

Article

# Dissecting the NK cell population in hematological cancers confirms the presence of tumor cells and their impact on NK population function

Running title: NK subsets and tumor cells

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**Abstract:** The lymphocyte lineage natural killer (NK) cell is part of the innate immune system and protects against pathogens and tumor cells. NK are the main cell effectors of monoclonal antibodies (mAbs) generating antibody-dependent cell cytotoxicity (ADCC). Hence it is relevant to understand NK physiology and status to investigate the biological effect of mAbs in the clinic. NK cells are heterogeneous with multiple subsets that may have specific activity against different attacks. The presence of viral-sculpted NK cell populations has already been described, but the presence of cancer-sculpted NK remains unknown. Cancer induces a broad NK cell dysfunction, which has not been linked to a specific population. Here we investigated the NK cell population by Uniform Manifold Approximation and Projection (UMAP) embed maps in Hodgkin lymphoma (HL) and acute myeloid leukemia (AML) patients at diagnosis and at least 30 days after treatment, which correlates with tumor cell clearance. We found that the NK lineage largely responded to the tumor by generating antitumor NK cells and renewing the population with a subset of immature NK cells. However, we failed to identify a specific “memory-like” subset with the NK cell markers used. Moreover, in patients in relapse we found essentially the same NK populations that those found at diagnostic, suggesting that NK cells equally respond to the first or second tumor rise. Finally, we observed that previous CMV infection largely affects the tumor-associated changes in NK population, but the CMV-associated CD57<sup>+</sup>NKG2C<sup>+</sup> NK cell population does not appear to play any role in tumor immunity.

Keywords: NK cells; UMAP; trogocytosis; AML; Hodgkin lymphoma

## 1. Introduction

The innate lymphocyte lineage natural killer (NK) cell protects against multiple pathogens and tumor cells <sup>1</sup>. The NK cell compartment is very heterogeneous <sup>2,3</sup> and contains more subsets than originally thought <sup>1</sup>. An individual may have more than 30,000 distinct NK cell phenotypes in blood <sup>2</sup>. Some of them are “natural”, as they are present in multiple healthy individuals. In contrast, others could have been induced by environmental factors, such as viral infection, and other pathological conditions, such as the tumor microenvironment <sup>4</sup>. Identification of new subsets that play, or can play, a significant role in pathological conditions is relevant to better understand NK cell physiology. There is an antitumor subset that has been described in several hematological cancers, including AML. This subset is characterized by expression of CD45RO (CD45RO cells) mostly together with CD45RA (CD45RARO cells). CD45RARO cells contain the cells that have degranulated and

performed trogocytosis of tumor-expressed antigens<sup>5-7</sup>. Trogocytosis is a process via which NK cells acquire antigens from targets through membrane exchange, implying a close contact with tumor cells. This, together with the fact that these NKs have degranulated, strongly suggests that these cells represent an anti-tumor population. However, if this antitumor NKs have effectively killed cancer cells is unknown. At the time of diagnosis, most hematological cancer patients, including acute myeloid leukemia (AML) patients, showed high levels of these CD45RARO cells. This increase was paralleled by a decrease in the CD45RA<sup>+</sup>RO<sup>-</sup> population (CD45RA cells)<sup>5-7</sup>. After treatment and tumor clearing, there was a marked decrease in the percentage of CD45RARO, which is logically associated to a decrease of NK cells that had performed trogocytosis or degranulated. The presence of this antitumor NK population has not been studied in Hodgkin lymphoma (HL) patients.

The impact of disease in NK subsets is not exclusive of cancer. Other diseases including infection and autoimmunity induce impaired NK function and the emergence of NK subsets rarely observed in healthy individuals<sup>8</sup>. However, little is known about the fate of the disease-specific subsets. One exception is virus-associated memory, or adaptive, NK cells. For example, human cytomegalovirus (HCMV) infection promotes expansion of NKG2C<sup>+</sup> NK cells with memory-like properties and the presence of NKG2C<sup>+</sup>CD57<sup>+</sup> NK cells is associated with prior CMV infection<sup>9,10</sup>. However, it is unknown if these NK have a specific activity against HCMV itself or if they are specifically activated after HCMV reactivation<sup>11</sup>. Previous viral infection can affect the NK response to a second danger such as tumor growth. For example, most glioblastoma express CMV proteins and CMV infection can imprint NK cells, perhaps because CMV alters the distribution of KIR–HLA ligand interactions and this modulates NK cell function<sup>12</sup>. In addition, KIR2DS2<sup>+</sup> and KIR2DS4<sup>+</sup> NK are more potent killers against glioblastoma cells than NK negative for those receptors<sup>13</sup>. Furthermore, the presence of the KIR allele KIR2DS4\*00101 is prognostic of prolonged survival in glioblastoma patients<sup>12</sup>. During the acute phase of Epstein Barr virus (EBV) infection there is accumulation of a proliferative NKG2A<sup>+</sup>KIR-CD57<sup>-</sup> NK cell subset that remains elevated up to 6 months after the acute symptomatic phase<sup>14</sup>. Hence, 2 different types of herpesviruses, i.e. HCMV and EBV, promote expansion of different NK cell subsets. Another example of long-term atypical NK distribution is found in HIV-infected humans with the expansion of anergic CD56<sup>-</sup>/CD16<sup>+</sup> NK<sup>15</sup>.

In cancer patients, the phenotype of the associated NK cell subsets may vary according to the tumor and its anatomical location<sup>16,17</sup>. The continuous presence of tumor cells impairs NK cell function<sup>18-20</sup>, which, in turn, helps tumor immune escape<sup>21,22</sup>. However, the persistence of specific subsets linked to the disease has not been shown yet.

Whereas NK are considered part of the innate immune system, they are lymphocytes, an immune population that contains the effector adaptive immune cells, i.e. T and B cells. These lineages rapidly react to the presence of targets. NK can mediate adaptive-like immune responses in humanized mice<sup>23</sup>, but how the whole NK population adapts to the chronic presence of targets such as tumor cells in humans is unknown. We investigated it in HL and AML patients by using multiparameter flow cytometry and processed data by Uniform Manifold Approximation and Projection (UMAP) maps<sup>24</sup>. We found that NK largely react to the chronic presence of targets with totally new populations appearing. In addition, we show that disease-related subsets profoundly evolve when targets disappear. This suggests that these populations depend on the presence of targets and the presence of long-lasting populations is unclear.

## 2. Materials, Subjects and Methods

### 2.1. Ethical statement

The use of human specimens for scientific purposes was approved by the French National Ethics Committee. All methods were carried out in accordance with the approved guidelines and regulations of this committee. Written informed consent was obtained from each patient or donor prior to surgery.

## 2.2. HL and AML patients

Data and samples from patients were collected at the Clinical Hematology Department of the CHU Montpellier, France, after patient's written consent and following French regulations. Patients were enrolled in the HEMODIAG\_2020 (ID-RCB: 2011-A00924-37) clinical program approved by the "Comités de Protection des Personnes Sud Méditerranée I" with the reference 1324. Samples were collected at diagnosis and kept by the CHU Montpellier<sup>6,25,26</sup>. Peripheral blood mononuclear cells (PBMCs) were obtained by ficoll gradient and stored frozen in liquid nitrogen until use.

## 2.3. Healthy donor (HD)

HD samples were obtained from written informed donors and collected by clinicians of the CHU Montpellier and collected and processed as patient's samples.

## 2.4. Clinical criteria for patients CMV seropositive

Previous CMV infection was resolved as follows: IgG < 12 U/mL => negative; IgG > 14 => positive; IgM < 18 => negative; IgM > 22 => positive; IgG + / IgM - => Profile for an old infection; IgM + => First infection or recent reactivation; IgG- IgM - => never in contact. At the moment of sampling, none of patients presented symptoms of CMV reactivation, however, patients were not properly tested to exclude this.

## 2.5. FACS analysis and antibodies

In brief, healthy donors and patient's total PBMC were stained for surface markers with the following fluorochrome conjugated antibodies: CD19-BUV737, CD107a-BUV395, CD3-BV786, CD33-BV711 & CD14-BV650 (for AML study), CD15-BV650 & CD30-BV605 (for HL study), PD1-FITC, CD62L-PerCP-Cy5.5, CD57-PE-CF594, CD16-AF700 from Beckton Dickinson, CD69-PE from Beckman Coulter, CD45RO-VioGreen, CD7-VioBlue, CD56-PE-Vio770, CD45RA-APC-Vio770 and NKG2C-APC from Miltenyi Biotec. Cell viability was determined using DAPI (BD Biosciences). Cells were stained with antibodies cocktail in FACS buffer (PBS, 2% FBS) on ice for 25-30 min and washed twice with the same FACS buffer and acquired on BD LSR-Fortessa instrument (Blue-Yellow/Green-Red-Violet-Ultraviolet) (BD Bioscience). FCS files were analyzed using FlowJo software v10.6.1 (Tree Star Inc.). The gating strategy for conventional flow cytometry and the determination of the "percentage" of selected cells in the figures is described in the supplemental Fig. 1.

## 2.6. High dimensional reduction analysis

To generate tSNE or UMAP embedding, a pre-gated NK cell population from each sample with the same number of cells per patient and timepoint was selected using FlowJo Downsample plugin (v3.1.0) and merged before loading into the respective high dimensional algorithms (Flt-SNE plugin v0.5, UMAP v2.2)<sup>27</sup>. All processes were performed under FlowJo software v10.6.1.

## 2.7. Correlation matrix analysis

The relationship between percentages of various NK cell and tumor cell populations was analyzed by Pearson correlation method with confidence interval (CI)= 95% and the result correlation matrix was visualized using the package corrplot (v0.84) in R environment (v3.6.1).

## 2.8. Statistical analysis

Experimental figures and statistical analysis were performed using GraphPad Prism (v8.0). All statistical values are presented as \* p<0.05; \*\* p<0.01; \*\*\* p<0.001 and \*\*\*\* p<0.0001. Mean values are expressed as mean plus or minus the standard error of the mean (SEM).

### 3. Results

#### 3.1. Antitumor NK cells in HL patients are identified by CD45RARO and CD107 expression.

To investigate how the chronic presence of tumor target cells and their subsequent clearance after treatment affect the NK cell population, we used a cohort of 21 patients from the Hematology Department, CHU Montpellier, France with Hodgkin Lymphoma (HL). We collected and analyzed blood samples at diagnosis and after treatment and compared them to those of healthy donors (HD). The treatment regime for these patients varied in terms of the chemotherapy used and the combination with radiotherapy (table 1). Since these patients received a variety of treatments we could not subgroup them based on treatment type with very few individuals and we analyzed the whole cohort. To analyze the NK cell population in these patients, we performed analysis based on both manual gating strategies and unsupervised UMAP mapping approach (Supplemental Fig. 2A). NK cells are usually detected by CD56 expression. However, some myeloid subsets can express CD56<sup>28</sup>. In agreement with previous results<sup>29</sup>, more than 90% of CD56<sup>+</sup> cells in our samples expressed CD7 (supplemental Fig. 3). Hence, we used CD7 as a marker of the lymphoid lineage to identify *bona fide* NK cells and analyzed CD7<sup>+</sup>/CD56<sup>+</sup> cells<sup>28</sup>.

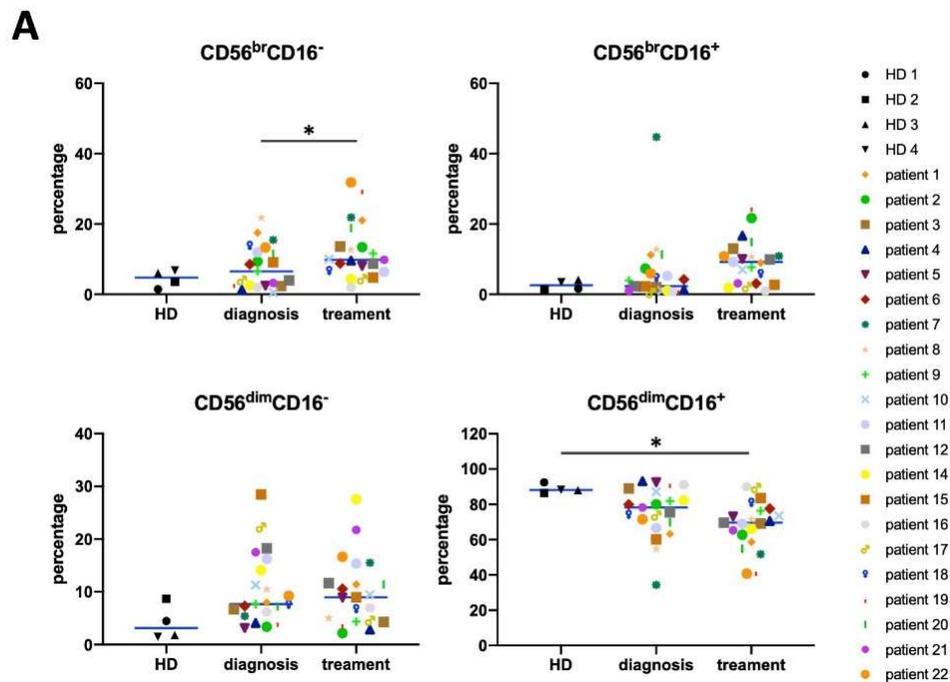
**Table 1. Clinical characteristics of the HL cohort.** The table describes the patients' subtype according to the Ann Arbor scoring system, the treatment and the outcome. The cohort of 22 patients is composed of 12 men (55%) and 10 women (45%). Patients were between 19 and 69 years old.

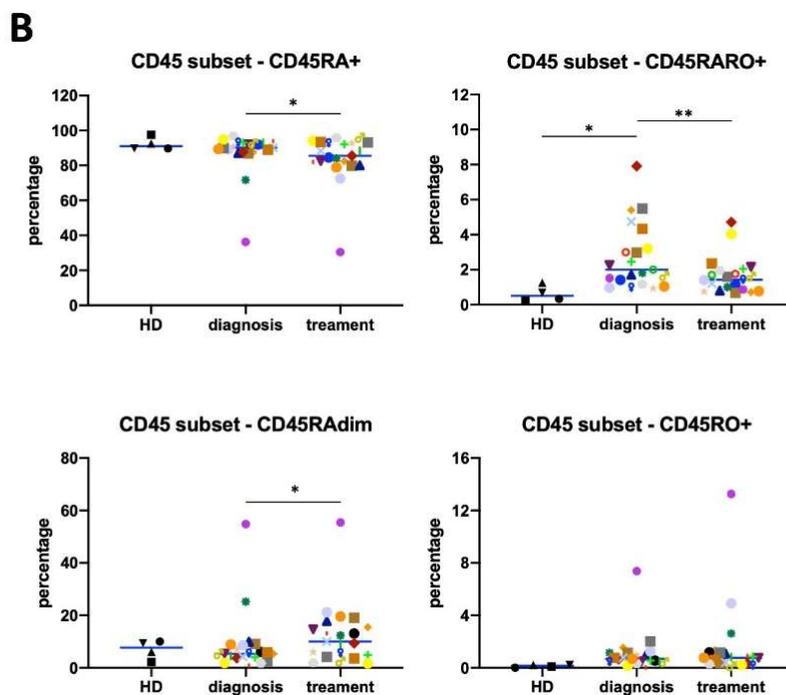
Patient number	Stage at diagnosis (Ann Arbor)	First treatment	Response (Lugano)	Relapse	First treatment on relapse	Response at the end of treatment	Second treatment on relapse	Last medical status	CMV / HIV
1	2	ABVD x3 + radiotherapy	CR	No	-	-	-	CR	na/-
2	3	BEACOPP x6 + AVD x2	CR	No	-	-	-	CR	-/-
3	1	ABVD x2 + R-DHAC x4 + BEAM + autograft	CR	No	-	-	-	CR	-/-
4	4	PVABx6	PR	Yes	DHAC x6	Progression	Brentuximab+ Bendamustine x6	CR	+/-
5	4	BVAPx6	CR	No	-	-	-	CR	+/-
6	3	BEACOPP x2 + ABVDx4	PR	No	-	-	-	PR	-/-
7	4	EACOPP x6	CR	No	-	-	-	CR	na/-
8	4	PVAB x4	CR	Yes	R-DHAC x4 + autograft	Progression	R-CHOP x4	CR	-/-
9	2	ABVD x3 + radiotherapy	CR	No	-	-	-	CR	na/-
10	3	BEACOPP x2 + ABVD-MP x4	CR	No	-	-	-	CR	-/-
11	1	ABVD x8	CR	No	-	-	-	CR	-/-
12	2	ABVD x4 + radiotherapy	CR	No	-	-	-	CR	-/-
13	3	ABVD-MP x6	CR	Yes	DHACx4	Progression	Brentuximab + bendamustine	CR	na/-
14	4	PVAB x6	CR	No	-	-	-	CR	+/-
15	2	ABVD x4 + radiotherapy	CR	No	-	-	-	CR	na/-
16	2	ABVD x6 + radiotherapy	PR	No	-	-	-	PR	na/-
17	2	ABVD x3 + radiotherapy	CR	No	-	-	-	CR	+/-
18	2	ABVD x4 + radiotherapy	CR	No	-	-	-	CR	+/-
19	3	PVABx6	CR	Yes	Nivolumab x13	CR	-	CR	na/-
20	1	ABVD x3 + radiotherapy	CR	No	-	-	-	CR	na/-
21	4	BEACOPP x2 + ABVD x4	CR	No	-	-	-	CR	na/+
22	4	BEACOPP x2 + ABVD x4	CR	No	-	-	-	CR	-/-

List of abbreviations: Complete Response to treatment (CR), Partial Response (PR). Treatments: BEACOPP = Bleomycin + Etoposide + Adriamycin + Cyclophosphamide + Vincristine + Prednisone + Procarbazine; EACOPP = BEACOPP without bleomycin; ABVD = Doxorubicin + Bleomycin + Vinblastine + Etoposide + Cytarabine + Melphalan; R-DHAC = Rituximab + Carboplatine + Cytarabine + Dexamethasone + Dacarbazine; PVA = Prednisone + Vinblastine + Doxorubicin; PVAB = PVA+ Bendamustine; MP = Melphalan + Prednisone ; BEAM = Carmustine; R-CHOP = Rituximab + Cyclophosphamide + Doxorubicin + Vincristine + Prednisone.

In general, HL patients show low numbers of tumor Reed Sternberg cells (RS), which are usually defined by CD15 and/or CD30 expression, in blood <sup>30</sup>. In agreement, we also found few CD15<sup>+</sup>/CD30<sup>+</sup> cells in our cohort (supplemental Fig. 2B and C), although we cannot affirm that these cells are tumor cells. Treatment modified the % of CD15<sup>+</sup>/CD30<sup>+</sup> cells, but the effect was highly variable, with patients showing increasing values while in others there was a decrease (supplemental Fig. 2B and C). This heterogeneity was also observed in B, T and NK cells, although T cell numbers usually decreased (supplemental Fig. 2C). The % of T cells also showed a tendency to decrease, whereas the % of NK increased (supplemental Fig. 2B).

Next, we analyzed changes in NK phenotype after patient treatment using a manual gating strategy. CD56<sup>bright</sup> cells represent an immature phenotype whereas CD56<sup>dim</sup>CD16<sup>+</sup> cells represent the most mature subpopulation <sup>5-7</sup>. Patients at diagnosis showed a tendency to decrease the % of mature NK cells compared to HD and this correlated with an increase in the most immature CD56<sup>bright</sup> subpopulation (Fig. 1A). After treatment, these changes were statistically significant. This suggests a most immature state of the NK cell compartment after treatment, as previously observed in diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) patients <sup>7</sup>.





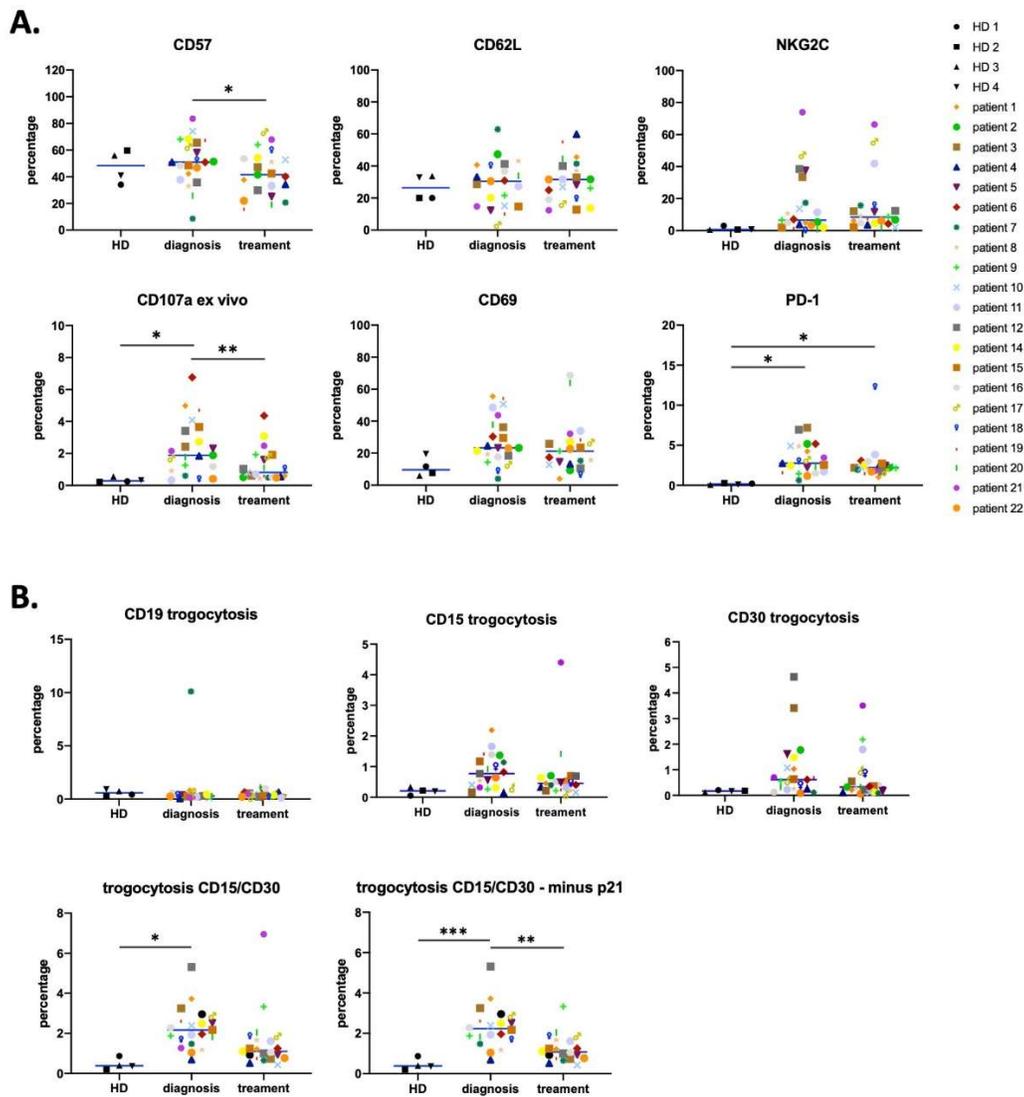
**Figure 1. HL NK cell subsets change after treatment.** NK cell phenotypes were analyzed regarding expression of CD56 and CD16 (A), or CD45RA and CD45RO (B). Statistical significance between HD (n=4) versus patients (n=21) were determined by one-way ANOVA (Dunnett's correction). Statistical significance between "diagnosis" and "after treatment" for each patient was compared by the paired t-test, n = 21, \* p ≤ 0.05, \*\* p ≤ 0.01 and \*\*\* p ≤ 0.001.

The antitumor NK cell population is recognized by expression of CD45RO (CD45RO cells) in general together with CD45RA (CD45RARO cells; <sup>5-7</sup>. At the time of diagnosis, hematological cancer patients show high levels of these cells together with CD45RO<sup>+</sup> cells, generally leading to a decrease in the CD45RA<sup>+</sup>RO<sup>-</sup> population (CD45RA cells) <sup>5-7</sup>. Patients in our cohort clearly showed this phenotype (Fig. 1B). At the end of treatment CD45RARO cells decreased. Taken together, these data suggest that elimination of target cells decreases NK cell activation status. The population that significantly increased was CD45RADimRO<sup>-</sup>, which is immature and are normally CD56<sup>bright</sup> <sup>5,6</sup>. This could partially explain the increase in immature NK cells (Fig. 1A).

We next analyzed expression of several NK markers. During *in vivo* maturation CD56<sup>bright</sup> cells become CD56<sup>dim</sup>CD62L<sup>+</sup>CD57<sup>-</sup> cells that produce perforin, while maintaining high IFN- $\gamma$  production in response to cytokines <sup>31,32</sup>. On the other hand, CD56<sup>dim</sup>CD62L<sup>-</sup>CD57<sup>+</sup> cells show lower responsiveness to cytokines and higher cytotoxic capacity <sup>31,33</sup>. CD69 is an activation marker <sup>5-7</sup>. NKG2C<sup>+</sup> NK cells accumulate in cytomegalovirus (CMV)-seropositive human adults <sup>9</sup> and NKG2C could be a marker of memory NK cells <sup>23</sup>. The presence of CD107 on the cell surface *ex vivo* is a sign of *in vivo* degranulation of NK cells <sup>5-7</sup>. PD-1 is an immune checkpoint, which is absent on NK cells isolated from healthy donors but it is expressed on those from certain hematological cancer patients <sup>34</sup>. However, NK cells from patients show a large heterogeneity of PD-1 expression and only a few of them constitutively express PD-1 <sup>7</sup>.

NK cells from patients did not statistically change expression of CD57, CD69, CD62L and NKG2C compared to those from HD (Fig. 2A). However, we observed a large heterogeneity, with some patients that largely increased the percentage of NKG2C<sup>-</sup> and CD69-expressing NK cells. Interestingly, in patients there were increased numbers of NK cells expressing CD107a compared to HD. Our data demonstrated that the NK population in HL is affected by the presence of tumor cells

as previously described in other hematologic cancers<sup>5-7</sup>. Surprisingly, certain patient's NK cells expressed PD-1, suggesting that they can be inhibited by PD-L1 expressing cells.



**Figure 2. NK cell phenotype analyzed by manual gating.** A) Percentage of cells expressing PD-1, CD69, NKG2C, CD57, CD62L and CD107a (degranulation ex vivo) on NK cells from HD and from HL patients at diagnosis and after treatment. B) Trogocytosis on tumor surface markers, i.e. CD19 (negative control), CD15 and CD30 (RS markers) by NK cell before and after treatment. In the bottom left graph, we represented NK cells expressing CD15 and/or CD30. The right graph represents the same but excluding patient 21, who was HIV+. Statistical significance between HD versus patients were determined by one-way ANOVA (Dunnett's correction), "diagnosis" versus "after treatment" for each patient was compared by paired t-test,  $n = 21$ , \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$  and \*\*\*  $p \leq 0.001$ .

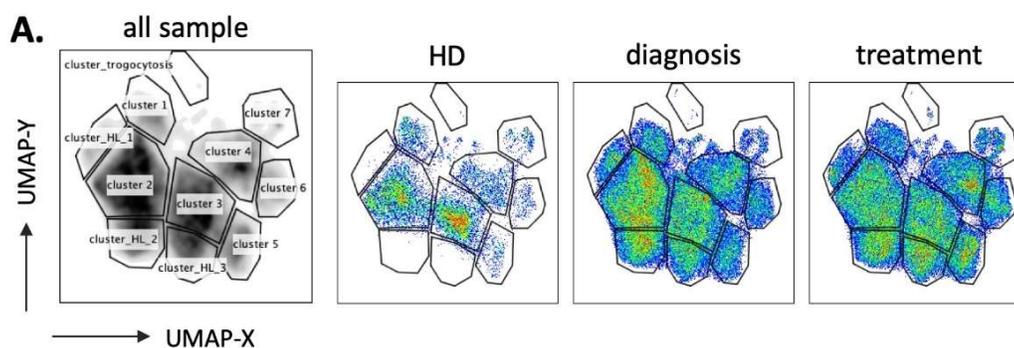
After treatment, the proportion of NK cells expressing CD57 decreased (Fig. 2A). This supports again an increase in the immature subset of the NK compartment. Percentages of CD62L, CD69 and NKG2C expressing cells did not change. On the other hand, PD-1 expression tended to decrease (Fig. 2A). Hence, the continued renewal of NK cells might preclude a larger PD-1 expression on NK cells

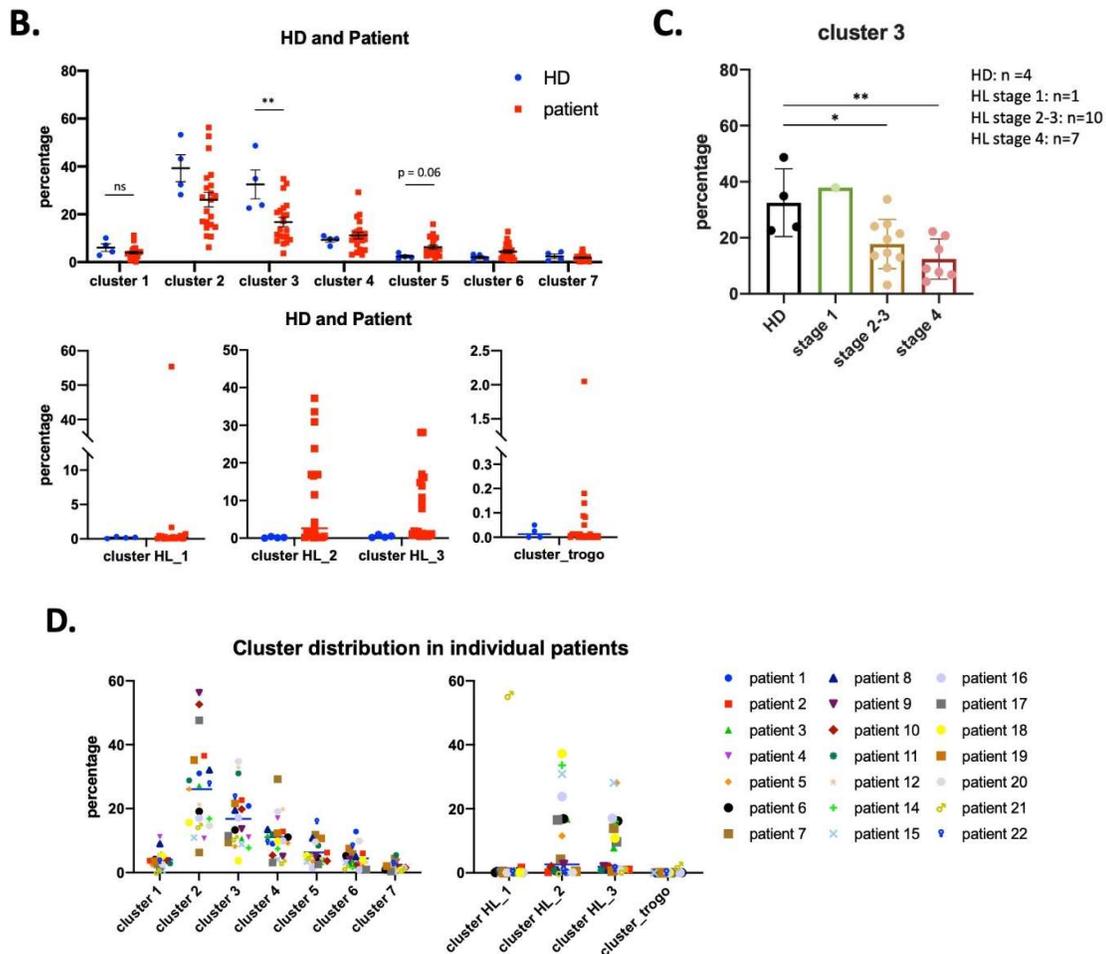
in some patients. In agreement with our previous observations<sup>5-7</sup>, the decrease of tumor target cells after treatment diminished CD107 expression on NK cells.

To further investigate the antitumor NK cell population, we looked at the gain of tumor markers by trogocytosis<sup>5-7</sup>. RS tumor cells usually express CD15 and CD30<sup>35</sup>, but lack CD19 or CD20 expression<sup>30</sup>. Interestingly, the percentage of NK cells carrying CD19 did not change in patients before or after treatment (Fig. 2B). In contrast, we observed that NK cells gained the CD15 and CD30 RS markers by trogocytosis. Differences were statistically significant when we analyzed cells that had trogocytosed on either CD15 or CD30 or both (Fig. 2B). The percentage of NK that have trogocytosed dropped after treatment. The degree of significance largely increased when patient 21 was taken out of the statistical analysis. In contrast to other patients, this patient showed a large increase in trogocytosis after treatment, and was the only one in the cohort that was HIV+ (Table 1). In summary, we found a contraction of the antitumor NK cell population after treatment, as we had previously observed in DLBCL and FL patients<sup>7</sup>. We next stratified patients regarding their treatment and analyzed their response (supplemental Fig. 4A). All groups equally responded to treatment regarding decrease in CD45RARO population, CD107a expression and trogocytosis. This shows that different treatments induced similar effects and supports the notion that a specific treatment does not lead to our observations.

### 3.2. UMAP (Uniform Manifold Approximation and Projection) identifies new NK cell subsets

To more precisely characterize how the presence of target cells, i.e. at diagnosis, or their absence, i.e. after treatment, affect the NK cell population, we used the high dimensional reduction algorithm UMAP to generate unsupervised UMAP embed maps with 14 NK surface markers (supplemental Fig. 5 and Fig. 3). As described in Figure 3A, we generated a UMAP plot for all patients at the 2 timepoints (n=42) and the 4 healthy donors (HD, n=4). Each sample contained 4000 NK cells. From this original map, we derived 3 daughter maps only showing the events from HD, patients at diagnosis or after treatment (Fig. 3A). We identified 7 clusters common for both HD and patients (1 to 7) and 4 clusters that exclusively appeared in patients (HL\_1 to HL\_3 and cluster trogocytosis). Cluster HL\_1 was almost exclusive of patient 21, who was HIV seropositive. Interestingly, this cluster contained mainly adaptive-like NK cells (CD57<sup>+</sup>NKG2C<sup>hi</sup>) (Supplemental Fig. 6 and Figs. 3B and 4C).



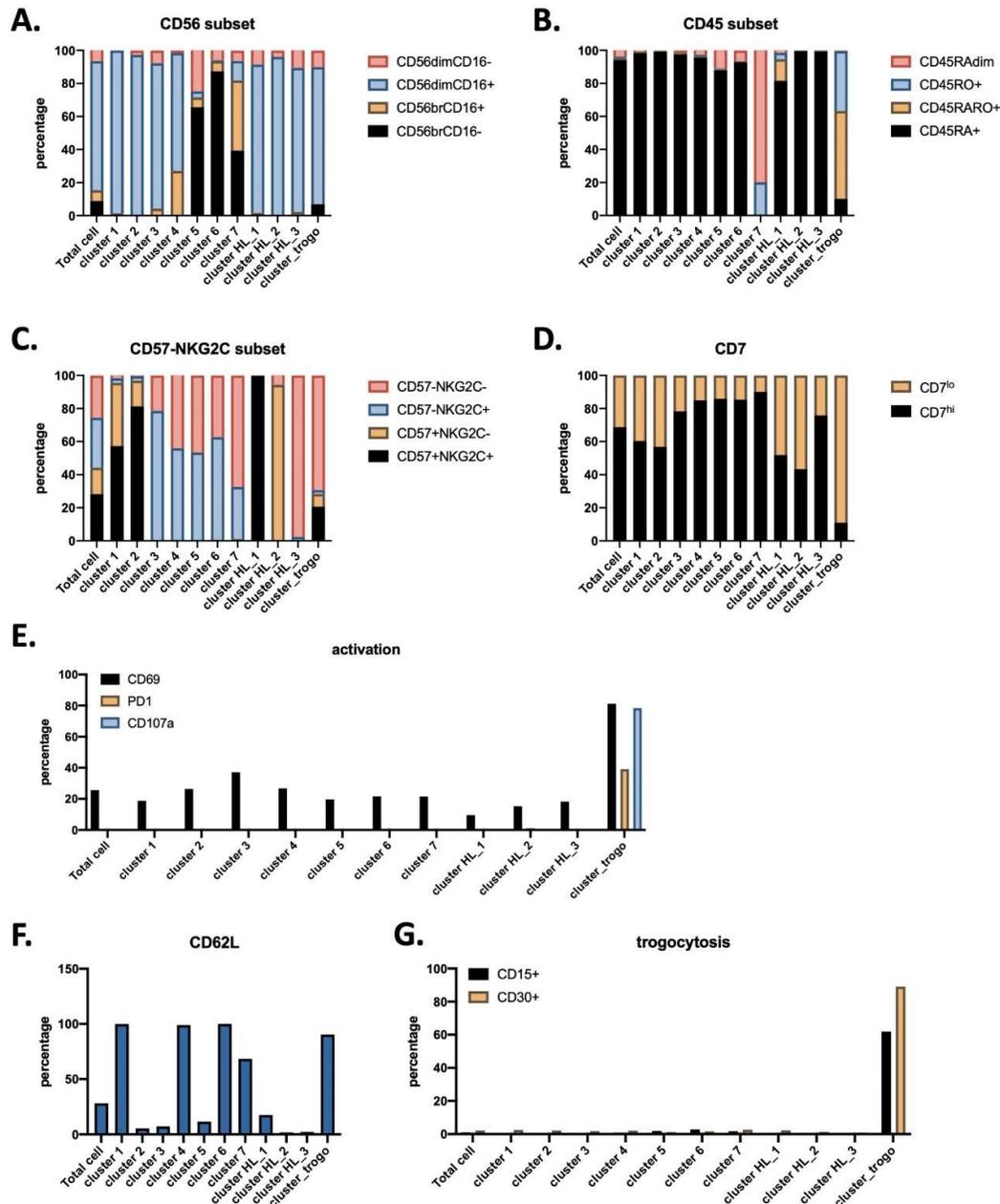


**Figure 3. Identification of NK cell clusters in HL patients via UMAP.** 4000 representative NK cells from HD (n=4) and HL patients (n=21, 2 timepoints per patient) were combined to generate an UMAP embed from expression level of 14 surface markers (total = 46). A) Original UMAP plot of all samples and daughter maps from HD, HL patients at diagnosis and HL patients after treatment. B) Each cluster was assigned an annotation and subsequently quantified in each HD and patient. C) Proportion of cluster 3 plotted against HD and various HL stages. D) Cluster quantification as shown in B with patient ID labelled. Statistical significance determined by unpaired t-test, n = 21, \* p ≤ 0.05, \*\* p ≤ 0.01 and \*\*\* p ≤ 0.001, unless exact value otherwise indicated.

Only cluster 3 significantly decreased in patients (Fig. 3B), whereas cluster 5 tended to increase. Cluster 3 mainly comprised CD56<sup>dim</sup>CD16<sup>+</sup> subset with CD57<sup>-</sup>NKG2C<sup>low</sup>CD7<sup>hi</sup> phenotype (Fig. 4). Interestingly, cluster 3 decreased more in advanced stages of HL (Fig. 3C). The specific HL\_ clusters were present in some, but not all, patients (Fig. 3B and 3D). This suggests that they are not representative of a “general” HL-associated NK population, but mainly of the status or the specific conditions of the patient.

To further interrogate the phenotype of these clusters, we plotted the heatmap of expression on each surface marker that constituted the UMAP embed map (supplemental Fig. 5). Clusters 1 to 4 represent mature CD56<sup>dim</sup>CD16<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup> populations (Fig. 4). Clusters 5 to 7 represent immature CD56<sup>bright</sup>CD16<sup>-/low</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup> populations, with cluster 7 containing the CD45RA<sup>dim</sup> population specific of hematological cancer patients<sup>5-7</sup>. The HL\_1 to 3 represent populations with CD56<sup>dim</sup>CD16<sup>low</sup> that can be discriminated by the expression of two markers NKG2C and CD57. HL\_1 expressed both, HL\_2 expressed only CD57 and HL\_3 did not express any of them. Finally, the antitumor population was found in a specific HL cluster that we called cluster

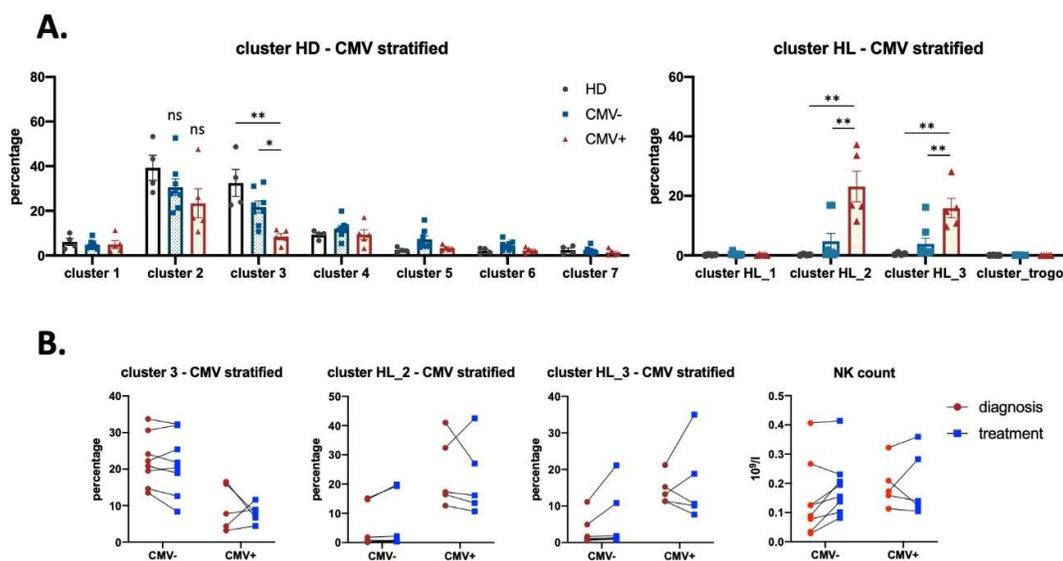
trogocytosis:  $CD56^{dim}CD16^{+}CD45RA^{low}CD45RO^{+}CD30^{+}CD15^{+}CD107^{+}PD-1^{+}$ . After treatment this cluster decreased when we removed the HIV+ patient 21 from the analysis (supplemental Figs. 6 and 7). Additionally, clusters 5 and 7 also changed, with a significant increase in the 7, which contained the immature  $CD45RA^{dim}$  cells (supplemental Figs. 6 and 7). Hence, the immature population increased after treatment although the exact markers are patient specific. Finally, correlation analysis showed a negative relationship between cluster 3 and clusters HL\_2/3 (supplemental Fig. 8). The trogocytosis cluster positively correlated with  $CD15^{+}/CD30^{+}$  cell counts.



**Figure 4. Phenotype of NK cell clusters identified via UMAP.** A-G) characterization of NK phenotype regarding to CD56 subsets (A), CD45 subsets (B), CD57 vs NKG2C (C), CD7<sup>hi</sup> (immature) vs CD7<sup>lo</sup> (mature) (D), activation markers (i.e. CD69, PD1, CD107a ex vivo) (E), CD62L (F) and trogocytosis markers (G).

### 3.3. Effect of CMV infection on NK subsets in HL patients

Several lines of evidence suggest that viral infection, i.e. EBV, CMV or HCV, influences NK repertoire<sup>36-39</sup>. In addition, a possible link between CMV infection and Hodgkin's lymphoma has been suggested<sup>40</sup>. Therefore, we examined the impact of previous CMV infection on the NK cell compartment in our HL cohort, which we divided in CMV-negative (patients 2, 3, 6, 8, 10, 11, 12 and 22, n=8) and CMV-positive (patients 4, 5, 14, 17 and 18, n=5) groups (table 1). None of the patients had symptoms of CMV reactivation at the moment of sampling. We used the same UMAP and analyzed CMV<sup>+</sup> vs CMV<sup>-</sup> patients. Cluster 3 was significantly lower and clusters HL\_2 and 3 were significantly higher in seropositive patients (Fig. 5A). However, treatment did not differently change these clusters in both type of patients (Fig. 5B). It is interesting to note that cluster 3 is mainly composed of CD57<sup>-</sup>NKG2C<sup>+</sup> cells, whereas clusters HL\_2/3 are NKG2C<sup>-</sup>. Hence, in HL, previous CMV infection does not seem to correlate with an increase in NKG2C<sup>+</sup> populations.



**Figure 5. Analysis of NK cells from HL patients stratified by CMV serotypes.** Based on CMV serotype, we analyzed separately NK cell phenotype of CMV<sup>-</sup> patients (n=8) and CMV<sup>+</sup> patient (n=5). A) NK cluster quantification between HD and both CMV<sup>-/+</sup> groups. B) Change in NK cluster proportion before and after treatment in the 2 CMV groups.

We analyzed single markers by manual gating in both CMV serotypes (supplemental Figs. 9 and 10). Treatment induced a higher increase of the CD45RA<sup>dim</sup> subset in the CMV<sup>-</sup> group and more interestingly a higher decrease of the antitumor CD45RARO subset (supplemental Fig. 9B). It is tempting to speculate that CD45RARO cells are kept in CMV<sup>+</sup> patients to fight against CMV-infected cells. In agreement with this idea, CMV<sup>-</sup> serotype showed a decrease in PD-1, CD69 and CD107a after treatment that was absent in CMV<sup>+</sup> serotype (supplemental Fig. 10A). Moreover, treatment decreased trogocytosis mainly in the CMV<sup>-</sup> serotype (supplemental Fig. 10B). This analysis also validated that seronegative patients had larger numbers of NKG2C<sup>+</sup> NK cells (supplemental Fig. 10A). In contrast, CMV serotype did not change the percentage of CD62L and CD57 positive cells. In summary, NK cell remodeling after anticancer treatment depends on CMV serotype and, remarkably, CMV<sup>-</sup> patients underwent a more robust shift in the NK cell compartment following treatment. Finally, we could analyze 2 patients in relapse: patient 4 (CMV<sup>+</sup>) and patient 8 (CMV<sup>-</sup>). Supplemental Fig. 11 showed that both patients equally recovered HL\_ clusters after relapse.

### 3.4. NK cells subsets in AML patients.

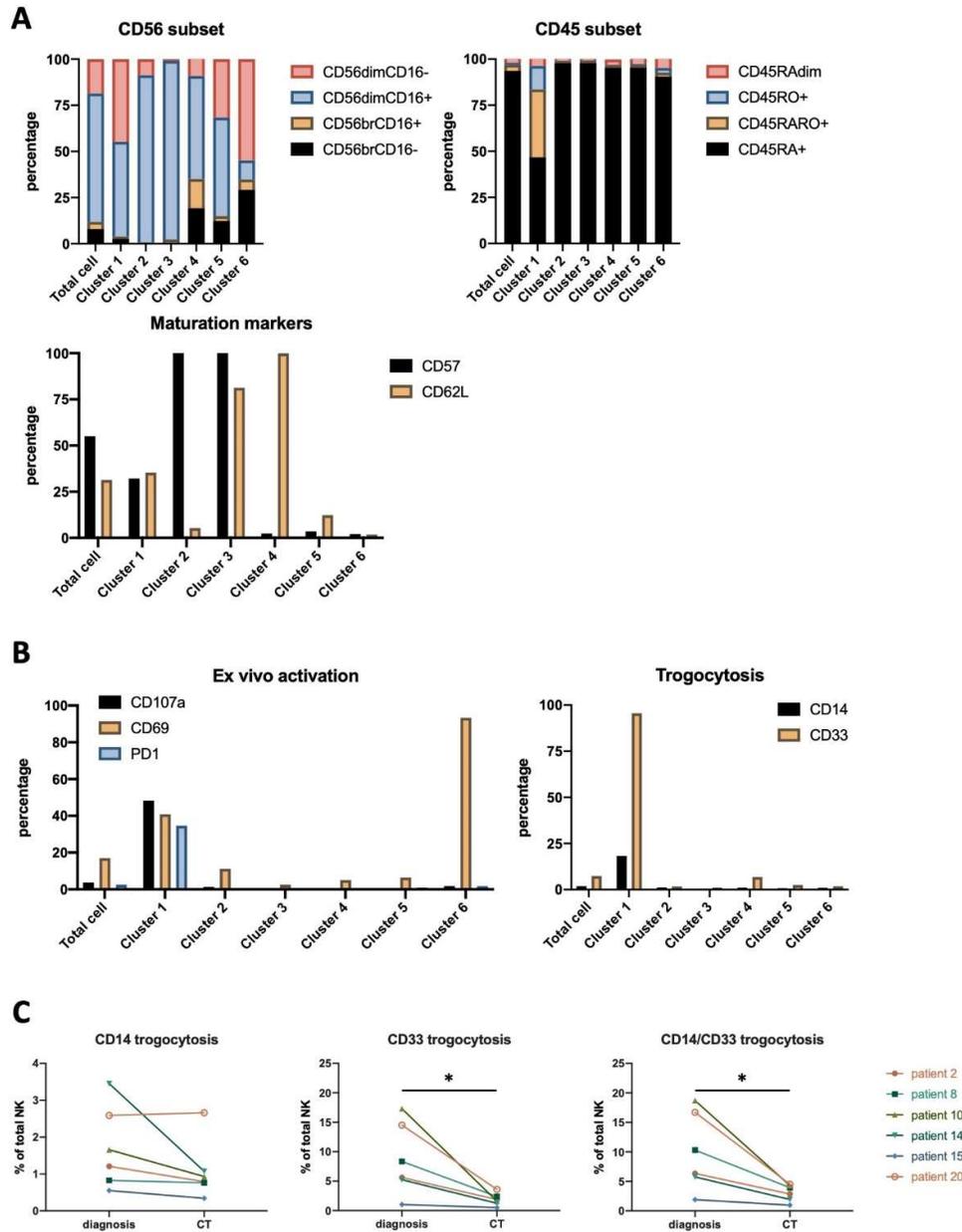
We had previously identified the antitumor NK cell population in AML patients<sup>5,6</sup>. As previously described, the NK compartment is enriched in CD45RARO and CD45RO cells that have degranulated, as evidenced by CD107a, and have performed trogocytosis. We investigated here the NK phenotypes linked to the presence of target cells. at the time of diagnosis, or their absence at the end of treatment. Thus, we collected and analyzed blood samples from 6 patients both at diagnosis and  $\geq 30$  days after chemotherapy treatment. For one patient, we got an additional sample after relapse, which occurred 6 months after the end of treatment. Table 2 describes patients' stage and treatment, which involved different chemotherapies. In AML the efficacy of treatment can be followed by the decrease in blood of myeloid CD33+ blasts, which can also express CD14 (supplemental Fig. 12A). CD33 is a myeloid marker commonly used for the diagnosis of AML and CD14 can be expressed in some AML types<sup>41</sup>. After treatment, NK cells decreased in patients with previous high blast numbers and increased in those with low numbers. Blast reduction was also observed using UMAP embed maps (supplemental Fig. 12B). To unveil NK cell subsets affected by the presence of target cells, we generated a UMAP embed map from total samples in which 2900 representative NK cells from each sample were included. NK cell subpopulations in the final UMAP map were clustered based on their similarities in the level of expression of 12 surface markers (supplemental Fig. 13 and Figure 6A, left panel). To assess the NK cell phenotypes associated with different disease stages, we extracted from the map containing all samples either those events from samples taken at diagnosis or from samples taken after treatment. This way, two additional daughter maps were generated describing diagnosis-related and post-treatment-related NK cell (Figs. 6A and B). We identified 6 clusters with notable differences in density (Fig. 6B). To further verify the differences in those 6 clusters between diagnosis and treatment samples, the percentage of cells in each of these clusters were quantified for the 6 patients (Fig. 6C). This revealed a decrease in cluster 1 and 2 and an increase in cluster 4 after treatment. Changes in other clusters were patient-dependent without a common pattern.

**Table 2. Clinical characteristics of the AML cohort.** Patients' subtype according to the French-American-British (FAB) classification, treatment and outcome of the AML cohort use in this study. Cohort of 6 patients is composed of 5 men (83%) and 1 woman (17%). Patients were between 25 and 59 years old. List of abbreviations: Complete Response to treatment (CR), High dose (HD), Medium Dose (MD); AMHAC = Amsacrine + Cytarabine.

Patient number	AML Subtype	Treatment induction	Consolidation	Response	Allograft	Relapse	Treatment on relapse	Last medical status
2	M4	1st induction : Idarubicine + cytarabine 2nd : cytarabine-HD	cytarabine HD	RC	Yes	6 months after graft	AMHAC + consolidation cytarabine-HD	deceased (myocard infarctus)
8	M2	1st induction : Idarubicine + cytarabine	cytarabine HD	RC	Yes	No	-	CR
10	M1	1st induction : daunorubicine + cytarabine	cytarabine HD	RC	Yes	1 month after graft	-	Deceased (AML relapse + GvH D)
14	M4	1st induction : Idarubicine + cytarabine	cytarabine HD	RC	Yes	2,5 years after graft	gemtuzumab ozogamicin + mitoxantrone + allograft	CR
15	M0	1st induction : idarubicine + cytarabine. 2nd : cytarabine HD	cytarabine HD	RC	Yes	No	-	Deceased (GvH disease and infections)
20	M1	1st induction : cytarabine + idarubicine + azacitidine	-	RC	No	before graft	azaticidine + sorafenib	Deceased



Accordingly, clusters 1 to 3 express CD57 cells whereas clusters 3 to 5 mostly expressed CD62L (Fig. 7A).



**Figure 7. Phenotyping of NK cell clusters identified from UMAP maps in AML patients.** A) Percentage of NK cells that express different levels of CD56 and CD16 (left), CD45RA and CD45RO (right) or CD57 and CD62L (below). B) Percentage of cells expressing ex vivo CD107a, CD69, PD-1 (left) or that have performed trogocytosis on CD33 and CD14 (right). C) Percentage of NK cells that performed trogocytosis of myelo-monocyte markers CD14 and/or CD33 at diagnosis and after therapy.

Cluster 1 contained the antitumor CD45RO<sup>+</sup> NK cells (Fig. 7A), including the NK cells that have degranulated, i.e. CD107a<sup>+</sup>, but also the PD-1<sup>+</sup> cells (Fig. 7B). Moreover, this cluster contained the cells that have trogocytosed the tumor antigen CD33 (Fig. 7B). The percentage of CD33<sup>+</sup> cells reached even 15 % in some patients and strongly diminished when the target population decreased after treatment

(Fig. 7C). Although some NK cell subsets could contain CD33 mRNA and express CD33<sup>42</sup>, we believe that we observed CD33 trogocytosis, because healthy donors NK cells express much less CD33. Moreover, after treatment, when tumor targets are sparser, CD33 decrease in patients NK cells. Finally, only cells that have degranulated express CD33. In summary, CD33 expression is closely related to a specific NK cell subset with antitumor properties.

CD14 trogocytosis was detected although at a lesser extent, likely due to the fact that not all tumor cells express CD14. CD14 expression is related to the AML type. M0 and M2 blasts do not express CD14, M4 always express it and M1 can express it<sup>41</sup>. Our cohort has patients of these 4 types (table 2). M0 and M2 patients showed the lowest CD14 trogocytosis, whereas the highest was shown by a M4 patient (Fig. 7C). This suggested that NK cells acquired CD14 by trogocytosis on tumor AML cells as they gained CD33. In summary, cluster 1 contains the *bonne fide* antitumor NK population<sup>5-7</sup> and cluster 2 are mature CD57<sup>+</sup> NK that decreased in percentage after treatment. In contrast, a similar population expressing CD62L did not decrease. Clusters 4 to 5 include immature NK cells, which arise when the target cells disappear.

The amount of AML blasts (CD33<sup>+</sup>CD14<sup>+</sup>) positively correlated with cluster 2 and negatively with cluster 4 in total samples or samples at diagnosis (supplemental Fig. 14). After treatment, AML blasts correlated negatively with clusters 1 and 5 and positively with cluster 3.

We next stratified patients regarding their treatment and analyzed trogocytosis before and after treatment (supplemental Fig. 4B). As in HL, all groups equally responded to treatment regarding trogocytosis, this supports again the notion that a specific treatment does not lead to our observations.

Finally, we obtained samples from the AML patient 2 after relapse. We generated a set of specific clusters (supplemental Fig. 15A) and observed that clusters 1 and 2 largely disappeared after treatment and reappeared in relapse (supplemental Fig. 15B). These clusters were largely enriched in the antitumor CD45RARO population, which contained PD-1- and CD69-expressing cells and had also gained by trogocytosis the tumor antigen CD33 (supplemental Fig. 15C). This clearly showed that the presence of target cells generated the presence of these clusters.

#### 4. Discussion

We have used here a non-supervised UMAP analysis of multiparametric FACS data to unveil new classical NK (cNK) subpopulations or clusters in HL and AML patients. We first verified whether HL patients possess similar NK subsets at diagnosis (Figs. 1 and 2) than those with other hematological malignancies such as AML<sup>5-7</sup>. For this purpose, we focused on *bona fide* peripheral blood NK cells that we identified by CD56 and CD7 expression<sup>28,29</sup>. We next used UMAP to unveil clusters that can be associated to disease before and/or after treatment. We could not compare clusters in samples from both diseases between them because in order to study trogocytosis, we used CD33 and CD14 in AML, and CD15 and CD30 in HL. Nonetheless, in both diseases NK subsets were deeply affected by the presence of tumor target cells.

Every individual has his own NK cell repertoire, which changes during development and is differentially distributed in different tissues. Moreover, multiple environmental factors can shape the NK cell compartment and generate new subsets<sup>1</sup>. This makes extremely difficult to identify disease-associated NK subpopulations shared by multiple patients. Although we revealed some disease-specific populations, they were not present in all patients. This could be due to the fact that each patient has his/her own NK cell subsets due to different genetic backgrounds that influence the expression patterns of multiple receptors. Additionally, environmental factors, such as the record of prior infections to which each individual is exposed, can differentially impact the NK cell populations<sup>43</sup>.

We have also shown that treatment largely modified the NK compartment. This could be due to the direct effect of treatment, i.e. chemotherapy, on NK cells or to the presence/absence of target cells. The lifespan of cNK cells is, in average, of 1 week in blood, implying a quick renewal<sup>8,44</sup>. Since treatment was stopped at least 30 days before analysis, we favor that the elimination of tumor target cells, rather than treatment itself, was the inducer of changes in NK populations. Moreover, all treatments in both diseases similarly affect NK cells (supplemental Fig. 4). Therefore, our data

provide evidence of a quick response and adaptation of the NK cell compartment to target absence: a large increase in immature population whereas antitumor NK cells disappeared.

The cytotoxic potential of NK cells was severely diminished at HL diagnosis, independently of the clinical stage. This deficiency is normalized 6 weeks after completion of treatment<sup>45</sup>. Failure to respond or evidence of early relapse do not improve NK function. Hence, NK activity could be a biomarker of clinical response and prognosis<sup>45</sup>. In agreement with this, and by manual gating, we identified that the antitumor NK subpopulation disappeared after treatment and that the NK population was normalized. In addition, using unsupervised analysis, we were able to identify new NK subsets that had not previously been described, e.g. cluster 3, which contained CD57<sup>+</sup>NKG2C<sup>+</sup> NK, or cluster trogocytosis, which gathered NK cells that had degranulated and performed trogocytosis. Hence, we revealed new NK cell subpopulations in this pathological context.

Similarly, in AML patients NK activity is suppressed at diagnosis, restored at remission, and suppressed again at relapse. Hence, the anti-tumor activity of NK cells inversely correlates to disease advancement<sup>46</sup>. Our unsupervised analysis revealed clusters clearly associated to diagnosis, i.e. cluster 1. This cluster is enriched in: i) CD45RARO cells, which we have shown to contain antitumor cells; ii) cells that have degranulated (roughly 50%) and iii) cells that have performed trogocytosis (100 % for CD33). In summary, these cells present all the characteristics of cells that have interacted with target cells and we consider this cluster the antitumor NK cell cluster, similar to the cluster trogocytosis in HL patients. Both antitumor clusters sharply decreased after treatment. Therefore, these data together with our previous results<sup>5-7</sup> support the notion that studying NK cell subsets could help to develop new methods on prognostic and/or diagnostic in cancer. A recent study also suggest that this would be the case in solid cancer patients<sup>47</sup>.

Analysis of other human NK populations is more challenging<sup>1</sup>; for example we did not investigate tissue-specific NK cells that will require biopsies. Hence, it is likely that some disease-associated populations escaped from our analysis. In addition, we have used an important, but limited panel of NK markers. Extending this panel in the future will likely help to identify new subpopulations. In any case, we were unable to identify tumor-associated NK cell populations that remained after treatment. We confirmed the presence of an antitumor NK population, but it disappeared or drastically decreased after target clearing. In hematological cancers, cNK should be in contact with tumor cells as these reside in the same organs that NK cells and their precursors, i.e. bone marrow and/or secondary lymphoid organs. This co-localization suggests a direct tumor effect on NK cell activation, maturation and/or development. For example, AML blasts infiltrate bone marrow and reduce NK cell development<sup>48,49</sup>. Remarkably, solid tumor patients also possess strong NK cell dysfunction<sup>20</sup>, indicating that invasion of NK cell niches is not a prerequisite for cancer-related NK dysfunction. In our study, the tumor/NK cell target interaction was revealed by the presence of NK cells that have trogocytosed tumor antigens (Figs. 2 and 5). Therefore, although we have not investigated a tumor infiltrating population such as that described in solid tumors, we are confident that we have investigated cNK that were in contact with cancer cells.

Previous infection with certain viruses, e.g. CMV, sculpts the NK population<sup>9,10</sup>. In cancer patients, the appearance of specific subsets in blood dictated by the presence of the tumor is not clearly defined<sup>16</sup>. Although it is clear that the NK lineage is dysfunctional<sup>18-20</sup>. Surprisingly, CMV-infected HL patients lost the CMV-related NK population, i.e. CD57<sup>+</sup>NKG2C<sup>+</sup> cells. This suggests that face to a new target challenge, new NK populations overshadow the “old” populations to respond to the new challenge. Interestingly, CMV<sup>8,16</sup> and EBV reactivation<sup>36</sup> favors patient survival after a cancer-related allogeneic transplantation. Hence, acute viral infection can improve NK cell-mediated antitumor function. However, in our study, patients did not show symptoms of CMV reactivation and our cohort is too small to reach any conclusion regarding the survival of previously infected CMV patients.

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**Author contributions:** Dang-Nghiem Vo, Michael Constantinides, Nerea Allende-Vega, Catherine Alexia performed the experiments. Guillaume Cartron and Martin Villalba perform study design and wrote the article.

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