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# Tumor-associated and immunochemotherapy-dependent long-term alterations of the peripheral blood NK cell compartment in DLBCL patients

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**Keywords:** ADCC, CD16, DLBCL, NK cells, NKG2D, R-CHOP immunochemotherapy, rituximab

**Abbreviations and acronyms:** ADCC, antibody-dependent cellular cytotoxicity; CNS, central nervous system; DLBCL, diffuse large B-cell lymphoma; FcγRIIIA/CD16, type III low-affinity Fcγ receptor; GrzB, Granzyme B; IFNγ, interferon γ; NK, natural killer cells; PBMC, peripheral blood mononuclear cell; PMLBCL, primary mediastinal large B-cell lymphoma; R-CHOP, rituximab with cyclophosphamide, doxorubicin, vincristine, and prednisone.

Natural Killer (NK) cells are a key component of tumor immunosurveillance and thus play an important role in rituximab-dependent killing of lymphoma cells via an antibody-dependent cellular cytotoxicity (ADCC) mechanism. We evaluated the phenotypic and functional assets of peripheral blood NK cell subsets in 32 newly-diagnosed diffuse large B-cell lymphoma (DLBCL) patients and in 27 healthy controls. We further monitored long-term modifications of patient NK cells for up to 12 months after rituximab-based immunochemotherapy. At diagnosis, patients showed a higher percentage of CD56<sup>dim</sup> and CD16<sup>+</sup> NK cells, and a higher frequency of GrzB<sup>+</sup> cells in CD56<sup>dim</sup>, CD56<sup>bright</sup>, and CD16<sup>+</sup> NK cell subsets than healthy controls. Conversely, DLBCL NK cell killing and interferon γ (IFNγ) production capability were comparable to those derived from healthy subjects. Notably, NK cells from refractory/relapsed patients exhibited a lower "natural" cytotoxicity. A marked and prolonged therapy-induced reduction of both "natural" and CD16-dependent NK cytotoxic activities was accompanied by the down-modulation of CD16 and NKG2D activating receptors, particularly in the CD56<sup>dim</sup> subset. However, reduced NK cell killing was not associated with defective lytic granule content or IFNγ production capability. This study firstly describes tumor-associated and therapy-induced alterations of the systemic NK cell compartment in DLBCL patients. As these alterations may negatively impact rituximab-based therapy efficacy, our work may provide useful information for improving immunochemotherapeutic strategies.

## Introduction

Natural killer (NK) cells are an essential component of innate immunity and surveillance against malignancies. Their anti-leukemic potential and ability to regulate normal and neoplastic haematopoietic precursors has recently raised considerable interest. Their effector functions also represent a crucial factor in determining the response to anticancer therapy.<sup>1-3</sup> Diffuse large B-cell lymphoma (DLBCL) is the most common aggressive

B-cell lymphoma, with heterogeneous genetic, phenotypic, and clinical features.<sup>4-6</sup> The immunochemotherapeutic association of anti-CD20 monoclonal antibody (mAb) rituximab, with cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) has significantly improved the survival of DLBCL patients, and represents the current standard-of-care with an effective outcome in 60–80% of cases.<sup>6,7</sup> NK cell-mediated, CD16-dependent antibody-dependent cytotoxicity (ADCC) likely plays a major role in determining the efficacy of rituximab-based

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therapy.<sup>8-10</sup> Indeed, structural and/or functional characteristics of CD16 receptor that affect anti-CD20 mAb-mediated ADCC *in vitro*,<sup>11-13</sup> also impact on NK cell activation status<sup>14</sup> and patient outcome *in vivo*.<sup>10,15-18</sup>

NK cells are resident in peripheral blood and in some lymphoid and non-lymphoid organs, and are promptly recruited to tumor sites.<sup>19</sup> The 2 main NK cell subsets in the periphery are thought to represent sequentially ordered differentiation steps. CD56<sup>dim</sup> NK cells with high cytotoxic potential account for the vast majority of peripheral blood NK cells, are primarily CD16<sup>+</sup> (FcγRIIIA low-affinity Fc receptor<sup>+</sup>), and most likely represent the final maturation step. CD56<sup>bright</sup> NK cells also home to secondary lymphoid organs, bear low or absent CD16, display higher capability to produce cytokines and lower cytotoxic activity, and are thought to represent the immediately preceding differentiation stage.<sup>20-22</sup>

Antitumor NK cell activation and effector functions are finely regulated by the balance between opposing signaling pathways initiated by activating and inhibitory, MHC Class I-specific, receptors.<sup>23-26</sup> While CD16 is responsible for ADCC against IgG-coated cells,<sup>27,28</sup> several activating receptors cooperatively trigger natural cytotoxicity against tumor cells.<sup>23-26</sup> Among these, killer cell lectin-like receptor K1 (KLRK1, better known as NKG2D) plays an important role in the immunosurveillance against human and mouse model lymphoma, by recognizing stress-induced ligands belonging to the MIC (MICA and MICB) and ULBP (ULBP1–6) families.<sup>29-35</sup> Expression and/or functional capability of activating receptors is orderly acquired during NK cell differentiation<sup>20-22</sup> and can be modulated upon activation and/or ligand engagement.<sup>29-37</sup> Activated NK cells also rapidly secrete a variety of cytokines and chemokines, such as interferon  $\gamma$  (IFN $\gamma$ ), that amplify the recruitment and activation of other participants to the antitumor response.<sup>19,38</sup>

Although peripheral blood lymphopenia at diagnosis and during treatment has been widely reported as a negative prognostic factor in DLBCL,<sup>39-41</sup> this phenomenon, a reflection of host systemic immunity, remains poorly characterized. Few studies have addressed the characterization of the NK cell compartment in DLBCL patients. A notable study suggested the relevance of a low NK cell count as an important prognostic factor in the pre-rituximab era,<sup>42</sup> while a recent report described the concomitant defect of CD16 expression and ADCC in NK cells of a small group of DLBCL patients at diagnosis.<sup>43</sup> While it has been shown that rituximab infusion rapidly (within hours) induces changes in the NK cell phenotype, activation status, and ADCC activity,<sup>14,44</sup> information on the long-term effects of rituximab-based therapy on NK cells are still mostly lacking.

In this prospective study, we have longitudinally assessed the phenotypic and functional long-term dynamics of the peripheral blood NK cell compartment, in a cohort of newly diagnosed DLBCL patients undergoing rituximab-based immunochemotherapy. As the phenotypic and functional profile of the host's NK cells might significantly contribute to the success of immunochemotherapy strategies, the novel information provided by this study may prove useful for the development of focused and innovative therapeutic approaches.

## Results

### Phenotypic and functional assets of the peripheral NK cell compartment in DLBCL patients at diagnosis

We initially assessed the profile of the peripheral blood (PB) NK cell compartment in a cohort of newly diagnosed DLBCL patients and age- and sex-matched healthy controls. Although patients showed significantly lower total lymphocyte counts (Fig. 1A), the absolute numbers of total CD3<sup>+</sup>CD56<sup>+</sup> NK cells and of CD56<sup>dim</sup>, CD56<sup>bright</sup>, and CD16<sup>+</sup> subsets were comparable between patients and healthy subjects (Fig. 1B). However, the relative frequency of total CD3–CD56<sup>+</sup> NK cells, and of CD56<sup>dim</sup> and CD16<sup>+</sup>, but not of CD56<sup>bright</sup>, NK cell subsets, were significantly higher in patients than in controls (Fig. 1C).

NK cells are endowed with cytotoxic activity and with the capability to promptly produce cytokines and chemokines.<sup>19,38</sup> A considerably higher frequency of cells expressing the cytotoxic granule marker Granzyme B (GrzB) characterized CD56<sup>dim</sup>, CD56<sup>bright</sup> and CD16<sup>+</sup> NK cell populations in patients' PBMC (Fig. 1D); nevertheless, either "natural" (anti-K562 erythroleukemia cell line) and CD16-dependent (anti-P815+anti-CD16 mAb) cytotoxic activities were comparable between patient and control-derived NK cells (Fig. 1E). NK cell capability to produce IFN $\gamma$ , as evaluated by the frequency of cytokine-producing cells upon short-term stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin, was also comparable between patients and controls (Fig. 1F).

Taken altogether, these data indicate that the peripheral blood NK cell compartment of newly diagnosed DLBCL patients (time point 1 [T1]), although being quantitatively and functionally normal, shows a higher representativity over lymphocytes, and displays a higher cytotoxic potential.

### Long-term dynamics of peripheral blood NK cell subsets in DLBCL patients undergoing rituximab-based immunochemotherapy

The absolute counts of CD3<sup>+</sup>CD56<sup>+</sup> NK cells, as well as their CD56<sup>dim</sup> and CD56<sup>bright</sup> subsets, were transiently decreased at mid-therapy time point (T2), and had recovered by the end of therapy (T3, within one month after the last treatment course); the diminution was significant, as compared to either healthy controls (Figs. 2A-C) or pre-therapy samples (T1, Table S1A-Clink>). Interestingly, the absolute count of CD16-expressing CD3<sup>+</sup>CD56<sup>+</sup> NK cells showed a marked and prolonged reduction, as it persisted till the end of therapy time point (T3), and had recovered by 3 months later (T4) (Fig. 2D; Table S1D).

The percentage of total, CD56<sup>dim</sup>, and CD16-expressing NK cells (over lymphocytes), that were higher at diagnosis (T1), became comparable to controls from T2 till the end of the following observation period (12 months) (Figs. 2E-F, and H). CD56<sup>bright</sup> NK cells were slightly elevated only at 12 months after therapy (T6, Fig. 2G).

Altogether, these results show that while circulating CD56<sup>dim</sup> and CD56<sup>bright</sup> NK cell counts transiently decrease during

therapy, the diminution of CD16-expressing NK cells is more prolonged.

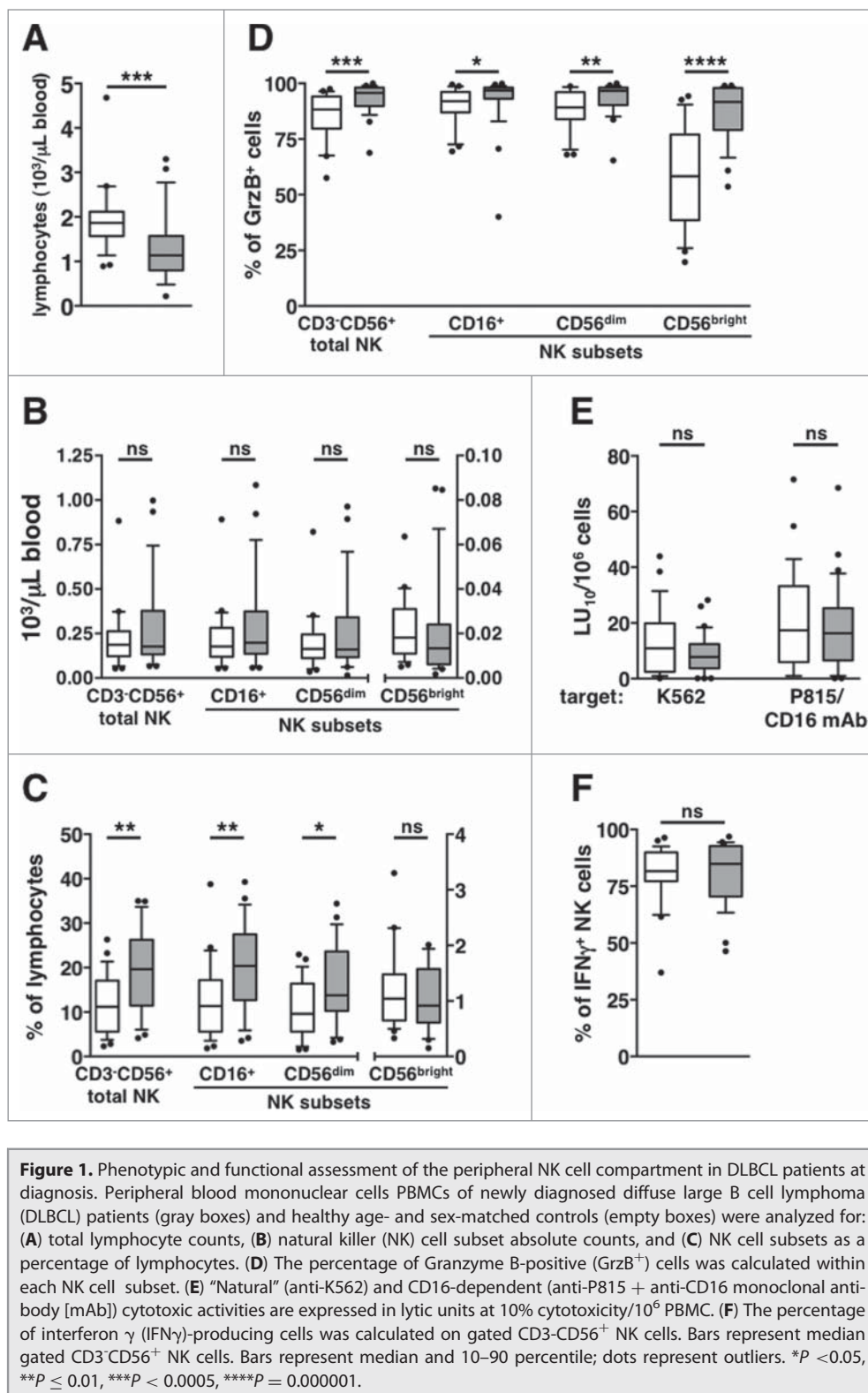
### Long-term dynamics of CD16 receptor expression on PB NK cells of DLBCL patients

Our findings suggest the occurrence of therapy-induced downregulation of CD16 receptor on NK cells in DLBCL patients. We next analyzed in depth the dynamics of CD16 expression on circulating NK cell subsets. Interestingly, the fraction of NK cells expressing CD16 receptor was markedly and significantly reduced at T3 (within one month upon therapy completion), with respect to healthy controls (Fig. 3A) or to pre-therapy levels (Table S2A-C). A significant diminution of CD16<sup>+</sup> cells selectively occurred on CD56<sup>dim</sup>, and not on CD56<sup>bright</sup> NK cells (Figs. 3B-C, respectively); however, CD16 receptor intensity (expressed as specific mean fluorescence intensity, MFI) was markedly reduced on CD56<sup>bright</sup> NK cells, at T3 (Figs. 3D-E, respectively).

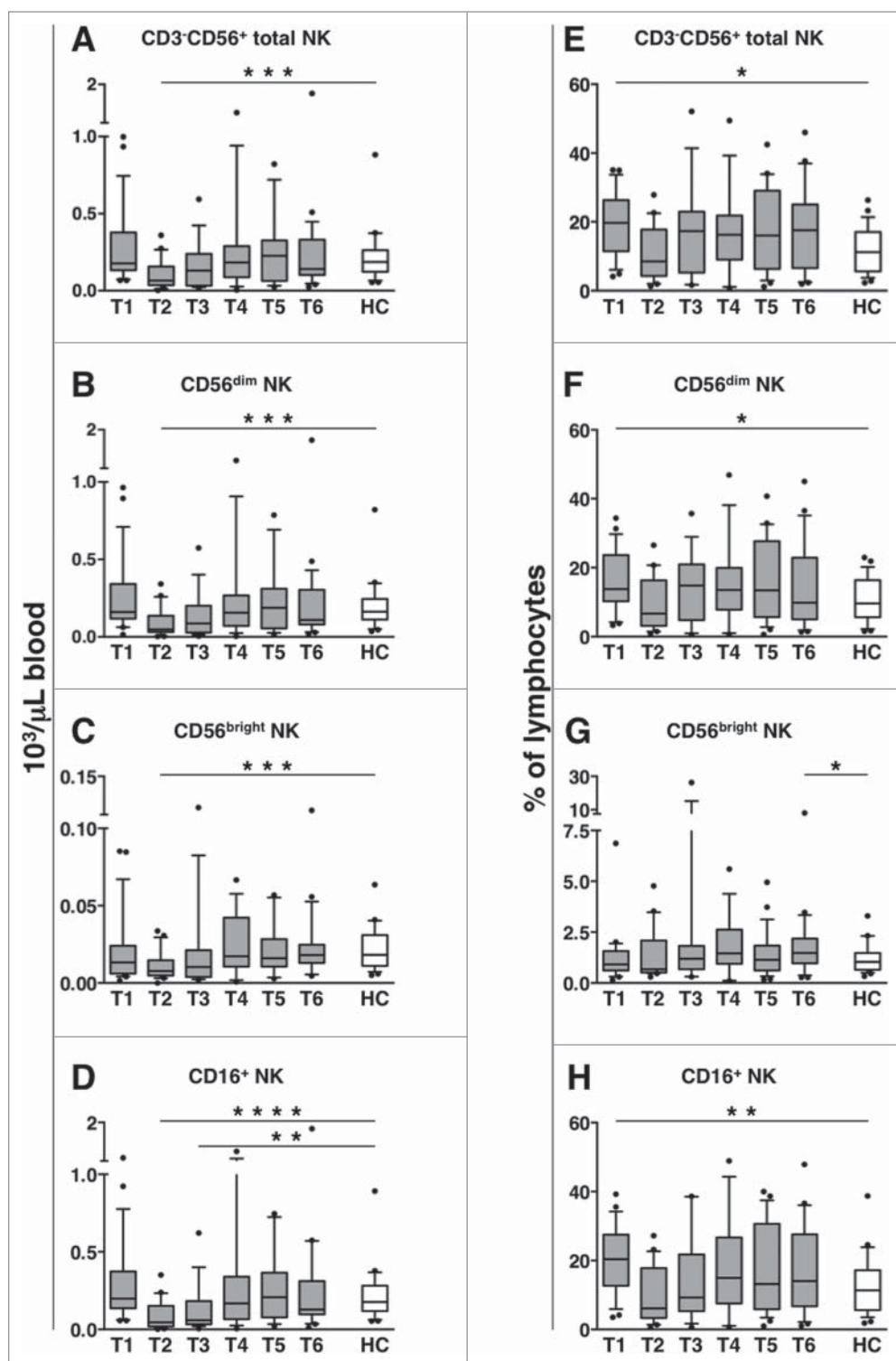
Taken altogether, these data suggest that immunotherapy induces the diminution of circulating CD16<sup>+</sup> NK cells. This decrease: a) persists over one month after therapy completion; b) more deeply affects the CD56<sup>dim</sup> subset; and c) is the combined result of diminished NK cell absolute counts, observed during therapy, and CD16 downregulation on NK cells, recorded after the completion of therapy.

### Transient down-modulation of NKG2D activating receptor on NK cells in DLBCL patients undergoing immunochemotherapy

NKG2D is an important activating receptor for “natural” cytotoxicity<sup>29-35</sup> and also participates in CD16-dependent killing.<sup>45,46</sup> The frequency of NKG2D<sup>+</sup> NK cells at diagnosis (T1) was comparable between DLBCL and healthy controls. However, it transiently decreased during therapy (T2), with the diminution more persistently



observed in the CD16<sup>+</sup> fraction (sustained until 3 months after the end of therapy, T4, Figs. 4A-B). NKG2D downregulation selectively occurred on CD56<sup>dim</sup> NK cells (Fig. 4Ci), notably, affecting only the CD16<sup>+</sup> fraction (Fig. 4Cii-iii). Conversely, NKG2D expression on either CD16<sup>+</sup> or CD16<sup>-</sup> CD56<sup>bright</sup> NK



**Figure 2.** CD56<sup>dim</sup> and CD16<sup>+</sup> NK cell absolute counts transiently decrease in DLBCL patients during immunochemotherapy. Peripheral blood mononuclear cells (PBMCs) of diffuse large B cell lymphoma (DLBCL) patients at different time points (T1-T6, gray boxes) and of healthy controls (HC, empty boxes) were analyzed for: (A-D) the absolute counts of total CD3<sup>+</sup>CD56<sup>+</sup> natural killer (NK) cells and their subsets, obtained by combining complete blood counts and immunocytofluorimetric analysis; (E-H) the percentage of total CD3<sup>+</sup>CD56<sup>+</sup> NK cells and their subsets within lymphocytes. Bars represent median and 10–90 percentile; dots represent outliers. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0005$  vs. controls.

cells did not vary significantly over the entire follow-up period (Fig. 4Di-iii).

Soluble NKG2D ligands can induce NKG2D receptor downregulation in cancer patients.<sup>29-33</sup> However, plasma levels of the NKG2D ligands MICA, MICB, ULBP1 and ULBP2 did not differ significantly between pre-therapy (T1) DLBCL patients and healthy controls. MICB diminution was the only variation observed during therapy (T2) (Fig. S1).

These results show that immunochemotherapy is associated with the down-modulation of NKG2D activating receptor on CD16<sup>+</sup> CD56<sup>dim</sup> NK cells, and suggest that circulating NKG2D ligands are not involved in this phenomenon.

#### Long-term dynamics of “natural” and CD16-dependent cytotoxic activities of DLBCL PBMCs

The aforementioned data show that immunochemotherapy is associated with a prolonged downregulation of CD16 and NKG2D activating receptors on NK cells, particularly the highly lytic CD56<sup>dim</sup> subset.<sup>20-22</sup> Interestingly, both “natural” and CD16-dependent cytotoxic activities were strongly reduced during and up to one month after the end of therapy (T2 and T3, respectively), relative to either pre-therapy levels (Table S3A and B) or healthy controls (Fig. 5). NK cytotoxic activities returned to levels comparable to those of control NK cells by 3 months after therapy completion (T4).

Taken together, our results show that immunochemotherapy-induced downregulation of CD16 and NKG2D activating receptors is temporally related to the impairment of both “natural”

and CD16-dependent *in vitro* cytotoxic capability. In accordance, CD16-dependent cytotoxic activity of NK cells derived from individual patients at T3 significantly correlated with NKG2D expression on CD16<sup>+</sup>, but not on CD16<sup>-</sup> CD56<sup>dim</sup> NK cells (Fig. S2A-C).

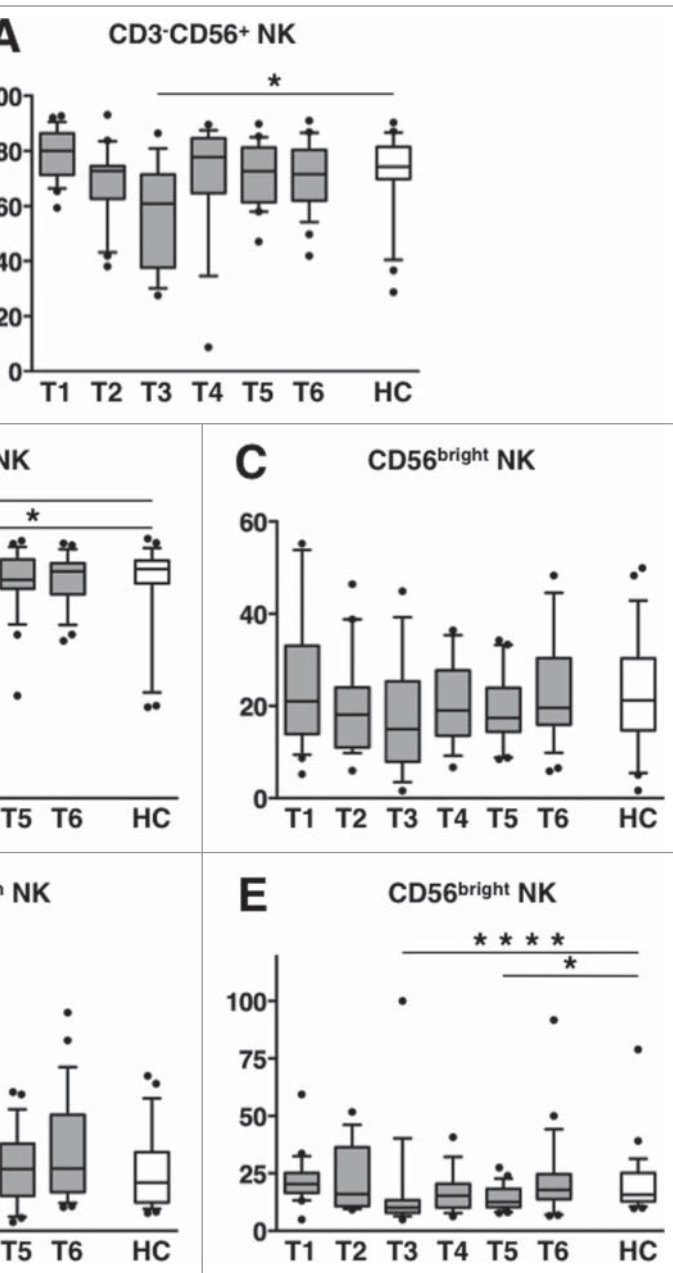
#### Long-term dynamics of NK functional competence in DLBCL patients

The frequency of GrzB<sup>+</sup> NK cells in patients remained significantly higher than in healthy controls till T4 and T5 (3 and 6 months after the end of therapy, respectively), in CD56<sup>dim</sup> and CD56<sup>bright</sup> NK cell subsets (Fig. 6A and B). At variance, the percentage of IFN $\gamma$ -producing NK cells was transiently elevated at T3, although comparable to NK cells derived from healthy controls at all the remaining time points (Fig. 6C).

These results indicate that the expanded pool of cytotoxic granule-containing cells that characterized the DLBCL NK compartment at diagnosis recalcitrantly returned to normal levels, albeit well after the end of the treatment.

#### Refractory/early-relapsed patients show defective "natural" cytotoxicity at diagnosis

The analysis of PBMC cytotoxic activity, after patients stratification, shows that patients that were resistant to therapy, or that underwent early relapse (within one year after diagnosis), displayed a significantly lower "natural," but not CD16-dependent, cytotoxic activity at diagnosis, as compared either with controls, or with patients attaining remission lasting more than 2 y (Fig. 7A and B). NK cell subset absolute counts and percentages were comparable between the 2 groups of patients (Fig. 7C). Clinical parameters of remitting and resistant patients are reported in Table S4. This observation, although based on a limited number of subjects, suggests that defective

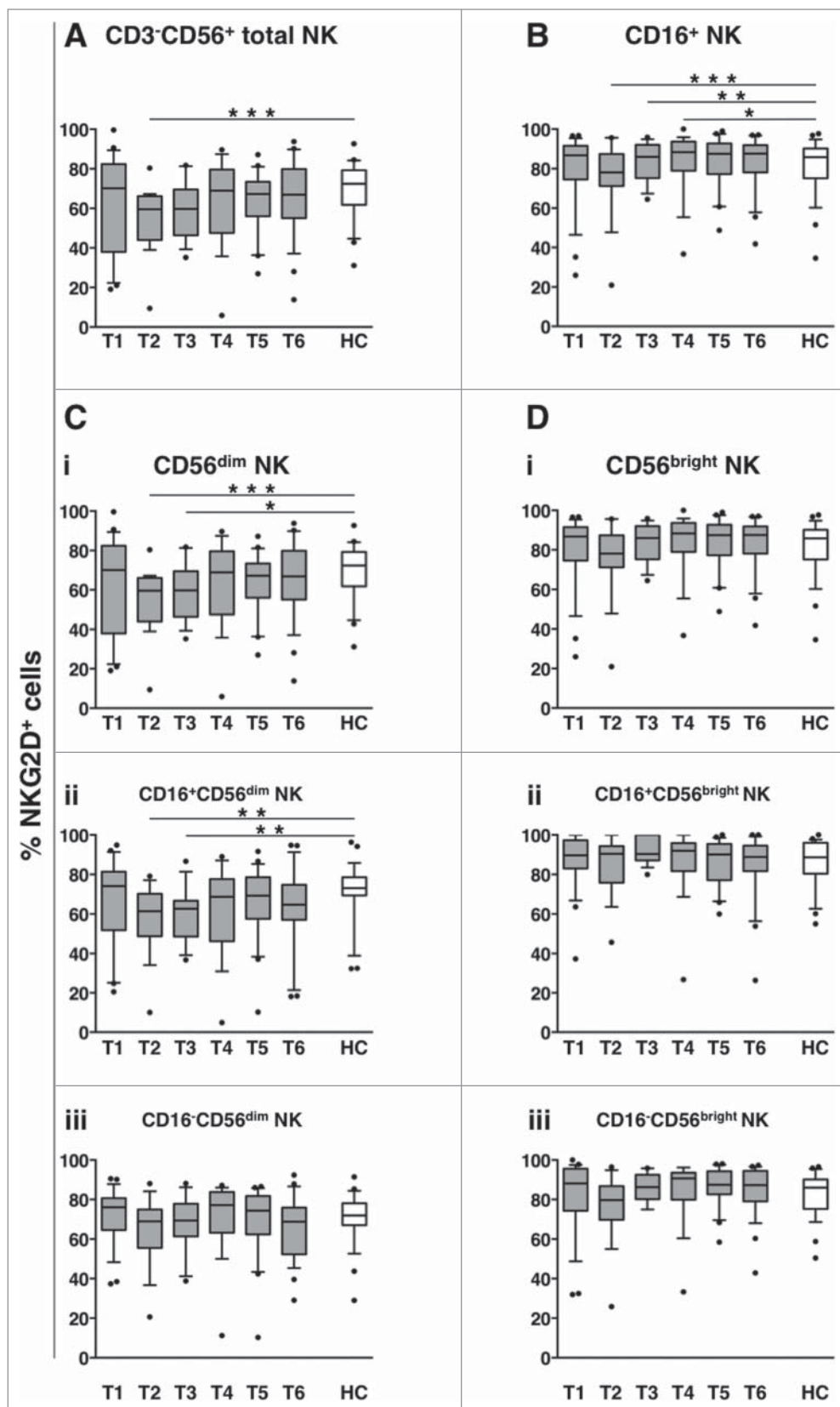


**Figure 3.** Long-term dynamics of CD16 expression on NK cell subsets in DLBCL patients upon immunochemotherapy. Peripheral blood mononuclear cells (PBMCs) of diffuse large B cell lymphoma (DLBCL) patients at different time points (T1-T6, gray boxes) and of healthy controls (HC, empty boxes) were analyzed for: (A) the percentage of CD16<sup>+</sup> cells over CD3<sup>+</sup>CD56<sup>+</sup> natural killer (NK) cells; (B-C) the percentage of CD16<sup>+</sup> cells and (D-E) the specific mean fluorescence index (MFI) of CD16 receptor calculated within CD56<sup>dim</sup> (B, D) and CD56<sup>bright</sup> (C, E) NK cell subsets. Bars represent median and 10–90 percentile; dots represent outliers. \**P* < 0.05, \*\*\*\**P* < 0.0005 vs. controls.

NK cell functional activity at diagnosis may be associated with immunochemotherapy failure.

#### Discussion

NK cells represent an important component of tumor immunosurveillance, and their effector functions may contribute



**Figure 4.** Long-term dynamics of NKG2D activating receptor on NK cell subsets in DLBCL patients upon immunochemotherapy. Peripheral blood mononuclear cells (PBMCs) of diffuse large B cell lymphoma (DLBCL) patients at different time points (T1-T6, gray boxes) and of healthy controls (HC, empty boxes) were analyzed for the percentage of NKG2D<sup>+</sup> cells in: total CD3<sup>-</sup>CD56<sup>+</sup> NK cells (A), CD16<sup>+</sup> NK cells (B), or within CD56<sup>dim</sup> (C) and CD56<sup>bright</sup> (D) NK cell subsets; the frequency of NKG2D<sup>+</sup> cells was evaluated in each subset, as a whole (i), and in the CD16<sup>+</sup> (ii) and CD16<sup>-</sup> (iii) fractions. Bars represent median and 10–90 percentile; dots represent outliers. \*P < 0.05, \*\*P < 0.01, \*\*\*P ≤ 0.001 vs. controls.

scarce. Here, we first to report baseline and long-term analysis of the phenotypic and functional dynamics of peripheral blood NK cell subsets in DLBCL patients. Our data reveal tumor-associated as well as therapy-dependent alterations of the systemic NK cell compartment.

At diagnosis, the major NK cell subsets (CD56<sup>dim</sup>, CD56<sup>bright</sup>, CD16<sup>+</sup>), although in the normal range of peripheral blood absolute counts, had a higher frequency compared to age- and sex-matched healthy individuals. This can be explained by the decreased absolute counts of B and CD4<sup>+</sup> T lymphocytes (MC Cox, G Palmieri et al., manuscript in preparation), which underlies the low absolute lymphocyte count observed in DLBCL patients. “Natural” and CD16-dependent cytotoxic activities were comparable between DLBCL and controls; however, refractory/early-relapsed patients showed a lower “natural” cytotoxic activity at diagnosis, as compared to either patients achieving durable remission or to healthy subjects.

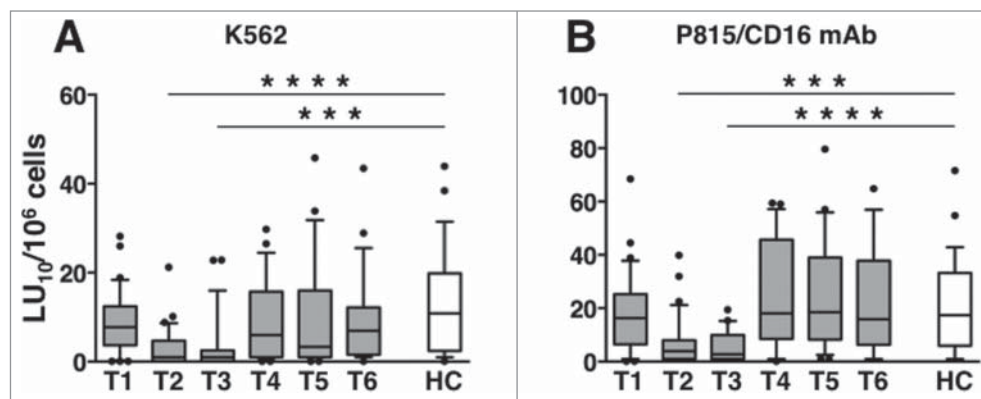
significantly to the success of rituximab-based anti-lymphoma therapies.<sup>8-10,15-18</sup> Characterization of NK cell phenotypic and functional assets in newly diagnosed DLBCL patients has been

Information on NK cell phenotypic and functional assets in newly diagnosed DLBCL patients has, so far, been scarce. In accordance with our data, an elevated NK cell frequency has

been previously reported in patients.<sup>47</sup> Furthermore, higher absolute NK cell counts and “natural” cytotoxicity at diagnosis have both shown to positively correlate with response to therapy, either in the pre-rituximab era,<sup>42,48-49</sup> and more recently in immunochemotherapy-treated patients.<sup>50</sup> Our results, although obtained in a limited number of patients, confirm that defective basal “natural” killing activity associates with treatment failure, thus stressing the role of NK cell cytotoxic capability in the success of rituximab-based therapy. A recent report described defective NK cell degranulation as a proxy for “natural” and ADCC activities in a group of newly diagnosed DLBCL patients.<sup>43</sup> As these authors did not provide information on demographic and clinical parameters of patients and controls, and on therapy outcome, the discrepancy with our results may depend on unknown patient characteristics and/or differences in the experimental assays employed (NK cell degranulation *vs.* killing and ADCC *vs.* CD16-dependent cytotoxicity).

The frequency of cytotoxic granule-containing cells was markedly elevated in all major NK cell populations of DLBCL patients, whereas the percentage of IFN $\gamma$ -producing cells was comparable to that of in healthy subjects. GrzB-containing cytotoxic granules are acquired during NK cell differentiation, and their biosynthesis is modulated by proinflammatory cytokines or members of the  $\gamma_c$  chain cytokine family.<sup>20-22</sup> The higher frequency of GrzB<sup>+</sup> NK cells, particularly in the CD56<sup>bright</sup> subset, may thus underlie a state of chronic activation and/or a perturbed differentiation process, which may stem from a tumor-dependent altered microenvironment.

Rituximab-based immunochemotherapy induced a complex phenotypic and functional perturbation of peripheral blood NK compartment. A profound and protracted impairment of both “natural” and CD16-dependent *in vitro* cytotoxic activities was observed during therapy, it persisted well over one month after the end of treatment, and had recovered 3 months later; this was paralleled by a significant reduction of CD16 and NKG2D activating receptors, that more markedly affected the CD56<sup>dim</sup> NK cell subset. Interestingly, neither the lytic potential nor the capability to produce IFN $\gamma$  were defective in DLBCL patients during the same time period. Moreover, the percentage of CD56<sup>dim</sup> and CD56<sup>bright</sup> subsets relative to total lymphocytes remained comparable to healthy subjects. However, NK absolute counts transiently diminished during therapy cycles, and returned to normal levels by one month after the end of therapy; this may be accounted by immunochemotherapy-induced NK cell margination and/or re-localization, or alternatively, drug-induced toxicity.



**Figure 5.** Long-term dynamics of “natural” and CD16-dependent cytotoxic activities in peripheral blood of DLBCL patients. (A) “natural” (anti-K562) and (B) CD16-dependent (anti-P815 + anti-CD16 monoclonal antibody [mAb]) cytotoxic activities of peripheral blood mononuclear cells (PBMCs) of diffuse large B cell lymphoma (DLBCL) patients at different time points (T1-T6, gray boxes) and of healthy controls (HC, empty boxes); data are expressed in lytic units at 10% cytotoxicity/10<sup>6</sup> PBMC. Bars represent median and 10–90 percentile; dots represent outliers. \*\*\*P < 0.0001, \*\*\*\*P < 0.00005 vs. controls.

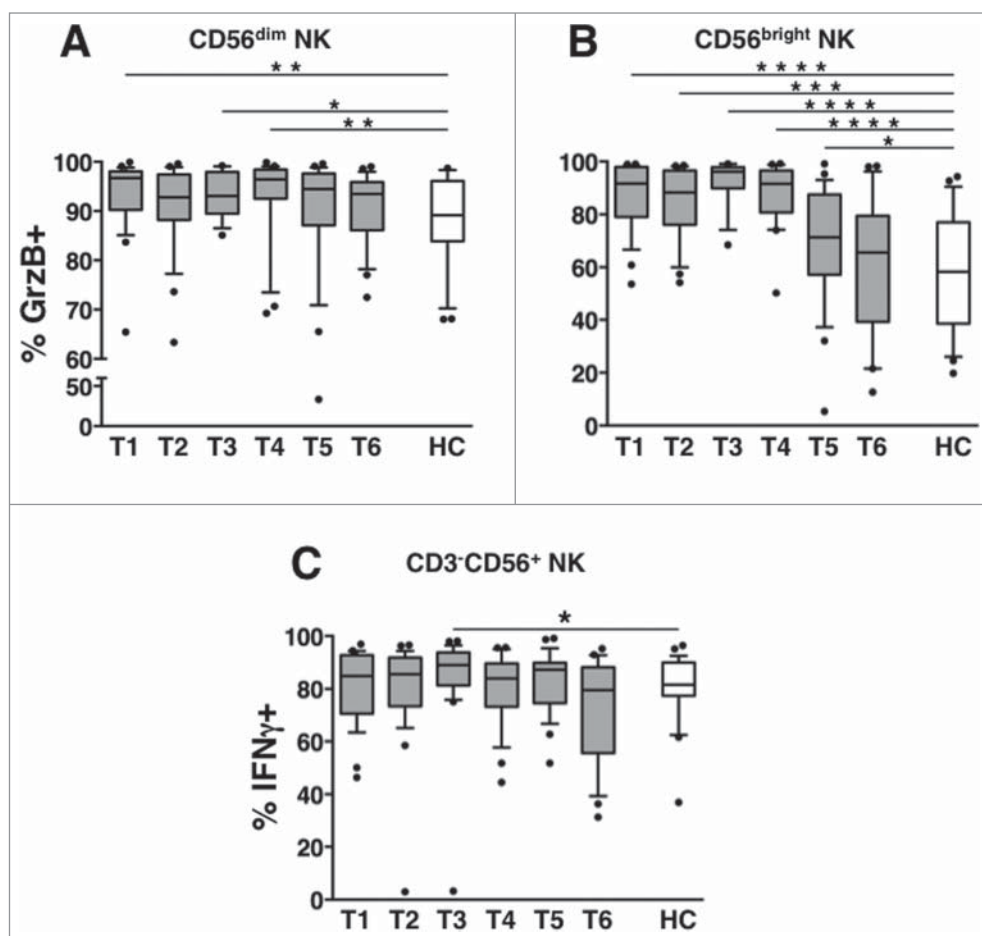
Taken together, these results suggest that the defective *in vitro* cytotoxic activity neither depends on a gross functional impairment, nor on the exhaustion of the lytic potential, nor on a reduced frequency of peripheral blood NK cells. Moreover, the short *in vivo* half-life of the chemotherapeutic drugs that were administered, together with the fast and reversible kinetics with which they partially affect *in vitro* and *in vivo* NK cell killing ability,<sup>51-53</sup> make it unlikely that the impaired NK cytotoxicity of DLBCL patients is attributable to the direct effect of cytotoxic drugs, especially for the end of treatment time point. In contrast, rituximab persists at detectable levels long after the end of therapy cycles.<sup>10,53,54</sup>

The downregulation of CD16 and NKG2D activating NK cell receptors on the more lytic CD56<sup>dim</sup> subset<sup>19-22</sup> suggests that defective recognition and/or activation mechanisms may rather play a role in the impairment of “natural” and CD16-dependent cytotoxic activities. The mechanisms underlying receptor down-modulation are presently unknown, although it has been widely reported that cell activation and/or ligand engagement can induce the modulation of NK activating receptors through several mechanisms.<sup>29-37</sup>

CD16 down-modulation, the upregulation of activation markers, and enhanced ADCC capability occur in a matter of hours after rituximab infusion, with some of these effects depending on the presence of CD16 high affinity polymorphism;<sup>14,44</sup> however, the long-term effects of rituximab-based immunochemotherapy on CD16 expression levels have not been previously studied in DLBCL patients.

Immunosuppressive cytokines, as well as chronic contact with cell-associated or soluble ligands, may induce NKG2D receptor downregulation, as shown *in vitro* and in tumor patients *in vivo*.<sup>29-34</sup> However, plasma levels of several NKG2D ligands were not increased in DLBCL patients. Moreover, NKG2D downregulation selectively affected the CD16<sup>+</sup> CD56<sup>dim</sup> NK subset. This finding argues against a role for soluble, systemic





**Figure 6.** Long-term dynamics of NK cell functional competence in DLBCL patients. Peripheral blood mononuclear cells (PBMCs) of diffuse large B cell lymphoma (DLBCL) patients at different time points (T1-T6, gray boxes) and of healthy controls (HC, empty boxes) were analyzed for: the percentage of GrzB<sup>+</sup> cells in (A) CD56<sup>dim</sup> and (B) CD56<sup>bright</sup> natural killer (NK) cell subsets. (C) The percentage of interferon  $\gamma$  (IFN $\gamma$ )-producing cells was determined by intracellular staining and cytofluorimetric analysis, and calculated on gated CD3<sup>+</sup>CD56<sup>+</sup> NK cells. Bars represent the median and 10–90 percentile; dots represent outliers. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.00005$ , \*\*\*\* $P < 0.000005$  vs. controls.

mediators in NKG2D downregulation. Our results suggest that a more effective, CD16-dependent, interaction with rituximab-coated, NKG2D ligand-expressing lymphoma cells may contribute to down-modulation of the NKG2D receptor. Indeed, DLBCL cells can constitutively express NKG2D ligands, that may be further enhanced by chemotherapy-triggered DNA damage response.<sup>29-34,56,57</sup>

Overall, our results suggest that the therapy-driven, CD16-dependent, continuous stimulation leads to a prolonged NK cell phenotypic modulation and *in vitro* functional impairment. These alterations may be highly significant also for *in vivo* NK cell cytotoxic activity against DLBCL cells, and as such, may impact rituximab-based therapy efficacy. Although CD16 engagement by IgG-coated target cells is the sole trigger for ADCC,<sup>26,27</sup> its activity can be enhanced by NKG2D engagement;<sup>45,46</sup> moreover, it has been shown that NKG2D<sup>+</sup> NK cells preferentially perform ADCC.<sup>44</sup> Indeed, the interplay between CD16 and NKG2D activating

receptors is undergoing active investigation for therapeutic purposes.<sup>58</sup>

Our study also revealed that the expanded pool of cytotoxic granule-containing NK cells in DLBCL patients at disease onset does normalize, albeit only after a prolonged interval following the completion of therapy. This delayed kinetics may reflect a slow NK cell turnover, possibly due to lymphopenia- and/or age-associated expansion of long-lived memory NK cells.<sup>20-22,59</sup> Additionally, a long-lasting perturbation in the NK differentiation/maturation process, either in the bone marrow and/or in the periphery, may also be involved in this process. This perturbation would be initially dependent on the presence of the tumor and afterward be sustained by chemotherapy-triggered alterations of the bone marrow microenvironment.<sup>60,61</sup>

In sum, our pioneering and comprehensive analysis has shown that the peripheral NK cell compartment in DLBCL patients is affected by both tumor-associated alterations that normalize late after the achievement of clinical remission as well as immunochemotherapy-dependent quantitative, phenotypic and functional alterations that recover with distinct kinetics. Immunochemotherapy-induced alterations of NK cell functionality might partly explain why a dose-dense rituximab regime may not lead to improved outcome.<sup>62,63</sup>

Innovative combination strategies aiming to potentiate NK cell effector functions and newly devised anti-CD20 antibodies to target malignant B cells are promising new tools in lymphoma therapy.<sup>64-68</sup> The novel information provided by our study may be relevant for the improvement of anticancer immunotherapy, as well as in other settings in which therapeutic efficacy relies on NK-dependent effector functions.

## Materials and Methods

### DLBCL patients and controls

All patients included in the prospective study were referred to the Hematology Unit of Sant'Andrea Hospital in Rome. Newly-diagnosed DLBCL patients with Stage II-IV or I bulky were

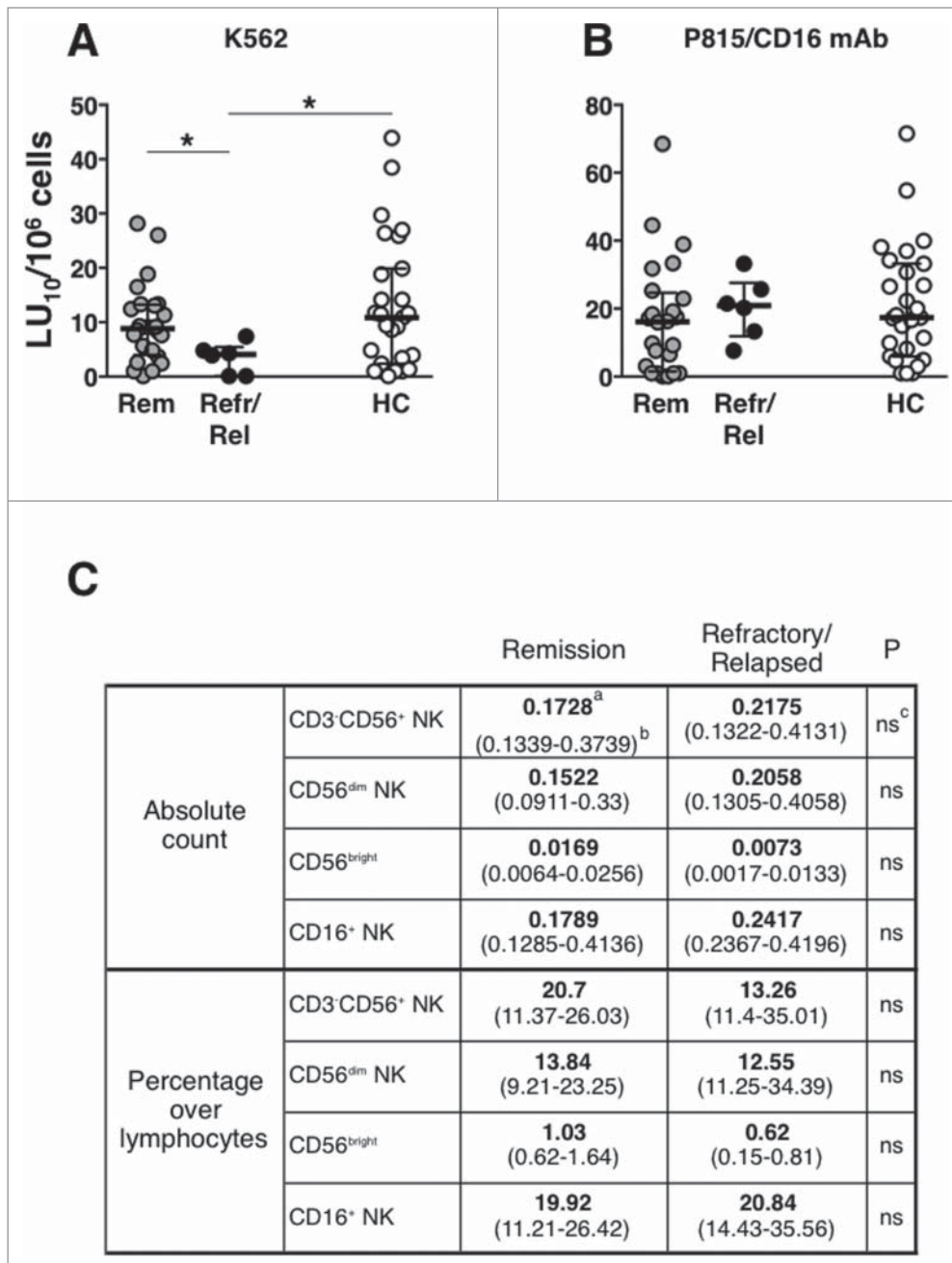
included in the study; exclusion criteria included positivity for human immunodeficiency virus (HIV) or hepatitis (HBV/HCV), presence of opportunistic infections, central nervous system (CNS) involvement, leukemic dissemination, or previous chemotherapy-treated neoplasia. Demographic and clinical characteristics of patients and healthy controls are in Table 1. Patients with conventional DLBCL were treated with 6 cycles of R-CHOP-21 or R-CHOP-14 (high risk <60 years); primary mediastinal large B-cell lymphoma (PMLBCL) patients were treated with 12 cycles of RMACOPB<sup>69</sup> and consolidation radiotherapy. In R-CHOP-treated, rituximab was administered at days 0 and +7 of cycle 1, and with chemotherapy cycles thereafter. The 8th and last rituximab infusion was administered 25-30 d after the last course of immunochemotherapy.

Patients were analyzed at diagnosis (T1), at mid-therapy (between 2nd and 3rd cycle of R-CHOP, and between 6th and 7th week of RMACOPB, T2), within one month after immunochemotherapy completion and immediately before last rituximab infusion (T3), at 3 (T4), 6 (T5), and 12 (T6) months after the end of immunochemotherapy. Patients that were considered to be non-responders after mid-treatment restaging,<sup>70</sup> or that relapsed during the study period, were not analyzed further.

The study was approved by our Institutional Review Board and was conducted in accordance with the regulations of health information protection policies, and with the Declaration of Helsinki. All patients and controls gave informed consent to the study.

#### PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by Lymphoprep (Cedarlane,



**Figure 7.** Defective "natural" cytotoxicity at diagnosis in refractory/early-relapse patients. (A) "natural" (anti-K562) and (B) CD16-dependent (anti-P815 + anti-CD16 mAb) cytotoxic activities of peripheral blood mononuclear cells (PBMCs) in diffuse large B cell lymphoma (DLBCL) patients that attained long-lasting remission (Rem, gray symbols, n = 25), or that were refractory/early-relapsed (Refr/Rel, black symbols, n = 6), and of healthy controls (HC, empty boxes, n = 27); data are expressed in lytic units at 10% cytotoxicity/10<sup>6</sup> PBMC. Bars represent median and 10–90 percentile. \*P < 0.05. (C) Natural killer (NK) cell subset absolute counts and percentages in remitting and refractory/relapsed patients; <sup>a</sup>median value; <sup>b</sup>(25–75 percentile range); <sup>c</sup>P value; ns, not significant.

Ontario, Canada) density gradient centrifugation. PBMCs were immediately used for cytotoxicity assay, and stored at  $-80^{\circ}\text{C}$  in 10% dimethyl sulfoxide (DMSO; Sigma Aldrich, St. Louis, USA) and 90% fetal calf serum (FCS; Euroclone, Milan, Italy), for phenotypic and functional analyses. Cells were rapidly thawed at

**Table 1.** Demographic and clinical characteristics of DLBCL patients and controls

	patients	controls	P
median age, y (range)	59 (37–79)	57 (29–73)	ns <sup>c</sup>
gender, M/F	20 <sup>a</sup> (62) <sup>b</sup> /12 (38)	12 (37)/15 (63)	ns
Ann Arbor stage			
I-II	10 (31)		
III-IV	22 (69)		
Histology			
DLBCL	28 (88)		
PMLBCL	4 (12)		
PS			
0-1	20 (62)		
2-4	12 (38)		
IPI			
0-2	18 (56)		
3-5	14 (44)		
BM involvement			
yes	12 (37)		
no	20 (63)		
Extra-nodal sites			
< = 1	21 (66)		
> 1	11 (34)		
Complete remission with first line treatment	28 (87)		

Abbreviations: BM, bone marrow; DLBCL, diffuse large B cell lymphoma; IPI, International Prognostic Index scores (0–2 and 3–5); PMLBCL, primary mediastinal large B cell lymphoma; PS, performance status of Eastern Cooperative Oncology Group (ECOG).

<sup>a</sup>number; <sup>b</sup>percentage; <sup>c</sup>not significant.

37°C, washed, and rested overnight in RPMI 1640 medium supplemented with 10% FCS and 1% glutamine (all from Euroclone; complete medium), at 37°C in 5% CO<sub>2</sub> atmosphere, prior to stimulation, immunostaining and cytofluorimetric analysis.

#### Evaluation of intracellular IFN $\gamma$

PBMCs were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 0.5  $\mu$ g/mL ionomycin at 37°C for 6h in complete medium, in the presence of the intracellular trafficking inhibitors Monensin (50  $\mu$ M, added at the beginning of stimulation) and Brefeldin A (10  $\mu$ g/mL, added after 1h) (all from Sigma-Aldrich Srl, Milan, Italy). Cell samples were then fixed, permeabilized, and stained with phycoerythrin (PE)-conjugated anti-IFN $\gamma$  antibody or isotype control mAb (both from BD Biosciences, Milan, Italy).

#### Immunostaining procedures and cytofluorimetric analysis

NK cell subsets were identified by a combination of physical parameters and surface staining with optimal dilutions of fluorochrome-conjugated mAbs (anti-CD3 Alexafluor488, anti-CD16 PE-Cy7, anti-CD56 APC, all from BD Biosciences), for 30 min at 4°C. The values of complete blood counts and cytofluorimetric analysis percentages were used to calculate the absolute number of lymphocyte subsets.

For intracellular staining, cell samples were stained for surface antigens, washed with phosphate-buffered saline, fixed with 2% paraformaldehyde (Sigma-Aldrich) for 20 min, permeabilized with 0.5% saponin (Sigma-Aldrich) in 1% FCS for 30 min, and

stained with PE-conjugated anti-IFN $\gamma$  or anti-Granzyme B (Enzo Life Science, Inc., USA) antibodies, or isotype control mAb, in the presence of 0.5% saponin/1% FCS.

All samples were analyzed with a FACScalibur (BD Biosciences) flow cytometer, using CellQuest Pro (BD Biosciences) and FlowJo v9.3.2 (Treestar, Ashland, OR) data analysis software. Lymphocytes were defined by gating on forward and side scatter physical parameters. The threshold for antigen positivity was set on the basis of a matched isotype control mAb-stained sample, whose positivity never exceeded 0.5% of gated events.

#### Cytotoxicity assay

“Natural” (anti-K562 erythroleukemia cell line) and CD16-dependent (anti-P815 murine mastocytoma cell line + anti-CD16 mAb) cytotoxic activities were measured with a 4 h <sup>51</sup>Cr-release assay, as previously described.<sup>71</sup> Briefly, K562 and P815 target cells were labeled with <sup>51</sup>Cr (PerkinElmer, MA, USA) (100  $\mu$ Ci/1  $\times$  10<sup>6</sup> cells) for 75 min at 37°C. Serial dilutions of PBMC, as effector cells, were plated in U-bottom 96-well plates in complete medium supplemented with 10 mM HEPES (Sigma-Aldrich), together with <sup>51</sup>Cr-labeled target cells (5  $\times$  10<sup>3</sup> cells/well), in the absence (K562) or in the presence (P815) of optimal concentration of anti-CD16 mAb (B73.1 clone, kindly provided by Dr G. Trinchieri, Cancer and Inflammation Program, NCI, Frederick, USA). After 4 h incubation at 37°C, released radioactivity in 100  $\mu$ L of supernatant was counted with a Cobra Gamma Counter (Packard-PerkinElmer). Percent specific lysis was calculated according to the formula: % specific lysis = (experimental release cpm - spontaneous release cpm)/(maximum release cpm - spontaneous release cpm)  $\times$  100. Spontaneous release never exceeded 5% of total release. Basal cytotoxicity against P815 cell line (in the presence of anti-CD56 mAb, as isotype control) never exceeded spontaneous release (data not shown).

Cytotoxicity is expressed in lytic units at 10% cytotoxicity/million PBMC (LU<sub>10</sub>/10<sup>6</sup>), calculated using D. Coggin’s software. One lytic unit is defined as the number of effector cells necessary to lyse 10% of target cells.

#### Evaluation of soluble NKG2D ligands

Enzyme-linked immunosorbent assays (ELISA) to detect soluble MICA, MICB and ULBP1 were obtained from R&D Systems (Minneapolis, MN, USA), and performed using 2  $\mu$ g/mL anti-MICA capture mAb (AMO1; BAMOMAB, Germany) essentially as previously described<sup>72</sup>. Soluble ULBP2 was detected as previously described.<sup>73</sup> Absorbance values of triplicate samples were obtained by subtracting readings at 540 nm from readings at 450 nm. Net absorbance was obtained by subtracting the reagent blank absorbance. Before the assay, plasma samples were diluted in PBS containing 0.1% Triton X-100 (vol/vol), and incubated for 30 min at 37°C.

#### Statistical analysis

Healthy controls and patients were compared with the 2-tailed Mann-Whitney *U* test. Patients at different time points

were compared with the Wilcoxon signed rank test. Pearson correlation test was employed for correlation analysis. Statistical analysis was performed with PRISM v.6 (GraphPad Software, San Diego, CA, USA) and SPSS v.20 (IBM Italia SpA, Segrate, MI, Italy) softwares. P values <0.05 (2-sided) were considered significant.

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## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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