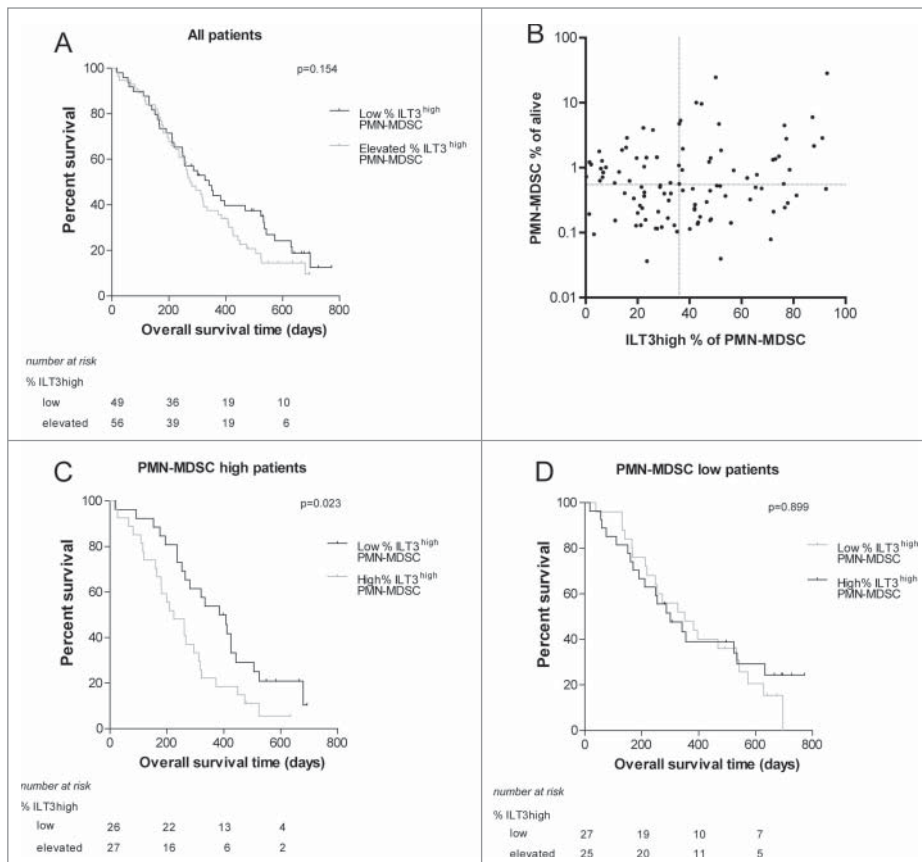


Figure 6. NSCLC patient survival based on ILT3 fractions of MDSC. (A) Survival of patients with an elevated percentage of ILT3^{high} PMN-MDSC versus patients with a low percentage of ILT3^{high} PMN-MDSCs. The survival curves were not significantly different. (B) No correlation was found between the level of total PMN-MDSCs and the percentage of ILT3^{high} PMN-MDSCs (Spearman rho; $P = 0.38$). For the curves in panels C and D, patients were divided based on the frequency of PMN-MDSC and the percentage of ILT3^{high} PMN-MDSCs. Cutoff values were the median value of all patients to create equally sized groups. (C) In patients with high levels of PMN-MDSC, the fraction of ILT3^{high} cells correlated significantly with overall survival. (D) In patients with low levels of PMN-MDSC, the percentage of ILT3^{high} PMN-MDSCs did not significantly contribute to overall survival. The curves were compared with a log-rank test, stratified for treatment arm. ILT3, immunoglobulin-like transcript 3; PBMC, peripheral blood mononuclear cells; MO-MDSC, monocytic myeloid-derived suppressor cell; PMN-MDSC, polymorphonuclear myeloid-derived suppressor cell.

ILT3^{high} and ILT3^{low} PMN-MDSC subsets or the suppressive capacity of MO-MDSCs.

The clinical relevance of both membrane-bound and soluble ILT3 has been demonstrated in studies of different cancer types. Membrane-bound ILT3 is expressed on tumor cells in B-cell chronic lymphocytic leukemia (B-CLL), myeloid leukemia, and pancreatic and gastric cancer, and in B-CLL its expression on tumor cells is associated with aggressive growth.^{13,37,38} Elevated expression on DCs was found in colorectal cancer patients compared to healthy controls.¹⁴ Moreover, strong infiltration of CD68⁺ILT3^{high} macrophages has been demonstrated in lymph nodes containing metastatic carcinoma cells.²² sILT3 is also elevated in serum of patients with melanoma, colorectal cancer, and pancreatic carcinoma^{21,22} In contrast, ILT3 expression is decreased on DCs of patients with systemic lupus erythematosus (SLE) and autoimmune thyroid disease, where, in contrast to

cancer, the immune system is overactivated^{15,39} and ILT3 was also reported to be decreased on circulating monocytes of patients with multiple sclerosis.¹⁶ In line with these results, we found elevated sILT3 levels and a higher percentage of ILT3⁺ PMN-MDSCs in NSCLC patients, which indicates a role for this molecule in tumor pathogenesis or progression, most likely by stimulating tumor immune escape. In this cohort of Stage IV NSCLC patients, the detrimental effect of the presence of elevated proportions of circulating MO-MDSC and PMN-MDSC was confirmed as elevated proportions of MDSC were correlated with a decreased overall survival. We were unable to demonstrate a significant effect of the percentage of ILT3^{high} PMN-MDSC on clinical outcome for the whole patient population, although the mean survival time was shorter in patients with a higher proportion of ILT3^{high} PMN-MDSCs (Fig. 6A). However, when patients with PMN-MDSC proportions above the median were selected, an elevated percentage of ILT3^{high} PMN-MDSC was correlated with reduced survival. This finding supports our hypothesis that ILT3 expression on MDSCs plays a role in immune suppression, but its influence is only large enough to be detected in survival analyses in patients with higher proportions of PMN-MDSCs. In contrast to PMN-MDSCs, all MO-MDSCs expressed ILT3 on their membrane. Given that the frequency of MO-MDSCs in peripheral blood was very

low but still yielded a significant correlation with overall survival, MO-MDSCs might be stronger immune suppressors than PMN-MDSCs in patients with Stage IV NSCLC. Although we did not provide evidence, one might speculate that this could be due to the constitutive expression of ILT3 on MO-MDSCs. Moreover, the proportion of ILT3^{high} PMN-MDSCs did not correlate with the proportions of other immunologic cell types, indicating that its clinical value is not a reflection of an altered balance in the rest of the immune system but instead might represent a tumor-promoting function, through either altering the functionality of the immune cells rather than cell numbers, or by acting locally on tumor progression or immune escape. The absence of evidence supporting any clinical relevance of the level of sILT3 in serum in this study might be due to the small number of serum samples measured, although the lack of any correlation with circulating immune cell populations provided no

further indication of its role in peripheral blood. sILT3 might, however, function more locally, since it has been shown to be produced by tumor-associated CD68⁺ macrophages.²¹ Given that sILT3 levels did not correlate with the expression level on circulating MDSCs or monocytes, it is not likely that sILT3 is produced in the periphery in high amounts, but rather might reflect the local immune composition and a suppressive microenvironment.

Taken together, our results show that ILT3 is expressed on MDSCs and indicate that this affects the clinical outcome. The relevance of ILT3 in cancer patients is supported by results from the literature^{14,37,38} and therefore further investigation of its mode of action should be performed. Nonetheless, it is debatable whether ILT3 on its own would determine the immunosuppressive status of MDSCs; rather, the sum of all immunosuppressive mechanisms and their relative contribution to the activity of MDSC will probably determine the extent of its unfavorable effects in cancer patients.

MDSCs play an important role in mediating immunosuppression and therefore represent a significant hurdle to successful immunotherapy in NSCLC.² Therefore, combining immunotherapeutic approaches with MDSC-inhibiting drugs like gemcitabine or VEGF blockers to elicit more potent anticancer effects is a promising approach.

To our knowledge this is the first study that demonstrates the expression of ILT3 on human MDSCs. Future studies will underscore the importance of this molecule on MDSC in other experimental or clinical settings.

Materials and Methods

Study population

The patients in this study were participating in the NVALT12 study (trial number NCT01171170), a randomized Phase II multicenter study on the effect of a nitroglycerin patch or placebo in patients with stage IV NSCLC treated with carboplatin, paclitaxel, and bevacizumab. Patients in the NVALT12 study were diagnosed with Stage IV non-squamous NSCLC and were not eligible for treatment with curative intent. Disease stage was determined in accordance with the American Joint Committee on Cancer (AJCC). Blood samples were collected before the start of treatment and analyzed by flow cytometry. In this study, 118 patients were included for analysis of ILT3 expression.

Twenty age-matched healthy controls (HC; mean age 54 years) with no history of malignancies and/or autoimmune diseases were also enrolled in the study.

Written consent was obtained from all individuals before blood sampling and the study was approved by the ethical committee of the Erasmus Medical Center (MEC-2012-048 [HC] and CCMO: NL33442.042.10 [NSCLC patients]).

Isolation of PBMCs

PBMCs were isolated using a Ficoll-Hypaque (GE Healthcare, Diegem, Belgium) density gradient. Blood was

supplemented to a volume of 50 mL with phosphate-buffered saline (PBS, Gibco, Breda, the Netherlands) before layering onto Ficoll-Hypaque. After centrifugation for 20 min at 1200 × g, PBMCs were collected from the plasma-Ficoll interphase. Cells were washed twice with 50 mL PBS and counted prior to further analysis. In previous research, we demonstrated that MDSC levels remain constant for the first 6 h, but decrease significantly when stored for a longer time.²³ Therefore, flow cytometry on PBMCs was performed immediately or within 6 h after blood was collected.

Flow cytometry

PBMCs were stained with the following conjugated monoclonal antibodies for analysis of MDSCs: anti-CD15-PE, anti-CD16-PERCP-Cy5.5, anti-CD33-PE-Cy7, anti-CD11b-APC, anti-HLA-DR-APC-Cy7 (all from BD Biosciences), anti-CD14-PE-Texas-Red (Invitrogen, Breda, the Netherlands), anti-ILT3-FITC (R&D Systems) and a live/dead marker 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes). Staining with anti-CD4-FITC, anti-CD8-APC, (BD Biosciences), anti-CD3-APC-eFluor 780 (eBioscience) antibodies and DAPI was performed for the analysis of T cells.

Cells were washed with FACS buffer (PBS, 0.25% BSA, 5 mM EDTA, 0.05% NaN₃) and stained for 30 min at 4 °C with the abovementioned antibodies, appropriately diluted in FACS buffer supplemented with 2% normal human serum. Flow cytometry was performed on a LSRII flow cytometer (BD Biosciences) and data were analyzed with FlowJo software (Tree Star).

MDSCs were characterized as previously described.²³ For MDSC staining, the CD16 marker was used to exclude mature granulocytes based on their CD16⁺⁺ expression, as previously described to be important for MDSC purity.^{5,24} PMN-MDSC were characterized as CD11b⁺CD14⁻HLA-DR⁻CD33⁺CD15⁺ and MO-MDSCs were characterized as CD11b⁺CD14⁺HLA-DR⁻CD33⁺CD15⁺. Total monocytes were characterized as CD11b⁺CD14⁺. For lymphocyte staining, T cells were characterized as CD3⁺ and divided into CD4⁺CD8⁻ (CD4⁺ T cells) and CD4⁻CD8⁺ (CD8⁺ T cells) subsets. B cells were characterized as CD19⁺ cells.

Measurement of soluble ILT3 in serum

For quantitative detection of ILT3 in serum, a commercially available enzyme-linked immunosorbent assay (ELISA) was used according to the manufacturer's specifications (Cusabio Biotech.). All samples were assayed in duplicate and quantified using a standard curve. The detection range was from 31.2–2,000 pg/mL.

Statistical analysis

Differences between healthy controls and lung cancer patients were evaluated by the Mann–Whitney *U* test. Correlations were assessed using the Spearman rho correlation test. The effects of size of immunologic cell populations and expression levels of ILT3 on survival were assessed with a log-rank (Mantel-Cox) test. In these analyses, patients were stratified for treatment

group. Statistical analysis was performed using the statistical program SPSS (version 21.0, SPSS Inc.). All *P* values were 2-sided and *P* values below the conventional level of significance (*P* < 0.05) were considered statistically significant. Figures were generated in GraphPad Prism (version 5.0, GraphPad Software).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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