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Malignant and non-malignant lung tissue areas are differentially populated by natural killer cells and regulatory T cells in non-small cell lung cancer

G. Esendagli[a,b,1], K. Bruderek[a,e,1], T. Goldmann[c,1], A. Busche[a,2], D. Branscheid[d], E. Vollmer[c], S. Brandau[a,e,*]

[a] *Division of Immunotherapy, Department of Immunology and Cell Biology, Research Center Borstel, Borstel, Germany*
[b] *Hacettepe University, Institute of Oncology, Department of Basic Oncology, Ankara, Turkey*
[c] *Division of Clinical and Experimental Pathology, Department of Clinical Medicine, Research Center Borstel, Borstel, Germany*
[d] *Department of Thoracic Surgery, Grosshansdorf Hospital, Grosshansdorf, Germany*
[e] *Department of Otorhinolaryngology, Head and Neck Surgery, University Duisburg-Essen, Essen 45122, Germany*

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Summary Even though the lung represents a special immune compartment with the capacity of a high inflammatory response, ineffective anti-tumour immunity is common in lung-associated malignancies. We asked whether a differential composition of the immune cell infiltrate in malignant (MLTAs) and non-malignant lung tissue areas (N-MLTAs) exists and might potentially contribute to this effect. We performed a comparative analysis of immune cells residing in MLTAs and N-MLTAs of non-small cell lung cancer (NSCLC) patients. To this end, we used immunophenotyping and functional analyses on directly isolated immune cells and tissue arrays on archived paraffin-embedded specimens. A strong T cell infiltration was prominent in both tissue compartments whereas $CD4^+CD25^+CD127^-$ T regulatory cells were present in MLTAs only. Nonetheless, concurrent functional *ex vivo* T cell analyses revealed no significant difference between T cells of MLTA and N-MLTA, suggesting that tumour-infiltrating T cells were not functionally impaired. Interestingly, T cell infiltration was less pronounced in specimens with a high neutrophilic infiltrate. NK cell infiltration was strikingly heterogenous between MLTA and N-MLTA. While NK cells were almost absent in the malignant tissue regions, non-malignant counterparts were selectively populated by NK cells and those NK cells showed strong cytotoxic activity *ex vivo*. We

* Corresponding author at: Department of Otorhinolaryngology, Head and Neck Surgery, University Hospital Essen, Hufelandstrasse 55, Essen D-45122, Germany. Tel.: +49 201 723 3193; fax: +49 201 723 5196.
E-mail address: sven.brandau@uk-essen.de (S. Brandau).
[1] These authors contributed equally to this study.
[2] Current address: Department of Virology, Hannover Medical School, Hannover, Germany.

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report that malignant and non-malignant tissue areas in NSCLC are selectively infiltrated by certain immune cell types with NK cells being displaced from the tumour tissue. These phenomena have important implications for tumour immunology of NSCLC and should be considered for the development of future immunologic intervention therapies.

1. Introduction

Lung cancer is one of the leading causes of cancer death for both men and women and its incidence continues to grow worldwide [1]. Since the survival rate remains low, besides the conventional cancer therapy the need for an adjuvant therapy is definitive. In this respect, immunotherapy and the activation of tumour-infiltrating immune cells represent promising approaches. However, the suppressive and heterogeneous nature of the tumour leads the success of the immunotherapy to differ individually at the patient level [2,3].

The lung represents a special immune compartment with the capacity of a high inflammatory response [4]. Even though being infiltrated by various immune cell populations, ineffective anti-tumour immunity is common in lung-associated malignancies. In non-small cell lung cancer (NSCLC), the presence of antigen presenting cells (APCs), tumour associated macrophages (TAMs) and dendritic cells (DCs), does not facilitate the immune responses [5], and defects in tumour-infiltrating lymphocytes (TILs) along with the presence of regulatory T cells (Treg) have been described [6—8]. The regulatory T cell subset in the tumour microenvironment contributes to the impediment of an effective anti-tumour immune response [9]. On the other hand, under physiological conditions, lung tissue is known to harbour relatively high numbers of NK cells which represent important potential anti-tumour effector cells [10]. Therefore, understanding the tissue distribution and functional role of NK cells as well as their interaction with other immune cells is important for further improvement of immunologic therapy of lung cancer.

NK cells have the ability for direct recognition and killing of tumour cells. Their activation depends on the balance between inhibitory signals mediated by MHC class I recognizing receptors and signals derived from activating receptors recognizing specific ligands [11]. Recently, the importance of pulmonary-NK activity in the control of tumour progression has been reported [12]. The impairment of NK cell infiltration and the downregulation of activating receptor expression are the major NK specific tumour evasion mechanisms [13,14]. On the other hand, activation of NK cells inhibited tumour growth in experimental models of human lung cancer [15].

In the present study, we investigated the immune cell populations infiltrating the malignant (MLTAs) and non-malignant lung tissue areas (N-MLTAs) in NSCLC. We report that MLTA and N-MLTA counterparts are selectively infiltrated by defined immune cell populations with differential tissue population by Tregs and NK cells. Low natural cytotoxicity together with the presence of Tregs might contribute to the compromised local immune response in malignant lung tissue areas.

2. Materials and methods

2.1. Patients and tissue specimens

NSCLC patients enrolled in this study were not subjected to preoperative radiotherapy and/or chemotherapy. Malignant and non-malignant lung tissue samples were obtained from 15 newly diagnosed patients undergoing surgical resection. Disease staging was defined according to international system for staging for lung cancer, stage I: $n = 6$; stage II: $n = 5$; stage III: $n = 4$. In addition to these freshly collected specimens, 26 HOPE (HEPES-glutamic acid buffer-mediated organic solvent protection effect)-fixed, paraffin-embedded tissue samples (stage I: $n = 7$; stage II: $n = 9$; stage III: $n = 8$; stage IV: $n = 2$) were obtained from Division of Clinical and Experimental Pathology archives at the Research Center Borstel. Histological sections of tissues were stained with hematoxylin—eosin and evaluated histopathologically under conventional light microscopy. Histopathological classification of the tumours was based on the World Health Organization criteria. Nine of the NSCLC tumour samples were classified as squamous cell carcinoma, whereas others were adenocarcinomas (patient summary in Table 1).

2.2. Isolation of infiltrating immune cells

Finely chopped tissue specimens were passed through CellTrics filters (100 μm; Partec, Münster, Germany) and subjected to discontinuous percoll gradient separation using the percoll dilutions 75%, 50% and 25%. Cells collected from the respective density fractions were directly prepared for flow cytometric analysis.

For the functional assays, chopped tissue was subjected to an additional step of enzymatic digestion (Dispase II (1.5 mg/g tissue, Roche Applied Science, Mannheim, Germany), Chymopapain (0.375 mg/g, Sigma, Taufkirchen, Germany), Collagenase Type I (0.75 mg/g, Worthington Biochemical Corp., Lakewood, USA), Elastase (1.79 mg/g, Worthington Biochemical Corp., Lakewood, USA)) for 4 h before the filtering and the gradient separation procedures. Then the cells were positively selected by magnetic activated cell sorting (autoMACS™ Separator, Miltenyi Biotec, Bergisch Gladbach, Germany). MACS was performed by using CD3 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for the purification of T cells according to the manufacturer's recommendations.

2.3. Flow cytometry

Freshly isolated immune cells were labelled with anti-human-CD3 (Ucht1), -CD19 (HD37), -CD25 (ACT-1), -CD56 (Moc-1) (Dako, Hamburg, Germany), -CD4 (S3.5) (Immuno-

Table 1 Summary of the patient data

Tumor histopathology	Gender		Age (mean ± S.D.)	Stage of the primary tumor			
	Male	Female		T1	T2	T3	T4
Squamous cell carcinoma	5	4	68 ± 5	2	3	3	1
Adenocarcinoma	22	10	59 ± 10	7	18	6	1

tools, Friesoythe, Germany), -CD69 (CH/4) (Biocarta, San Diego, USA), -CD66b (80H3), -NKp30 (Z25), -NKp44 (Z231), -NKp46 (BAB281), -CD127 (R34.34) (Beckman Coulter, Fullerton, USA), -NKG2D (149810) (R&D Systems, Minneapolis, USA) antibodies. Cells were analysed on a FACSCalibur flow cytometer (Becton Dickinson). The percentage of positive cells was calculated by comparison with the appropriate isotype-matched antibody controls. Before evaluation a gate on tissue-infiltrating lymphocytes was set according to forward scatter and sideward scatter values.

2.4. Enzyme-linked immunosorbent assay (ELISA)

$CD3^+$ T cells (10^6 cells/ml) were cultured in 96-well plates coated with 1 μg/ml mouse anti-human CD3 antibody (X35, Beckman Coulter, Fullerton, USA) in RPMI1640 (PAA, Pasching, Austria) media supplemented with 10% fetal bovine serum (FBS) (Pan-Biotech, Aidenbach, Germany), 1% penicillin and streptomycin (PAA, Pasching, Austria). Cells were further stimulated by the addition of 1 μg/ml mouse anti-human CD28 antibody (CD28.2, Beckman Coulter, Fullerton, USA) and goat anti-mouse cross-linking antibody (Jackson Immunoresearch, West Baltimore, USA) for 3 days. Supernatants were collected and used in IFN-γ, IL-10 (R&D Systems, Minneapolis, USA) and IL-4 (e-bioscience, San Diego, USA) ELISA assays according to manufacturer's instructions.

2.5. Proliferation assay

$CD3^+$ T cells (10^6 cells/ml) were stimulated in 96-well plates coated with 1 μg/ml mouse anti-human CD3 coated wells in the presence of 1 μg/ml mouse anti-human CD28 antibody and goat anti-mouse cross-linking antibody. After 3 days of incubation, cells were incubated over night with (^{3}H)-thymidine (Hartmann Analytik, Braunschweig, Germany) (0.1 μCi per well) and incorporated (^{3}H)-thymidine was detected using a beta-counter. Assays were performed as triplicates.

2.6. Cytotoxicity assay

Cytotoxicity was determined in a standard 4 h chromium release assay. Target K562 cells were labelled with $Na_2{}^{51}CrO_4$ (Hartmann Analytik, Braunschweig, Germany) for 1.5 h at 37 °C, washed, and resuspended at 5×10^4 cells/ml. After 3-day-preincubation in the presence of IL-2 (250 U/ml) and IL-12 (10 U/ml), effector cells were added to a total of 100 μl of target cells at the ratios indicated. The radioactive content of the supernatant was measured in a gamma counter (Berthold, Wildbad, Germany). Specific lysis was determined according to the following formula: specific lysis (%) = 100 × (Exp − Spo)/(Max − Spo), where Exp is the experimental release, Spo is the spontaneous release, and Max is the maximum release. Assays were performed as quadruplicates. Effector cells derived from healthy donor (HD) PBMCs were used as positive controls.

2.7. Immunohistochemistry (IHC)

Immunohistochemical staining of HOPE-fixed paraffin-embedded human lung tissues was performed as described earlier [16]. CD68 (PG-M1, Dako, Hamburg, Germany), CD3 (PC3/188A, Dako, Hamburg, Germany), CD66b (80H3, Beckmann Coulter, Fullerton, USA) primary antibodies were diluted to ratio 1/100 in Tris-buffered saline. Primary antibodies were applied for 1 h at ambient temperature. Detection was performed using the LSAB2-HRP technique (Dako, Hamburg, Germany) with AEC as a chromogen. Individual specimens were analysed by light microscopy and subjected to semiquantitative scoring (no, rare and moderate, high and maximal infiltration).

2.8. Statistical analysis

All values are expressed by arithmetic mean ± standard deviation (S.D.). Statistical difference between experimental groups was determined using Student's paired or unpaired *t*-test where appropriate. Differences were regarded as statistically significant when $P \leq 0.05$.

3. Results

3.1. Malignant and non-malignant lung tissue areas are strongly infiltrated by T cells

T cells are regarded as major effector cells in anti-tumour immune responses. Therefore, at first, the status of local T-cell infiltration in NSCLC tissue was determined. The lymphocytic fraction of freshly isolated immune cells contained high percentages of CD3 positive cells in both MLTAs (65.5 ± 14.5%) and N-MLTAs (61.4 ± 11.5%) (Fig. 1A). Furthermore, immunohistochemical analyses showed that more than 80% of the NSCLC samples were considerably infiltrated by T cells (Fig. 1B). Consequently, $CD3^+$ T cells were found to represent the predominant immune cell subpopulation infiltrating the tumour but there was no evident difference in the status of T cell infiltration between malignant and non-malignant tissue counterparts.

Interestingly, we frequently observed that high numbers of polymorphonuclear (PMN) cells correlated with a

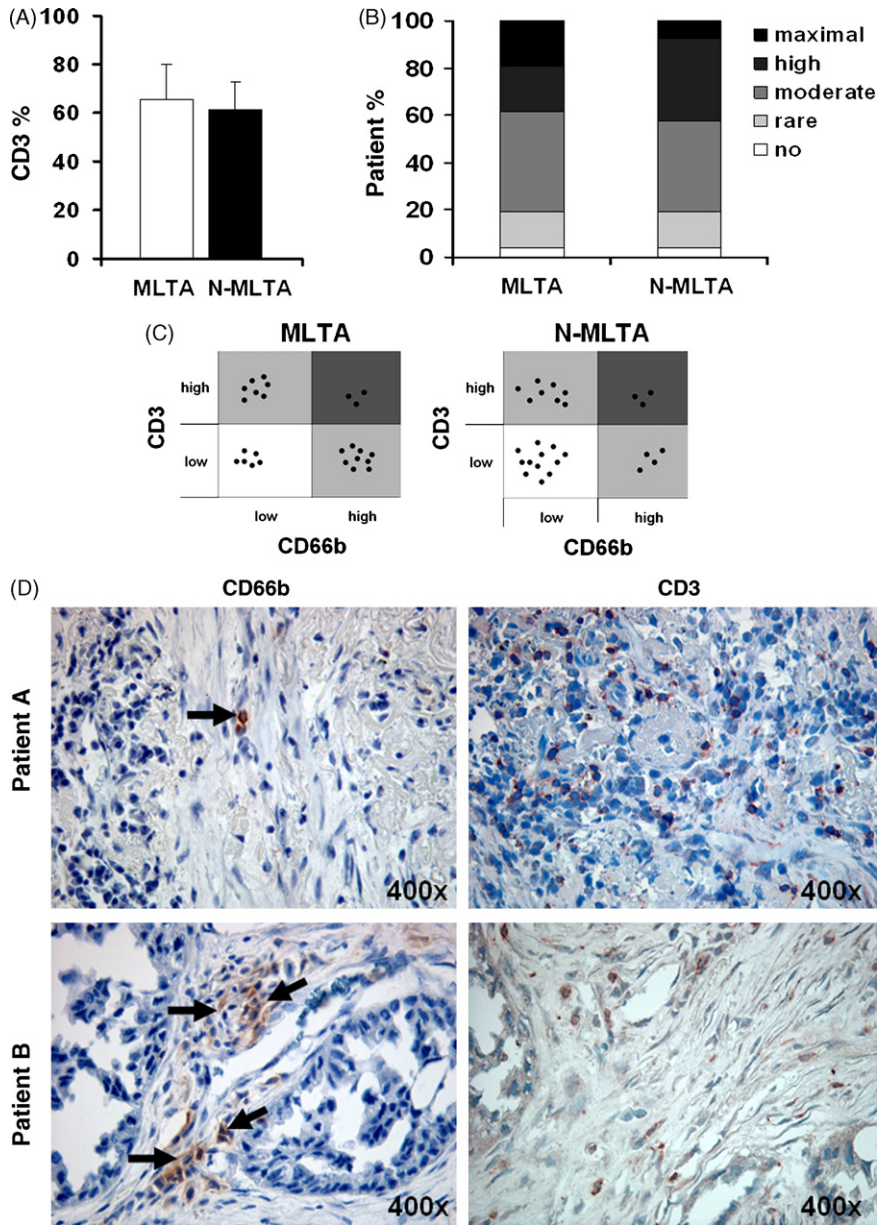

Fig. 1 $CD3^+$ T cell infiltration in MLTAs and N-MLTAs. T cell infiltration was determined in freshly isolated tissue samples by flow cytometry ($n = 9$) (A) or in paraffin-embedded tissue sections by IHC ($n = 26$) (B—D). For panel (C) scatter diagrams are plotted according to high and low infiltration status by $CD66b^+$ and $CD3^+$ cells. In panel (D) CD66b and CD3 IHC staining on MLTAs of two representative patients is shown. Patient A has a low infiltration by PMNs and a high infiltration by T cells whereas in Patient B higher PMNs counts can be observed together with a low T cell infiltration.

low T cell infiltration. In Fig. 1C T cell infiltration was categorized as high (high and maximal) or low (no, rare, moderate). These data were then plotted against the CD66b positivity of the specimens. Note that MLTA specimens with high neutrophil counts are frequently infiltrated by relatively low numbers of T cells. Fig. 1D shows representative examples to illustrate the pattern of neutrophil and T cell infiltration. Patient A shows a low infiltration by PMN and a high T cell count, while patient B has a stronger neutrophilic infiltrate and relatively small numbers of T cells.

We also determined distribution and infiltration by macrophages and IL-8 gene expression. Constitutive expression of IL-8 and irregular scattered distribution of macrophages was observed in more than 90% of the samples. However, no difference between MLTAs and N-MLTAs was apparent (data not shown).

3.2. Regulatory T cells infiltrate MLTAs but not N-MLTAs

Infiltration by regulatory T cells is frequently observed in solid tumours and may lead to substantial suppression of anti-tumour immune responses. The absence or low cell-surface expression of CD127 on $CD4^+CD25^+$ T cells has been

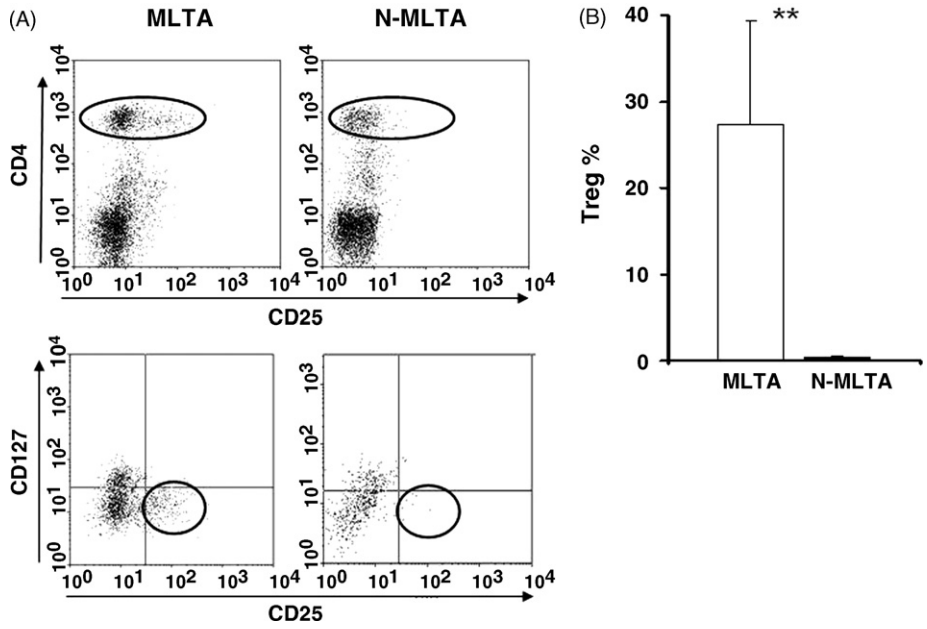

Fig. 2 Infiltration of MLTAs and N-MLTAs by regulatory T cells. Percentage of Tregs in NSCLCs was determined by flow cytometry CD4/CD25/CD127 triple staining. CD4/CD25 and CD25/CD127 dot plots of a representative patient are shown (A), note the presence of $CD4^+/CD25^+$ and $CD25^+/CD127^-$ Tregs, respectively, in MLTA. (B) Quantification of Tregs in MLTA vs. N-MLTA (mean percentage of Treg phenotype within $CD4^+$ cells, $n = 3$, $^{**}P < 0.01$).

reported as a novel Treg marker [17,18]. When the infiltrating T cells were further analysed $27.4 \pm 11.9\%$ of the $CD4^+$ cells were found to be $CD25^+CD127^-$ indicating the presence of Treg cells in the malignant lung tissues, whereas there were almost no T cells with this phenotype in the non-malignant counterparts (Fig. 2A and B).

3.3. Tumour-infiltrating T cells retain *ex vivo* functional activity

The expression of activation markers, CD69 and NKG2D, were examined to investigate the activation status of infiltrating T cells. CD69 expression was significantly increased

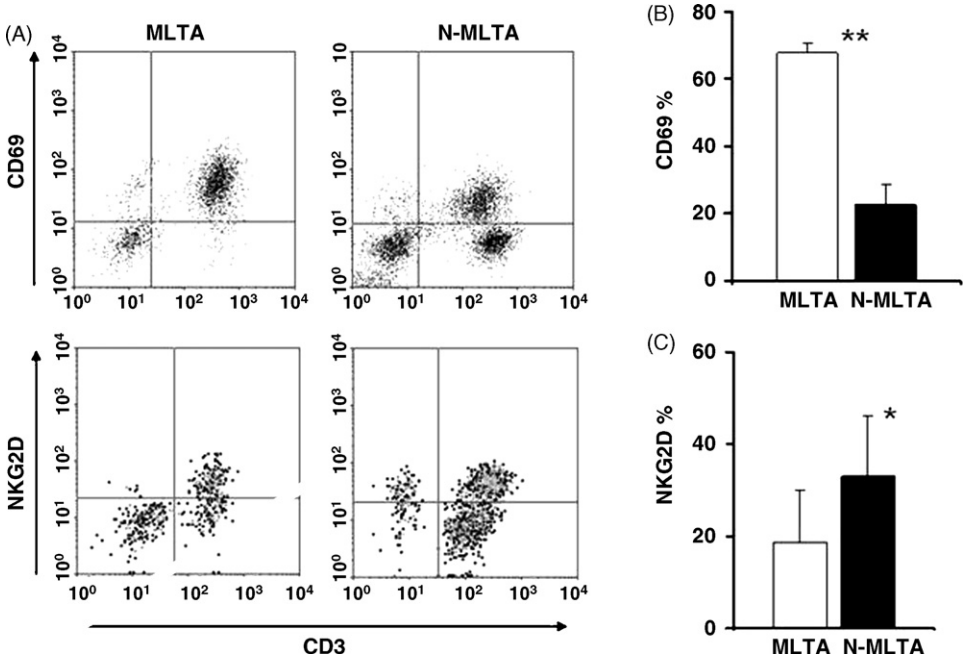

Fig. 3 Expression of activation markers on tissue-infiltrating lymphocytes. (A) Representative flow cytometry dot plots showing CD69 and NKG2D expression. Mean percentage values of (B) CD69 ($n = 3$, $^{**}P < 0.01$) and (C) NKG2D ($n = 5$, $^{*}P < 0.05$) expression on $CD3^+$ T cells is shown.

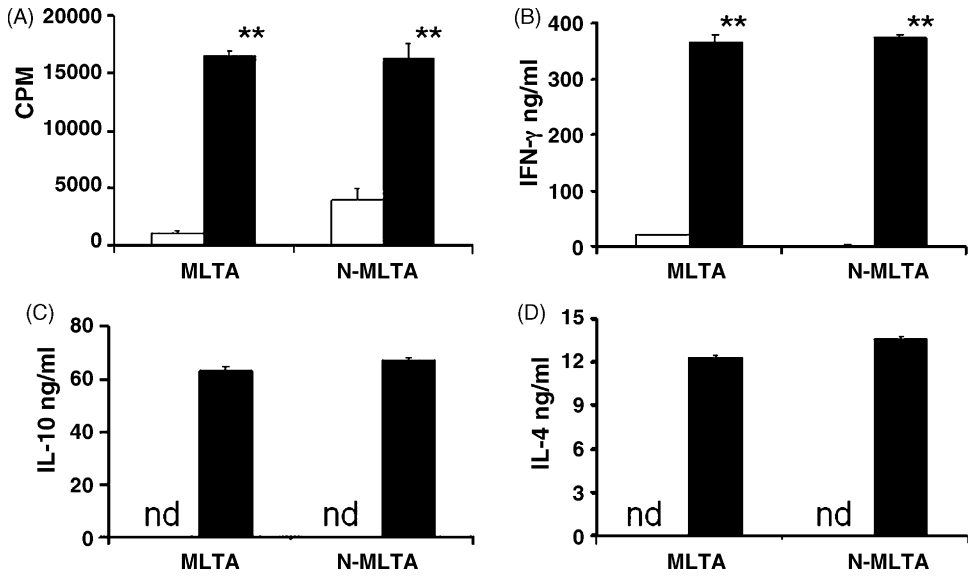

Fig. 4 Functional *ex vivo* analyses on T cells obtained from MLTAs and N-MLTAs. T cells were subjected to *in vitro* stimulation with anti-CD3/anti-CD28 mAbs (filled bars) or cultured unstimulated (open bars). Proliferation was determined by (^{3}H)-thymidine incorporation (A), and production of IFN-γ (B), IL-10 (C) and IL-4 (D) was determined by ELISA (nd, not detected). One representative experiment out of three is shown.

on MLTA T cells (67.6 ± 2.8%, $P < 0.01$) (Fig. 3A and B). On the other hand, NKG2D receptor expression on T cells was lower in malignant tissues when compared to non-malignant counterparts (33.2 ± 13.2%, $P < 0.05$) (Fig. 3A and C).

We further carried out *ex vivo* functional T cell assays to test the possible functional consequences of this distinct expression pattern of the activation markers. Directly *ex vivo* isolated T cells displayed no or little proliferation and no cytokine production. In some cases N-MLTA T cells showed some proliferation without further *in vitro* stimulation. Following *in vitro* stimulation by anti-CD3/-CD28 antibodies both T cells obtained from MLTAs and N-MLTAs started to proliferate and produced substantial amounts of cytokines (Fig. 4).

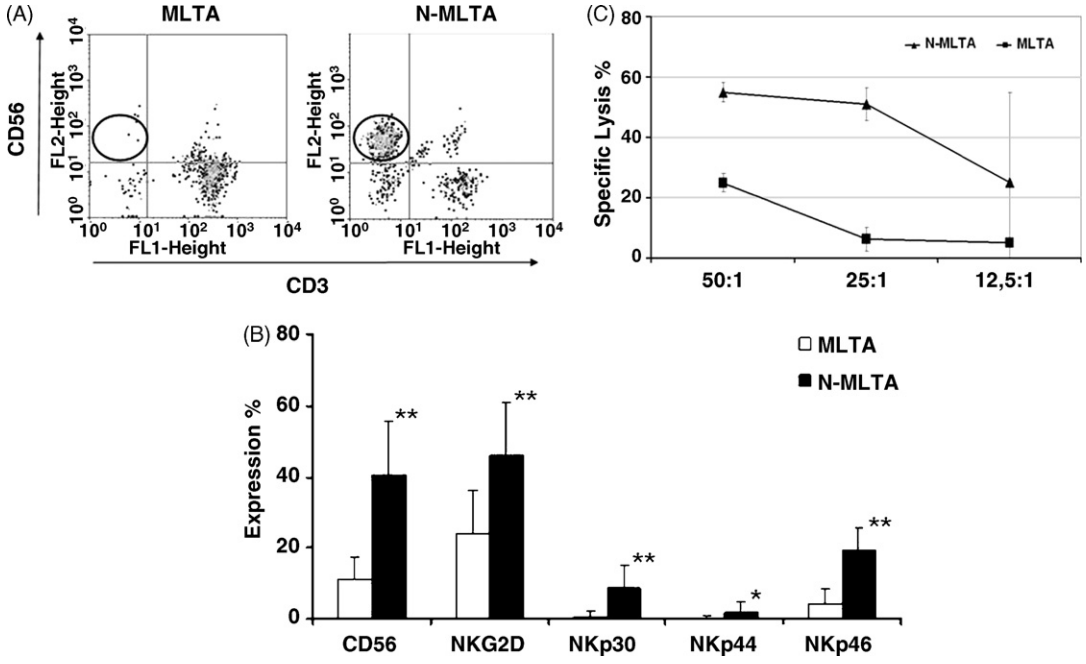

Fig. 5 NK cell infiltration and natural cytotoxicity in NSCLC specimens. (A) Representative dot plot illustrating expression of CD56 and CD3 on tissue-infiltrating lymphocytes, note the low number of NK cells in MLTA. (B) Mean percentage of tissue-infiltrating lymphocytes expressing NK surface markers ($n = 9$, $^*P < 0.05$, and $^{**}P < 0.01$). (C) Natural cytotoxicity of lymphocytes infiltrating MLTA and N-MLTA was tested against NK-sensitive K562 cells.

3.4. NK cells are largely absent in MLTAs

In addition to reduced NKG2D expression on $CD3^+$ T cells, we observed that this marker was virtually absent on CD3-negative cells (Fig. 3A). NKG2D was initially described as a potent activating receptor on NK cells which represent major effector cells of the innate immunity, generating cytotoxic responses against tumours [11]. Consequently, we continued our study with the analysis of the NK cell infiltrate. Most interestingly, it turned out that NK cells showed the most selective distribution between MLTAs and N-MLTAs in our study. Considerable amounts of $CD56^+$ cells were present in the non-malignant tissues ($40.5 \pm 11.9\%$, $P < 0.001$) while in the malignant tissues, they consistently remained at a low level (Fig. 5A and B). The overall expression of NK-related activation markers, namely NKG2D, NKp46, NKp30 and NKp44, was also found to be decreased in all malignant lung tissue samples (Fig. 5B). In functional experiments, we used NK-sensitive K562 as target cells. We consistently observed that effector cells derived from malignant tissue areas exhibited significantly lower cytotoxic activity compared with their counterparts derived from N-MLTAs (Fig. 5C). Altogether, these data demonstrate considerably reduced numbers of NK cells, reduced expression of NK activation markers and low natural cytotoxicity in MLTA.

4. Discussion

The lung is exposed to the exterior space and harbors a specialized immune network which has to cope with various infectious and pathological conditions. Although lung cancer remains a clinically challenging tumour entity, the local interaction between malignant tissue and host immune components is still poorly characterized in human subjects. In the present study, we performed a comparative analysis of the immune cell populations infiltrating malignant and non-malignant lung tissue counterparts in NSCLCs. To this end, we immunophenotypically and functionally analysed immune cell populations freshly isolated from MLTAs and N-MLTAs of NSCLC patients. We supported our data set by series of archived tissue array samples. With this study design we showed clear differences between the local immune cell composition of malignant and non-malignant tissue areas. NK cells were expelled from MLTA resulting in low natural cytotoxicity of MLTA infiltrating immune cells. While in general T cells populated MLTAs and N-MLTAs to a similar extent, malignant tissue areas were preferentially infiltrated by Tregs, which were largely absent in N-MLTA. This is one of the few studies directly analysing local tumour—host interaction in human lung cancer patients. Therefore, our data are complementary to larger groups of studies which focus on somewhat distant and more accessible compartments such as peripheral blood or pleural cavity [19,20]. While those compartments are important to analyse they do not directly reflect the local tumour microenvironment.

It is now widely accepted that the developing and progressing tumour negatively modulates local and systemic antitumoural immune responses. Consequently, possible recovery of T cell functions forms the basis of current immunotherapy approaches [21]. Our data on tissue distribution of $CD3^+$ T cells revealed no significant difference between MLTAs and N-MLTAs, as both compartments were infiltrated almost to the same extent in NSCLC patients. Notably, in MLTAs, PMN infiltration was inversely correlated with T cell density. This is of interest as PMN are important cellular components of tumour-associated inflammation, shape the local tumour microenvironment and may promote tumour progression [22]. PMN are rarely infiltrating healthy lung tissue under normal physiologic conditions. However, under various pathophysiological conditions neutrophils are rapidly recruited to the site of infection or inflammation [23]. We observed that in NSCLCs, the resident macrophages and PMNs were heterogenously distributed through MLTAs and N-MLTAs. IL-8 expression did not seem to be the primary chemokine guiding the high myeloid infiltrations as we found no correlation between tissue distribution of IL-8 and myeloid cell infiltration (data not shown). While the role of PMN is well defined in infection and inflammation, surprisingly little is known about the exact role of PMNs in tumour immunology [24]. Our descriptive observations may stimulate more detailed investigations on the functional interaction of T cells and PMN in pulmonary malignancies.

Intriguingly, two T cell activation markers, CD69 and NKG2D, were differentially expressed between the malignant and non-malignant tissue areas. In a study addressing the role of CXCL12 in lung adenocarcinoma, Wald et al. showed an increase in tumour-infiltrating $CD69^+CD4^+$ T cells with potential immunoregulatory function [25]. It has also been reported that CD69 is implicated in the modulation of immune responses whereas its deficiency has been shown to augment anti-tumour responses [26]. The relatively high CD69 levels observed on MLTA T cells in our study might therefore reflect local immunomodulatory processes.

NKG2D is a receptor for MHC class I chain-related A and B molecules, which are frequently expressed by epithelial cancers. Ligation of NKG2D induces antitumoural effector functions in both NK and T cells. Thus, the downregulation of NKG2D may indicate another immune impediment mechanism in MLTAs. We performed experiments with T cells directly purified from MLTA and N-MLTA environments. T cells from both compartments retained functional capacity, secreted cytokines and proliferated after CD3/CD28 stimulation. In our study, effector functions of tumour-infiltrating T cells were maintained despite the selective presence of Tregs in MLTA only. Woo and colleagues used sort-purified $CD4^+/CD25^+$ subpopulations from NSCLC patients and found that those cells inhibited autologous but not allogeneic T cell proliferation *ex vivo* [27]. In an alternative approach Semino and colleagues isolated NSCLC-associated T cells and demonstrated weak expression of HLA-class-I specific natural killer cell receptors along with fully preserved functional capacity [28]. A number of studies has highlighted the potential immunosuppressive role of Tregs in rodent and human tumours [7,29] and there is no doubt that manipulation of Treg functions holds great promise in the treatment of solid cancers. Nevertheless, in our study we did not observe a general functional impairment in directly *ex vivo* isolated bulk T cell cultures containing a subpopulation of regulatory T cells.

Most recently, an attractive alternative function of Tregs in tumour immunosurveillance is emerging. A number of studies have suggested a critical role for Tregs in dampening NK cell effector functions [30]. In addition to the

downregulaton of the adaptive effector phase of immunity, in this way Tregs would also modulate early and innate immune reactivity. NK cells are dynamic sentinels of immune surveillance, which resist to airborn intracellular infections and transformed cells [31] and thus contribute to the first line of defense in lungs. Our previous study has revealed the importance of NK-mediated tumour rejection by NKG2D stimulation in experimental models of human lung cancer [15]. In agreement with other reports on the lymphocytic infiltrate in NSCLCs [25,32] in the current study we observed a clear exclusion of NK cells from the malignant lung tissue areas whereas non-malignant counterparts were selectively populated by NK cells. *Ex vivo* cytotoxicity experiments revealed low natural cytotoxicty against K562 targets in MLTA specimens. In contrast, N-MLTA-derived immune cells exerted considerable natural cytotoxicity and displayed higher levels of the activating receptor NKG2D. In addition to NKG2D, we investigated expression of the natural cytotoxicity receptors (NCRs) NKp46, NKp30 and NKp44. NKp46 and NKp30 are expressed both on resting and activated NK cells, whereas NKp44 is induced following activation [33]. Again, in MLTAs, expression of these NK-activating receptors was significantly diminished.

Collectively, our data show differential tissue distribution of Tregs and NK cells along with differential natural cytotoxicity in malignant and non-malignant tissue areas in NSCLC. Currently an important role for Tregs in tumour immunosurveillance and exciting new data on the interaction of Tregs and NK cells are emerging. At present, the mechanism underlying the functional impediment of NK cells in tumours and the role of Tregs in this process are only beginning to be elucidated. However, Treg—NK interactions and Treg-derived TGF-β expression seem to be involved [13,34,35].

In this context, our study adds new information on the tissue distribution and function of these cell types in human lung cancer. Tissue distribution and activation status of regulatory and effector immune cells in tumour-bearing patients have important implications for intrinsic or therapeutically induced antitumour immunity. In the future, novel approaches targeting Treg-mediated suppression and improved recruitment and activation of local NK cells in MLTAs may be considered as potential immunotherapeutic strategies for NSCLCs.

Conflict of interest

None declared.

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