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Dampening of cytotoxic innate lymphoid cells: A new tumour immune escape mechanism in B cell non-Hodgkin's lymphoma

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ABSTRACT

The role and regulation of innate immune cells is poorly understood in B-cell non-Hodgkin lymphoma (NHL). As natural killer (NK) cells, helper innate lymphoid cells (ILCs) are lymphocytes endowed with either anti- or pro-tumour activity and involved in inflammatory processes.

In our *ex vivo* analysis of NK cells and ILCs from NHL patients, we observed that, in comparison to healthy donors (HD), the frequency of the cytotoxic subset of NK cells, the $CD16^+$ NK, decreased in patients' peripheral blood. In general, circulating NK cells showed a pro-tumorigenic phenotype, while ILCs displayed a more activated/cytotoxic phenotype. Conversely, at the tumour site, in patients' lymph nodes, ILCs showed a low expression of granzyme. *In vitro* mixed lymphocyte-tumour cell cultures with HD PBMCs and NHL cell lines demonstrated that ILC cytotoxic potential was lowered by the presence of tumour cells but, in the absence of T regulatory cells (Tregs), their cytolytic potential was recovered.

Our data shed novel light on dysfunctional innate immunity in NHL. We suggest a new mechanism of tumour immuno-escape based on the reduction of cell cytotoxicity involving ILCs and likely controlled by Tregs.

1. Introduction

Natural killer (NK) cells are innate lymphoid cells endowed with potent cytolytic function, capable of killing infected cells and tumour cells. Circulating NKs could be distinguished in three subsets considering the expression of two cell-surface markers: CD16 and CD56. $CD56^{bright}$ NKs are mainly inflammatory cytokine producing cells which represent about the 10–15 % of total NK in the human blood [1]. $CD16^+$ NKs constitute about 90 % of total circulating NK cells and are highly cytotoxic, in particular they are responsible of antibody dependent cell cytotoxicity (ADCC) [2,3]. Another subset of NK cells is represented by $uCD56^{dim}$ NKs [4–6] which have been recently discovered to have cytotoxic activity, especially against hematologic malignancies *in vitro* [7,8].

Helper innate lymphoid cells (ILCs) are mainly tissue-resident cells [9,10] representing the most recent innate lymphocytes identified [11]. ILCs lack the lineage cell-surface molecules physiologically expressed by leucocytes, therefore they are referred as lineage negative (Lin-) and do constitutively express the IL-7 receptor α (CD127) [12]. In tissues, based on cell surface marker and transcription factor expression and cytokine production, ILCs are divided in three main subsets: ILC1s, ILC2s and ILC3s. In peripheral blood (PB), their progenitors (ILCPs) give rise to

Abbreviations: HD, healthy donor; ILC, innate lymphoid cell; ILCP, innate lymphoid cell progenitor; LN, lymph node; MLTC, mixed lymphocyte tumour culture; NHL, non-Hodgkin's lymphoma; NK, natural killer, PB, peripheral blood; PBMCs, peripheral blood mononuclear cells; TME, tumour microenvironment.

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ILC1, ILC2 and NK cell [13]. In humans, ILC1 development relies on the transcription factor *T*-bet; these cells mainly produce TNF-α and IFN-γ [14]. ILC2 is a GATA3 dependent subset characterized by the expression of CRTH2, also known as prostaglandin D2 receptor, IL-33 receptor (ST2) and a variable level of CD117 (also known as c-Kit) [15,16]. ILC3s are characterized by CD117 marker expression [17] and their development relies on RORγt expression. Cells which are phenotypically stackable to ILC3 in terms of surface marker expression in human peripheral blood were defined as ILCP and they were found to be multipotent progenitors able to give rise both to ILCs and NK cells [10,13]. Despite ILCs are very rare cells in the peripheral blood [18–19], they have pleiotropic functions such as tissue repairing [21], homeostasis maintenance, response against pathogens and are responsible of T cell response modulation [3,22]. ILCs can produce cytokines and chemokines which might result harmful in cancer by boosting the formation of an immunosuppressive environment that in turn supports tumour growth or progression [12,24,24]. The role of ILCs in cancer strongly depends on the tumour microenvironment (TME), thus on the ILC-tumour and ILC interactions with other immune cells [25].

Among hematologic malignancies, ILCs have been studied so far in acute promyelocytic leukaemia (APL), acute myeloid leukaemia (AML) and chronic lymphocytic leukaemia (CLL). ILC2s are increased in the blood of APL patients in which they over-produce IL-13, resulting in monocytic myeloid derived suppressor cell recruitment and in the establishment of an immunosuppressive environment [26]. In AML (non-APL) patients, it has been reported that an hypofunctional ILC1 subset is expanded compared to healthy donors (HD) [27]. A population of ILC1-like cells, possessing both properties of helper innate lymphoid cells and NK cells, was found to possess impaired cytotoxic abilities in AML patients at diagnosis [28]. Importantly it has also been demonstrated that the AML-mediated activation of the aryl hydrocarbon receptor (AHR) drives ILCP toward ILC1 and not toward NK cell, thus decreasing their number [29]. In AML, allogenic hematopoietic stem cell transplant represent a widely used treatment for hematologic malignancies [30]. After treatment, it has been found that ILCs are slower reconstituted compared to NK in a manner which is independent from the proportion of $CD34^{+}$ stem cells given in the graft [31]. However, both donor and recipient circulating ILCs show expression of activation markers and markers of cell recruitment to tissues. ILCs expressing these markers were associated to a lower incidence of graft versus host disease (GVHD) [32]. Moreover, the lower level of gut-homing $NKp44^{+}$ ILCP affects positively GVHD [31]. In CLL, an expansion of functionally altered ILCs has been described [33]. While their role in B-cell non-Hodgkin's lymphomas (NHL) is completely unexplored.

In the present study we investigated ILC and NK subset distribution and phenotype in PB and lymph nodes (LNs) of NHL patients. Our data indicate that, in the NHL TME, ILCs and NK cells display a less functional phenotype, and that T regulatory cells (Tregs) are at least in part responsible for the inhibition of potential anti-tumour activity of cytotoxic ILCs.

2. Materials and methods

2.1. Samples

PB samples from healthy donors (HD, gender, age distribution not significantly different from NHL patients) were provided anonymously by Ospedale Maggiore Policlinico of Milan with the authorization of Ethical committee and in compliance with the Italian Regulation. Patient samples were provided by European Institute of Oncology (Supplementary Table S3) (IEO, Project IEO886) in collaboration with the clinicians of the division of haematology-oncology. Patients' recruitment was authorized by the ethics committee in the context of the Project IEO886. Patients recruited in this study had a diagnosis of B-cell non-Hodgkin lymphoma and did not show any other concomitant tumours or pathologies affecting the immune system.

2.2. Cell isolation from primary samples

Peripheral blood mononuclear cells (PBMCs) of HD and patients isolated from venous peripheral blood were lysed with red blood cell lysis solution (0.4 % Ammonium Chloride, Potassium Bicarbonate and EDTA) for 5 min at 37 °C in a humidified chamber. Lymph nodes from NHL patients were provided by the division of Pathology at the IEO. Fresh samples were mechanically smashed with the bottom of a syringe piston and a 150 μm cell strainer with the addition of RPMI 1640 supplemented with 10 % FBS. Cells were washed in PBS, and, if necessary, red blood cells were lysed for 5 min at 37 °C in a humidified chamber.

2.3. Cell characterization, phenotyping, and sorting

Either whole blood or isolated PBMCs from HD and patients were stained with different panels of monoclonal antibodies. ILCs were identified as lineage (CD3, CD4, CD8, CD14, CD15, CD16, CD19, CD20, CD33, CD34, CD203c, FcεRI) negative and CD127 positive. In peripheral blood, ILCs were analyzed among $CD56^{-/dim}$ cells. CD117 and CRTH2 were used to distinguish the different ILC subsets. NK subsets were identified combining CD16 and CD56 markers among CD3 and CD19, CD20 negative cells.

2.4. Intracellular staining

For intracellular staining IntraStain-Dako™ (K2311, Dako) has been used. First, cells were stained with monoclonal antibodies for surface molecules and then washed and stained with IntraStain-Dako reagents.

2.5. Cell sorting

Cells were sorted at the IEO (Milan, Italy) and UNIL (Lausanne, Switzerland) flow cytometry facilities. BD FACSAria fusion and BD sorter Melody were used. Cells were sorted in 1.5 ml Eppendorf with 10 % FBS RPMI, on ice.

Samples were acquired with BD FACSCelesta (12-colour), BD LSRFortessa and BD LSR-Sorp or BD FACSLyric. Flow cytometry data were analysed with FlowJo software (Version 10) [34] and with Kaluza released by Beckman-Coulter [35]. List of antibodies used for flow cytometry analysis and cell sorting: see Supplementary Table 1.

2.6. Cell lines and cell cultures

Human cell lines SU-DHL-4 and SU-DHL-10 were bought from American Type Culture Collection (ATCC) and handled by the IEO Tissue culture facility. All cell lines were cultured and stored accordingly to manufacturer's instructions. Cells were tested every 6 months for Mycoplasma by means of the ATCC Universal Mycoplasma Detection Kit 30–1012, cultured for no more than 2 weeks, and used for no longer than 15 passages.

2.7. Mixed lymphocyte-tumour cell culture (MLTCC) experiments

HD PBMCs were cultured with or without SU-DHL-4 and SU-DHL-10 cell lines. Experiments were conducted using 24 or 48 well plates, maintaining a cell concentration of 800 cells/μL in RPMI supplemented with 10 % FBS (Euroclone®), 1 % penicillin–streptomycin (Euroclone®-ECB30010) and 1 % HEPES (Sigma®-H0887). The ratio between PBMCs and tumour cell lines was 2:1. SU-DHL-4 and SU-DHL-10 were marked with CellTrace Far Red Cell Proliferation Kit to be excluded from flow cytometry analysis. Experimental controls were designed using B cells separated from HD PBMCs by magnetic bead selection (CD19 MicroBeads, cat. 130–050-301, Miltenyi-biotech) following manufacturer's instructions.

Co-culture experiments excluding cell–cell contact were performed using 0.3 μm inserts for 24 or 12-well transwell plates (costar®-ref

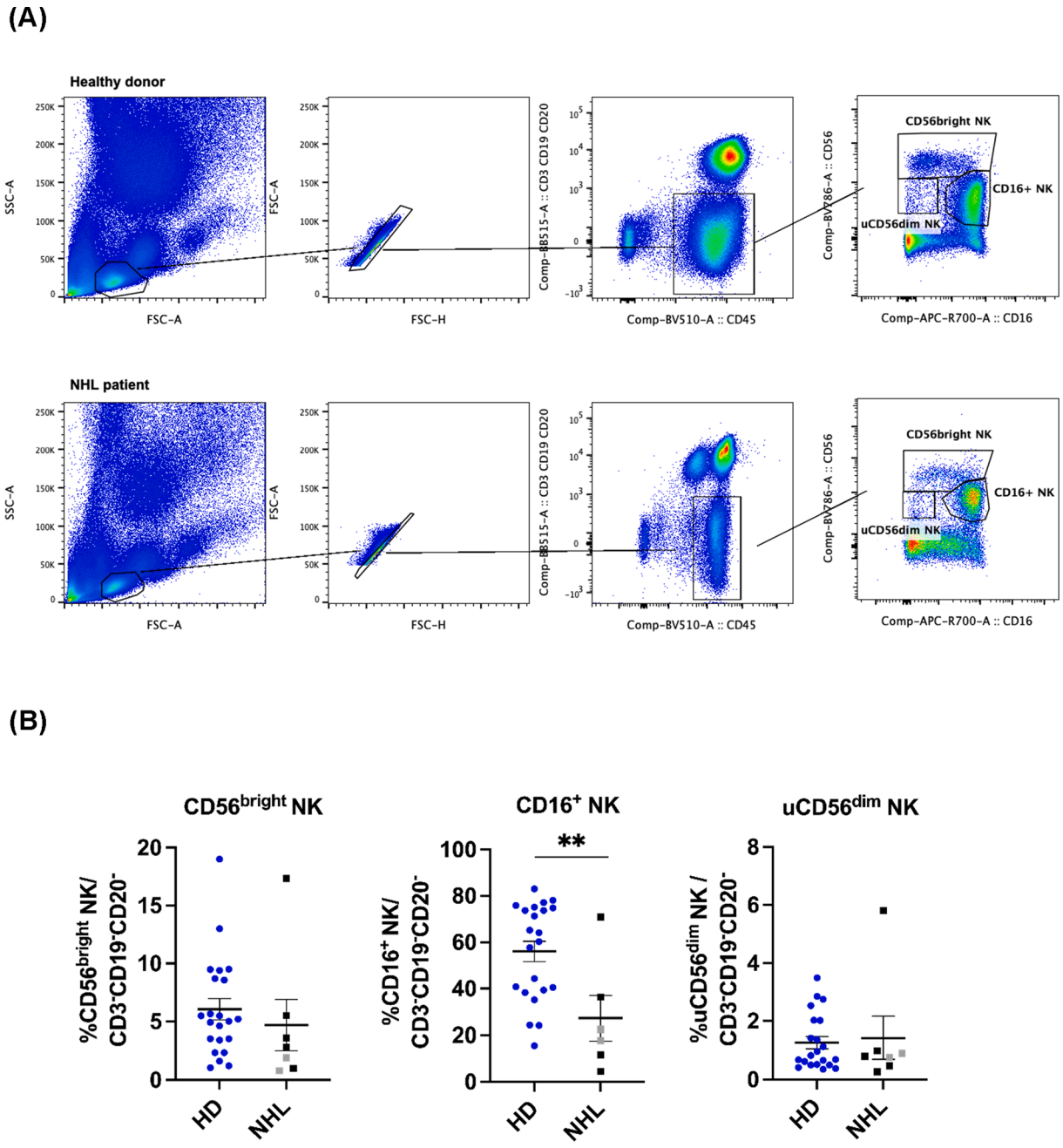

Fig. 1. NK distribution in HD and NHL patients' PB. After red blood cell lysis, whole peripheral fresh blood from patients and HDs was stained with antibodies to detect the three populations of NK cells ($CD56^{bright}$ NK, $CD16^{+}$ NK, $uCD56^{dim}$ NK) among $CD45^{+}CD3^{-}CD19^{-}CD20^{-}$ lymphocytes after doublet exclusion. **(A)** Representative dot plots showing the gating strategy used to distinguish NK populations in healthy donors (top) and in patients affected by NHL (bottom). **(B)** Comparisons of NK subset distribution in the PB of 22 HDs (blue) and 7 NHL (grey squares for aggressive NHL and black squares for indolent NHL), 21 HDs were evaluated for $uCD56^{dim}$ NK. Each subset was represented as percentage of $CD3^{-}CD19^{-}CD20^{-}$ lymphocytes. Statistical significance was calculated using Wilcoxon (Mann-Whitney) non-parametric test after testing the not-normal distribution of data through Shapiro-Wilk test. For normal distributed data set, parametric *t* test was applied.

3460).

2.8. MLTCC experiments with sorted cells

NK and ILCs were sorted by FACSAria fusion. NK were sorted as live, $CD45^{+}$, $CD3^{-}$ cells and $CD56^{bright}$, $CD16^{+}$ and helper ILCs as Lineage negative $CD127^{+}$ lymphocytes and put in culture with allogenic healthy B cells or with tumour cell lines at a 1:1 ratio in 96-well plates V-bottom in RPMI 10 % FBS, 1 %HEPES and with 10U/ml IL-2.

HD CD3 positive T cell exclusion was performed using cell sorting purification. PBMCs were stained with PerCP-Cy5.5 anti-human CD3 mAb. Both $CD3^{+}$ and $CD3^{-}$ T cells were collected and then co-cultured alone or with SU-DHL-4 and SU-DHL-10 tumour cell lines marked with CellTrace Far Red Cell Proliferation Kit, for 48 h.

For T regulatory cell exclusion, $CD4^{+}$ T cells were enriched with magnetic separation (Miltenyi-biotech) and then sort-purified as $CD25^{+}$ and $CD127^{low/-}$. PBMCs without Tregs were co-cultured alone or with SU-DHL-4 and SU-DHL-10 tumour cell lines marked with CellTrace Far Red Cell Proliferation Kit, for 48 h.

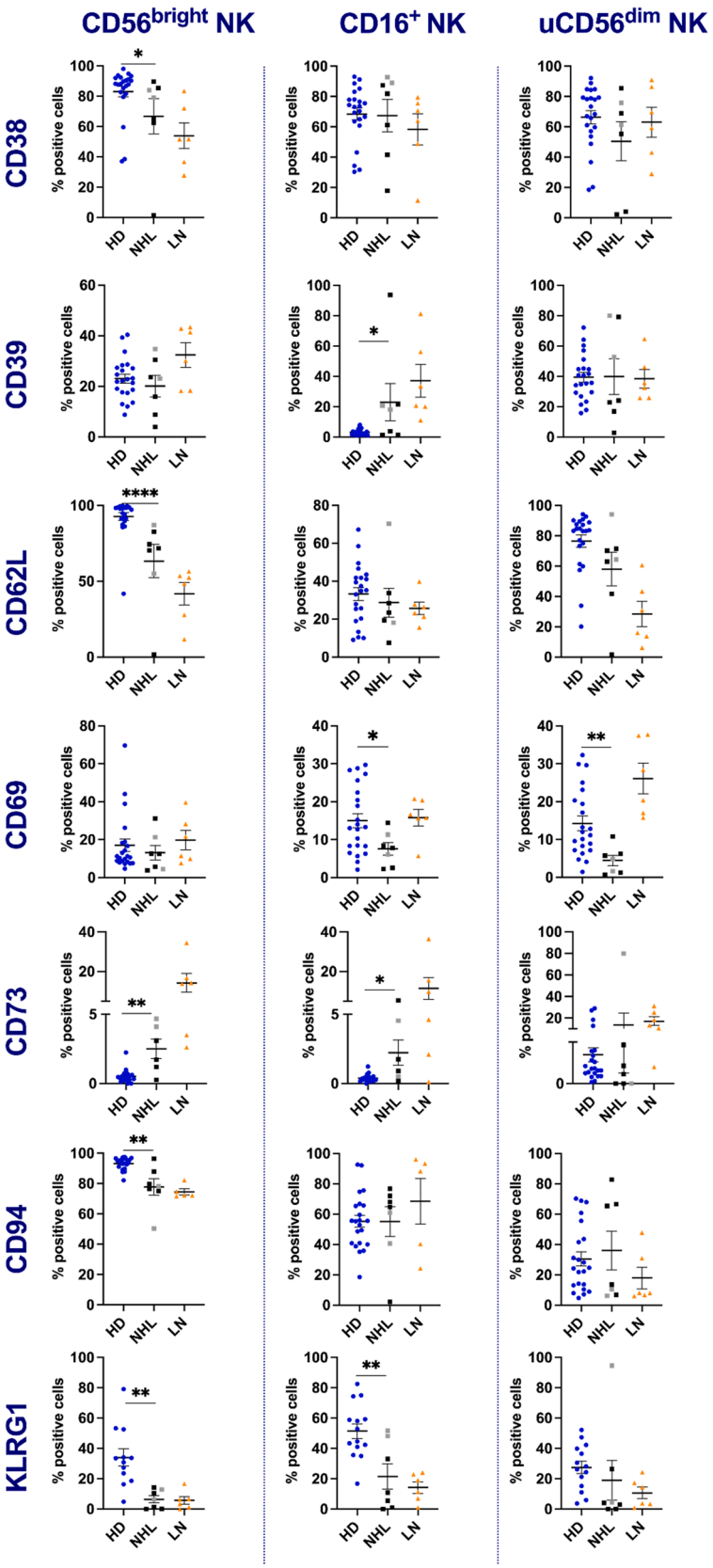

Fig. 2. NK phenotype in NHL patients' PB and LNs. The three populations of NK cells ($CD56^{bright}$ NK, $CD16^{+}$ NK, $uCD56^{dim}$ NK) were detected in whole peripheral fresh blood from NHL patients and HD as reported in Fig. 1A and in patients' LN with the same gating strategy. Expression of the indicated proteins, showed as percentage of positive cells, and considered as independent, was analysed among each subset. Sample size for PB was HD n = 22 (blue dots) and NHL n = 7 (grey squares for aggressive NHL and black squares for indolent NHL), except for KLRG1 in which n = 12 HD and n = 7 NHL were analysed. Orange triangles represent data obtained from single cell suspensions of tumoral LN (n = 6) of non-matched NHL patients (5 LNs were analysed for CD94 in $CD56^{bright}$ NK and $CD16^{+}$ NK). Statistical significance was tested only considering data from PB of HD and NHL and not LN, using Wilcoxon (Mann-Whitney) non-parametric test after testing the not-normal distribution of data through Shapiro-Wilk test. For normal distributed data set, parametric *t* test was applied.

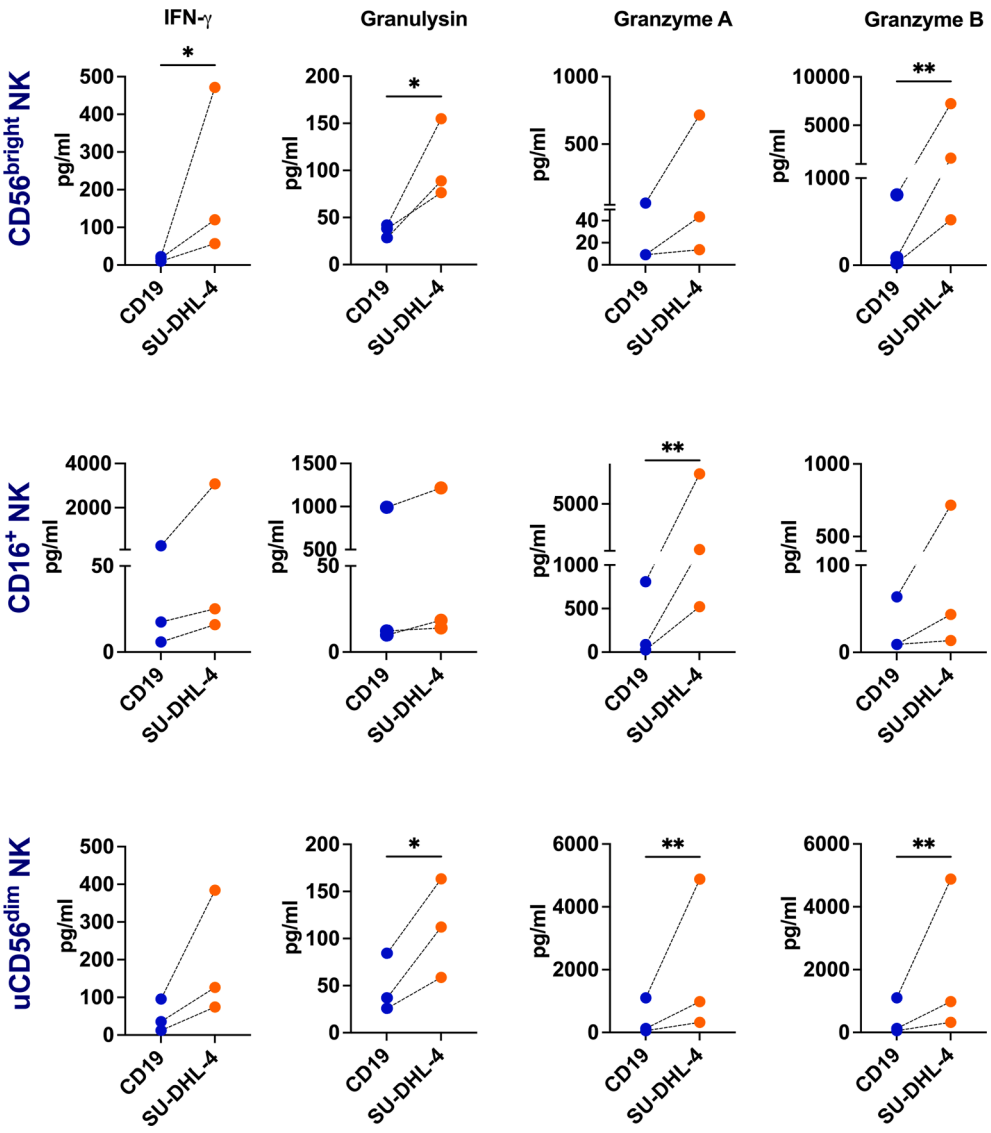

Fig. 3. Co-culture experiments with sorted NK cells and NHL tumour cells. The experiments were performed using PBMCs from 3 independent HDs, cytokines were measured using LEGENDplex™ Human CD8/NK Panel. NK cells were sorted as CD56^{bright} NK, CD16^{+} NK, uCD56^{dim} NK (Fig. 1A for gating strategy applied). Release of IFN-γ, Granulysin and Granzyme (pg/ml) by NK cell subsets was measured in the supernatants of cell co-cultures. Graphs show NK + CD19 allogenic B cells (CD19) vs NK + SU-DHL-4 (SU-DHL-4) co-cultures. Statistical analysis was conducted using ratio paired *t*-test.

2.9. Plasma and supernatants analysis

To detect cytokines in supernatants of co-cultures and to analyse plasma composition, we performed analyses using LEGENDplex™ (LEGENDplex™ Human CD8/NK Panel, LEGENDplex™ Human Cytokine Panel 2). Both supernatants and plasma were collected and stored at −20 °C for a month or at −80 °C for longer time. Samples were thawed only once or twice. After 10 min they reached 20–25 °C they were used for the immunoassay. The samples were read on Beckman CoulterNavios flow cytometer. Each sample was acquired at low flow rate and a maximum of 5000 events were recorded. The analysis was performed using LEGENDplex™ Data Analysis Software. Beads were distinguished for SSC-A and FSC-A; beads classification was reported on FL6 signal (APC) and report signal on FL2 (PE). Bead concentration was adjusted depending on the kit batch.

2.10. Data analysis

Flow cytometry data were analysed with FlowJo software [34] and with Kaluza released by Beckman-Coulter [35]. Statistical analyses were performed using Prism GraphPad 9.1 [36]. Data normal distribution was tested with Shapiro-Wilk test. Pairwise comparisons were performed using either the Student *t* test or the Mann-Whitney non parametric test according on normal or not normal distributions respectively.

For paired co-culture experiments the normality was tested as before, and normal multiple ordinary-one-way ANOVA or Friedman test was applied accordingly using Bonferroni correction.

For original data, please contact francesco.bertolini@ieo.it.

3. Results

3.1. NK cells in NHL patients showed an immunosuppressive phenotype

The distribution of the three subsets of NK cells, CD56^{bright} NK, CD16^{+} NK and uCD56^{dim} NK was evaluated in NHL patients' peripheral blood (PB) and compared to that of HDs (Fig. 1A for gating strategy). Only the frequency of CD16^{+} NK cells, which represent the highly cytotoxic subset of NK cells, was significantly reduced compared to HD (20.17 % median value of total NK cells in NHL patients, compared 62.20 % median in HD ($p < 0.01$)), indicating a down-modulation of cells displaying cytotoxic activity in NHL patients (Fig. 1B). The phenotype and activation status of NKs were also analysed.

A multiparametric flow cytometry panel, including markers for cell activation and recruitment to tissues, was designed (Supplementary Table 2, NK phenotype panel) and applied to PB and tumoral LN. As reported in Fig. 2, in patients' PB CD56^{bright} NK cells exhibited a

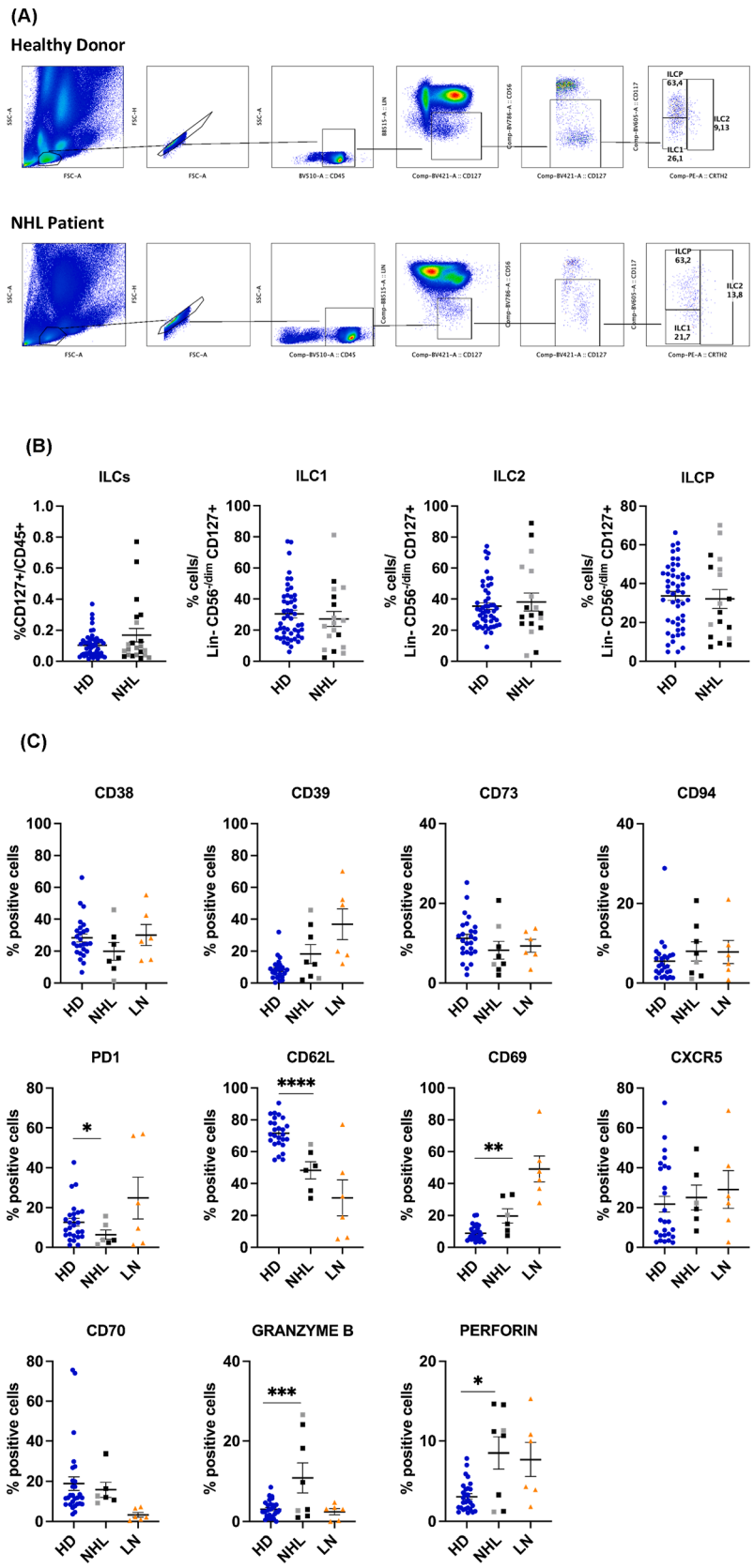

Fig. 4. ILC distribution and phenotype in HD and NHL patients. **(A)** After red blood cell lysis, whole peripheral fresh blood from NHL patients (n = 18) and HD (n = 50) was stained with antibodies to detect the three populations of ILCs (ILC1, ILC2 and ILCP) among $CD45^+$ $LIN^-CD127^+CD56^{-/dim}$ lymphocytes after doublet exclusion. Dot plots show the gating strategy used to distinguish ILC populations in one representative HD (top) and one NHL patient (bottom). **(B)** Comparisons of ILC subset distribution in HD (blue) and NHL patients (grey squares for aggressive NHL and black squares for indolent NHL). ILC1, ILC2 and ILCP were represented as percentage of positive cells among total $CD127^+Lin^-$ $CD56^{-/dim}$ lymphocytes, while total ILCs as $CD127^+$ $Lin^-/CD56^{-/dim}$ cells in the $CD45^+$ gate. Normality of the data distribution was tested using Shapiro-Wilk test. Statistical significance was tested using Wilcoxon (Mann-Whitney) non-parametric test if the data were not-normally distributed, otherwise unpaired *t* test was used. **(C)** ILC phenotype was evaluated analysing the indicated proteins considering them independent. Percentage of expression was reported inside total ILCs (Lin^- $CD56^{-/dim}$ $CD127^+$) in the PB of HD (blue) and NHL patients (grey squares for aggressive NHL and black squares for indolent NHL). Single cell suspensions of NHL LN were obtained, and the same panels and gating strategy used for PB was applied (orange triangles for LN). For CD38 NHL n = 7, for CD39, PD-1, CD73, CD94, granzyme B and Perforin NHL n = 8, for CD62L, CD69 and CXCR5 NHL n = 6. Statistical significance was tested only considering data from PB of HD and NHL using Wilcoxon (Mann-Whitney) non-parametric test after testing the not-normal distribution of data through Shapiro-Wilk test. For normal distributed data set, parametric unpaired *t* test was applied.

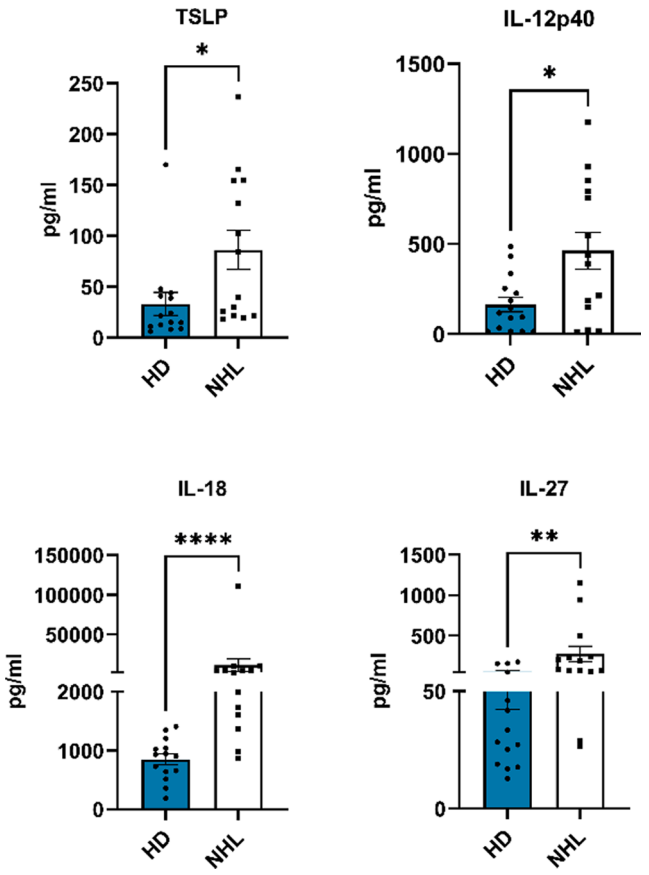

Fig. 5. Inflammatory cytokines upregulated in the plasma of NHL patients compared to HD. Plasma of 14 HD and 14 NHL patients were evaluated by using LEGENDplex™ for the release of the indicated cytokines measured in pg/ml. Only the cytokines significantly changed between HD and NHL were reported in figure. Statistical analyses were performed applying Mann-Whitney test.

significant decrease of the activation markers CD38, CD62L and CD94 and also of the maturation molecule KLRG1; while CD73, an ectoenzyme that, if over-expressed, participates in the establishment of an immunosuppressive environment [37], was upregulated. As for $CD56^{bright}$ NK, also $CD16^{+}$ NKs showed a slight increase of CD73, that paralleled CD39 expression. CD39 is an integral membrane protein that, together with CD73, phosphohydrolyzes ATP and becomes upregulated in inflammatory environments. Instead, CD69 and KLRG1 resulted downregulated. In $uCD56^{dim}$ NK, only CD69 expression was decreased in the patients' PB compared to HD. These data might suggest a pro-tumorigenic role of NK cells in NHL and strength the hypothesis that their cytotoxic potential could be counteracted in the TME. Therefore, investigating the phenotype of NK cells in tumoral lymph nodes of NHL patients, we found that, at tumour site, NK cells showed an even higher expression of the immunosuppressive markers CD73 and CD39, compared to PB, especially in $CD56^{bright}$ and $CD16^{+}$ NKs (Fig. 2, orange triangles).

3.2. NK cells are functionally activated in the presence of the tumour cells

To study if NK phenotype and function can be directly modulated by NHL cells, we performed *in vitro* co-culture. The three NK cell subsets were sorted from HD PB based on CD16 and CD56 expression in the $CD3^{-}CD19^{-}CD20^{-}$ gate and co-cultured with the SU-DHL-4 NHL cell line (or with allogenic HD $CD19^{+}$ B cells, as a non-tumour-related B cell control). After 48 h of co-culture, among the markers analysed, only the CD39 marker was upregulated in the presence of the tumour cell line in the $CD56^{bright}$ subset, while no significant changes were detected for CD69 and CD94 in any subtype of NK cells (Supplementary Fig. S1). Instead, an increased level of IFN-γ, granzymes and granulysin were measured in the co-culture supernatants in the presence of tumour cells in all the three subsets compared to the B cell allogenic controls (Fig. 3). These data suggest that NK cells could acquire cytotoxic capacities when encountering tumour cells, thus in the TME.

3.3. ILCs with activated/cytotoxic phenotype are expanded in NHL patients' PB but not in tumour LNs

To investigate the distribution of the other ILC family members in HD and NHL patients' PB, we designed a 12-colour multiparametric flow cytometry panel (gating strategy in Fig. 4A). No differences between HD and NHL patients, either in terms of ILC frequency or subset distribution, have been observed (Fig. 4B). Noteworthy, the percentages of ILCs were less than 1 %, supporting the evidence that these cells constitute a rare subset also in NHL patients.

To characterize the phenotype of patients' circulating total ILCs, three multiparametric flow cytometry panels, including markers of cell activation, exhaustion and cell recruitment to tissues were applied (Supplementary Table 2). Among the markers analysed, total ILCs in NHL patients' PB showed a slight but significant decrease of PD-1 and CD62L expression and a concomitant increase of activation/functional markers, such as CD69, granzyme B and perforin (Fig. 4C), suggesting a potential involvement of ILC in direct anti-tumour activity.

Taking advantage of LN samples from non-matched NHL patients, we compared ILC subset distribution among PB and LN. In the PB the three ILC subsets were quite equally distributed, while ILC1 were highly represented in the LNs (Supplementary Fig. S2). Moreover, total ILCs displayed a peculiar phenotype pattern in LNs. CD39, PD-1 and CD69 were found to be highly expressed, while granzyme B was almost absent (Fig. 4C, orange triangles). Taken together, these data suggest that circulating ILCs in patients have been triggered by cancer cells but, at tumour site, they might be deregulated and thus less functionally active.

To define if ILC-modulating cytokines were quantitatively altered in NHL patients, we compared NHL patient and HD plasma content, founding some inflammatory cytokines upregulated in patients: TSLP ($p < 0.05$), IL-12p40 ($p < 0.05$), IL-18 ($p < 0.00001$) and IL-27 ($p < 0.01$). IL-1β was also upregulated, although barely detectable (Fig. 5). The upregulation of these molecules in NHL patients might be due to the inflammatory process promoted by the tumour. These cytokines could directly or indirectly modulate the patients' innate response and promote ILC and NK recruitment to tumour tissues.

3.4. ILC activation is affected by NHL cell contact and by Treg presence

Since ILC phenotype and functions can be affected by the TME, we tested the effects of tumour cells on ILCs. PBMCs from HD were cultured alone or with SU-DHL-4 or SU-DHL-10 cell lines. After 48 h of co-cultures in the presence of NHL cell lines were harvested and analysed by multiparametric flow cytometry analysis. Total ILCs showed a significant down-modulation of the activation marker CD38 in the presence of the SU-DHL-4 cell line compared to the control (total PBMCs only), while CD62L reduction was found only after the co-culture with SU-DHL-10. CXCR5 was the only marker found to be upregulated with both cell lines (Fig. 6A); while the expression of granzyme B was significantly diminished with both the models if compared to the control (total PBMCs only) (Fig. 6B). Moreover, the same experiments performed with transwells revealed that the effect was mediated by cell–cell contact between PBMCs and tumour cells (Supplementary Fig. S3).

Since other immune cells present in the TME could directly downregulate the potential cytotoxic anti-tumour activity of ILCs at tumour site, we decided to deplete Tregs from HD PBMCs by sorting purification. Then Treg-depleted PBMCs were co-cultured for 48 h with the two NHL cell lines or alone, as control (PBMCs-Tregs only). Interestingly, granzyme B expression in ILCs resulted upregulated in presence of both the

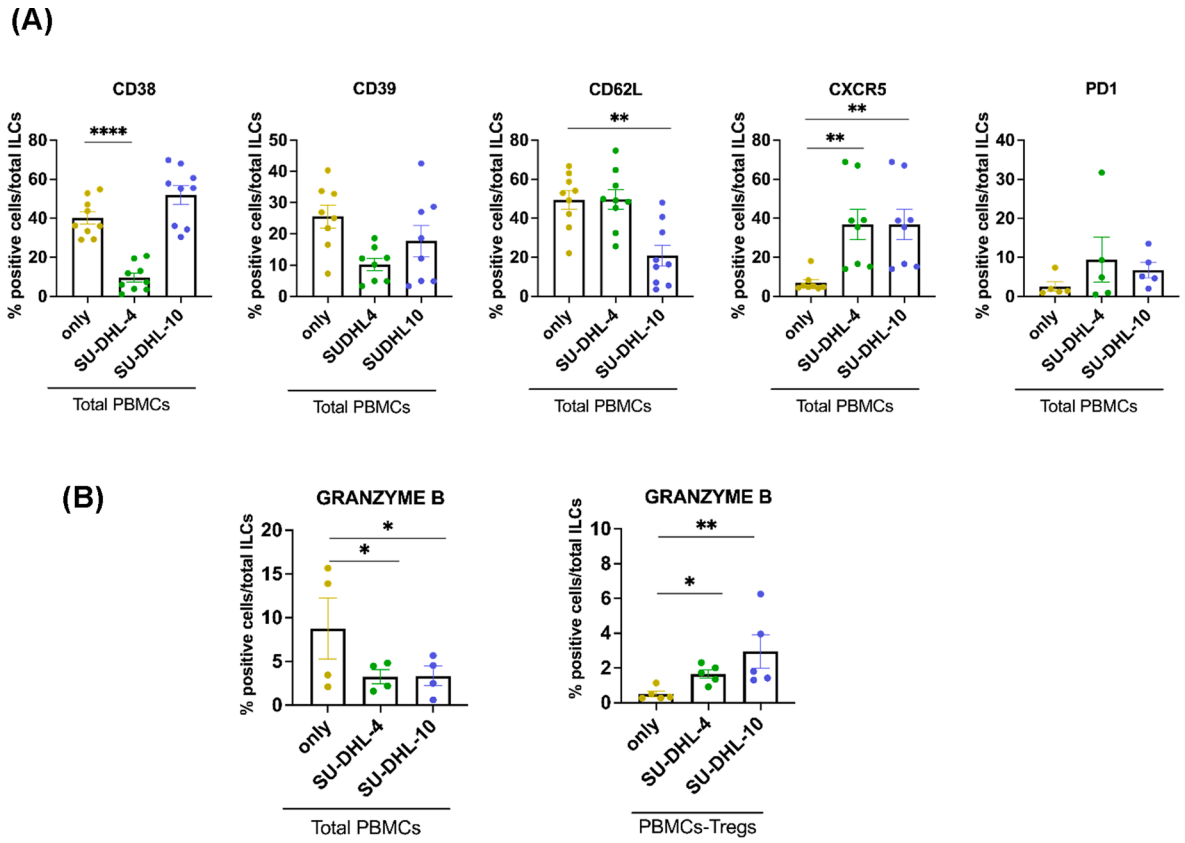

Fig. 6. MLTCC experiments with PBMCs and NHL cell lines. (A) MLTCC experiments were conducted using total HD PBMCs (n = 9) and two tumour cell lines (SU-DHL-4 and SU-DHL-10); Histograms show the percentage of the indicated markers in total ILCs in untreated PBMCs (PBMC only) and in PBMCs/cell lines co-cultures (PBMCs + SU-DHL-4 or SU-DHL-10). Comparisons were performed between untreated PBMCs and PBMCs with tumoral cell lines. Statistical significance was tested using paired one-way ANOVA in case of normal distribution of the data set or multiple paired non-parametric Friedman. **(B)** Intracellular granzyme B expression in ILCs was evaluated in total HD PBMCs (Total PBMCs n = 4) and in PBMCs after Treg depletion (PBMCs-Tregs, n = 5). Comparisons were performed between total PBMCs (only condition) and total PBMCs in the presence of the two tumour cell lines (left panel) or between Treg-depleted PBMCs (only condition) and Treg-depleted PBMCs after cell line co-cultures (right panel). Statistical analysis was performed using ratio paired *t* test and Mann-Whitney *t* Test; p values were considered significant when $p < 0.05$.

cell lines if Treg were depleted from total PBMCs (Fig. 6B, right), while it was significantly diminished in total PBMCs in the presence of tumour cells (Fig. 6B, left). These findings highlight a potential role of Tregs in ILC functional control of NHL, since they could directly or indirectly participate in the inhibition of ILC cytotoxic activity at tumour site.

4. Discussion

The role of ILCs in cancer is on debate and remains completely unexplored in NHL. In our study, we described NK and ILC distribution and phenotype in PB and LNs of patients affected by NHL.

We found that $CD16^{+}$ cytotoxic NK cells were downmodulated in patients' PB and that NK cells upregulated CD39 and CD73 expression in tumoral LNs. These markers define immune-checkpoints expressed by tumour infiltrating NK cells and collaborate in the establishment of an immunosuppressive environment, as demonstrated for other tumours such as breast cancer, ovarian cancer and melanoma [38–39], indicating a potential pro-tumorigenic role, not yet described, for NK cells in NHL. Also ILCs have been described to contribute to the formation of ATP catabolite, adenosine (Ado) through CD73 expression, and in melanoma Ado contributes also to the ILC suppression [41].

Comparing circulating ILCs from NHL patient and HD, we found the same frequency of total ILCs and the same distribution. While, investigating the phenotype of circulating ILCs, we found that ILCs showed an increased expression of activation markers in NHL patients compared to HD; in particular, CD69, important for cell retention in tissues, and granzyme B and perforin were upregulated, thus suggesting that ILCs in patients could be endowed with cytotoxic functions. Concomitantly, PD-1, one of the most important immune-checkpoint, was downregulated, thus strengthening the hypothesis that these cells might be ready to be activated and potentially endowed with anti-tumour activity. Cytotoxic ILCs were described in human to develop from helper innate lymphoid cells; in particular, the cytotoxic functions of these cells were found to be likely dependent on the secretion of IL-12 by dendritic cells in response to pathogens, viruses and other signals [42]. Their role in cancer is still unexplored. Instead, the role of PD-1 in ILC biology is under intense investigation. It has been reported that its expression vanished during ILC differentiation [43] but resulted upregulated during inflammation involving the lungs [44]. PD-1 expression reduced the type 2 cytokine release by ILC2 [45], while ILC3 were found to express high levels of functional PD-1 receptor in the pleural effusions of patients affected by metastatic cancers [46].

Considering the importance and the role of cytokines in affecting ILC functions and plasticity, we explored plasma molecules both in patients and HDs. TSLP is known to induce ILC2s, giving them a survival advantage [47]. Among the cytokines upregulated in NHL, data from literature show that IL-12p40 and IL-18 can induce cell cytotoxicity [48]. In particular, IL-12 can promote ILC cytotoxicity and could be likely displayed when NK cell functions are silenced or reduced [42]. Thus, the accumulation of these two cytokines in NHL patients' plasma could be linked to the higher expression of granzyme B and perforin observed in patient ILCs compared to HD. We also detect an

accumulation of IL-27 in patients. This cytokine mediates Th1 development from naïve $CD4^+$ T cells [49] but also it has been found to enhance the activity of Tregs by inducing the expression of the Lag3 molecule, involved in Treg suppressor activity of Tregs [50,51].

Compared to PB, total ILCs in the LNs barely express granzyme B and perforin, indicating that ILCs could be dysfunctional at tumour site. Through *in vitro* experiments, we studied ILC behaviour in the presence of NHL cells. Results show that ILC activation was affected by tumour cells in a contact-dependent manner in the presence of other immune cells. Our results also show that ILC phenotype could be different depending on the NHL cell line used. To our knowledge, SU-DHL- 4 and SU-DHL- 10 have distinct expression of PDL-1 and PDL-2. SU-DHL-10 derive from a more indolent lymphoma cells and do not express PDL-1 or PDL-2, while SU-DHL-4 show high expression of PDL-1 and are more invasive *in vitro* [52]. Our experiments suggest that the immunosuppressive microenvironment set by the tumour could negatively regulate ILCs. Treg-depleted PBMCs co-cultured with NHL cell lines have a distinct impact on ILCs as compared to total PBMCs. Granzyme B expression in ILCs increased in the presence of tumor cells if Tregs were depleted. These findings imply that Tregs present in the TME might inhibit cytotoxic ILC functions and potentially downregulate their anti-tumour activity in NHL patients.

Declarations

All methods were carried out in accordance with relevant international and national guidelines and regulations. All experimental protocols were approved by IEO and Ospedale Maggiore Policlinico Ethical committees.

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CRediT authorship contribution statement

Stefania Roma: Conceptualization, Formal analysis, Investigation, Writing – original draft. **Chiara Camisaschi:** Methodology, Conceptualization, Writing – review & editing. **Patrizia Mancuso:** Conceptualization. **Sara Trabanelli:** Conceptualization, Writing – review & editing. **Anna Vanazzi:** . **Stefania Villa:** Resources. **Daniele Prati:** Resources. **Stefano Fiori:** Resources. **Daniele Lorenzini:** . **Valentina Tabanelli:** Resources. **Stefano Pileri:** Resources. **Corrado Tarella:** Resources. **Camilla Jandus:** Conceptualization, Writing – review & editing. **Francesco Bertolini:** Supervision, Project administration, Funding acquisition, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2022.104615.

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