Circulating NKp46⁺ Natural Killer cells have a potential regulatory property and predict distinct survival in Non-Small Cell Lung Cancer

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ABSTRACT
Natural killer (NK) cells are innate effector lymphocytes widely involved in cancer immunosurveillance. In this study, we described three circulating NK cell subsets in patients with non-small cell lung cancer (NSCLC). Compared to healthy donors (HD), lower rate of the cytotoxic CD56bright CD16⁺ NK cells was found in NSCLC patients (76.1% vs 82.4%, \( P = 0.0041 \)). In contrast, the rate of CD56dim CD16⁺ NK cells was similar between patients and HD. We showed in NSCLC patients a higher rate of a NK cell subset with CD56dim CD16⁺ phenotype (16.7% vs 9.9%, \( P = 0.0001 \)). The degranulation property and cytokines production were mainly drive by CD56dim CD16⁺ NK cell subset in patients. Analysis of natural cytotoxicity receptors (NCRs) expression identified four distinct clusters of patients with distinct NK cell subset profiles as compared to one major cluster in HD. Notably the cluster characterized by a low circulating level of NKp46⁺ NK cell subsets was absent in HD. We showed that the rate of circulating NKp46⁺ CD56dim CD16⁺ NK cells influenced the patients’ survival. Indeed, the median overall survival in patients exhibiting high versus low level of this NK cell subset was 16 and 27 months respectively (\( P = 0.02 \)). Finally, we demonstrated that blocking NKp46 receptor in vitro was able to restore spontaneous tumor specific T cell responses in NSCLC patients. In conclusion, this study showed a distinct distribution and phenotype of circulating NK cell subsets in NSCLC. It also supports the regulatory role of NKp46⁺ NK cell subset in NSCLC patients.

Introduction
Natural killer (NK) cells, the most important effectors of innate lymphoid cells (ILCs), play a fundamental role in tumor immunosurveillance.¹-³ NK cells are subdivided into several subsets based on CD56 and CD16 relative expression, with different antitumor functions.⁴ CD56dim CD16⁺ NK cells are largely predominant in blood and have high cytotoxic properties mediated by a strong production of granzymes and perforin.⁵ This function is regulated by a balance between a set of inhibitory and activating receptors such as CD16 which is critical to mediate antibody dependent cell mediated cytotoxicity (ADCC). The recognition of tumor cells by NK cells involves other main activating receptors such as NKp2D and natural cytotoxicity receptors (NCRs) NKp30 and NKp46, which bind their respective ligands expressed on tumor cells.⁶-⁸ CD56bright CD16⁺ NK cells (CD56 bright NK) which are poorly cytotoxic and have a regulatory function.⁹,¹⁰ contribute also in tumor immune control by secretory large amounts of IFN-γ.¹¹ More recently NK cell subsets such as CD56bright CD16⁺ NK cells and CD56dim CD16⁺ NK cells have been described but they are poorly found in healthy individuals and their functions are largely unknown.¹²,¹³,¹⁴,¹⁵ Tumor-infiltrating NK cells are associated with a better prognosis in several tumors¹⁶,¹⁷ including in lung cancer, suggesting their implication in tumor control.¹⁸ Thus, NK cells were characterized within the tumors and their phenotype and function were mainly investigated in patients’ blood due to an easier access. A downregulation of NKG2D, NKp30 and NKp46 expression on NK cells was found in tumor microenvironment as well as in blood of patients with breast cancer, colorectal cancer, gastric and pancreatic cancer and melanoma.¹⁹-²² This NK cells altered phenotype is correlated with a defective functionality highlighted by a reduction of both perforin level¹⁹ and IFN-γ production.²⁰,²¹ It has also been reported in melanoma and colorectal cancer that the decrease of NKp46⁺ NK cells in advanced disease is associated with a decreased survival.¹⁸,²² In this study, we described in...
NSCLC patients three main circulating NK cell subsets such as CD56\textsuperscript{dim} CD16\textsuperscript{+}, CD56\textsuperscript{dim} CD16\textsuperscript{−} and CD56\textsuperscript{bright} NK cells. Subsequently, we evaluated their cytotoxic potential, analyzed the NCRs expression and also addressed the issue of their clinical influence.

**Results**

**Distribution of peripheral NK cell subsets in NSCLC patients**

NK cell subsets were measured in 176 NSCLC patients free of any treatment and 41 HD. Patients’ main clinical characteristics at the time of sampling are indicated in Supplementary Table S1. For the flow cytometry analysis, total NK cells were gated among the lymphocyte population on CD3\textsuperscript{−} CD56\textsuperscript{+} cells. No differences were observed between the rate of total circulating NK cells from NSCLC patients and HD (15.1x vs 15.5% respectively) (Figure 1A). NK cells were further divided according to the expression of CD56 and CD16 markers. We found that both HD and NSCLC patients exhibited three distinct NK cell subsets: CD56\textsuperscript{dim} CD16\textsuperscript{+}, CD56\textsuperscript{dim} CD16\textsuperscript{−} and CD56\textsuperscript{bright} NK cells (Figure 1B). Like in HD the majority of circulating NK cells in patients were of CD56\textsuperscript{dim} CD16\textsuperscript{+} phenotype (76.1%). But, there was a substantial decrease of the percentage of CD56\textsuperscript{dim} CD16\textsuperscript{+} NK cells in NSCLC patients as compared to HD (76.1 vs 82.4% respectively, \(P\) = 0.0041) (Figure 1B-C). The CD56\textsuperscript{dim} CD16\textsuperscript{−} and CD56\textsuperscript{bright} NK cell subpopulations represented 16.7\% and 5.6\% of total NK cells respectively in NSCLC patients (Figure 1B). While we found a significant higher rate of CD56\textsuperscript{dim} CD16\textsuperscript{−} NK cells in NSCLC patients (16.7 vs 9.9\%, \(P\) = 0.0001) (Figure 1D), no difference was observed between the frequency of CD56\textsuperscript{bright} NK cells from patients and HD (Figure 1E). We confirmed that the three NK cell subsets found in the blood of NSCLC patients differ from NCRs\textsuperscript{−} ILC3 by their lack of CD127 expression (Supplementary Fig. S1). These different subsets were homogeneously distributed according to patients’ main clinical characteristics (Figure 1F and Supplementary Table S1). Thus, our results indicate that NSCLC patients exhibit high level of CD56\textsuperscript{dim} CD16\textsuperscript{−} NK cells and low rate of CD56\textsuperscript{dim} CD16\textsuperscript{−} NK cells in blood.

**Cytotoxic and regulatory functions of NK cell subsets in NSCLC patients**

Next, we asked for the cytotoxic property of each NK cell subsets. To this end we evaluated their degranulation capacity when exposed to NK-sensitive K562 tumor cells. As for HD, we showed that the percentage of total CD107a\textsuperscript{+} NK cells from patients significantly increased in presence of K562 target cells (\(P\) < 0.0001 and \(P\) = 0.003 respectively) (Figure 2A). Conversely, the rate of total Granzyme B\textsuperscript{+} NK cells similarly decreased after K562 exposition both in patients and HD (\(P\) = 0.004 and \(P\) = 0.015 respectively) (Figure 2A). However, at the steady state, NSCLC patients exhibited higher circulating rate of Granzyme B\textsuperscript{+} NK cells than HD (\(P\) = 0.003). Next, we evaluated K562 cell death after culture with PBMCs from patients and HD. We found similar rates of annexin V\textsuperscript{−} 7-AAD\textsuperscript{−} (early apoptosis) and annexin V\textsuperscript{−} 7-AAD\textsuperscript{+} (late apoptosis) K562 cells, between patients and HD (Figure 2B).

When we focused on each NK cell subset, the degranulation activity characterized by CD107a\textsuperscript{+} expression was mainly driven by CD56\textsuperscript{dim} CD16\textsuperscript{−} NK cells as compared to CD56\textsuperscript{dim} CD16\textsuperscript{+} commonly described as high cytotoxic subpopulation (23.4 vs 9.4\% respectively, \(P\) = 0.041) (Figure 2C). However, the ability to produce Granzyme B against K562 was preferentially relied on CD56\textsuperscript{dim} CD16\textsuperscript{+} NK cell subset in NSCLC patients (Figure 2D). As expected CD56\textsuperscript{bright} NK cells, a subset with regulatory properties,\textsuperscript{10} showed a lower level of Granzyme B compared to CD56\textsuperscript{dim} CD16\textsuperscript{+} NK (19.3 vs 54.8\% respectively, \(P\) = 0.0002), that was associated with a poor degranulation property against K562 cells (Figure 2C-D).

We next investigated the property of NK cell subsets to produce effector cytokines in response to K562 cells. Overall, circulating total NK cells both from NSCLC patients and HD were able to significantly produce IFN-\(\gamma\) and TNF-\(\alpha\) in response to K562 cells (Figure 3A). We found that IFN-\(\gamma\) was mainly produced by CD56\textsuperscript{dim} CD16\textsuperscript{−} NK cells as compared to CD56\textsuperscript{bright} NK cells (14.2 vs 6.3\% respectively, \(P\) = 0.048) (Figure 3B). Similar observation was made about TNF-\(\alpha\) production (5.5 vs 1.1\% respectively, \(P\) = 0.015) (Figure 3C). As expected, CD56\textsuperscript{dim} CD16\textsuperscript{−} NK cell showed a low production of IFN-\(\gamma\) and TNF-\(\alpha\) following K562 cells activation (Figure 3B-C). Of note, no obvious difference was observed between patients and HD about the capacity of NK cell subsets to produce IFN-\(\gamma\) and TNF-\(\alpha\) (Figure3A-C). Moreover, we analyzed the capacity of NK cell subsets to secrete effector cytokines in response to different conditions of activation using cytokines involved in NK cell homeostasis and function.\textsuperscript{5} We showed that the two main CD56\textsuperscript{dim} CD16\textsuperscript{−} and CD56\textsuperscript{bright} NK cell subsets particularly secreted IFN-\(\gamma\) and TNF-\(\alpha\) following stimulation with IL-2, IL-12 or IL-21 (Figure 3D). Collectively our results show that NK cells and especially CD56\textsuperscript{dim} CD16\textsuperscript{−} NK cells display both regulatory and degranulation functions in NSCLC patients.

**Distinct profile of ncr-expressing NK cell subsets in NSCLC patients**

To further characterize these circulating NK cell subsets, we analyzed the cell-surface expression of NKGD2 and NCRs (NKp30, NKp44, NKp46) activating receptors. About NKGD2, only the rate of NKGD2\textsuperscript{+} CD56\textsuperscript{bright} NK cells was found increased in NSCLC patients as compared to HD (72.5 vs 61.4\% respectively, \(P\) = 0.0001) (Figure 4A). No obvious difference was observed between patients and HD about NKp30\textsuperscript{+} and NKp44\textsuperscript{+} NK cell subsets (Figure 4B-C). In contrast, the percentage of each NKp46\textsuperscript{+} NK cell subset was significantly lower in NSCLC patients than in HD (Figure 4D). Notably, we distinguished two groups of patients according to the low (< 37\%) or high rate (> 37\%) of circulating NKp46\textsuperscript{+} NK cells (referred as NKp46\textsuperscript{+} NK\textsuperscript{low} or NKp46\textsuperscript{+} NK\textsuperscript{high} cells respectively) (Supplementary Fig. S2A). Of note, NSCLC stages did not seem to influence the rate of circulating...
Figure 1. Distribution of NK cell subsets in the blood of NSCLC patients.

Representative flow cytometry gating strategy and proportion of circulating (A) total CD3\(^-\) CD56\(^+\) NK cells among PBMCs in HD (n = 41) and NSCLC patients (n = 176); and (C) CD56\(^{dim}\) CD16\(^{+}\), (D) CD56\(^{dim}\) CD16\(^{-}\) and (E) CD56\(^{bright}\) CD16\(^{-}\) NK cell subsets among total NK cells in HD (n = 41) and NSCLC patients (n = 176). (B) Frequency of each NK cell subsets in blood of HD (n = 41) and NSCLC patients (n = 176) represented by stacked bars. (F) Proportion of total NK cells among PBMCs and proportions of CD56\(^{dim}\) CD16\(^{+}\), CD56\(^{dim}\) CD16\(^{-}\) and CD56\(^{bright}\) CD16\(^{-}\) NK cell subsets among total NK cells in NSCLC patients according to the disease stage. Statistical analysis were performed using the Mann-Whitney test (two groups) or the Kruskal-Wallis test (more than two groups) (ns = not significant; ** P < 0.01; *** P < 0.001).
NKp46+ NK cell subsets (Supplementary Fig. S2B). As expected, we observed a strong positive correlation between NKp46 on NK cells and the other activating receptors among NSCLC patients (Supplementary Table S2).

Unsupervised hierarchical clusterings (heatmap) according to the NCRs-expressing NK cell subsets identified four clusters of NSCLC patients (Figure 5A). The cluster 1 (CL1) gathered patients with globally high level of NCRs on all NK cell subsets. The cluster 2 (CL2) was characterized by a lower rate of NKp44+ NK cells compared to CL1. Patients from cluster 3 (CL3) have both a reduced level of NKp44+ and NKp46+ NK cells. Finally, the last cluster (CL4) with heterogeneous expression of NCR included only 5 patients, thus it was not included for further analysis. In contrast, the heatmap showed one major cluster in HD corresponding to CL2 found in patients (Figure 5B).

Remarkably, the cluster 3 from NSCLC patients characterized by a low/moderated level of circulating NKp46+ NK cells, was totally absent in HD. The cluster distribution was similar for patients’ main clinical characteristics (data not shown). We further analyzed the expression of co-inhibitory receptors such as TIM3 and PD-1 known to be expressed by NK cells. TIM3 was significantly higher in NK cells from NSCLC patients than in HD (19.7 vs 5.9% respectively, \( P < 0.0001 \)) and was preferentially expressed by CD56dim CD16+ NK cell subset (Supplementary Fig. S2C). Similarly, the percentage of PD-1+ NK cells was significantly increased in NSCLC patients as compared to HD (1.6 vs 0.4% respectively, \( P = 0.008 \)) and also for the whole of subsets (Supplementary Fig. S2C).

These results show distinct levels of NCRs+ NK cells in NSCLC patients and especially a subgroup that exhibiting a low rate of circulating NKp46+ NK cells.

The level of circulating nkp46+ NK cells influences the overall survival

We investigated the prognostic value of circulating NK cell subsets on four-years-overall survival (OS) of patients. There was no association of the level (low or high according to the...
Figure 3. Regulatory function of NK cells in NSCLC patients.

PBMCs from patients and HD were activated with K562 cells for 6 hours at a ratio E:T of 1:1. (A) Cytokines production of total NK cells among PBMCs was assessed by flow cytometry: percentages of total NK cells producing IFN-γ (left panel) and TNF-α (right panel) in HD (n = 10) and NSCLC patients (n = 10), Mann-Whitney test (ns = not significant). Spontaneous (open circles) and K562-activated (solid circles) values are indicated, paired t-test (** P < 0.01). On the left panels, (B) IFN-γ and (C) TNF-α production by NK cell subsets in response to K562 stimulation of a representative patient are showed. The percentages indicate rates of IFN-γ+ and TNF-α+ NK cells after gating on CD56dimCD16+ and CD56dimCD16− or CD56bright cells. On the right panels, are summarized rates of (B) IFN-γ+ and (C) TNF-α+ among CD56dimCD16+, CD56dimCD16− and CD56bright NK cell subsets of either both HD (grey n = 10) and patients (black n = 10), Mann-Whitney test (ns = not significant), or only patients (black n = 10), Friedman test (ns = not significant; * P < 0.05; ** P < 0.01; *** P < 0.001). Red bars represent the mean of each group. (D) PBMCs from patients were activated with IL-2, (1000U/mL), IL-12 (10ng/mL) or IL-21 (50ng/mL) for 20 hours. Proportions of IFN-γ+ and TNF-α+ among total, CD56dimCD16+, CD56dimCD16− and CD56bright NK cell subsets in NSCLC patients (n = 10), Friedman test (ns = not significant; * P < 0.05; ** P < 0.01; *** P < 0.001). Red bars represent the mean of each group.
mean percentage) of total circulating NK cells or the three described NK cell subsets with the OS (Supplementary Fig. S3A-D). Next we wondered whether the level NCRs+ NK cell subsets influenced the outcome. To this end we defined two groups of patients according to the low or high circulating level of each NCRs+ NK cell subset. No association of the level of circulating NKp44+ or NKp30+ NK cell subsets with survival was observed (data not shown).

However, we showed that the level of NKp46+ NK cells influenced the OS. Indeed the total NKp46+ NKlow is significantly associated with a better OS (median OS, 24 vs 16 months, P = 0.04) (Figure 6A). Notably, we found that this association with OS was significantly driven by the NKp46+ CD56dim CD16+ subset (median OS, 27 vs 16 months, P = 0.02) (Figure 6B-D). Indeed, no association between the level of NKp46+CD56bright or NKp30+ CD56− NK cells and survival was observed (data not shown).

Collectively, our results demonstrate that low level of peripheral NKp46+ CD56dim CD16+ NK cells is associated with better survival in lung cancer and also highlights a potential regulatory property of this NK cell subset.

**Discussion**

Tumor-induced modifications of NK cell phenotype and function is a well-known mechanism contributing to the tumor escape. Here we described three circulating NK cell subsets in NSCLC patients such as CD56dim CD16+, CD56dim
Figure 5. Hierarchical clustering of NK cell activating receptors.
Hierarchical clustering of (A) NSCLC patients (n = 176) and (B) HD (n = 41) was realized according to NKp44, NKp30, NKG2D and NKp46 expression on NK cell subsets. The percentage values obtained for the Figure 3 were used and the clustering was performed using Morpheus Software.
CD16− and CD56bright NK cells. While the cytotoxic and regulatory functions of NK cells, mainly driven by CD56dim CD16− NK cell subset, were similar between patients and HD, we found differences about NK cell phenotype. Indeed, the rate of all of these NK cell subsets expressing NKp46 is significantly lower in NSCLC patients as compared to HD. Notably, we showed in NSCLC patients that a low rate of NKp46+ CD56dim CD16+ NK cells is significantly associated with a better OS.

The analysis of NK cells based on CD56 and CD16 expression highlighted three main NK cell subsets in both NSCLC patients and HD. Although similar rate of the regulatory CD56bright NK cell subset12 was found between patients and HD, lower circulating level of the cytotoxic CD56dim CD16+ NK subsets10 was observed in NSCLC patients. We also observed in peripheral blood of NSCLC patients a higher rate of NK cells expressing CD16+ NK cells in our study (76.1%). Moreover, Bruno et al. described around 17% of CD16− NK cells in the blood of NSCLC patients which is very close to the value of the rate of CD56dim CD16− NK cells in our cohort (16.7%).35 All of these studies support our results that NSCLC patients display a high rate of circulating CD56dim CD16− NK cells. Nevertheless, although recent studies identified these CD56dim CD16− NK cells as a distinct subset their ontogeny remains unclear.10,12,13 Indeed, several studies described a quick shedding of CD16 from NK cell surface upon activation due to metalloproteases.36,37 Future investigations are required to assess whether CD56dim CD16− NK cells represent a specific differentiation of circulating NK cells or derived from another mechanisms.35–40

While we found a similar NK cells-cytotoxic and regulatory functions in both patients and HD, our results also demonstrated that these CD56dim CD16− NK cells exert a superior degranulation capacity against K562 cells and produce more amounts of IFN-γ and TNF-α than the two others circulating NK cell subsets found in NSCLC. Although CD56dim NK cells are well known for their cytotoxic activity,
CD56\textsuperscript{dim} CD16\textsuperscript{+} NK cells were rather described as responsible for ADCC and CD56\textsuperscript{dim} CD16\textsuperscript{−} NK cells for natural cytotoxicity.\textsuperscript{41,42} The high capacity of response of CD56\textsuperscript{dim} CD16\textsuperscript{−} NK cells to K562 cells may suggest a potential involvement of this subset in cancer immunosurveillance.

Analysis of NCRs expression on NK cells revealed several groups of patients and HD. Two clusters CL1 and CL2 were shared by both HD and NSCLC patients with a main cluster represented by CL2. Another third cluster CL3 was only found in lung cancer patients. This cluster showed a lower expression of NCRs and particularly the activating receptor NKp46 on NK cells. Grouped analysis of NCRs and NKG2D expression on NK cells confirmed the heatmap results by revealing significant lower rates of NKp46\textsuperscript{+} NK cell subsets in NSCLC patients as compared to HD. Nonetheless, the disease stage didn’t have impact on the rate of activating receptor expression on NK cell subsets.

Although NKp46 is the main activating receptor involved in NK cell functions,\textsuperscript{43,44} the downregulation of this receptor observed in our study has also been reported in many other cancers.\textsuperscript{17–19,24,45,46} This suggests an involvement of tumor-related factors in the phenotypic alteration of NK cells. Indeed, some immune suppressive cytokines or soluble factors such as TGF-\(\beta\) or IDO secreted by tumor cells or stromal cells have been shown to decrease the expression of NCRs or NKG2D on NK cells.\textsuperscript{47,48} Moreover, tumor cells are able to release a soluble form of MIC ligands which keeps away NK cells and downregulates NKG2D expression.\textsuperscript{49,50} Thus possible NKp46 ligands or immunosuppressive factors released from tumor cells and could also promote a downregulation of NKp46 on NK cells. On this view, we investigated the correlation between the rate of NKp46\textsuperscript{−} NK cells and the concentration of several cytokines in the serum of patients. We found a positive correlation between the concentration of TGF-\(\beta\) and the level of NKp46\textsuperscript{+} NK cells and the concentration of several cytokines in the serum of patients. While other circulating NK cells expressing NCRs or NKG2D don’t have any prognostic role, we notably found an inverse correlation between patients’ survival and the rate of NKp46 CD56\textsuperscript{dim} CD16\textsuperscript{+} NK cells. Thus, we showed that patients with NKp46\textsuperscript{+} NK\textsuperscript{low} cells had a better survival than those with NKp46\textsuperscript{+} NK\textsuperscript{high} cells. This observation is...
unexpected because previous studies reported conflicting results in melanoma and colorectal cancer where a low rate of NKp46+ NK cells in blood was associated with poor prognosis.18,24 Several parameters could explain this discrepancy. A very low number of patients have been evaluated in these studies.18,24 Furthermore, in the study by Fregni et al., the association between the level of NKp46+ NK cells and the survival had only found in metastatic melanoma patients.24 Moreover, the cut-off value of NKp46+ NK cells rate used to define the groups of patients is around 80% in melanoma and colorectal cancer versus 37% in our study.18,24

One hypothesis that could explain the worse prognosis of patients with high rate of NKp46+ NK cells likely involved the crossstalk between NKp46+ NK cells and adaptive T cell immunity. Indeed, it has been reported that NK cells are able to regulate T cell immunity by soluble factors or by direct cell contact.29,51 For example, the IFN-γ production by NK cells is well known to promotes the priming of Th1-polarized immunity.52 A recent study reported that a subset of tumor-infiltrating NKp46+ CD3−CD56+ cells prevent the expansion of antitumor T cells by mechanism involving NKp46.29 In line with this study, we also showed that the blockade of NKp46 receptor in vitro effectively reinvigorates the IFN-γ production by TERT-specific CD4 Th1 cells. We previously demonstrated that the presence of circulating anti-TERT Th1 cells in cancer patients is associated with better survival.53-55 Thus we believed that the worse prognostic value found in population with NKp46+ NKhigh cells could be due to the ability of these NK cells to suppress anti-tumor Th1 immunity. Although our observation also suggests that NKp46+ NK cells could exert its negative control on antitumor T cells in a contact depend-manner, we demonstrated a positive correlation between the rate of NKp46+ NK cells and the level of TGF-β in the serum of patients, which is a cytokine known to be of poor prognosis in NSCLC.56 In contrast to immune profile shown in patients with NKp46+ NKhigh cells, we showed a trend of inflammatory signature profile in patients with NKp46− NKlow cells. Another hypothesis that could explain the worse prognosis of patients with high rate of NKp46+ NK cells is the expression of co-inhibitory receptors such as PD-1 or TIM-3. Indeed, a previous study in lung cancer reported that a high expression of TIM-3 on NK cells was associated with poor survival of patients.57 However in our study, no obvious difference of TIM3 and PD-1 levels was found regardless NKp46 expression (data not shown). Thus, we speculated that NKp46+ NK cells could drive an immunosuppressive environment which prevents adaptive T cell immunity. However, future investigations are needed to address the mechanisms by which NKp46 acts on antitumor T cell responses.

In conclusion, our study describes the presence of distinct circulating NK cell subsets and highlights an alteration of NKp46 pathway in NSCLC patients. This study also supports a regulatory role of NKp46+ NK cells in lung cancer.

Materials and methods

Patients and healthy donors

Non-Small Cell Lung Cancer (NSCLC) patients were from Telocap01 cohort, a prospective multicenter immunomonitoring study conducted in the European Hospital Georges Pompidou (Paris) and the University Hospital of Besançon between July 2010 and January 2014 (N°EUDRACT: 2009-A00642-55). Patient blood samples were collected before any treatment including surgery. Information about patients’ survival was collected at one and two-years after the inclusion. All patients had given their written consent and the protocol was approved by local and national ethic committees. For healthy volunteer donors, peripheral blood mononuclear cells (PBMCs) were collected from anonymous donors at the Etablissement Français du Sang (EFS, Besançon, France) as apheresis kit preparation after the signature of the informed consent and following the EFS guidelines. Patients’ and donors’ blood samples were isolated and frozen until use.

Flow cytometry analysis

PBMCs were stained for 20 minutes at 4°C with conjugated antibodies: anti-CD3-V500 (Biologend, 613406), anti-CD16-APC H7 (BD Biosciences, 554529), anti-CD56-eFluor 710 (eBioscience, 46–0567-42), anti-NKp30-PE (BD Biosciences, 558407), anti-NKp44-APC (R&D System, IC0041P), anti-NKp46-V450 (BD Biosciences, 562099), anti-NKG2D-PeCy7 (BD Biosciences, 557924), anti-PD-1-Pecy7 (Biolegend, 367414) and anti-TIM3-APC (Biolegend, 345012). Cells were analyzed with BD Facs Canto II flow cytometer (BD Biosciences) and data were analyzed with BD Facs Diva software.

Cell culture and cd107a degranulation assay

PBMC were cultured in RPMI 1640 medium (Gibco Invitrogen) supplemented with 10% human serum and 1% Penicillin-Streptomycin. K562 cells derived from human leukemia cell line were cultured in RPMI 1640 medium (Gibco Invitrogen) supplemented with 10% fetal calf serum and 1% Penicillin-Streptomycin.

For CD107a degranulation assay, PBMCs were cultured with K562 cells at 1:1 effector/target (E/T) ratio, for 6 hours at 37°C with golgi stop (BD Biosciences, 554724) and anti-CD107a-PE (BD Biosciences, 555406). After cell surface staining, cells were fixed and permeabilized using Cytofix/CytoPerm kit (BD Biosciences, 560842) and anti-CD16-FITC (BD Biosciences, 555406).

Cytokines secretion assay

For measurement of intracellular IFN-γ, TNF-α and Granzyme B, PBMCs were cultured with K562 cells at 1:1 E/T ratio for 6 hours or were stimulated with 1000U/mL IL-2 (Peprotech, 200–02), 10ng/mL IL-12 (Miltenyi Biotec, 130–096-704) or 50ng/mL IL-21 (Shenandoah Biotechnology Inc, 100–90) for 20 hours, at 37°C. Golgi plug (BD Biosciences, 555029) was added to cultures during the last 6 hours of stimulation. PBMCs were then stained for 20 minutes at 4°C with anti-CD3-PB (BD Biosciences, 558117), anti-CD56-eFluor 710 (eBioscience, 46–0567-42) and anti-CD16-FITC (BD Biosciences, 555406). After cell surface staining, cells were fixed and permeabilized using Cytofix/CytoPerm kit (BD Biosciences, 554714) and stained with anti-IFN-γ-APC (BD
Biosciences, 554702), anti-TNF-α-PE (Biolegend, 502909) and anti-Granzyme B-BV510 (BD Biosciences, 563388).

Cytotoxicity assay
K562 cells were labeled with CFSE (CellTrace CFSE Cell proliferation Kit, Molecular Probe) at 5µM in PBS 1X (Gibco Invitrogen) for 15 min under agitation. Then 2 volumes of fetal calf serum were added and cells were again incubated under agitation for 10 min. Finally, cells were washed twice with PBS 1X before culture. K562 cells were cultured with PBMCs at 1:1 E:T ratio, for 6 hours at 37°C. Cells were then stained for 15 minutes at room temperature with anti-Annexin V-APC (BD Biosciences, 556454) and anti-7-AAD (BD Biosciences, 559925) in 1X Annexin V binding buffer (BD Biosciences, 556454). Dead cells were analyzed after gating on CFSE positive K562 cells.

Synthetic peptides
We previously reported four HLA-DR and four HLA-DP4-restricted telomerase (TERT)-derived peptides that can be used for the monitoring of anti-TERT Th1 responses. These peptides were purchased from CTL (Cellular Technology Ltd, Germany).

Assessment of tumor antigen-specific T-cell responses
Tumor-specific Th1 response was assessed using a short-term in vitro stimulation with TERT-derived peptides as described previously. Briefly, PBMCs from patients or HD were cultured in 24-well plates with TERT-derived peptides (5µg/mL) in presence of anti-NKp46 mAb (5µg/mL, BD Biosciences, 557847) or IgG1 isotype control (5µg/mL, BD Biosciences, 554721). At day 10, the TERT-specific T cell specificity was investigated by IFN-γ ELISpot assay. Cells were seeded in triplicates in anti-human IFN-γ monoclonal antibody precoated ELISpot plates with TERT-derived peptides (5µg/mL) or with medium in X-VIVO 15 (control) for 18 hours at 37°C. The IFN-γ spots were revealed following the manufacturer’s instructions (Diaclone). Spot-forming cells were counted using the CTL Immunospot system. The number of specific T-cells expressed as spot forming cells per 10⁶ cells was calculated after subtracting negative control values (background).

Cytokine assay by CBA
Concentrations of IL-1β, IL-10, IL-17A, IL-21, TNF-α and TGF-β in undiluted sera samples of patients were measured using Cytometric Bead Array (CBA) Flex Set kits according to the manufacturer’s instructions (BD Biosciences, 558279, 558274, 560383, 560358, 560112, 560429 respectively). Samples were acquired using BD Facs Canto II flow cytometer (BD Biosciences) and data were analyzed with FCAP Array Software (BD Biosciences).

Statistical analysis
Data were analyzed using GraphPad Prism 6.0 software. Descriptive analyses are expressed as mean or median. For two-group comparisons, the nonparametric Mann-Whitney U-test was used. For multiple-group comparisons, the nonparametric unpaired Kruskal-Wallis test or paired Friedman test were used. Proportions were compared using the X2-test. Correlations were performed using the nonparametric Spearman test. Hierarchical cluster analysis and dendrograms were performed using the online Morpheus software and robust Z-score normalization (https://software.broadinstitute.org/morpheus/). Overall survival (OS) was calculated from the date of study enrollment to the date of death from any cause. Patients known to be alive were censored at the time of their last follow-up assessment. The survival curves were plotted according to the Kaplan-Meier method and compared using the Log-Rank test. P values less than 0.05 were considered significant (* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001).

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Disclosure of interest
The authors report no conflict of interest.

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References
1. Waldhauer I, Steinle A. NK cells and cancer immunosurveillance. Oncogene. 2008;27(45):5932–5943. doi:10.1038/onc.2008.267.
2. Guillerey C, Mj S. NK Cells and Cancer Immunodecting. Curr Top Microbiol Immunol. 2016;395:115–145. doi: 10.1007/82_2015_446.
3. Del Zotto G, Marcenaro E, Vacca P, Sivori S, Pende D, Della Chiesa M, Moretta F, Ingegnere T, Mingari MC, Moretta A, et al. Markers and function of human NK cells in normal and pathological conditions. Cytometry B Clin Cytom. 2017;92(2):100–114. doi: 10.1002/cyto.b.21508.
4. Nagler A, Lanier LL, Cwirla S, Phillips JH. Comparative studies of human FcRII-positive and negative natural killer cells. J Immunol. 1989. 143(10):3183–3191.
5. Cooper MA, Fehninger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol. 2001;22(11):633–640. doi:10.1016/S1471-4906(01)02060-9.
6. Brandt CS, Baratin M, Yi EC, Kennedy J, Gao Z, Fox B, Haldeman B, Ostrander CD, Kaifu T, Chabannon C, et al. The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. J Exp Med. 2009;206(7):1495–1503. doi: 10.1084/jem.20090681.
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7. Hecht M-L, Rosenthal B, Horlacher T,ershkovitz O, De Paz JL, Noti C, Schauer S, Porgador A, Seeberger P.H. Natural cytotoxicity receptors NKP30, NKP44 and NKP46 bind to different heparan sulfate/heparin sequences. J Proteome Res. 2009;8(2):712–720. doi:10.1021/pr800477c.

8. El-Gazzar A, Groh Y, Spies T. Immunobiology and conflicting roles of the human NKG2D lymphocyte receptor and its ligands in cancer. J Immunol. 2013;191(4):1509–1515. doi:10.4049/jimmunol.1301071.

9. Cooper MA, Fehniger TA, Turner SC, Chen KS, Ghahter B.A, Ghayur T, Carson WE, Caliguri MA. Human natural killer cells: a unique innate immunoregulatory role for the CD56 (bright) subset. Blood. 2001. 97(10):3146–3151.

10. Poli A, Michel T, Thérèse M, Andrès E, Hentges F, Zimmer J. CD56bright natural killer (NK) cells: an important NK cell subset. Immunology. 2009;126(4):458. doi:10.1111/j.1365-2664.2008.03027.x.

11. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol. 2008;9(5):503–510. doi:10.1038/ni1582.

12. Michel T, Poli A, Cuapiou A, Briqueumont B, Isérentant G, Ollert M, Human Zf, CD56bright NK Cells: an Update. J Immunol. 2016;196(7):2923–2931. doi:10.4049/jimmunol.1502570.

13. Penczek A, Sienczyk M, Wirtz CR, Burster T. Cell surface cathepsin G activity differs between human natural killer cell subsets. Immunol Lett. 2016;179(80–84): doi:10.1016/j.imlet.2016.09.010.

14. Coca S, Perez-Piqueras J, Martinez D, Colmenarejo A, Saez MA, Vallejo C, Martos JA, Moreno M. The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma. Cancer. 1997. 79(12):2320–2328.

15. Ishigami S, Natsumtech I, Tokuda K, Nakajo A, Che I, Iwashige H, Aridome K, Hokita S, Aikou T. Prognostic value of intratumoral natural killer cells in gastric carcinoma. Cancer. 2000. 88(3):577–583.

16. Villegas FR, Coca S, Villarribua VG, Jiménez R, Chillón MJ, Jareño K, Zuil M, Callol L. Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. Lung Cancer Amst Neth. 2002. 35(1):23–28.

17. Nieto-Velázquez NG, Torres-Ramos YD, Muñoz-Sánchez JL, Estepa-Medrano C, Sánchez-López L, Gómez-Godoy I, Gómez-Cortés S, Moreno J, Moreno-Eutimio MA. Altered Expression of Natural Cytotoxicity Receptors and NKG2D on Peripheral Blood NK Cell Subsets in Breast Cancer Patients. Transl Oncol. 2016;9(5):384–391. doi:10.1016/j.tranon.2016.07.003.

18. Rocca YS, Roberti MP, Juliá EP, Pampena MB, Bruno L, Rivero S, Juliá EP, Pampena MB, Bruno L, Rivero S. Activating NK receptor is associated with impaired NK cell cytotoxicity in metastatic melanoma patients. Cancer Immunol Res. 2016;4(18):3923–3931. doi:10.1158/2326-6066.CIR-15-0757.

19. Konjević G, Mirjacić Martinović K, Vuletić A, Jović V, Jurisić V, Babović N, Spuzić I. Low expression of CD161 and NKGD2 activating NK receptor is associated with impaired NK cell cytotoxicity in metastatic melanoma patients. Clin Exp Metastasis. 2007;24(1):1–11. doi:10.1007/s10601-006-9043-9.

20. Mamessier E, Sylvin A, Bertucci F, Castellano R, Finetti P, Jacquemier J, Castellano R, Gonçalves A, André P, Romagné F, Cremer I, Avril M-F, et al. Phenotypic and functional characteristics of blood natural killer cells from melanoma patients at different clinical stages. PloS One. 2013;8(10):e76928. doi:10.1371/journal.pone.0076928.

21. Beldi-Ferchiou A, Lambert M, Dogniaux S, Vély F, Vivier E, Oliver D, Dupuy S, Levasseur F, Zucman D, Lébert C, et al. PD-1 mediates functional exhaustion of activated NK cells in patients with Kaposi sarcoma. Oncotarget. 2016;7(45):72961–72977. doi:10.18632/oncotarget.12150.

22. Liu Y, Cheng Y, Xu Y, Wang Z, Xu D, Li C, Peng J, Gao L, Liang X, Ma C. Increased expression of programmed cell death protein 1 on NK cells inhibits NK-cell-mediated anti-tumor function and indicates poor prognosis in digestive cancers. Oncogene. 2017;36(44):6131–6133. doi:10.1038/tj.2017.209.

23. Wang Y, Sun J, Gao W, Song B, Shao Q, Zhao L, Zhang Y, Wang Q, Zhang Y, Qu X. Preoperative Tim-3 expression on peripheral NK cells is correlated with pathologic TNM staging in colorectal cancer. Mol Med Rep. 2017;15(6):3810–3818. doi:10.3892/mmr.2017.6482.

24. Ip S, Gaiero E, Jimenez-Baranda S, Khan S, Ac A, Vek K, Ollert M, Bhardwaj N. Reversal of NK-Cell Exhaustion in Advanced Melanoma by Tim-3 Blockade. Cancer Immunol Res. 2014 Feb 11. doi:10.1158/2326-6066.CIR-13-0171.

25. Crome SQ, Nguyen LT, Lopez-Verres S, Yang SYC, Martin B, Yam JY, Johnson DJ, Nie J, Pniak M, Yen PH, et al. A distinct innate lymphoid cell population regulates tumor-associated T cells. Nat Med. 2017;23(3):368–375. doi:10.1038/nm.4278.

26. Du N, Zhou J, Lin X, Zhang Y, Yang X, Wang Y, Shu Y. Differential activation of NK cells by influenza A pseudotype H5N1 and 1918 and 2009 pandemic H1N1 viruses. J Virol. 2010;84(15):7822–7831. doi:10.1128/JVI.00609-10.

27. Zitvogel L, Tiseiere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Cancer. 2006;6(10):715–727. doi:10.1038/nrc1936.

28. Platonova S, Cherfils-Vicini J, Damotte D, Corsez L, Villiard V, Validire P, André P, Mc D-N, Alifano M, J-F R, et al. Profound coordinated alterations of intratumoral NK cell phenotype and function in lung carcinoma. Cancer Res. 2011;71(16):5412–5422. doi:10.1158/0008-5472.CAN-10-4179.

29. Le Maux Chanasc B, Moretta A, Vergnon I, Opolon P, Lécluse Y, Brunewald N, Kabin M, Soria J-C, Chouaib S, Mami-Chouaib F. NK cells infiltrating a MHC class I-deficient lung adenocarcinoma display impaired cytotoxic activity toward autologous tumor cells associated with altered NK cell-triggering receptors. J Immunol. 2005. 175(9):5790–5798.

30. Carrega P, Morandi B, Costa R, Frumento G, Forte G, Altavilla G, Ratto GB, Mingari MC, Moretta L, Ferlazzo G. Natural killer cells infiltrating human nonsmall-cell lung cancer are enriched in CD56 bright CD16(-) cells and display an impaired capability to kill tumor cells. Cancer. 2008;112(4):863–875. doi:10.1002/cncr.23239.

31. Bruno A, Focaccetti C, Pagani A, Imperatori AS, Spagnolletti M, Rotolo N, Catelmo AR, Franzì F, Capella C, Ferlazzo G, et al. The proangiogenic phenotype of natural killer cells in patients with non-small cell lung cancer. Neoplasia. N Y. N. 2013. 15(2):133–142.

32. Grzywacz B, Kataria N, Verneris MR. CD56(dim)CD16(+) NK cells downregulate CD16 following target cell induced activation of matrix metalloproteinases. Leukemia. 2007;21(2):356–359. doi:10.1038/leu.2004.499.

33. Romee R, Foley B, Lenvik T, Wang Y, Zhang B, Ankarlo D, Luo X, Cooley S, Vermeulen MJ, Walcheck B, et al. NK cell CD16 surface expression and function is regulated by a disintegrin and metalloprotease-17 (ADAM17). Int J. 2013;121(18):3599–3608. doi:10.1082/blood-2012-04-425397.
