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Role and regulation of human innate lymphoid cells and natural killer cells in lymphoma

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List of abbreviations

- AML acute myeloid leukemia
- APC antigen presenting cell
- APL acute promyelocytic leukemia
- AREG amphiregulin
- ADCC antibody dependent cell cytotoxicity
- BC breast cancer
- BCR B cell receptor
- BM bone marrow
- CD cluster of differentiation
- CHILIP common helper innate lymphoid progenitor
- CILP common innate lymphoid progenitor
- CLL chronic lymphocytic leukemia
- CLP common lymphoid progenitor
- COPD chronic obstructive pulmonary disease
- CRC colorectal cancer
- DC dendritic cell
- DLBCL diffuse large B cell lymphoma
- DNAM DNAX accessory molecule
- Eomes eomesodermin
- FL follicular lymphoma
- GM-CSF granulocyte-macrophage colony-stimulating factor
- HL Hodgkin lymphoma

- HLA human leukocyte antigen
- HSC hematopoietic stem cell
- iBC innate B cell
- IBD inflammatory bowel disease
- Id2 inhibitor of DNA binding 2
- ieILC intra epithelial ILC
- IFN interferon
- Ig immunoglobulin
- ILC innate lymphoid cell
- IL-n interleukin
- ITIM immunoreceptor tyrosine-based inhibition motif
- KIR inhibitory killer cell immunoglobulin-like receptor
- LDHL lymphocytes-depleted Hodgkin lymphoma
- LN lymph node
- LRHL lymphocytes-rich Hodgkin lymphoma
- LTi lymphoid tissue inducer
- MALT mucosa-associated lymphoid tissue
- MCHL mixed Cellularity Hodgkin lymphoma
- MCL mantle cell lymphoma
- MDSC myeloid derived suppressor cell
- MHC major Histocompatibility Complex
- MLTC mixed lymphocyte-tumor cell culture
- MZL marginal zone lymphoma
- NCR natural cytotoxicity receptor
- NHL non-Hodgkin lymphoma

- NK natural killer
- NKT natural killer T
- NLPHL nodular lymphocyte predominant Hodgkin lymphoma
- NMZL nodal marginal zone lymphoma
- NSCLC non-small cell lung cancer
- NSHL nodular Sclerosis Hodgkin lymphoma
- PB peripheral blood
- PD-1 programmed cell death protein 1/2
- PDL-1/2 programmed cell death protein ligand 1/2
- PGD2 prostaglandin receptor 2
- PMBCL primary mediastinal large B-cell lymphoma
- SLL small-cell lymphocytic lymphoma
- SLO secondary lymphoid organ
- SMZL splenic marginal zone lymphoma
- Tc T cytotoxic
- Tfh T follicular helper
- Th T helper
- TNF tumor necrosis factor
- Treg T regulatory
- TSLP thymic stromal lymphopoietin
- VDJ variable diversity joining
- WHO World health organization

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To my grandma F.

1. Abstract

Innate immune system can be helpful or harmful in cancer regression depending on tumor microenvironment. Innate lymphoid cells are constituents of innate immune system with helper innate lymphoid cells (ILCs) that are mainly tissue-resident lymphocytes and natural killer cells (NK) that are pro-inflammatory cells displaying cytotoxic activity. In our project, we explored the role and regulation of helper ILCs and NK cells in B-cell non-Hodgkin lymphoma and Hodgkin lymphoma, hematologic malignancies derived from mature B-lymphocytes. We found that in peripheral blood of non-Hodgkin lymphoma patients, helper ILCs showed higher expression of activation markers compared to healthy donors while NK cells show a potential pro-tumour role. Moreover, analysing patients' plasma we observed some upregulated chemokines and cytokines compared to healthy donors. We performed *in vitro* mixed lymphocyte-tumour cell cultures of peripheral blood mononuclear cells (PBMCs) from healthy donors and lymphoma cell lines, using healthy B-lymphocytes and PBMCs grown alone as controls. Our experiments showed a contact dependent downregulation of activation markers on helper ILCs in presence of tumour cells. However, in absence of all other immune cells, ILCs showed high cell activation and cytokine production in presence of lymphoma cell lines, thus meaning that among the immune cells there is a cell family able to counteract the ILC activity, thus contributing potentially to the formation of an immunosuppressive environment. We suggested that T regulatory cells are at the base of this mechanism, since their removal from PBMCs resulted in a rescue of ILC cytotoxic potential. Altogether, these data suggest a mechanism of innate lymphoid cell regulation that could become a new possible therapeutic strategy for non-Hodgkin lymphoma patients.

In parallel, we also investigate the phenotype and distribution of ILCs and NK in Hodgkin lymphoma. Results show that in HL, ILCs were increased compared to healthy donors, in particular ILC1. Moreover, patient circulating ILCs express higher level of granzyme B and perforin. These preliminary data suggest an involvement of ILCs in HL biology.

2. Introduction

2.1. The Immune system

The immune system is a complex network of cells, organs and many other components which allows a multicellular organism to resist and protect itself against potentially dangerous microbes. Depending on the type of response and on its speed, immunity can be named as innate or adaptive[1]. The innate immunity is the first and fast used barrier against pathogens, it acts immediately without pre-conditioning of the environment by killing or eliminating the pathogens. The nature of innate immune response is highly conserved throughout animals, thus empowering its importance in guaranteeing survival[2]. It was in 1882 that Eli Metchnikoff described the white blood cells as the responsible for pathogen destruction[3].

Adaptive immunity is the trait of higher animals and refers to the ability of generating a specific however less rapid response to a pathogen[4]. In 1890 Emil von Behring and Shibasaburō Kitasato studying the body fluids (also named humors), discovered components of the fluids which confer immunity after the transfer from an animal to another: these were the antibodies[4]. Along with humoral immunity, there is the cellular immunity, mediated by cellular components.

2.1.1. Components of the immune system

Organs, cells, cytokines are all components of the immune system. The organs are responsible of immune cell production and release. The bone marrow (BM) is the site in which all the blood cells derive, generated from a common hematopoietic precursor stem cell, however the immune cells undergo a process of maturation in different sites[5]. B cells and T cells start their maturation in the primary immune organs, which are BM and thymus. Then, these cells end up into secondary immune organs (lymph nodes, spleen, and mucosa-associated lymphoid tissue -MALT-). Cells can easily move from on organ to another thank to lymphoid fluids and blood[4].

2.1.2. Cells of the innate immune system

Cells are the other components of the immune system. Different types of cells guarantee the quick responses of the innate immune system: granulocytes, macrophages (derived from monocytes), mast cells, dendritic cells, helper ILCs and NK cells.

Granulocytes include three different cell types endowed with a short lifespan; they are neutrophils, basophils and eosinophils. *Neutrophils* are phagocytic cells able to produce granules full of cytokines that act displaying their cytotoxicity for bacteria and fungi.

Basophils represent the key players in allergic diseases, which use the molecule histamine to attack multicellular parasites, while *eosinophils* are able to kill multicellular parasites.

Macrophages is a group of efficient phagocytic cells, derived from monocytes which circulate in the blood[6]. These cells are able to uptake and destroy microbes and also to release molecules, cytokines and chemokines, acting as signals to recruit other immune cells in the infected area[7]. Macrophages undergo polarization process depending on the stimuli received in the environment in which they are. Classical activated M1 macrophages can be generated in presence of infection by bacteria or viruses. This type of macrophages produce pro-inflammatory cytokines, such as interleukin (IL) IL-1, IL-6, IL-12, IL-23, TNF[8]. Fungi and helminth infections polarize the macrophages to alternative activated M2 type, which produce IL-10 and TNF[9].

Mast cells are responsible of inflammation; they release histamine and heparin in presence of an infection and are also involved in allergic diseases promoting vasodilatation[10].

Dendritic cells are the bridge between the adaptive and the innate immunity they are endowed with the ability to internalize and destroy microbes[11], however they are also antigen presenting cells (APCs), therefore they activate adaptive immune cells through their ability to present antigen peptides on their cell surface[4].

NK cells and *helper ILCs* will be discussed in the section **2.2**.

2.1.3. Cells of the adaptive immune system

The *B cells* are usually considered cells that produce antibodies. Antibodies are proteins defined as the secreted form of the B cell receptor (BCR), namely the typical surface marker of B cells[12]. Antibodies, also named immunoglobulin (Ig) are Y shaped and consist in heavy chains and light chains bounded together through disulfide bonds. The apical part of Y shaped antibody is the most variable region that gives specificity to the pathogen. The light chain can be either type κ or λ and the ratio in human between these two types is 2:1.

Different ratios are due to an abnormal B clonal expansion that might associate with a B hematologic malignancy[12].

Concerning the heavy chain, it can be μ, δ, γ, α or ε giving therefore rise to IgA, IgD, IgE, IgG or IgM whose properties are independent to the light chain and are conferred by the constant region, located in the carboxyl-terminal part rather than the apical part of the protein[12].

In human, the development of B cells starts in the bone marrow from the hematopoietic stem cell (HSC). B cell transcriptional program occurs in concomitance with the contiguity between maturing B cells and the stroma of BM cells which support their development through growth factors and cytokines[13]. For B cell maturation it occurs a process named V (variable) D (diversity) and J (joining) (VDJ) recombination that consists in DNA segments rearrangement involving the exons encoding for antigen binding domains[14]. The binding between the transmembrane protein VLA-4 expressed in B stem cell and VCAM expressed by BM cell induces V(D)J recombination of the heavy chains while the production of IL-7 by BM cells induces V(D)J light chain recombination in pre-B cell stage of maturation.

During B cell maturation the different stages of development are characterized by consequent rearrangements of the heavy (H) and light (L) gene segments of immunoglobulin (Ig)[15] (**Figure 1**). In the BM there is also a clonal selection of BCR in which B cells carrying autoreactivity undergo either to other light chain rearrangements or are counter selected, therefore only the B cells which are not excluded by this process migrate to the spleen and are able to complete the process of maturation[13].

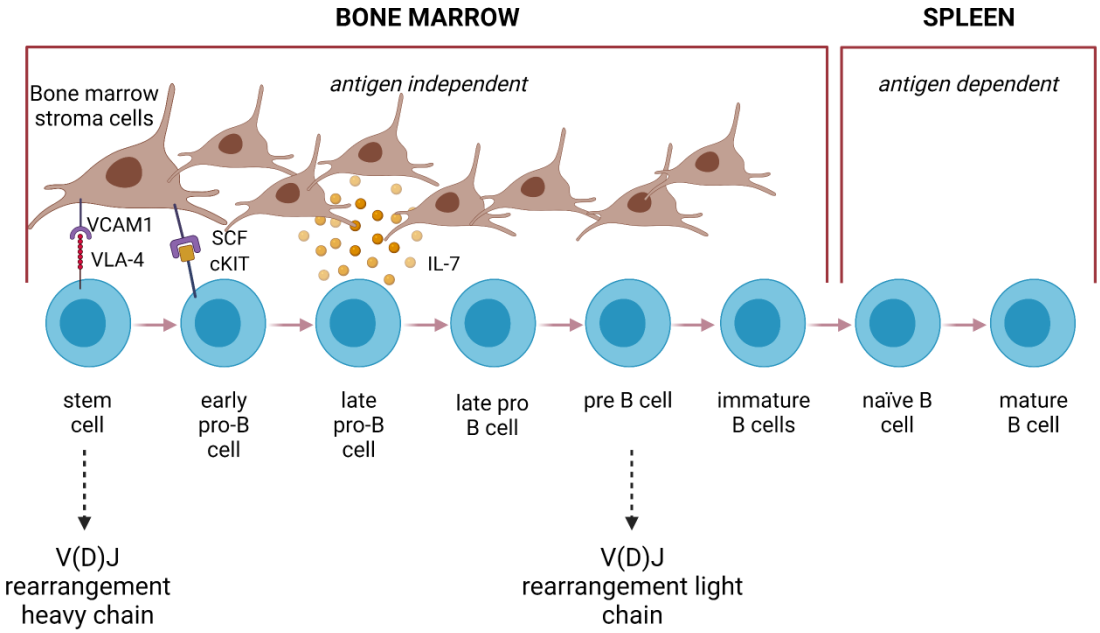

Figure 1. B cell development and BCR formation

The illustration represents the maturation of B cells in bone marrow and in spleen through V (variable) D (diversity) and J (joining) (VDJ) recombination, responsible of BCR formation. (Figure created with BioRender.com with reference to [16]).

T cell development takes part in the thymus, thanks to the interaction between cortical epithelial cells of thymic stroma and thymocytes defined to be the pre -T cells (**Figure 2**). Initially the thymocyte cells do not express the markers which will define their role (either CD4 or CD8), therefore are named double negative. After the interaction with thymic nurse cells, they become double positive thymocytes because they express both CD4 and CD8. They undergo to a positive selection mediated by cortical epithelial cells. Depending on the response of double positive thymocyte to MHC it will become CD8 if the response will be made in presence of MHC class I or CD4 if the response will be to MHC class II. Negative selection of $CD8^+$ or $CD4^+$ cells is mediated by dendritic cells and BM-derived macrophages which will help self-reactive T cells to undergo apoptosis, while the self-tolerant T cells can reach the periphery. Mature T cells also express CD3 cells which requires the presentation of an antigen through MHC in order to become activated. T cells can recognize the MHC associated with the specific antigen peptide and not only the antigen alone[1].

CD4+ T cells

Th1 is a T-bet transcription factor dependent subset of T cells, which regulates cell immunity through the production of IFNγ, in addition, these cells activate macrophages, thus contributing to protect against intercellular pathogens[17]. Moreover, they are responsible of cell-mediated immunity and the clearance of tumor cells[18].

IL-12 produced by either macrophages or APCs induces the commitment of CD4 naïve T cells into Th1. This is enhanced by the production of IFN-γ that leads to T-bet expression and Th1 differentiation while Th2 suppression[19].

Th2 are dependent on Gata-3 transcription factor; they produce IL-4, IL-5 and IL-10[1] and contribute to humoral responses but also to allergic responses and play an important role in B cell class switching for IgE production[20].

Th17 are dependent on Ror-γ, they protect against bacteria and fungi and target pathogens which are not recognized by Th1 and Th2. Th17 commitment happens in absence of IL-12 and IL-4, which drives Th1 and Th2 development and in presence of IL-6, IL-23 and TGF-β[21].

Th9 are important helper T cells in protecting from helminths and parasites by IL-9 and IL-10 production[20].

Prominent role in mucosal immunity is provided by both *Th22* and *Th25*. The former intervein also in repair processes while the others are able to limit Th1 and Th17 induced inflammation. Other important T helper cells are the *T follicular helper*, which help the B cells in high affinity antibody production[20]. *T regulatory cells* (Treg) are $CD4^+$ T cells which protect against auto-immunity and serve as immune response suppressors[20].

$CD8^+$ T cells.

They are mainly cytotoxic T cells to cells carrying the antigen for which they are specific[1]. They play important role against tumors and intracellular pathogens [22,23] and in autoimmune and allergic diseases[24,25]. Naïve $CD8^+$ T cells are activated by antigen presenting cells carrying MHC class I, present in all the cells of our body except erythrocytes, in peripheral lymphoid organs[26]. The most common $CD8^+$ T cells are the Tc1 which are endowed with the capability of killing the target antigen through the release of granzyme and perforin and the production of TNFα and IFNγ that fasten the innate immune responses[27]. Other groups of $CD8^+$ T cells include Tc2 cells; they release granzyme and perforin and produce also IL-4, IL-5 and IL-13 [26], thus contributing to the enhancement of allergic responses[25]. Tc9 $CD8^+$

T cells contribute to anti-tumor responses, for example in melanoma through IL-9 production[28], while they can suppress $CD4^+$ T cells responses in the intestine[26]. Another group of T $CD8^+$ T cells are the Tc17, which have been shown to contribute to autoimmune disorders but also to immunity against cancer[29] and viruses[26]. Another population of $CD8^+$ T cells is the $CD8^+$ Treg with important properties of regulation for T cell responses[30] and the ability to produce TGFβ, IL-10, perforin and granzyme[26].

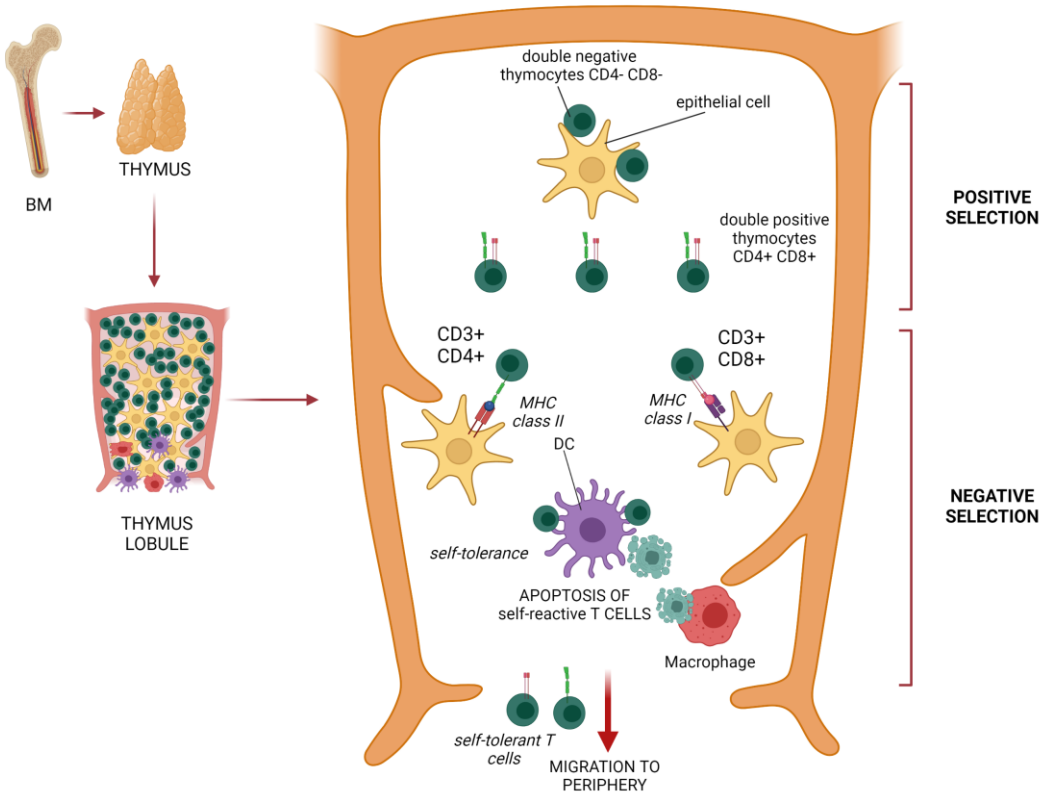

Figure 2. T cell development

Starting from bone marrow pre-T cells reach the thymus in which they start their maturation. T cells undergo positive selection and negative selection therefore becoming $CD4^+$ or $CD8^+$ T cells. (Figure created with BioRender.com)

2.1.4. Acellular components of the immune system

Complement system is an acellular component of immune system, consisting of about 20 glycoproteins, which are activated by a cascade process and are able to mutually interact. It has important roles in immunity, it is responsible of phagocytosis of opsonized antigens, the

clearance of apoptotic cells, it helps the modulation of cellular immune responses; it represents a bridge between innate and adaptive immunity since it also promotes humoral immunity[31–33].

Cytokines are small peptides, glycoproteins and proteins that serve for intercellular communication and display a huge regulatory role in immunity and inflammation[34]. Cytokines can be either soluble, therefore released by cells and bind to their receptor on recipient cells, or membrane-bound[1]. Most of them were referred to be produced by leukocytes therefore were called interleukins, however it has been seen that many other cells of the immune system respond to and produce these signaling molecules[4]. Cytokines endowed with chemoattractant ability are named chemokines. Interferons are cytokines able to interfere with viruses and cancer; while tumor necrosis factor family includes cytokines with structural similarities involved in cell survival, regulation of cell differentiation and mostly in tumor cytotoxicity[4]. The list of cytokines treated in this thesis is reported in the table below (**Table 1**).

Table 1. Cytokines of the immune system

The table reports all the cytokines, which will be mentioned in the project. Table adapted from Akdis et al. [35].

Cytokine	Cell source	Cell target	Functions
IL-1α/ IL-1β	Macrophages, monocytes, lymphocytes, megakaryocytes, neutrophils, fibroblasts etc	T cells, fibroblasts, epithelial cells	Induce proinflammatory proteins; hematopoiesis; differentiation of Th17 cells
IL-2	T cells, DCs, NK, NKT cells, ILCs	T cells, NK, B cells, ILCs	Induce proliferation of effector T and B cells; development of Tregs; differentiation and proliferation of NK cells; proliferation and cytokine production in ILCs

IL-5	Th2 cells, activated Eosinophils, T cells, NK, NK T cells ILC2s	Eosinophils, basophils, Treg cells, neutrophils and monocytes	Induce myeloid cells; increment of eosinophils activity; involved in remodeling and wound healing
IL-7	Epithelial cells, DCs, B cells, monocytes/ macrophages	Developing B and T lymphocytes, mature T cells, NK cells, ILCs	V(D)J recombination in BM; proliferation of thymocytes; development and maintenance of ILCs
IL-9	Th2, Th9, Th17, Treg cells, ILCs	B, T, mast cells; hematopoietic cells; airway epithelial cells, intestinal epithelial cells	inhibits Th1 cytokines; promotes proliferation of $CD8^+$ T cells
IL-10	T cells, B cells, monocytes, macrophages, DCs	Macrophages, monocytes, T cells, B cells, NK cells, mast cells, DCs, granulocytes	Immunosuppressive effects through APCs or direct effects on T cells; suppression of IgE and induction of IgG by B cells in human
IL-12 (p35/p40)	Monocytes, macrophages, neutrophils, DCs, B cells	Th1 cells, NK cells	Development and maintenance of Th1 cells; activation of NK cells; DC maturation; induction of cytotoxicity
IL-15	Monocytes, macrophages, DCs and activated $CD4^+$ T cells, bone marrow stromal cells,	NK, NKT cells, monocytes, macrophages, DCs, neutrophils, eosinophils, mast cells, T cells and B cells	T-cell activation; proliferation and activation of NK cells; NK, and NKT cells; of Th2 differentiation

IL-17A	Th17 cells, $CD8^+$ T cells, NK cells, NKT cells, ILCs	Epithelial/endothelial cells, fibroblasts, osteoblasts, monocytes, macrophages, B and T lymphocytes	Induction of proinflammatory cytokines, chemokines, recruitment and activation of neutrophils
IL-18	Macrophages, DCs, epithelial cells, chondrocytes, osteoblasts, Kupffer cells, keratinocytes, astrocytes, renal tubular epithelial cells	T cells, NK cells, macrophages, epithelial cells, chondrocytes	Induction of IFN-g in the presence of IL-12; enhancement of NK cell cytotoxicity, promoting TH1 or TH2 cell responses depending on cytokine milieu
IL-22	Th17 and Th22 cells, NKT cells, activated NK cells, LTi, ILCs	Keratinocytes and epithelial cells of kidney, small intestine, liver, colon, lung, pancreas and skin	Contribute to pathogen defense; wound healing; tissue reorganization
IL-23	Phagocytic cells, macrophages, and activated DCs	Th17 cells, NK and NKT cells, eosinophils, monocytes, macrophages, DCs, epithelial cells	Production of proinflammatory IL17; enhancement of T cell proliferation, promotion of memory T cells; activation of NK cells; antibody production regulation
IL-27	Activated DCs, macrophages, epithelial cells	T cells, NK cells	expression of T-bet, promoting Th1 cell differentiation; inhibition of Th17 cells
IFN-α/β	all nucleated	All cells express	Defense against viral

	cells can produce IFN-α/β in presenceof viral infection	IFN receptors	infection by orchestrating adaptive immune responses; stimulation of DC, of macrophage antibody-dependent cytotoxicity; activation of naïve T cells; apoptosis of tumor cells and virus infected cells
IFN-γ	NK and NKT cells, macrophages, Th1 cells, cytotoxic T cells, and B cells	Epithelial cells, macrophages, DCs, NK cells, T, B cells	Antiviral properties; promotion of cytotoxic activity, Th1 differentiation; upregulation of MHC class I and II; inhibition of cell growth; proapoptotic effects; induction of epithelial apoptosis in skin and mucosa
TGF-β	large variety of cells, including eosinophils, macrophages, and Treg cells	Epithelial, endothelial, mesenchymal cells, CD8+ and CD4+ T cells, NK cells, etc.	balance of proinflammatory and anti-inflammatory effects; regulation of the differentiation of several Th cell subsets, induction of Treg cells; immune tolerance
TNF-α	Activated macrophages, monocytes, CD4+ T cells, B cells, neutrophils, NK cells and mast cells, etc.	Nucleated cells	Host defense; proinflammatory mediator and immunosuppressive mediator; inhibition of the development of autoimmune diseases and tumorigenesis; epithelial apoptosis

2.1.5. The innate side of adaptive immunity

Platelets are usually referred to be cell fragments with a restricted role in coagulation; however, it has been observed that they can also protect against pathogens and are implicated

in autoimmune disease like systemic lupus erythematosus[36]. They have been shown to produce pro-inflammatory molecules able to induce plasmacytoid dendritic cells to produce IFNα.

Innate B cells (iBC) contribute in the production of natural antibodies, immunoglobulins which are present in absence of imunization[37]. Their main function consists in response to infections, apoptotic cell elimination and antigen presentation; in addition, they avoid inflammation worsening through anti-inflammatory cytokine production[37].

NKT cells are innate-like T cells expressing a semi-invariant form of T-cell receptor, able to recognize glycolipid antigens presented by APCs through the major histocompatibility complex class I-related protein CD1d[38]. NKT cells act as modulators of immune responses against allergens, tumors, infection agents[38].

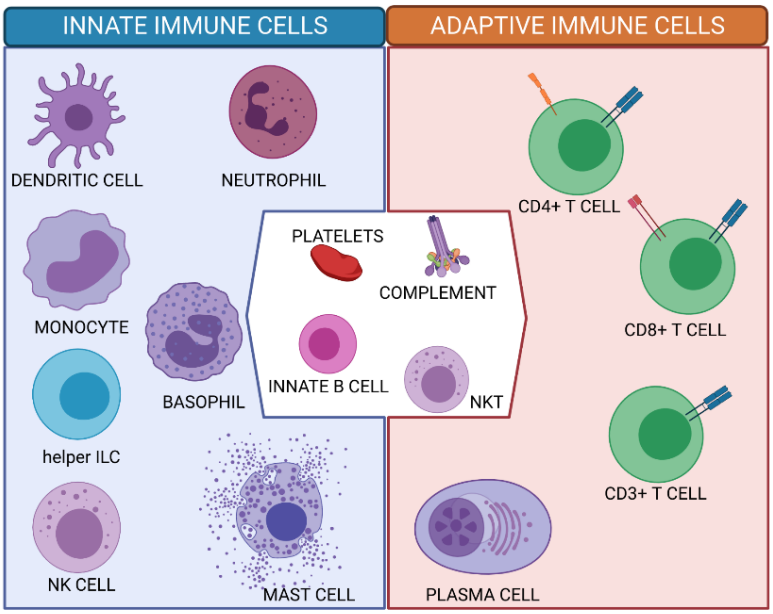

Figure 3. A comprehensive image of cells of the immune system and other immune components

The immune system is divided in innate and adaptive immune system. The group of components in the middle represents the bridge between adaptive and innate arms of the immune system. (Created with BioRender.com)

2.2. Helper ILCs and NK cells

2.2.1. Developmental pathways

Helper ILCs are the most recent family of innate lymphocytes that have been identified[39]. They lack the cell-surface molecules physiologically expressed by leucocytes, therefore are defined to be lineage negative (Lin^-) and are CD127 positive (IL7 receptor α) [40,13]. In human, the development of all lymphocytes initiates in the bone marrow (**Figure 4**), from a common lymphoid progenitor (CLP) which can give rise also to B cell and T cell lineages[41,42]. Both NK cells and helper ILCs rise from common innate lymphoid progenitor (CILP) which differentiates in NK precursor (NKP) for NK development and in common helper innate lymphoid progenitor (CHILIP)[43] for ILC progenitor (ILCP). CHILIP is characterized by Id2 and variable PLZF expression: $PLZF^+$ ILC precursor can generate all the ILC subsets but not NK cells or LTi (lymphoid tissue inducer)[44]. Each stage of innate lymphoid cells development depends on the expression of specific transcription factors. The early differentiation of CLP is driven by TOX and NFIL3 transcription factors, which are not involved in B and T cells development[45,46]. Expression of Id2 (inhibitor of DNA binding 2) is fundamental for ILCs and NK development, indeed mice Id2-deficient are characterized by complete ablation of ILCs/NK[47]. Other two important transcription factors required for ILCs development are GATA-3 and TCF-1[48,49]. The absence of GATA-3 in hematopoietic stem cells inhibit helper ILCs and T cells, however not B cell or NK cells[50]. Differently in mature helper ILCs, GATA-3 affects the maintenance and the survival only for ILC2[50]. T-bet is an important transcription factor for T_H1 cells [51]and drives the development of helper ILC1s. Together with eomesordermin (Eomes), T-bet also has a role in NK function and migration[52]. ROR-γt is fundamental for ILC3s[53], but also for T_H17 cells[54]. These two cell types also share another transcription factor, AHR (aryl hydrocarbon receptor) for the functional control and their survival[55,56].

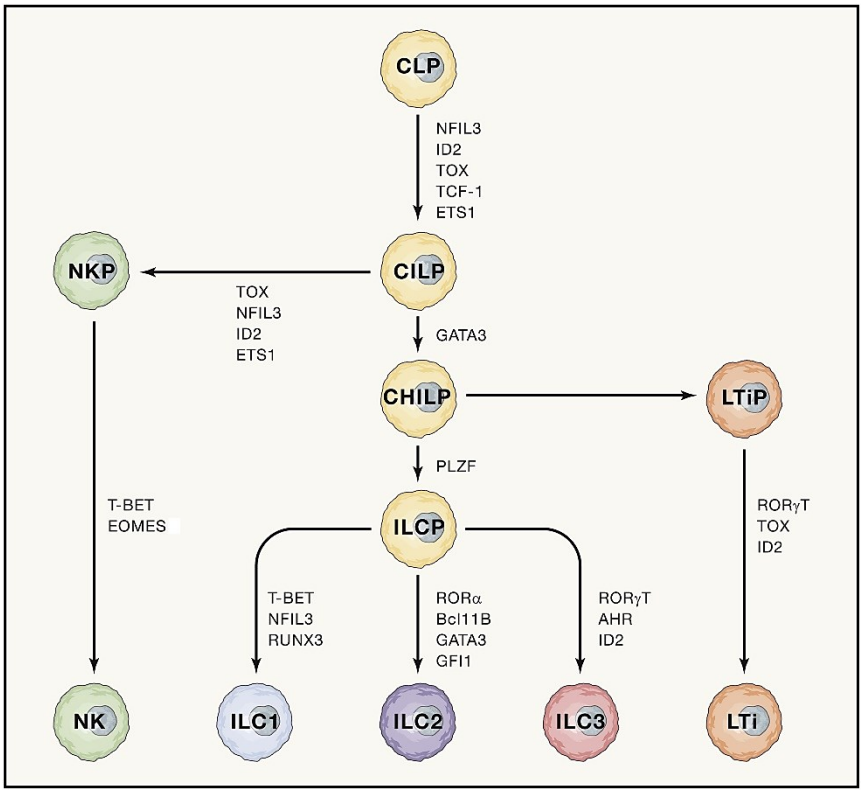

Figure 4. ILC development

The illustration taken and adapted from Vivier, E. et al. [43] represents the ILC developmental pathway in human.

2.2.2. NK cells classification

Innate lymphoid cells are classified in five main subsets including LTi, cytotoxic ILCs mainly known as NK cells, ILC1s, ILC2s and ILC3s.

Circulating NK cells are divided in different subsets based on CD16 and CD56 marker expression. $CD56^{bright}$ NK represent about 10-15% of total NK and are characterized by high pro-inflammatory cytokine production and low cytotoxic activity[57]. In particular they represent a source of IFN-γ, TNF-β, IL-10, IL-13, and GM-CSF[58]. $CD56^{bright}$ NK cells respond to different stimuli, especially to IL-1β, IL-2, IL-12, IL-15 and IL-18 which are released by dendritic cells an T cells [59, 60]. $CD16^{+}$ NKs constitute about the 90% of total NK cells, and show a low production of immunomodulatory cytokine upon stimulation; however they display high cytotoxicity trough the production of lytic molecules such as granzymes and perforin[61]. Recently, a subset of NK has been described to be highly cytotoxic, especially

against hematologic malignancies[62,12], the unconventional NK (uCD56^{dim} NK) which are phenotypically CD56^{dim}/CD16^{-} [63,64,65].

2.2.3. Receptors and physiological functions of NK cells

NK cells have a prominent role in immune responses against bacteria, viruses and parasites. They also play modulation activity[66], such as displayed in lymph nodes, in which NK cells enter in inflammatory conditions, controlling T cell responses[67]. Among the main functions of NK cells there are cytokine production and cytotoxicity; this latter function is carried out with requirement of cell-cell contact between the NK and the target cell[68]. NK cells show activating and inhibitory receptors on their cell surface that can either activate or restrict NK cells depending on the stark result of the stimuli[69,70]. Within the NK receptors, natural cytotoxicity receptors (NCRs) are the most expressed by NK cells, they belong to the immunoglobulin superfamily[68], proteins that contain a transmembrane domain which interacts with singling adaptor protein endowed by immunoreceptor tyrosine-based activation motifs (ITAMs)[71,72]. There are three different NCRs: NKp46, NKp44 and NKp30[71]that can recognize ligands of different origin (for example viral, parasite, bacterial, cellular derived[71]).

Another important receptor expressed by most NK cells is NKG2D[72]. Its ligands are proteins which become overexpressed under stress conditions by normal cells or that are commonly expressed by tumour/malignant cells[72,73]: MHC class I chain related proteins A and B (MICA and MICB), UL16-binding proteins[74]. The effect of NKG2D stimulation results in IFN-γ production and NK cell cytotoxicity[73]. Oppositely, NKG2A is an inhibitory receptor beard by around one-half of circulating NK cells[75]. It has an intracytoplasmic tyrosine-based inhibitory motif (ITIM) and becomes upregulated in response to cytokines. NKG2A forms a heterodimer with the marker CD94[72]. NKG2A/CD94 binds to human leukocyte antigen E (HLA-E)[76]. The inhibitory receptors in NK cells are fundamental to block the cytolytic activity when they recognize a self-MHC-I molecule on the target cell[76]. CD94 forms heterodimers also with other NKG2 receptors, however with different effects and behaviours[72]. The affinity of the different heterodimer for HLA-E is variable and is higher for the inhibitory ones compared to the activator ones[77]. In summary, all the nucleated healthy cells express classical MHC-I molecules binding the inhibitory killer immunoglobulin-like receptors (KIRs) expressed on NK cells, thus protecting themselves from cytotoxic attack[78,79]. NK cells are also able to recognize and kill the malignant cells

which downregulate the expression of MHC-I, hiding from T $CD8^+$ cells (missing-self hypothesis)[80].

DNAX accessory molecule-1 (DNAM-1) is another NK receptor is accessory molecule-1 (DNAM-1). It recognizes and binds ligands which become upregulated after a cellular stress, with the effect of inducing NK cell cytotoxic functions [81,82].

2.2.4. Role of NK cells in cancer

The cytotoxic activity of NK drives many anti-tumour responses[83], indeed in many tumours the downregulation of NK cell functions is related to a higher incidence of metastasis[72,84], or in other tumours such like colorectal cancer, a low number of NK cells increases the incidence of cancer recurrence after resection[72,85]. Despite in non-small cell lung cancer (NSCLC)[86], the infiltration of NK cells does not have impact on cancer prognosis, in renal cell cancer it is related with a better prognosis[87]. This is also because $CD56^{bright}$ NK cells found in NSCLC were localized in the stroma rather than in contact with the tumour cells and they also present high expression of inhibitory receptors[88]. In hematologic malignancies, NK cells display different behaviours, depending on the modulation of inhibitory and activating receptors. For example in myelodysplastic syndrome (MDS) NK cells with a low expression of NKG2D at diagnosis, display a very low cytotoxicity thus contributing to high-risk disease[89]; similarly in acute myeloid leukaemia (AML) low NKG2D expression impairs cell cytotoxicity and IFNγ production[90]. Also a low expression of DNAM1 is related to low blasts killing and high blast infiltration in MDS[91]. Especially in HL and less also in DLBCL[92], NK $CD16^-$ have high expression of PD-1 in patients showing a possible mechanism acted by malignant B cells to inhibit NK activities[72,83]. Similarly, in multiple myeloma at the stage of diagnosis a high expression of PD-1 on NK cells is related to a low displayed cytotoxicity and low IFN- γ production[93]. In AML patients with high expression of NKG2A on NK cells show a low TNF production and a decreased overall survival[94]. Baseline NK cell count at diagnosis of FL and DLBCL assessed in peripheral blood is considered to have a prognostic impact on the outcome of immunotherapy treatment with anti-CD20[95]. Moreover CD70 on NK cells is triggered by CD27 expressed on NHL tumour cells resulting in NK cell activation, IFN-γ production and therefore NHL cells elimination[96].

2.2.5. Helper ILC classification and their physiological functions

ILCs are divided in different groups: conventional NK cells, LTi, ILC1 dependent on T-Bet transcription factor, ILC2s dependent on GATA-3 and ROR-γt dependent ILC3s.

LTis were the first ILCs discovered. They have important roles in the formation of secondary lymphoid organs (SLOs) during embryogenesis[97]. In adult human, LTi-like cells are majorly abundant in the intestinal lamina propria, exerting important functions in gut, such as the maintenance of its homeostasis trough the repression of CD4+ T cells specific for commensal bacteria antigens[98].

Helper ILCs are mainly tissue-resident cells acting as critical mediators in tissue repairing and remodelling, homeostasis, response against pathogens, allergies and tumours[99]. In tissues, based on cell surface marker expression, transcription factors and cytokine production, helper ILCs can be divided in different subsets: ILC1s, ILC2s and ILC3s. Initially these three subsets had been identified to parallel T helper cell functions[40]. Each tissue presents its own ILC subset distribution, for example, the small intestine is enriched in ILC1s, while skin and lung tissues are populated majorly by ILC2s, tonsils and colon of ILC3s [100,101]. In peripheral blood (PB), ILCs are rare, and their progenitors (ILCP) give rise to both ILC1, ILC2 and NK cells. In humans, ILC1s development is dependent on the transcription factor T-bet; these cells mainly produce TNFα and IFNγ[102]. 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)[103]. ILC3 development relies on RORγt expression and these cells are characterized by CD117 expression[104]. Alterations in ILC number has been shown to associates with inflammatory conditions and diseases. Examples of these conditions are the increment of ILC1 found in peripheral blood of patients suffering for Chronic Obstructive Pulmonary Disease (COPD); in this condition, it has been suggested that ILC2 are converted to ILC1 after stimulation with IL-12 and IL-18 in inflamed areas of the lung[105]. Moreover, in patients with psoriasis there is a significant increment of IL-22 producing ILC3 both in peripheral blood and in the skin[106].

ILCregs are a recent identified group of IL-10-producing ILCs in the lamina propria of both mice and humans in presence of intestinal inflammation. ILCregs may suppress ILC1 and ILC3 via IL-10 secretion, protecting against intestinal inflammation[107]. They also produce TGF-β, which is required for their survival. In summary, it seems that ILCregs are regulatory ILCs that promote the resolution of intestinal inflammation[107]. In contrast, more recently it

has been proposed that ILCs which produce IL-10 during intestinal inflammation are activated ILC2s rather than an innate counterpart of Tregs[108], as instead described in Wang S. et al. [107].

ILC1

ILC1s are characterized by cells producing Th1- like cytokines, such as IFN-γ and TNF-α[109]. They are found in different tissues, including spleen, fat, secondary lymphoid organs (SLOs), skin and blood[106,110]. Human ILC1s represent a very complex subsets of ILCs, which includes also $CD127^+$ ILCs, NK displaying low cytotoxicity and a subset of intra epithelial ILC1 (ieILC1) which are cytotoxic cells[111]. These cells lack the expression of CD127 and express T-bet and Eomes[112]. IeILC1s are absent in peripheral blood, bone marrow and in lymph nodes, as demonstrated by high-dimensional flow cytometry profiling in healthy organs[112]. It has been demonstrated that ILC1s, similarly to NK cells, need C-C chemokine receptor 7 (CCR7) and CD62L in order to migrate in tissues and produce also IFNγ[113]. At steady state, ILC1s are found in small numbers in the intestines, skin, lung and liver and their increment is associated with inflammatory conditions[114]. Through RNA velocity analysis it has been also found an intermediate IL3-to-ILC[115] confirming the possibility that ILC1s $CD127^+$ derive from ILC3s in the gut, after exposure to inflammatory conditions [102,116].

ILC2

In human ILC2s are characterized by the production of Th2-like cytokines[109] and by the expression of CD161 and CRTH2, which is the prostaglandin D_2 receptor [117].

Depending on cKit (CD117) expression ILC2 can be distinguished in two different populations : $cKit^{hi}$ and $cKit^{low}$ [103,118]. The former are endowed with ILC3 characteristics and the ability of secreting IL-17, moreover they express RORγt, while the latter produce exclusively type 2 cytokines[117, 119].

ILC2s produce cytokine Th2 for a type 2 immune response (IL-4, IL-5, IL-9, amphiregulin and IL-13) after stimulation with TSLP, IL-25 or IL-33[120]. Skin and adipose tissue are the most populated sites by ILC2[110], however this ILC subset also represents the most important in peripheral blood[121, 117]. Analysis conducted by mass cytometry show an homogenous phenotype of ILC2 across the healthy tissues[100]. In inflammatory conditions involving airways, intestine and skin, ILC2s are potent inducers through their Th2- responses[122]. In

particular, considering the pathogenesis of allergen-induced asthma, ILC2s were seen to persist also after the inflammation resolution responding to unrelated allergens, these were referred to be memory-like ILC2s and may help to explain the persisting sensitization to multiple allergens displayed by patients with asthma[123].

ILC3 and ILC progenitors

ILC3s can produce Th-17 like cytokines. Human circulating ILCs, which are phenotypically stackable to ILC3, express CD45RA and CD62L, that are commonly expressed by naïve T cells[124, 125]. This is why recently this subset was referred to as ILC progenitors endowed with the ability to give rise both to ILCs and NK cells, depending on the environmental signals[126]. The expression of KLRG1 on ILCP indicates a commitment to ILC2, while $Nkp46^+$ ILCP tends to become ILC3s and to a less extend ILC1s[127]. $Nkp44^-$ ILC3 is the most abundant population of ILC3 found in SLOs, in skin and lung; in the resting lymph nodes, this population express CXCR5 and CCR7, but not a typical ILC3 signature[128] and are probably an ILC3 reservoir[129]. Both in mice and humans, ILC3 are abundant in the gut in which they contribute to confine the commensal bacteria[43]; indeed in mice it has been demonstrated that ILC3 depletion in gut determines the shift of these bacteria to peripheral organs[130]. ILC3 are also able to limit the activity of Th17 cells against commensal bacteria through interactions with MHC class II[131], moreover another T cell modulation done by ILC3 is exerted through GM-CSF in the gut[132].

2.2.6. Plasticity in mature ILCs

ILCs are endowed by great plasticity, similarly to T helper cells, this means that they can change their developmental commitment or their phenotype depending on the microenvironment[133]. For example, *in vitro*, under the influence of IL-23 and IL-1β, it has been shown that ILC3s can convert into ILC1s, endowed with the ability of producing IFN-γ[134]. The conversion of IL-22-producing ILC3s into ILC1s has been shown in mouse intestine[135] and it is regulated by inflammatory signals[136]. This process is reversible, therefore ILC1s can convert into ILC3s[116], as observed in human intestine. This process *in vitro* happens under the influence of IL-12 that contributes to the down-regulation of ROR-γt and the upregulation of T-bet[133].

NK cells, in presence of TGF-β signal, can convert into ILC1-like cells[137]. In this process IL-12 is involved, however completely unable to prompt the conversion alone, as shown in mouse model infected by *Toxoplasma gondii*[138].

Human and mouse ILC2s can convert into IFN-γ-producing ILC1s under the stimulation of IL-12 and IL-1β as shown *in vitro*[139]. ILC2s do not respond to IL-12 directly since they need IL-1β for their IL-12 receptor expression and IFN-γ production[133]. The conversion of ILC2s to ILC1s is driven by IL-4 which is produced mostly by basophils and eosinophils and induces GATA3 and CRTH2 expression[120].

Stimulation of ILC2 with IL-1β and IL-23 induces the conversion into ILC3-like cells[140], which were found to be accumulated in skin lesions of psoriasis patients as the major source of IL-17[118]. In human peripheral blood, two subsets of ILC2s have been identified: $CD117^+$ ILC2s and $CD117^-$ ILC2s[103,118]. $CD117^+$ ILC2s show some features of ILC3s, for example the expression of ROR-γt and the production of IL-17 upon the stimulation with IL-1β and IL-23, while $CD117^-$ ILC2s need also TGFβ to produce IL-17[133]. This suggests that the down modulation of CD117 is linked to a less plastic type of ILC2s[133]. A summary of ILC subset plasticity are summarized in the **Figure 5**.

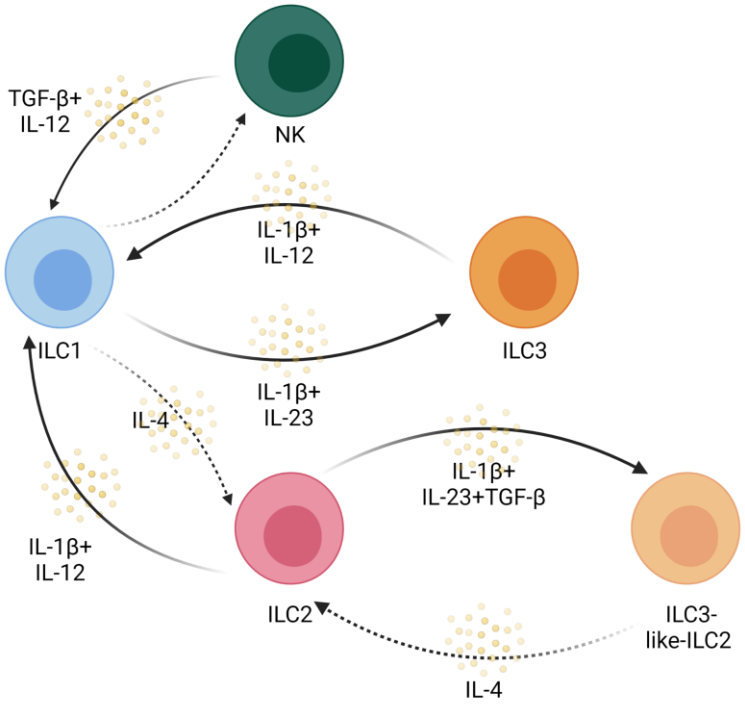

Figure 5. ILC plasticity

Plasticity of mature ILCs depends on cytokines present in the immune environment, which can drive transcriptional reprogramming and plasticity. NK cells transdifferentiate in ILC1 under the influence of TGF-β. ILC1-ILC3 plasticity is governed by signals of IL-23 and IL-1β /IL-12. *In vitro* stimulation of ILC2s with IL-12 and IL-1β determines their conversion into ILC1, for CD117- ILC2 also TGF-β is required, while IL-1β and IL-23 induce their transformation into ILC3-like cells. Dashed arrows represent the less defined pathways while the solid ones the proven ones. Figure created with references and adaptation to [114,133] .(Created with BioRender.com).

2.2.7. Role of helper ILCs in cancer

ILC1 in cancer

Tumour microenvironment affects helper ILC behaviour (**Figure 6**). Total ILCs have been shown to be significantly dysregulated in terms frequency, subset distribution and function in patients affected by acute myeloid leukemia (AML), in particular hypofunctional ILC1 were expanded in patients compared to healthy donors[141]. ILC1

numerical and functional dysregulation has been described also in CRC[142] and chronic lymphocytic leukaemia (CLL)[143]. Moreover, an increment of ILC1s has been detected in human gastric and oesophageal cancer tissues compared to healthy surrounding tisues[144] and also in the bone marrow of patients affected by multiple myeloma[145]. Additionally, in patients with myelofibrosis ILC1 are increased in peripheral blood compared to healthy donors, however showing a low functional capacity[146]. In CLL, ILC1s show a pro-tumour role, since they are induced to produce IFN-γ and TNF-α, thus forming an immunosuppressive environment [143]. For example, in mice IL-15 rich tumour environment, tissue resident ILC1s-like result in activation and secretion of granzyme B with a mechanism that may involve NKG2D, thus limiting tumour progression[147]. ILC1 are able to produce IFN-γ, thus suggesting an anti-tumour function, in fact, IFN-γ promotes Th1 cell polarization and induce cytokine production[148]. Moreover, in melanoma patients it has been described an impairment of ILC1 proinflammatory functions both in peripheral blood and lymph nodes infiltrated by the tumour, causing a immunosuppressive tumour microenvironment[149]. Oppositely, IFN-γ has also the ability again in mouse model; in TGF-ß rich tumours ILC1s can promote tumour growth. Indeed, TGF-ß can promote the conversion of NK cells to ILC1s, which have higher NKG2A, KLRG1 and other inhibitory receptors[83], thus limiting the surveillance on tumour growth and boosting methastatization[83]. In Chron's[116] disease the percentage of ILC1 is particularly very high in intestinal tissues characterized by inflammation. Data from literature show that these ILC1s are derived from ILC3s after the downregulation of the transcription factor ROR-γt and the upregulation of T-bet and IFN-γ, they can contribute to gut inflammation[150].

ILC2 in cancer

ILC2s can cooperate with dendritic cells (DCs) stimulating T cell anti-tumour responses[151] (**Figure 6**). In mice it has been shown that the absence of ILC2 results in higher incidence of tumour growth and easier development of metastasis[83]. Increment of ILC2 number was described in human breast cancer tissues compared to healthy breast tissue[144]. ILC2s were also found in urinary immune infiltrate in non-muscle-invasive bladder cancer[151]. In mouse models of metastatic melanoma, it has been described an anti-tumour activity of ILC2s activated through IL-33 activation; these ILC2s release IL-5 which in turn recall the eosinophils exerting anti-tumour functions[152]. Moreover, in patients affected by melanoma the ILC2 infiltration in the tumour correlated with a good prognosis. However, infiltrating

ILC2s in tumour express high level of PD-1 which causes a reduction anti—tumour effects that may be overcome through the synergic blockade of PD-1 and the concomitant administration of IL-33 which contributes to ILC2 activation [153]. Similarly, through *in vivo* experiments considering pancreatic ductal adenocarcinoma (PDACs) Moral J. A. et al., showed that IL-33 induces activation of tissue-specific immunity, such as of tissue ILC2s and CD8+ T cells, endowed with the ability of limiting tumour growth. Tissue ILC2s in the tumour show great expression of PD-1, thus the blockade this immune checkpoint may represent a new potential strategy in immunotherapy [154].

In vivo ILC2 infiltrate in colorectal tumour has been found to be responsible of tumour burden reduction, moreover it was also described to be associated with improved overall survival in patients affected by this cancer type[155].

ILC2 are considered harmful, since they produce type 2 cytokines that promote tumour formation and tumour progression; for example, ILC2s produce AREG, which is a potent inhibitor of tumour responses via Treg recruitment and activity promotion[156,157] and it associates with a bad prognosis in breast, ovarian and gastric cancer[157]. Considering hematologic malignancies, ILCs have been studied with particular attention for acute promyelocytic leukaemia (APL) and AML. ILC2s are increased in the PB of APL patients, recruited by prostaglandin D2 (PGD2) released by APL blasts. The endogenous ligand B7H6, present on APL blasts, engages CRTH2 NKp30^{+} ILC2s, inducing their activation and IL-13 release. This allows the expansion of monocytic myeloid derived suppressor cells(M-MDSCs) and the establishment of an immunosuppressive environment[158]. As in APL, also in AML patients it has been found that ILC2 produce IL-13 and recruit MDSCs establishing an immuno-suppressive environment[83]. In lung cancer, tissue ILC2s increase metastasis potential and mortality, since they suppress via IL-5 and IL-33 secretion the NK cytotoxic functions and IFN-γ production [159].

Moreover, studying non-muscle-invasive bladder cancer it has been found that ILC2s control the local ratio of T cells/MDSCs and through the production of IL-13 they induce a preferential recruitment of MDSCs thus decreasing the recurrence-free survival in patients affected by this tumour[160]. In lung and liver, the production of IL-13 by ILC2s promotes tissue fibrosis, that often culminates in cancer[161,162]. Also in gastric cancer patients it has been studied that ILC2s may favour the establishment of an immunosuppressive microenvironment, since in peripheral blood of these patients ILC2- associated cytokines were found upregulated[163].

ILC3 in cancer

The number of ILC3 has been found increased in the lymphoid infiltrate of human NSCLC[164]. Moreover in patients affected of Chron's disease an accumulation of ILC3 in the intestine has been found to have a role in inflammation through IL-22, IL-17 and IFN-γ production [165]. In CLL, an expansion of functionally altered ILCs has been described[143].

Group 3 ILC is involved in chronic inflammation related to IL-23 production. This cytokine plays a role in autoimmunity, host defence and chronic inflammatory diseases; however high concentration of IL-23 and of IL-23 receptor (IL-23R) relates to different types of human cancer, such like skin, breast, gastric, liver[166–168].

In some cases, ILC3 role could be helpful in terms of cancer elimination.

ILC3s might also have a role in cancer control, as described in a mouse model of melanoma. In this study an IL-12 increase in TME promoted the expansion of NKp46$^+$ CD49b-RORγt$^+$ ILC3s which, in turn, enhanced leukocyte infiltration and tumor suppression [169]. Moreover, in NSCLC, the accumulation of ILC3s NCR$^+$ at the tumor-associated tertiary lymphoid structures has been associated with a better clinical outcome [164].

For example, in a mouse model of melanoma the increment of IL-12 promotes the expansion of NKp46$^+$ CD49b$^-$ RORγt$^+$ ILC3s which help leukocytes infiltration and therefore tumor suppression[169]. Moreover in NSCLC (non-small cell lung cancer), an accumulation of ILC3s NCR$^+$ at the tumor-associated tertiary lymphoid structure is related to a more favorable clinical outcome[164].

Inflammatory bowel disease (IBD) pathogenesis is linked to an increment of IL-23R signaling which then in turn promotes tumor growth in the gut [170]. In mice models the expression of IL-23 transgene leads to the formation of intestinal tumors by the stimulation of an ILC3 population expressing IL23R and THY1 membrane glycoprotien[171].

ILC3s without NCR expression have a harmful role in hepatocellular carcinoma. Indeed, patients affected by this tumor show a high level of IL-23 in serum which also correlates with a poor outcome and prognosis; in response to IL-23, ILC3s NCR$^-$ initiate the production of IL-17 which contributes to tumor progression by limiting CD8+ T cells immunity[172]. ILC3s that produce IL-17 are associated with poor prognosis even considering squamous cervical carcinoma [173].

ILC3s play an important role in host-microbiota homeostasis [43,174] and in the gut ILC3s have a role in chronic inflammation and contribute to the gastrointestinal cancer development[175]. Despite IL-22 producing ILC3s help epithelial tissue repairing process, a continuous

secretion of IL-22 induces excessive inflammation[176]. Indeed, colon rectal cancer (CRC), in particular, has be shown to be linked to high intra-tumour levels of IL-22 which affects the upregulation of anti-apoptotic an pro-proliferating genes[177] and induce an abnormal epithelial cell proliferation therefore contributing to cancer progression, as demonstrated in CRC mouse model [178]. IL-22 concentration in serum of patients affected by CRC correlates with chemo resistance, a possible effect of ILC3 activation[179]. Moreover, considering CRC again, through the stimulation of TGF-β ILC3s can convert into ILCregs [180] which support cancer spreading by IL-10 secretion in the gut[107].

Furthermore, in breast cancer (BC) ILC3s are increased in tumour tissue and have a pro-tumorigenic behaviour; indeed, *in vitro* studies show that ILC3 interact with stoma cells increasing tumour invasiveness and formation of metastasis in lymph nodes which also associate with IL-17 production by ILC3 in the tumour infiltrate[181].

Additionally, it has been described that ILC3 may originate a rare form of NHL, that is the anaplastic large cell lymphoma, giving the suggestion that also ILCs can originate hematologic malignancies[182] (**Figure 6**).

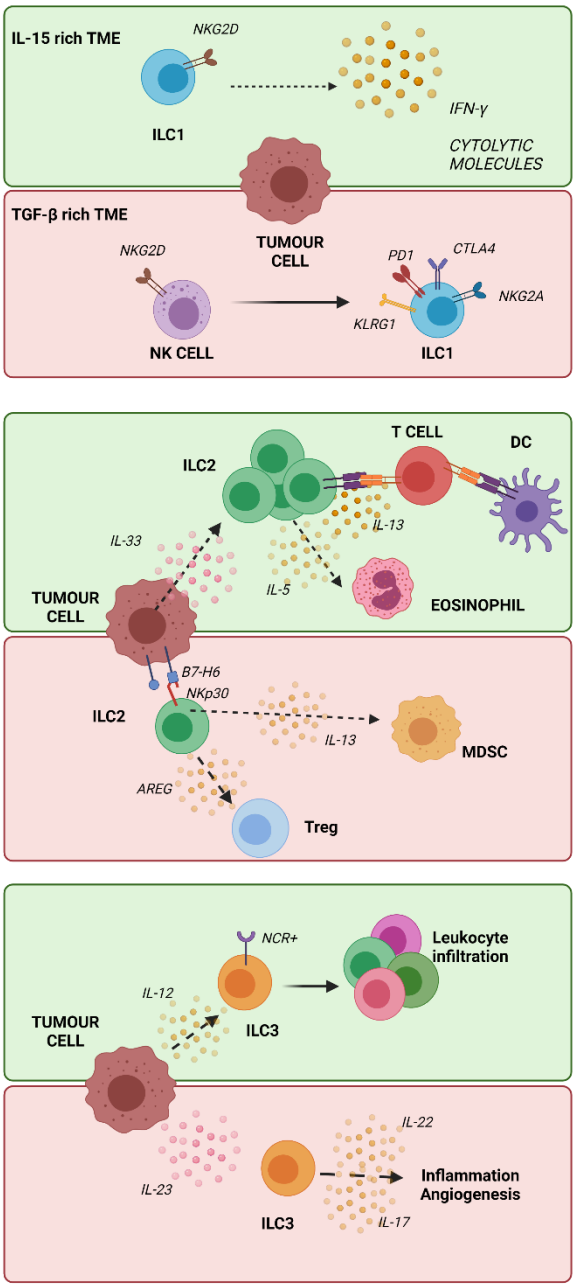

Figure 6. ILCs in tumour microenvironment

The figure reports the roles of helper ILCs in cancer. In green how ILC1s, ILC2s or ILC3s may be helpful for cancer control; in red how ILCs are harmful and support cancer progression. Many of the pathways represented in this illustration were described in Crinier et al., 2019 [183]. In IL-15 rich TME, ILC1s produce lytic molecules and contribute to tumour cell inhibition and elimination. Oppositely, TGF- β causes the trans differentiation of NK cells to ILC1s, less cytotoxic, this may sustain tumour growth/progression. ILC2s can help tumour regression through the recruitment of T cells and eosinophils, however, on the other side through IL-

13 production, they recruit MDSCs and through AREG they recruit Tregs, contributing to tumor immune escape. ILC3s contribute to tumour regression by IL-12 induced leukocyte infiltration; on the other hand, they display pro-tumour role through the production of IL-17. ILC can be helpful or harmful in terms of cancer responses. (Adapted from Roma S. et al, Cancers 2021[72]).

2.3. Non-Hodgkin B cell lymphomas

Non-Hodgkin lymphomas (NHL) represent a heterogeneous group of hematologic malignancies, originated from mature lymphocytes at different stages of their differentiation. NHL ranks 8th for men and 11th for women most diagnosed cancer[184]. Europe, North America, Oceania are geographic areas with the highest distribution of NHL cases worldwide[185]. Risk factors comprehend immune disorders, medications and medical interventions, familiar history and infectious agents.

2.3.1. Aetiology and risk factors of B cell non-Hodgkin lymphoma

The 85/90% of NHL derive from B cells, while T- and NK cell-derived lymphomas represent the minority percentage. Autoimmune disorders, such as rheumatoid arthritis, systemic lupus erythematosus, represent a risk factor for NHL development, probably because of the effects of immunosuppressive therapies given to these patients[186]. Moreover, patients who suffered of breast cancer or skin cancer resulted more prone to develop NHL. This increased susceptibility may be explained as the result of chemotherapy or exposure to immunosuppressive therapies[184]. Patients affected by immunosuppressive disease or immunosuppressed for different reasons (HIV infection, organ transplants) are more prone to develop NHL[187].

Chronic inflammatory conditions such as Hashimoto thyroiditis, Sjögren's syndrome or chronic gastritis increase the risk of NHL development[188]. The same happens for infections by bacteria and viruses. Human T-cell lymphotropic virus 1 (HTLV-1), which is diffuse in Japan, South America, West Africa [189], is responsible for adult T-cell lymphoma, since it causes uncontrolled stimulation for T-cells. The *Epstein-Barr* virus is associated with NK-T-cell lymphoma[190], while *Hepatitis C* with diffuse large B cell lymphoma (DLBCL) and marginal lymphoma. *Helicobacter pylori* is known to be the cause of most of mucosa-associated lymphoid tissue lymphoma (MALT)[191], indeed the solely eradication of the

bacterium results in regression for large proportion of MALT lymphomas, especially if early staged and low-grade[192].

Alcohol has been reported to seem protective against NHL [193], potentially because it improves immune responses[194], in the same way sun exposure has been found to be protective[195].

2.3.2. Non-Hodgkin B cell lymphoma classification, staging and therapies

Staging of NHL

The gold standard for lymphoma classification is currently the WHO (World Health Organization) classification system. It distinguishes the lymphomas according to their origin (T cells, NK cells or B cells) and the grade of development of the involved cells. In order to classify the non-Hodgkin B cell lymphoma according to WHO classification system the patient must be evaluated with different approaches, comprehending the knowledge of the following factors:

-clinical information, based on anatomic staging

-morphologic

-immunophenotyping

-molecular characteristics.

Clinical staging is based on Ann Arbor/Cotswold staging system, revised after the 11th International Conference on Malignant Lymphoma in Lugano. The system describes four different stages of disease[196] (**Figure 7**) (**Table 2**).

Table 2. Stages of NHL according to Ann Arbor/Cotswold staging system.

The table reports the staging system proposed by Ann Arbor/Cotswold. Each stage is described according to the regions affected by the tumor. Adapted from Andersson, A.et al. [197].

Stage I	**Single lymph node region or single extranodal organ or site**
Stage II	Two or more regions on the same side of diaphragm, nodal and/or extra nodal sites
Stage III	Nodal involvement on both sides of the diaphragm and localized extralymphatic extension or splenic involvement
Stage IV	Dissemination to one or more extranodal tissues or organs, with or without nodal involvement
A	Asymptomatic
B	Night sweats, unexplained fever >38°C, unexplained weight loss (> 10%)
E	Extranodal disease
X	Bulky disease (>10 cm maximum diameter or mediastinal mass > one third of the maximal chest diameter
S	Engagement of the spleen

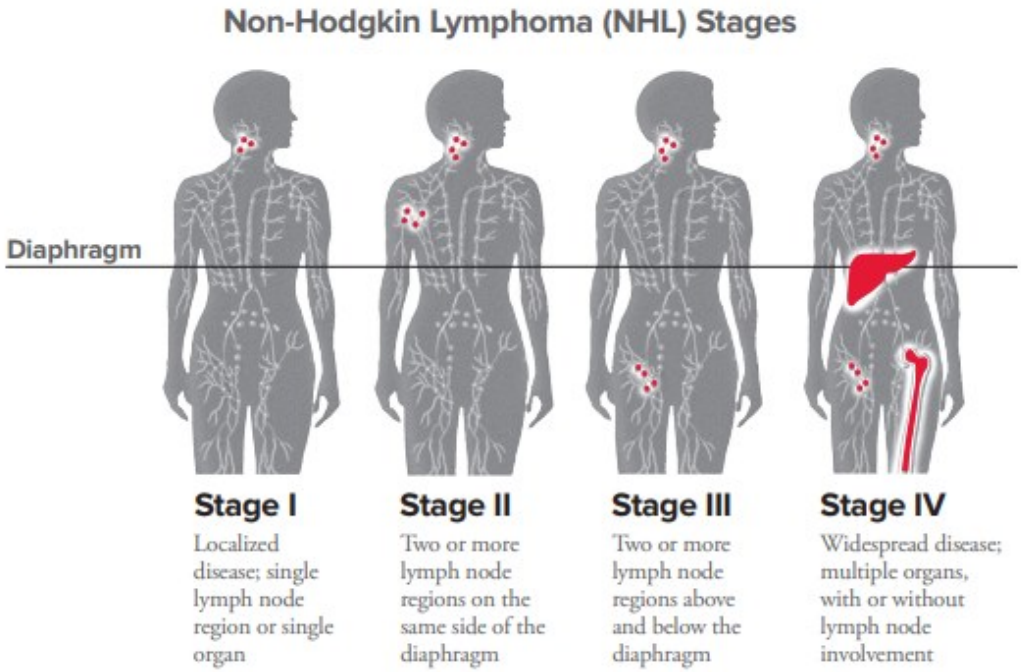

Figure 7. Stages of NHL disease

According to the position and the type of organs involved by NHL spread, it is possible to define four stages of disease, characterized by increased malignancy. Image from Leukemia & lymphoma society[198].

For disease staging, different clinical exams have to be performed. According to AIOM guidelines, the first step is the complete anamnesis with the evaluation of superficial lymph node stations. The patient is evaluated basing on the clinical history (age, sex, familiarity of the disease) and presented symptoms, which can include fatigue, fever, unexplained weight loss, night sweats (that constitute the so-called B-symptoms), and enlargement of lymph nodes. Computer tomography (CT) and PET (positron emission tomography) total body are usually required for anatomical staging PET is more efficient that CT especially for the detection of extra nodal localization [199]. Bone marrow biopsy is required for NHL staging especially for follicular lymphoma but it is not mandatory for diffuse large B cell lymphoma in presence of a positive PET in the bones however it might be important in PET negative patients in case the bone marrow involvement either is discordant with histology or represents a key point for therapy choice[200].

The International Non-Hodgkin Prognostic Index is a tool able to assign a specific score predicting the clinical outcome of a specific NHL considering all the risk factors (age, stage of disease) of the patient[201].

2.3.3. Subtypes of NHL

Non-Hodgkin lymphoma can be classified in two main groups, depending on how the disease progresses [202]: indolent and aggressive lymphoma. Focusing on B cell NHL the main types of NHL for subgroups could be summarized as in the table below (**Table 3**) (**Figure 8**).

Table 3. NHL types and subtypes

The table represents the classification of NHL according to their aggressiveness and characteristics.

AGGRESSIVE NHL	INDOLENT NHL
• Diffuse Large B-cell lymphoma (DLBCL) • Mantle cell lymphoma (MCL) Lymphoblastic lymphoma • Primary mediastinal large B-cell lymphoma (PMBCL) • Transformed follicular and transformed mucosa-associated lymphoid tissue lymphoma	• Follicular lymphoma (FL) • Marginal zone lymphoma (MZL) • Chronic lymphocytic leukemia/small-cell lymphocytic lymphoma (CLL/SLL) • Gastric mucosa-associated lymphoid tissue (MALT) lymphoma • Lymphoplasmacytic lymphoma Nodal marginal zone lymphoma (NMZL) • Splenic marginal zone lymphoma (SMZL)

Diffuse large B cell lymphoma:

It represents the most common subtype of NHL, generating either *de novo* or as a transformation of an indolent lymphoma[203]. DLBCL either can originate from B cells in germinal centers or from activated B cells that have exited the germinal center[204]; the latter usually show a worse prognosis.

DLBCL could be a double hit lymphoma when presenting a double mutation: either BCL2 or BCL6 with MYC. This type of mutations lead to a poor prognosis and a high probability of cancer progression even during the chemotherapy administration[205]. The standard treatment for DLBCL is one dose every three weeks of a R-CHOP, a chemotherapy composed by rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone[206] . Another treatment is the therapy based on dose adjusted EPOCH-R, (etoposide, prednisone, vincristine, cyclophosphamide, hydroxydoxorubicin plus rituximab) which might be more effective than R-CHOP[207]. Rituximab is not a chemotherapeutic agent, but a monoclonal antibody, which targets B cells. Different studies have demonstrated that rituximab is

metabolized more rapidly in male patients compared to females[208], therefore in order to avoid a poor outcome in men treated with this monoclonal antibody an increased dose has to be administrated to male patients to equilibrate the difference[209].
Some indication combine chemotherapy with radiotherapy but evidences show that chemotherapy alone is sufficient to cure most of the patients[210].

Mantle cell lymphoma

This type of aggressive lymphoma shows an incidence of 4% in US and 7-9% in Europe of all lymphomas[211] with a distinctive prevalence in males compared to women[212]. Patients usually present a high stage disease showing involvement of blood, bone marrow, lymph nodes, spleen[213] and in some cases also of extra nodal sites, especially the gastrointestinal tract (stomach, colon, and liver)[214]. MCL diagnosis is made on a lymph node or tissue biopsy in which tumor cells present, in most of the cases, a chromosomal translocation t(11:14) (q13;32), responsible for the anomalous expression of cyclin D1[215]. Another important marker for aggressive MCL diagnosis is the over-expression of SOX11[216]. Most of patients with MCL need to be treated soon, but 'watch and wait strategy' for patients with low prognostic index or without symptoms is usually applied[212]. Common therapy for MCL are based on R- CHOP and on the combination of rituximab with cyclophosphamide, vincristine, and prednisone (CVP-R); R-CHOP resulted not to be sufficient for MCL treatment[217]. Clinical trials revealed that treatments based on bendamustine combined with rituximab (BR) are more effective compared to R-CHOP or R-CVP[218]. Other approaches include bortezomib instead of vincristine in R-CHOP [219] and therapies with high doses of cytarabine[220]. Patients with relapsed MCL show to have improvements with treatments based on lenalidomide[221] or lenalidomide plus rituximab[222].

Marginal zone lymphoma

In Western world area MZLs represents the 5-15% of non-Hodgkin lymphomas[223]. MZL has different subtypes: extra-nodal MZL, mucosa associated MZL (also known as MALT), splenic marginal zone lymphoma. MALT is the most frequent type of extra nodal MZL, when localized in gastric site it is principally linked to an infection by Helicobacter pylori, thus a simple eradication with antibiotics of this bacteria may lead to the complete clinical remission of the patients[224]. Despite the grade, a triple-therapy for *Helicobacter pylori* eradication should be given to the patients with gastric MALT[225]. This treatment based on

proton-pump inhibitor, amoxicillin and clarithromycin shows to be effective in a huge portion of patients[226]. The presence of t(11;18) translocation represents a higher risk for relapse[227]. For nodal MZL, especially when the patient is symptomatic, a therapy based on rituximab plus bendamustine or R-CHOP, R-CVP should be adopted [223]. Splenic MZL requires usually a therapy based on rituximab which shows a rapid and long lasting response[228].

Follicular lymphoma

FL represents approximately the 22% of all the NHL in the western area of the world[211]. In most of the cases FL derives from B cells carrying the chromosomal translocation t(14;18)(q32;q21) responsible for Bcl-2 overespression[229]. There are four grades of FL and WHO distinguishes the third stage as 3A, in which the centrocytes are the location of most large B cells and 3B, consisting exclusively in centroblasts[230]. The 3B stage FL is similar to DLBCL and has to be treated in a similar way. For stages 1 and 2 usually the approach could either be a 'wait and watch' or a therapy based either on radiation or on rituximab[231]. Treatments for higher stages of the disease include chlorabucil in combination with rituximab or lenalidomide plus rituximab[232]. Relapse of follicular lymphoma is commonly treated with inhibitors of B-cell receptor pathway (ibrutinib plus idelisilib)[233] or with BCL-2 inhibitors (venetoclax)[234] and PD-1/PDL-1 (programmed cell death protein 1) inhibitors[235].

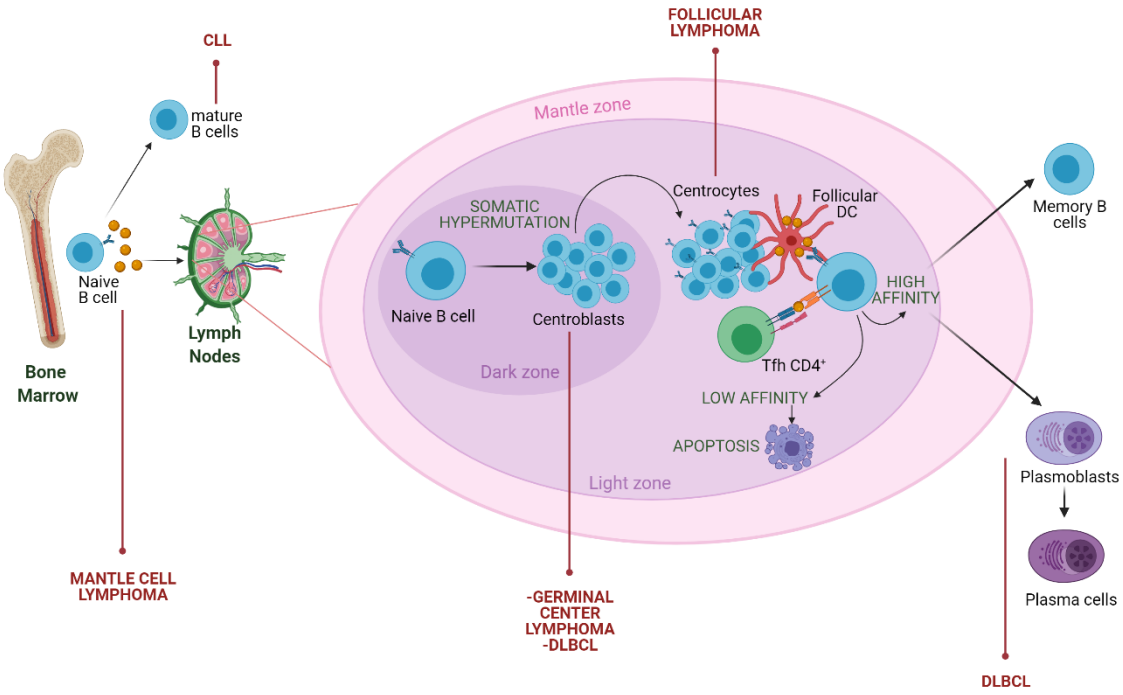

Figure 8. B cells and linked malignancies

Figure shows the development of B cells from bone marrow and proceeding in lymph nodes. Fails during the process may bring to the development of a hematologic malignancy. (Figure created with BioRender.com with reference to[236])

2.4. Hodgkin lymphoma

HL shows to be different from NHL, especially for its morphological appearance at the microscope and presents particular characteristics compared to other malignancies, such as the rarity of malignant cells, the incidence in young patients[237]. The incidence of HL varies with age: it increases in the age of 15-35[238]. It represents the 15-20% of all lymphomas in western world[239].

2.4.1. Aetiology and risk factors of B cell Hodgkin lymphoma

HL is mostly characterized by Hodgkin (H) cells, Reed-Sternberg neoplastic cells (RS)[240] , which constitute only the 10% of the tumor in contrast to all the other NHL[239]. HRS cells are known to derive from B cells in germinal center; these cells lack B-cell receptors on the surface and do not undergo to programmed B-cell death[241,242], the inhibition of apoptosis is probably connected to NF-kB which becomes constitutively expressed in HL[243]. Reed-

Sternberg cells are characterized also by the ability to evade immune responses through alterations in genes encoding PD-L1 and PD-L2 (PD-1 receptor ligands)[244]. RS cells are usually found in tissue biopsy for they morphology, since they present a bilobed nucleus and an abundant cytoplasm[245].

Epstein-Barr virus infection is associated with classic HL in about the 40% of the cases[246] . HIV infection is a risk factor for HL development, especially for the EBV positive subtype[247] Risk factors for HL development include heritability. Specifically, variants in genes which control HLA class II expression are associated with HL EBV negative, while variants in HLA class I link to HL EBV positive[248].

2.4.2. Staging of HL

Differently from NHL, patients with HL show involvement of lymph-node chains. Symptoms usually include fever, drenching night sweats, weight loss, pain in the chest[249]. For HL diagnosis and staging, chest X-ray and CT scan for neck, chest and abdomen are required[243], while bone marrow biopsy is not indicated if PET-CT gives consistent results to show possible bone marrow involvement[250]. HL staging also needs a blood test in which C-reactive protein, lactate- dehydrogenase abundance is measured. As for NHL the Ann Arbor/Cotswold staging system is applied to HL[200], however in the case of HL the spleen is not considered to be extra nodal involvement of the disease. Patients are classified depending on their prognostic score; commonly either the Lymphoma Study Association and European Organization for Research and Therapy in Cancer or the German Hodgkin Study Group (GHSG) score are applied[251]. The main difference within these two score systems relies in the approach to advanced stage of HL. The GHSG system counts the stage IIB with extra nodal or bulky involvement as an advanced stage disease (III or IV).

2.5. Subtypes of HL

Table 4. HL types and subtypes

The table represents the classification of HL according to its characteristics.

CLASSIC HL	NODULAR LYMPHOCYTES PREDOMINAT HL
• Nodular Sclerosis HL (NSHL) • Mixed Cellularity HL (MCHL) • Lymphocytes-rich HL (LRHL) • Lymphocytes-depleted HL (LDHL)	

Classic HL

All classical HLs do arise from peripheral lymph nodes and commonly in the mediastinum, very rarely HL originates from the inguinal lymph nodes which can be involved during the disease progression together with spleen, liver and other extra nodal sites [239].

Nodular Sclerosis HL (NSHL)

This lymphoma subtype represents the 73% of all cases of HL and it is defined to be the most common especially in young adults. Its name derives from the typical histological characteristic in which it appears as nodules divided by bands of collagen. Symptoms frequently include weight loss, night sweats, pain in the lymph nodes which are usually enlarged especially in the areas above the diaphragm. Histology revealed that in this tumor the RS cells are surrounded by lymphocytes and other immune cells such as histocytes eosinophils, neutrophils. Eosinophils are responsible of TGF production together with RS cells; TGF stimulates the production of collagenous bands found in this malignancy[252].

Mixed Cellularity HL (MCHL)

It represents about the 20% of all HL with an incidence that does not differ depending on the age. Symptoms usually include the enlargement of lymph nodes in spleen and abdomen accompanied frequently with implication of mediastinum. Histologically RS cells appear to be surrounded by eosinophils, lymphocytes, epithelial cells, however in this case no fibrosis or nodules are found[252].

Lymphocytes-rich HL (LRHL)

This HL subtype is characterized by high aggressiveness and a rare incidence. Histologically RS cells appear to be surrounded by a very consistent number of lymphocytes[252].

Lymphocytes-depleted HL (LDHL)

This subtype of relatively rare classic HL is characterized by a high prevalence of neoplastic cells and a lower representation of all the other immune cells. The diagnosis of LDHL is usually in advanced stage disease and represents the most common HL diagnosed in patients affected by immunodeficiencies. The disease involves BM, abdomen included the spleen[252]. EBV infection is very common in this type of HL and its detection during diagnosis is extremely helpful to discriminate HL from other B- or T- cell non-Hodgkin lymphomas with similar characteristics[239].

Nodular lymphocytes predominant HL (NLPHL)

This type of HL has an incidence of 5% of all HL cases and is mostly diffused in patients with median age of 30-50 years[252]. NLPHL differs from the classic form of HL since it is characterized by neoplastic lymphocyte predominant (LP), a variant of RS cells, named 'popcorn cells'. These cells are located in nodules and display expression of B cell markers such as OCT2 and are surrounded by T follicular cells expressing PD-1[230]. No extra nodal location is described for this type of HL[239]. Lymphadenopathies show a slow growth rate and the involved lymph nodes contain fibrous tissue, known as sclerosis.

Treatment for HL must be contextualized with patient clinical situation which includes the evaluation of disease stage, clinical prognostic factors, risks and other pathologies[253]. For early stage classical HL risk of cardiac toxicity, pulmonary damages and the development of secondary malignancies has to be taken into consideration[254]. The usual therapy corresponds to four cycles of ABVD (Adriamycin-doxorubicin-, bleomycin, vinblastine, decarbazine), followed by 30Gy radiation therapy, which shows 84% cases of progression-free patients after 10 years of follow-up[255].

For advanced stages of HL disease, a treatment based on blomycin-etoposide-doxorubicin-cyclophophamide-vincristine-procarbazine and prednisone (eBEACOPP) show an incomparable progression free survival however concomitantly with an increased risk of

secondary malignancies[256]. An alternative for relapsed classic HL is the brentuximab vedotin[257] (an anti CD30 antibody) therapy after six cycles of ABVD. This treatment has the aim to reduce toxicity however maintaining a good efficacy[258].

2.6. Aim of the project: ILCs and NK cells as potential players in hematologic malignancies

Our project of unravelling NK and ILC role in lymphoma raised from the hypothesis to employ these two cell types of the immune system as a cell therapy in hematologic malignancies concomitantly with the standard therapies. The unexplored regulation of ILCs in patients could be at the base of new potential therapies for NHL and HL, overcoming chemotherapy resistance or boosting chemotherapy efficacy. Our research aims to be translational finding the mechanisms at the base of innate immunity regulation.

3. Material and methods

3.1. Primary human samples

Ospedale Maggiore Policlinico of Milan, with the authorization of Ethics committee, provided peripheral blood (PB) samples from healthy donors (HD) anonymously. Samples derived from patients (n=43) were provided by IEO (European Institute of Oncology, Project IEO886 approved by Ethics committee) in collaboration with the clinicians of the division of hematology-oncology. A detailed list of patients involved in the study is reported in **Table 5**. Patient recruitment was authorized by the Ethics Committee as 'clinical trial, code IEO886'. Patients recruited in this study were recruited if either affected by B-cell non-Hodgkin lymphoma or Hodgkin lymphoma and did not show other concomitant tumors or pathologies affecting the immune system. All the patients were treatment-naïve and were first diagnosed with the above-mentioned lymphomas. HD gender and age distribution was not significantly different from NHL patients.

Table 5. Detailed division of samples in IEO 886 clinical trial

NUMBER OF PATIENTS/SAMPLES*	DIAGNOSIS	TISSUE
12	Indolent NHL (9 follicular lymphomas, 3 marginal zone lymphoma)	Peripheral blood
10	Aggressive NHL (4 DLBCL, 1 mantle cell, 5 primary mediastinal large B-cell lymphoma)	Peripheral blood
5	Indolent lymphoma	Lymph nodes
6	Indolent lymphoma	Bone marrow
6	Aggressive lymphoma	Bone marrow
12	Hodgkin Lymphoma	Peripheral blood

*****_Please note that BM and lymph node in many cases derive from the same patient who also donated peripheral blood/lymph node._

3.2. Cell isolation from primary samples

For each patient recruited in IEO886 clinical trial we received 3 tubes of whole blood with the addition of EDTA in order to avoid blood coagulation for a total of 18 ml of blood. BM and lymph nodes were not provided as a sample for this study; however, we used the leftover of the samples used for diagnostic purpose. For each HD we received 2 tubes of 6 ml of peripheral whole blood in EDTA.

Peripheral blood mononuclear cells (PBMCs) of healthy donors and patients were isolated from venous peripheral blood by density gradient centrifugation with Ficoll/Hypaque (Lymphosep). Peripheral blood was diluted with PBS solution, overlaid on Ficoll/Hypaque in ratio 2:1 and centrifuged for 20 minutes with no brake. After centrifugation, the white layer corresponding to lymphocytes and monocytes was collected in a new tube and washed with PBS. If necessary, the red blood cells left in the samples were lysed with lysis solution (0.4% Ammonium Chloride, Potassium Bicarbonate and EDTA) for 5 minutes at 37°C in a humidified chamber. The same procedure was applied for patients' bone marrow samples, which were previously filtered with a 150µm cell strainer to remove possible fragments.

Lymph nodes from NHL and HL patients were provided by the division of pathological anatomy in IEO. Fresh samples were mechanically smashed with the bottom of a syringe piston and a 150µm cell strainer with the addition of RPMI 1640 supplemented with 10% FBS. Cells were washed in PBS and only if necessary, sample red blood cells were lysed with lysis solution for 5 minutes at 37°C in a humidified chamber.

Table 6. List of reagents used for human samples

FBS heat inactivated	Euroclone	ECS5000CH
Lymphosep	Biowest	L0560-100
RPMI 1640	Euroclone	ECM2001L
Dulbecco's Phosphate Buffered Saline 1X	Microgem	TL-1005-3000ML

3.3. Cell characterization, phenotyping and sorting purification

Table 7 and **Table 8** reported below summarize the panels and the antibodies used for this purpose. Whole blood from healthy donors and patients was stained with different panels of monoclonal antibodies. The blood was previously treated with ammonium chloride solution

in order to remove red blood cells, except if the sample was stained with IntraStain-Dako™ solutions, in this case the lysis was not recommended in order to avoid cell killing and stress.

ILCs were identified as lineage negative (CD3, CD4, CD8, CD14, CD15, CD16, CD19, CD20, CD33, CD34, CD230c, FcεRI) and positive for CD127. In peripheral blood ILCs were taken as CD56 $^{low/dim}$. CD117 and CRTH2 were used to distinguish ILC subsets. NK cells were identified as CD3, CD19/CD20 negative and the NK populations were distinguished by CD16, CD56 antibodies.

Intra-nuclear and intracellular staining

For intracellular staining IntraStain-Dako™ (K2311, Dako) has been used. First cells were stained with monoclonal antibodies for surface-cell molecules. Cells were washed with PBS/EDTA/FBS buffer and centrifuged for 3 minutes at 2000 rpm. The supernatant was removed, and the pellet was resuspended with left buffer and 100 µl of IntraStain Reagent A-Fixative- for 15 minutes. Cells were washed with buffer and centrifuged 2000 rpm for 3 minutes. The supernatant was removed, and the pellet resuspended in the left buffer. 100µl of IntraStain Reagent B was added for 15 minutes with the fluorochrome-conjugated antibodies for intracellular staining. Cells were then washed and centrifuged before reading the samples to the flow cytometer.

For nuclear staining Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, cat. 00-5523) was used. After the staining for cell surface markers cells were washed with buffer and 1ml of Foxp3 Fixation/Permeabilization buffer at working dilution was add or 30 up to 60 minutes at room temperature protected from light. Cells were washed with 2ml of 1x Permeabilization buffer and centrifuged for 5 minutes at 600 x g. The supernatant was discarded, and the pellet resuspended in the left-over buffer in the tube with the conjugated antibodies for the detection of intra-nuclear markers for 30 minutes. After the staining, the cells were washed twice with 1x permeabilization buffer and then resuspended in appropriate flow cytometry staining buffer to be analyzed by flow cytometer.

Samples were acquired with BD FacsCelesta (12-colour) or with DxFLEX by Beckmann Coulter (15-coulours). In the host laboratory of Doct. Camilla Jandus in Lausanne, BD Fortessa, Gallios by Beckmann Coulter and BD LSR-Sorp were used.

Cell sorting

Cells were sorted in IEO flow cytometry facility and in Lausanne CHUV flow cytometry facility. BD Facs Aria fusion and BD sorter melody were used. Cell were sorted with a pressure of 4 PSI, in 1.5ml Eppendorf with 10% FBS RPMI and in ice.

NK and ILCs were sorted by FACS Aria fusion. For dead cell discrimination, we used Live-Dead Fixable Aqua Dead Cell Stain Kit, which was applied after the surface marker staining in pure PBS solution. NK cells were sorted as Live-Dead Fixable Aqua negative, $CD45^{+}$, $CD3^{-}$, $CD19^{-}$, $CD20^{-,}$ $CD56^{bright/dim}$, $CD16^{-/+}$. Helper ILCs as live cells, lineage negative (Lin^{-}) $CD127^{+}$ lymphocytes. The sorted cells were put in culture with healthy B cells or with tumour cell lines. Sorted cells were put in MLTC in 1:1 ratio with tumour cell lines in V-bottom 96-well plates with RPMI supplemented with 10% FBS, 1% HEPES and with 10U/ml IL-2. For each million of PBMCs, about 300/400 ILCs were sorted.

HD T cell exclusion was performed using cell sorting purification. Fresh PBMCs were stained with PerCP-Cy5.5anti-human CD3 mAb. Both $CD3^{+}$ and $CD3^{-}$ T cells were collected in RPMI medium supplemented with 10% FBS.

Myeloid cells exclusion was performed through sorting purification from fresh PBMCs, using anti-human FITC CD33 and APC CD13 mAbs. Non-myeloid cells were collected in RPMI medium supplemented with 10% FBS.

For Treg cell exclusion, $CD4^{+}$ T cells were enriched with magnetic bead separation (miltenyibiotec kit) and then sorted as $CD25^{+}$ and $CD127^{low/-}$. This strategy avoided the occupation of CD127 on helper ILCs for cell phenotyping after mixed lymphocyte-tumor cell cultures (MLTC) experiment. PBMCs without Tregs were collected in RPMI medium supplemented with 10% FBS.

Table 7. List of antibodies used for flow cytometry, cell sorting purification

ANTIBODY	SOURCE	CLONE	IDENTIFIER
FITC anti-human CD3	BD	SK7	345764
PerCP anti-human CD3	Biolegend	OKT3	317336
APC-AF750 anti-human CD3	Beckmann Coulter		A94680
FITC anti-human CD4	BD	SK3	345768

APC anti-human CD4	BD	SK3	345771
FITC anti-human CD8	BD	RPA-T8	557085
PerCP anti-human CD8	Biolegend	SK1	344710
PC7 anti-human CD8	Beckmann Coulter	SFCI21Thy2D3	737661
APC anti-human CD13	Beckmann Coulter	Immu103.44	200042
FITC anti-human CD14	BD	MøP9	345784
FITC anti-human CD15	BD	MMA	332778
FITC anti-human CD16	BD	3G8	556618
AlexaFluor700 anti-human CD16	Biolegend	3G8	302026
ECD anti-human CD16	Beckmann Coulter	3G8	B49216
FITC anti-human CD19	BD	SJ25C1	345788
APC-H7 anti-human CD19	BD	SJ25C1	641395
FITC anti-human CD20	BD	L27	345792
APC-H7 anti-human CD20	BD	L27	641414
PC7 anti-human CD25	Beckmann Coulter	B1.49.9	200054
FITC anti-human CD33	BD	P67.6	345798
APC-AF750 anti-human CD33	Beckmann Coulter	D3HL60.251	A70200
FITC anti-human CD34	BD	581	555821
APC anti-human CD34	BD	8G12	345804
PE-Cy7 anti-human CD39	Biolegend	A1	328212
FITC anti-human CD203c	Biolegend	E-NPP3	324614
BV605 anti-human CD203c	Biolegend	E-NPP3	324620
FITC anti-human FcεRI	Biolegend	AER-37	334608
BV605 anti-human FcεRI	Biolegend	AER-37	334628
BV510 anti-human CD45	BD	HI30	563204
BV786 anti-human CD56	BD	NCAM16.2	564058
PerCP anti-human CD56	Biolegend	HCD56 (NCAM)	318322
FITC anti-human CD56	BD	B159	562794
FITC anti-human CD94	Biolegend	DX22	305504
APC anti-human CD94	BD	HP-3D9	559876
BV421 anti-human NKG2A	BD	131411	747924
PE anti-human CD117	Beckmann Coulter	104D2D1	IM2732
BV605 anti-human CD117	Biolegend	104D2	313218

BV421 anti-human CD127	Biolegend	A019D5	351310
APC-AF700 anti-human CD127	Beckmann Coulter	R34.34	A71116
BV421 anti-human CD69	Biolegend	FN50	310930
PE-Cy7 anti-human CD69	Biolegend	FN50	557745
PE-Cy7 anti-human CD70	Biolegend	113-16	355112
BV421 anti-human CRTH2	Biolegend	BM16	350112
PE anti-human CRTH2	BD	BM16	563665
BV421 anti-human NKG2A	BD	131411	747924
Alexa Fluor700 anti-human PD-1	Biolegend	EH12.2H7	329951
Alexa Fluor 700 anti-human GRANZYME B	BD	GB11	560213
BV650 anti-human CD38	Biolegend	HB-7	356620
BV650 anti-human CXCR5	BD	RF8B2	740528
BV605 anti-human CD62L	Biolegend	DREG-56	304834
BV605 anti-human NKp44	BD	P44-8	744301
PE-CF594 anti-human CD73	BD	AD2	562817
PE-CF594 anti-human T-BET	BD	04-46	562467
PE-CF594 anti-human CD62L	BD	DREG-56	562301
PE-CF594 anti-human PERFORIN	BD	δG9	563763
PE anti-human KLRG1	Biolegend	14C2A07	368610
APC-Cy7 anti-human Bcl-6	BD	K112-91	563581
BV421 anti-human Lag3	BD	T47-530	565720
BV650 anti-human ROR-γt	BD	Q21-559	563424
PE anti-human Foxp3	eBioscience	236A/E7	12-4777-42
Live-Dead Fixable Aqua dead Cell Stain Kit	ThermoFisher	-	L34957
CellTrace Far Red Cell Proliferation Kit	ThermoFisher	-	C34564

Table 8. (a, b, c) Panels of antibodies most frequently used

(a)

ILCs phenotype panel A	ILCs phenotype panel B	ILCs phenotype panel C	NK phenotype panel
Lineage (CD3, CD4, CD8, CD16, D14, CD15, FcεRI, CD203c, CD19, CD20) CD45, CD127, CRTH2, CD117, CD56, CD94, PD1, CD73, CD39	Lineage (CD3, CD4, CD8, CD16, D14, CD15, FcεRI, CD203c, CD19, CD20) CD45, CD127, CRTH2, CD117, CD56, CD94, CD62L, CD69, CXCR5	Lineage (CD3, CD4, CD8, CD16, D14, CD15, FcεRI, CD203c, CD19, CD20) CD45, CD127, CRTH2, CD117, CD56, CD94, CD70, perforin, Granzyme B	CD3, CD19, CD20 CD45, KLRG1, CD73, CD39, CD94, CD16, CD56, CD69, CD62L, CD38

(b)

MLTC cell-cell contact ILCs	MLTC cell-cell contact NK	MLTC cell-cell contact ILC/NK
Lineage, CRTH2, perforin, CD69, CD94, Granzyme-B, CD127, CD45, CD117, CD56	CD16, perforin, CD70, Granzyme-B, CD3, CD45, CD56	Lineage (without CD16), CRTH2, CD16, CD70, CD94, PD1, CD19, CD20, CD127, CD45, CD117, CD56

(c)

MLTC transwell ILCs	MLTC transwell NK	MLTC transwell ILCs	MLTC transwell NK
Lineage, CRTH2, CD73, CD39, CD94, PD1, CD127, CD45, CD117, CXCR5, CD56	CD3, CD19, KLRG1, CD73, CD39, CD94, CD16, CD69, CD45, CXCR5, CD56	LIN, CRTH2, CD69, CD94, CD127, CD45, CD117, CD56	CD16, perforin, CD94, Granzyme-B, CD3, CD45, CD56

3.4. Cell line and cell cultures

Human cell lines SUDHL-4 and SUDHL-10 were bought from American Type Culture Collection (ATCC). All cell lines were culture and stored accordingly manufacturer's instructions. SUDHL-4 and SUDHL-10 were cultured in RMPI-1640 supplemented with 10% FBS, (100U/ml penicillin/streptomycin, Euroclone), 1% penicillin/streptomycin and

2mM L-Glutamine (Euroclone). Since glutamine is unstable at 4°C even if already present in commercial media, it was periodically freshly added to the media. All the cell lines were in suspension therefore after reaching confluence; they were harvested and centrifuged at 1500 rpm for 5 minutes. Cultures were grown at 37°C in a humidified chamber supplied with 5% CO_2. All the cell manipulations were performed in a laminal flow hood to avoid cell contaminations. For long-term storage, cells (preferably with lower passage) were frozen in a solution composed by 90% FBS and 10% DMSO (dimethyl sulfoxide, Alchimia, Italy) first in crioboxes and after 24 hours in frozen nitrogen tanks.

3.5. *In vitro* experiments

ILC Cloning

This experiment has been performed in Dr. Jandus Laboratory. OP9 cells are a stromal cell line from murine model which provide growth support to ILCs without modifying their plasticity[126]. ILCs were seeded in terasaki cell culture plates (Nalgene, cat 757-10171-434) at the amount of $1x10^5$ cells for each well. After 24 hours, ILCs were sorted purified with FACSAria Fusion II. They were put in RPMI 8% human serum supplemented with cytokines: 100 U/mg IL-2, 5 ng/ml IL-7, 50 ng/ml IL-12p40, 50 ng/ml IL-18, 50 ng/ml IL-27. Cells were plated in terasaki cell culture plates onto OP9 cells, counting half a cell for each well in 20µl of medium. Plates were covered with an aluminum foil and grown in a humidified chamber at 37°C, 5% CO_2 for up to 15 days. Each week cell growth and cloning were checked with a microscope.

MLTC experiments

Peripheral blood mononuclear cells were cultured with or without SU-DHL-4 and SU-DHL-10 cell lines, using PBMCs alone and PBMCs co-cultured with healthy B cells as controls. PBMCs were separated from the other blood cells by density gradient centrifugation with Ficoll/Hypaque. Experiments were conducted using cell-cell contact in multiwell plates, maintaining a cell concentration of 800 cells/µL. RPMI supplemented with 10% FBS, 1% HEPES was used. The ratio between PBMCs: tumor cell lines and PBMCs: healthy B cells was 2:1 in each experiment. $1x10^6$ PBMCs MLTC with $5x10^5$ tumor cell lines were seeded in 24-well plates and about $5x10^5$ PBMCs MLTC with about $2.5x10^5$ healthy B cells were co-cultured in 48-well plates. PBMCs alone were seeded in 24-well plates at a density of

$1x10^6$ cells. SUDHL-4, SUDHL-10 and healthy B cells were marked with CellTrace Far Red Cell Proliferation Kit in order to be excluded from the analysis and the cell counts during flow cytometry acquisition. These conditions were maintained for all the experiments with PBMCs with exception for the ones performed with sorted ILCs/NK.
B cells were separated from PBMCs of healthy donors using magnetic labelling. For this purpose, CD19 MicroBeads (cat. 130-050-301, Miltenyi-biotech) were added to PBMCs and then the cells were magnetically separated using MiniMacs Separator by using the correct column adapter. All the protocol foresees different washes, which were performed using a cold buffer composed by phosphate-buffered saline (PBS), 0.5% bovine serum albumin and 2 mM EDTA.

MLTC experiments excluding cell-cell contact were performed using 0.3 μm inserts for 24-well transwell. The transwell inserts was put between PBMCs and tumour cells (in the top of the membrane) or healthy B cells. Ratio was maintained 1:2.

MLTC experiments with sorted ILCs.
Sorted NK and ILC cells were sorted purified and put in MLTC with healthy B cells or with tumour cell lines. After sorting cells were maintained in RPMI 10% FBS, 1%HEPES and with 10U/ml IL-2. Sorted cells were put in MLTC 1:1 ratio with tumour cell lines in 96-well plates V-bottom to increase cell-cell contact reducing cell dispersion in the medium.

Sorted PBMCs selected as negative for CD3 or negative for CD19 or for CD13/CD33 were put in MLTC in 1:2 ratio with B cells sorted from healthy donors. Depending on the amount of ILCs sorted the volumes and the amount of tumour cells/ healthy B cells were adjusted.

MLTC experiments with immune cell type exclusion

$CD3^-$ PBMCs, sorted purified for $CD3^+$ cell exclusion, were collected and then cultured 48 hours alone or with SUDHL-4 and SUDHL-10 tumour cell lines marked with APC-cell trace After 48 hours culture supernatants were collected and cells stained with panels for ILCs phenotyping.

PBMCs purified for myeloid cells were collected and then cultured alone or with SUDHL-4 and SUDHL-10 tumour cell lines marked with APC-cell trace, for 48 hours. At the end of the experiment the supernatants were collected and cells stained with panels for ILCs phenotyping.

T regulatory cell exclusion was performed combining magnetic beads cell separation and cell sorting purification. Cells were enriched with magnetic separation since positive for CD4 expression and then sorted for $CD25^{+}$ and $CD127^{low/-}$. This strategy avoided the occupation of CD127 on helper ILCs for cell phenotyping after MLTC experiment. T regulatory negative cells were collected and then cultured alone or with SUDHL-4 and SUDHL-10 tumour cell lines marked with APC-cell trace, for 48 hours. After 48 hours culture supernatants were collected, and cells stained with panels for ILCs phenotyping.

In vitro stimulation of ILCs and NK.

Experiments of sorted cell stimulation were conducted by using different cytokines: IL-2 (10 U/ml), IL-12p40 (50 ng/ml), IL-18 (50 ng/ml), IL-27 (50 ng/ml) and chemokines MIG (50 ng/ml), MIP 1β (50 ng/ml), IP10 (50 ng/ml) in RPMI medium 10% FCS, 1% HEPES. List of cytokines used is reported in **Table 9.**

Table 9. List of cytokines and chemokines used

Cytokine/chemokine	Source	Identifier
Recombinant Human IL-2	Peprotech	200-02
Recombinant Human IL-7	Peprotech	
Recombinant Human IL-12p40	Peprotech	200-12P40
Recombinant Human IL-18	InvivoGen	rcyec-hil18
Recombinant Human IL-27	Peprotech	200-38
Recombinant Human MIG	Peprotech	300-26
Recombinant Human MIP 1β	Peprotech	300-09
Recombinant Human IP10	Peprotech	300-12

Recombinant Human TSLP	Peprotech	300-62

3.6. Cytokine analysis

In order to detect cytokines in supernatants of MLTCs and to analyse plasma composition, we performed LEGENDplex, an immunoassay based on beads, specific for different analytes. 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 minutes they reached 20-25°C they were used for the immunoassay. For each LEGENDplex experiment a standard curve made of 8 standard serial dilution was performed. Samples were left for two hours to let each bead bind the analyte. Samples were washed with washing buffer provided by the LEGENDplex kit and centrifuge at 1050 rpm for 5 minutes. After removing the wash buffer, an incubation on a shaker (800 rpm) with biotinylated detection antibody and then streptavidin-phycoerytrin (SA-PE) which bound the biotinylated detection antibody. After the staining, the beads were spin down and resuspended in wash buffer. The samples were read on BeckmanCoulter Navios 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 batch of the kit.

Table 10. List of LEGENDplex used.

LEGENDplex	Source	Identifier
Human CD8/NK Panel	Biolegend	740267
LEGENDplex Human inflammation Panel 1	Biolegend	740809
LEGENDplex cytokine Panel 2	Biolegend	740102

3.7. Data analysis

Flow cytometry data were analysed during acquisition at flow cytometer with BD FacsDiva (LSR-Sorp, FacsCelesta) or with BD FACSuite (FacsLyric) software. After acquisition at

flow cytometer FCS files were analysed with FlowJo software[259] and with Kaluza released by Beckmann-Coulter[260]. Statistical analyses were performed using Prism GraphPad, version 9.1[261]. 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 MLTC experiments, the normality was tested as before, and normal multiple ordinary one-way ANOVA or Friedman test was applied accordingly using Bonferroni correction.

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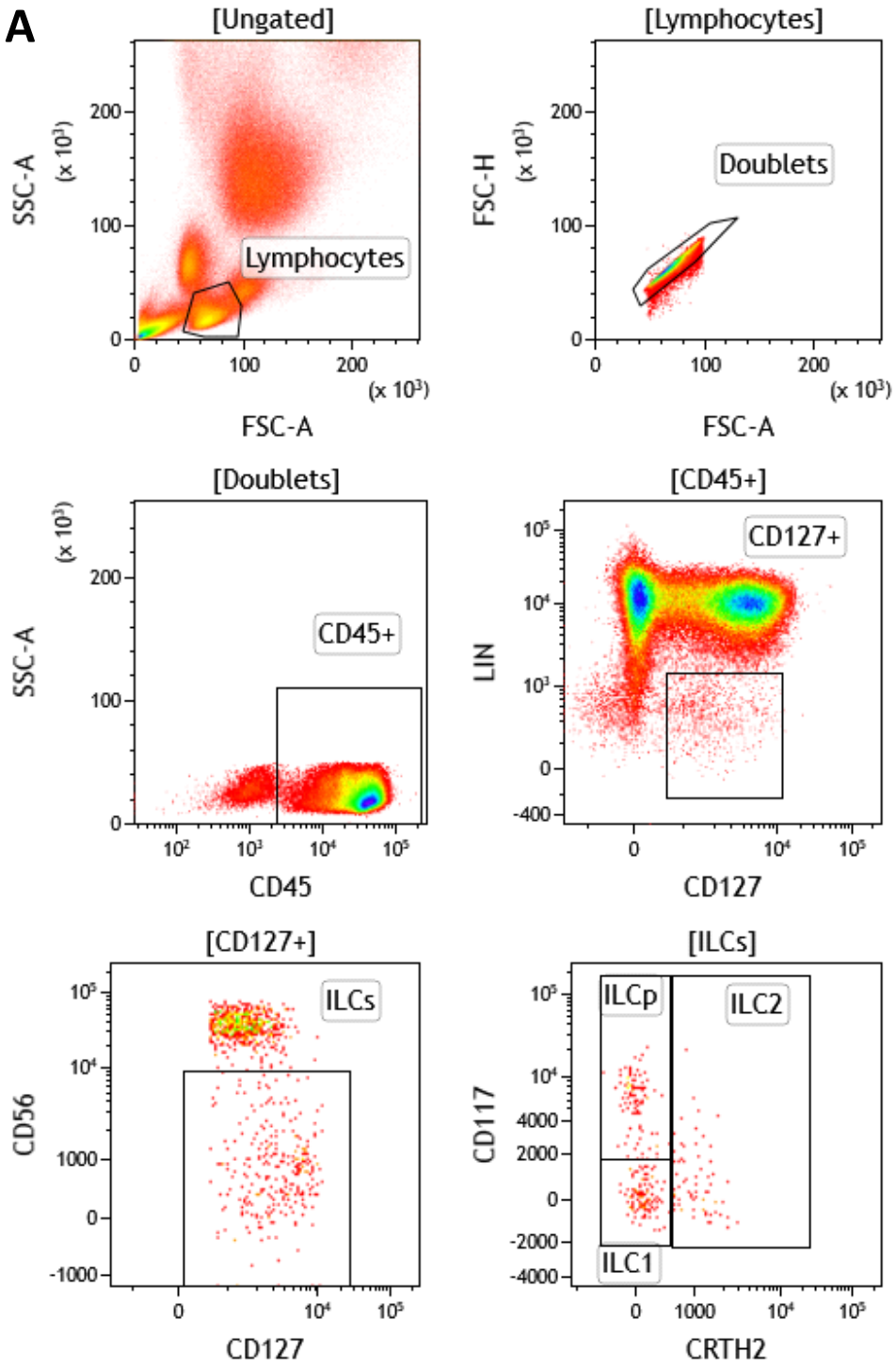
4. Results

4.1. Helper ILC frequencies are not altered in NHL patients

Our first main aim was to describe these cells starting to see if there were differences between healthy donors and NHL patients in terms of ILC and NK distribution in peripheral blood. To do this, we designed a 12-colour multiparametric flow cytometry panel and gating strategies to detect both NK cells and helper ILCs in peripheral blood (**Figure 9**). The gating strategies were designed considering data from literature, indeed helper ILCs were gated as lineage negative and CD127 (IL-7R) positive cells and identified as $CD56^{low/-}$. The three ILC subsets were distinguished by the expression of CRTH2 and CD117 (c-Kit): ILC1s were negative for both these markers, ILC2 positive for CRTH2 and variable expression of CD117 (therefore $CD117^{+}$ and $CD117^{-}$) and ILCP subset as positive for CD117 expression while negative for CRTH2 marker. NK cells were gated as CD3 negative and distinguished on the expression of CD56 and CD16. $CD56^{bright}$ NK cells expressed CD56 and were $CD16^{low/-}$, $CD16^{+}$ NK were gated as $CD16^{+}$ $CD56^{dim}$ and unconventional NK, named as $uCD56^{dim}$ NK, were gated as $CD16^{-}$ $CD56^{low/dim}$.


A
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SSC-A
(x 10³)
Lymphocytes
FSC-A
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[Lymphocytes]
FSC-H
Doublets
[Doublets]
CD45+
CD45
[CD45+]
CD127+
LIN
CD127
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ILCs
CD56
CD127
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ILCp
ILC2
ILC1
CD117
CRTH2

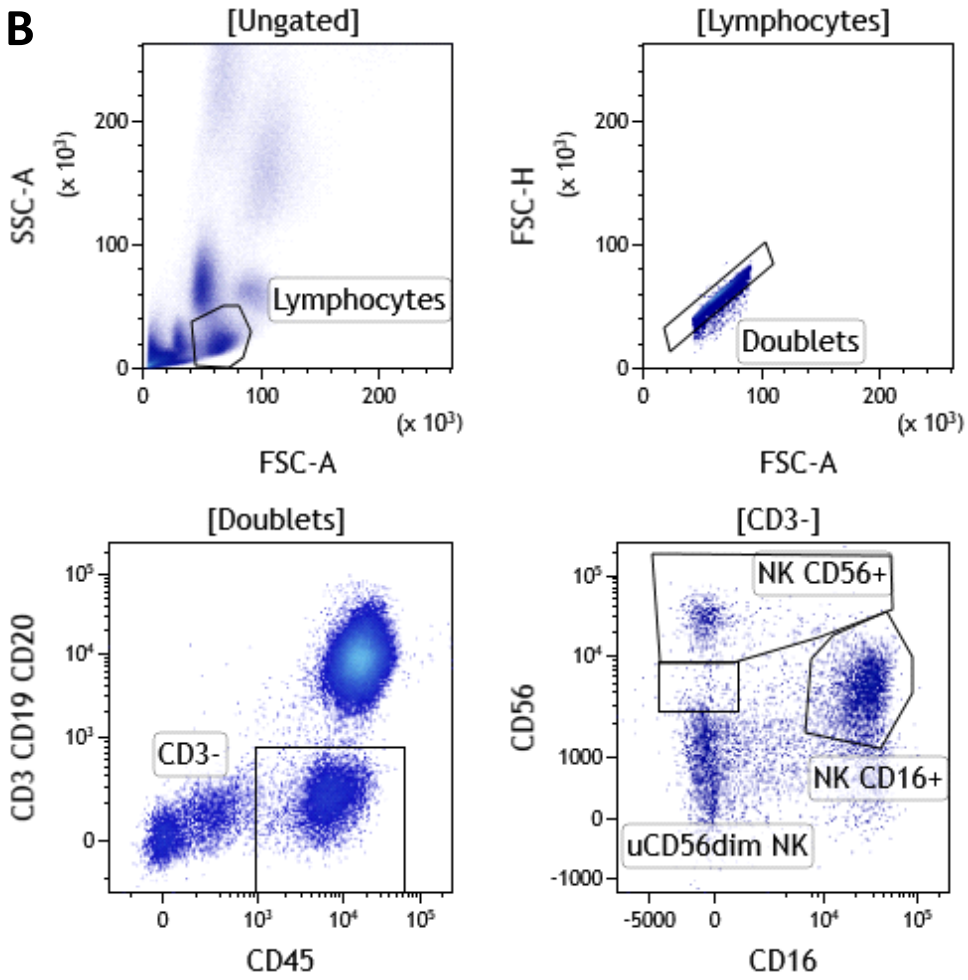

Figure 9. Gating strategy for helper ILCs and NK cells

(A) Helper ILCs were gated in whole peripheral blood after red blood cell lysis. After gating total lymphocytes, $CD45^+$ cells were chosen. Taking the gate of $CD45^+$ immune cells, lineage positive lymphocytes were excluded, using CD3, CD4, CD8, CD14, CD15, CD16, CD19, CD20, CD33, CD34, CD203c, FcεRI antibodies. $CD127^+$ cells were chosen and among them $CD56^{+/bright}$ cells excluded from the gate of total helper ILCs. CD117 and CRTH2 antibodies, considering the CD127+ CD56low/- gate, distinguished the three subsets of helper ILCs. (B) NK cells gating strategy starts from choosing lymphocytes gate among the whole cells of peripheral blood, excluded red blood cells, which were lysed. NK cells were selected as $CD3^-$ $CD19^-$ $CD20^-$cells and gated using the expression of CD56 and CD16 markers.

We compared 50 healthy donors (25 females and 25 males, with a mean age 47 years) with 21 patients affected by NHL, of which 11 females and 10 males (mean age 55 years). ILCs

are very rare cells in peripheral blood, therefore we used more than 1 million of cells from whole blood to detect about 200-550 total ILCs. Our analysis revealed no differences neither in terms of frequencies of helper ILCs between patients and healthy donors, nor in terms of subset distribution. In contrast, among NK cells, $CD16^{+}$ NK subset in patients was found reduced in percentage compared to healthy donors. $CD16^{+}$ NK cells represent the highly cytotoxic subset of NK cells; their reduction in NHL patients, 20.17% median value, compared to healthy donors, 62.20% median ($p<0.01$) suggests a down-modulation of cells displaying anti-tumor cells and a potential mechanism of tumor immune escape in NHL patients. However, no significant differences in distribution were recorded in other NK cell subsets (**Figure 10**).

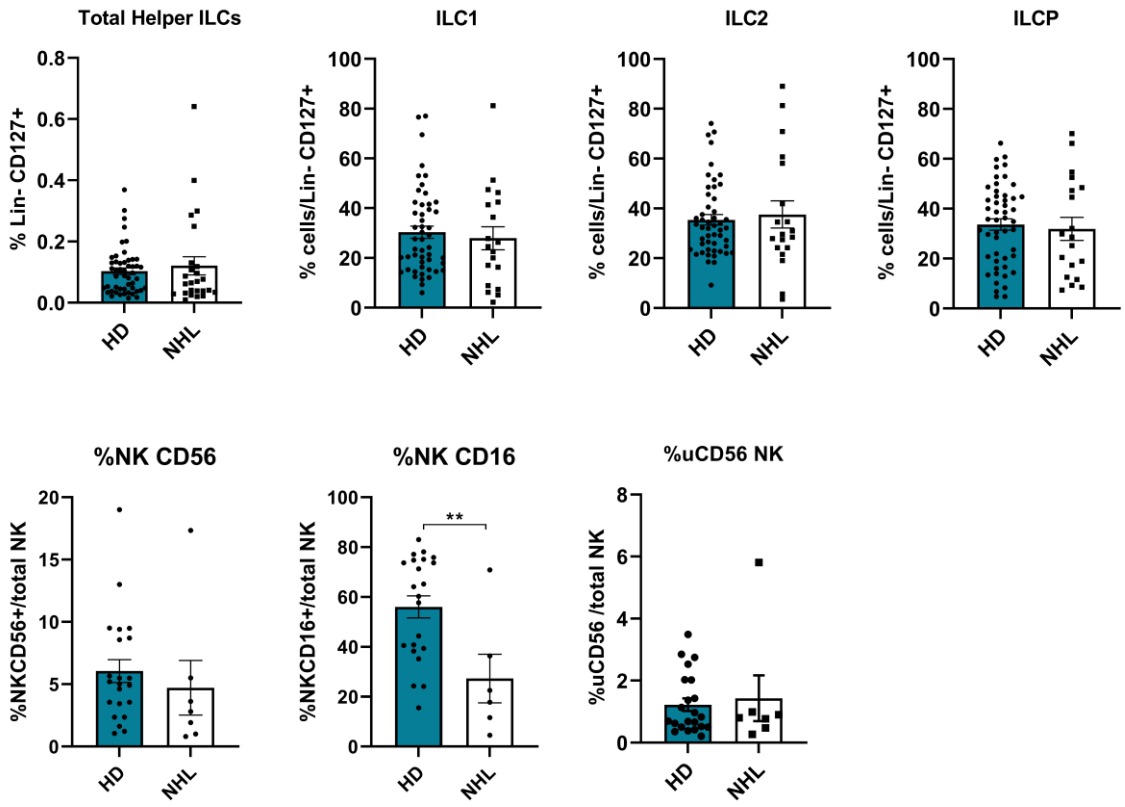

Figure 10. Helper ILCs and NK distribution in patients and healthy donors

Whole fresh peripheral blood was stained with antibodies to detect NK cells and helper ILCs. Considering helper ILCs, we compared n=50 healthy donors (HD) and n=21 patients affected by B-cell non-Hodgkin lymphoma (NHL). Helper ILC distribution was represented considering $Lin^{-}/CD56^{dim/-}$ /$CD127^{+}$ flow cytometry gate. The 3 subsets of helper ILC s were shown in the graphs as part of the whole helper ILCs. Considering NK cells, comparisons

were made between 22 HDs and 7 NHL and each subset has been represented as percentage of the whole NK cells. 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.

Going into details, we divide the patient cohort in two main groups, indolent and aggressive NHL to exclude that our observation was dependent from tumor aggressiveness. We did not show any difference among indolent and aggressive lymphoma, thus confirming that the two patient cohorts present a similar behavior in terms of ILC distribution in PB (**Figure 11**). For this reason, considering the low availability of NHL patient samples, we decided to consider all the NHL types as part of the same group.

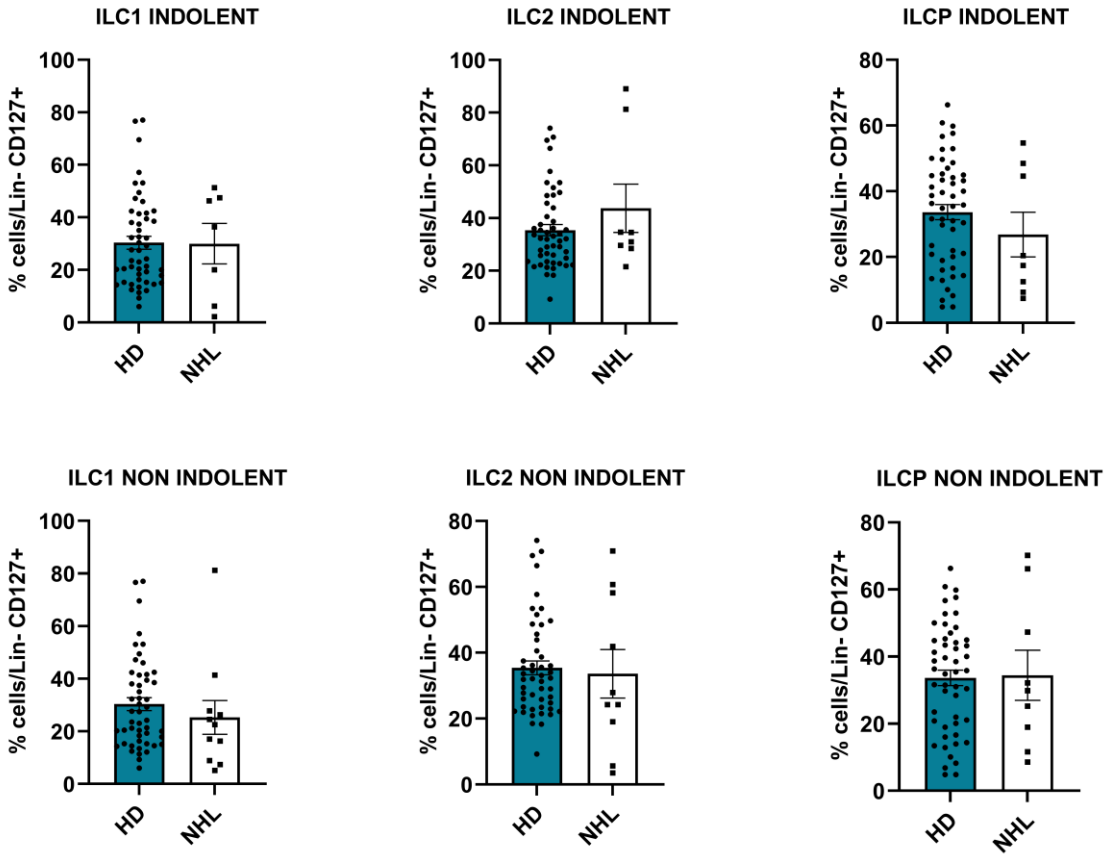

Figure 11. Distribution of helper ILCs is not altered neither in indolent nor in non-indolent NHL patients

Comparisons between NHL and healthy donors show no differences in terms of ILC subset distribution considering NHL patients split in 7 indolent and 10 non-indolent (aggressive) NHL. Percentages of helper ILC subsets were calculated considering the total Lin^- $CD127^+$ as the reference gate.

4.2. In NHL patients' blood, NK cells are less phenotypically active compared to healthy donors

In order to reveal NK cell phenotype in peripheral blood of NHL and HD, we designed a flow cytometry panel including activation markers and markers of cell exhaustion. Overall, in patients, $CD56^{bright}$ NK cells, therefore the subset of NK cells which has a role in cytokine production, exhibited a significant decrease of the explored activation markers such as CD38, CD62L and CD94, but also of the co-inhibitory molecule KLRG1 (**Figure 12A**). Additionally, $CD56^{bright}$ NK show grater expression of CD73, an ectoenzyme that, if over-expressed, participates to the establishment of an immunosuppressive environment[262] and therefore contribute to tumour progression.

Similarly, to what found for $CD56^{bright}$ NK, even $CD16^+$ NK showed a slight upregulation of CD73 that parallels the recorded increment of CD39 expression, an integral membrane protein that, together with CD73, phosphor-hydrolyzes ATP molecules and which is upregulated in inflammatory environments. Concomitantly, both CD69 and KLRG1 were downregulated on the surface of $CD16^+$ NK cells (**Figure 12B**).

Regarding uCD56dim NK, only CD69 expression was downregulated in NHL patients compared to HD, thus suggesting a lower activation of these cells in the context of NHL patient's peripheral blood (**Figure 12C**).

These data, despite the low expression of KLRG1 in two of the three subsets of NK cells, might suggest a pro-tumorigenic role for NK cells in NHL patients, strengthening the hypothesis that the NK cytotoxicity and cytokine production could be counteracted and impaired in presence of NHL.

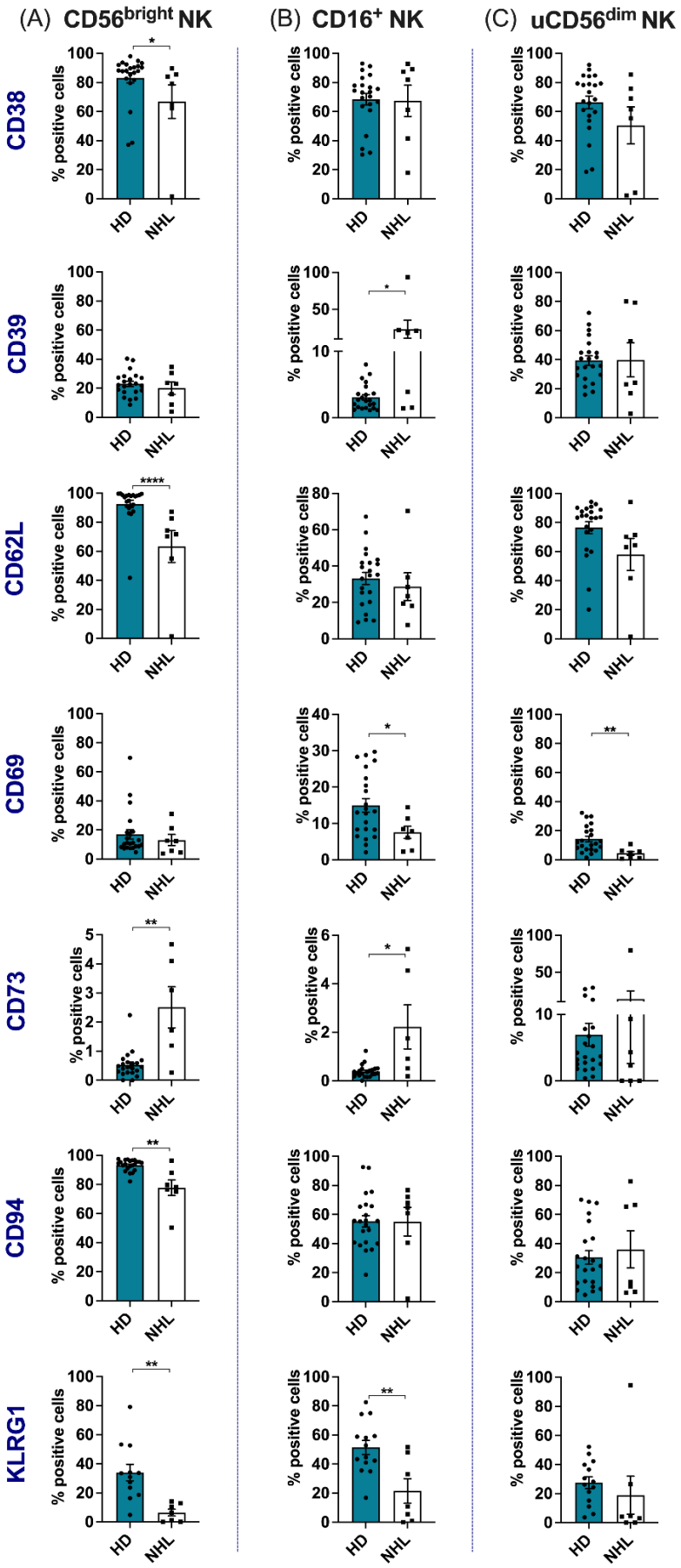
(A) CD56bright NK
(B) CD16+ NK
(C) uCD56dim NK
CD38
CD39
CD62L
CD69
CD73
CD94
KLRG1
% positive cells
HD
NHL
*
**
****

Figure 12. NK phenotyping reveals NK cell reduced activation

Whole fresh peripheral blood from patients and healthy donors was stained with monoclonal antibodies in order to study NK cell phenotype. Each marker was considered independent. For all the markers sample size was HD n=22 and NHL=7, except for the immune checkpoint KLRG1 in which n=12 HD and n=7 NHL were considered. Percentages were expressed considering the positivity for each marker referred to the gate of NK cells ($CD3^-$, $CD19^-$ and $CD20^-$). Statistical significance was tested using Wilcoxon (Mann-Whitney) non-parametric test after testing the not-normal distribution of data through Shapiro-Wilk test. For normal distributed data sets, parametric t test was applied.

4.3. Upregulated chemokines and cytokines potentially influence helper ILC and NK cell activation or exhaustion in NHL

We wanted to explore the possible contribution of cytokines and chemokines in affecting ILCs and NK. We decided to investigate chemokines and cytokines related to inflammation and cell recruitment to tissues. We measured a total of 26 between cytokines and chemokines in the plasma of 15 healthy donors and 15 NHL patients. We found that in NHL patients' plasma 4 out of 13 cytokines tested were upregulated compared to healthy donors, specifically it was the case of TSLP ($p<0.05$); IL12p40 ($p<0.05$); IL-18 ($p<0.00001$) and IL-27 ($p<0.01$). Also, IL-1β was found to be upregulated in plasma of NHL patients but its values were near to the lower threshold of test sensitivity (**Figure 13A**), for this reason we did not use the IL-1β for our further experiments.

Additionally, 3 out of 13 chemokines tested were upregulated in patients, specifically IP-10 ($p<0.01$), MIP1β ($p<0.05$) and MIG ($p<0.0001$) (**Figure 13B**). The three chemokines found upregulated in patients are responsible for cell recruitment and cell retention to tissues. Moreover, MIG and IP-10 are known to stimulate $CD4^+$ T cells to increase the expression of Rorγ-t and T-Bet, which not only contribute to T cell polarization toward Th1 or Th17 [263] but are also fundamental transcription factors for ILC1 and ILC3 polarization.

These data suggest that not only chemokines but also the cytokines found upregulated in patients' plasma could be responsible for innate lymphoid cell modulation. Studying the role of IL-27 we found that it mediates Th1 development from naïve $CD4^+$ T cells[264]and that its

role in terms of T regulatory (Treg) cell function is contrasting, since it is able to induce T regulatory cell expansion and proliferation, but is also implicated in anti-inflammatory function thus inhibiting Treg cells [265]. Tregs are able to block anti-tumour response by creating an immunosuppressive environment. Taking together these data explanation, we noticed a possible correlation between the accumulation of certain chemo/cytokines in patients' plasma and the helper ILC phenotype in NHL.

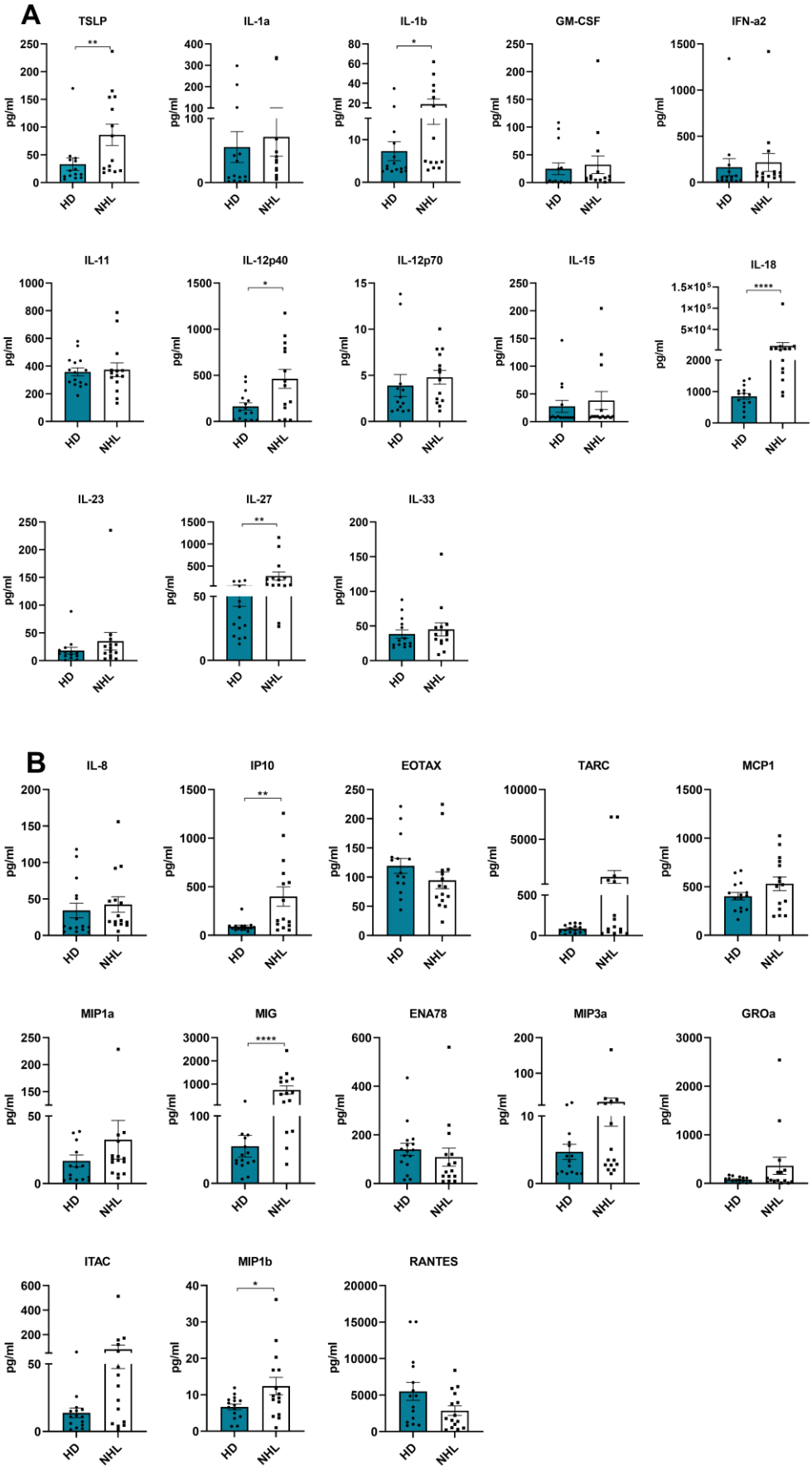
A
TSLP
IL-1a
IL-1b
GM-CSF
IFN-a2
IL-11
IL-12p40
IL-12p70
IL-15
IL-18
IL-23
IL-27
IL-33
B
IL-8
IP10
EOTAX
TARC
MCP1
MIP1a
MIG
ENA78
MIP3a
GROa
ITAC
MIP1b
RANTES
pg/ml
HD
NHL

Figure 13. Inflammatory cytokines and chemokines for cell recruitment to tissues increase in NHL plasma compared to healthy controls

Plasma was collected from whole peripheral blood of 15 patients (NHL) and 15 healthy donors (HD). LegenPlex analysis was performed on plasma after they were frozen. Results are expressed as pg/ml indicating the concentration for each analyte. (**A**) Cytokines increased in NHL patients were TSLP, IL-1b, IL-12p40, IL-18 and IL-27.

(**B**) Chemokines increased in NHL patients were: IP-10, MIP-1b, MIG. Statistical significance was tested 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.

4.4. NK cells are primed in NHL patients and change their phenotype among BM, PB and LN

We analysed and compared the phenotype of NK cells in bone marrow (BM), peripheral blood (PB) and lymph nodes (LN) of NHL patients. As we could know by the clinical records, LNs were the tissue in which cancer cells of NHL were localized. Comparing the three different compartments, all the three NK population showed an enhanced expression of CD73 ectoenzyme and a lower expression of KLRG1 in LNs; CD62L was found upregulated only in PB for the three NK subsets. Considering CD38, it was more expressed in the BM showing a trend of decrement in PB and BM, as observed when considering KLRG1. $CD56^{bright}$ NK express less CD38 in the LN and higher CD62L and CD94 in the PB. $CD16^{+}$ NK from LN displayed lower CD38 but enhanced CD39, CD69 and CD94. While in $uCD56^{dim}$ NK CD69 was increased in LN (**Figure 14 A, B, C**). Altogether, these findings suggest a possible pro-tumour role of NK cells in the lymph node sites, by meaning that NHL tumour cells were able to perturb NK cell phenotype.

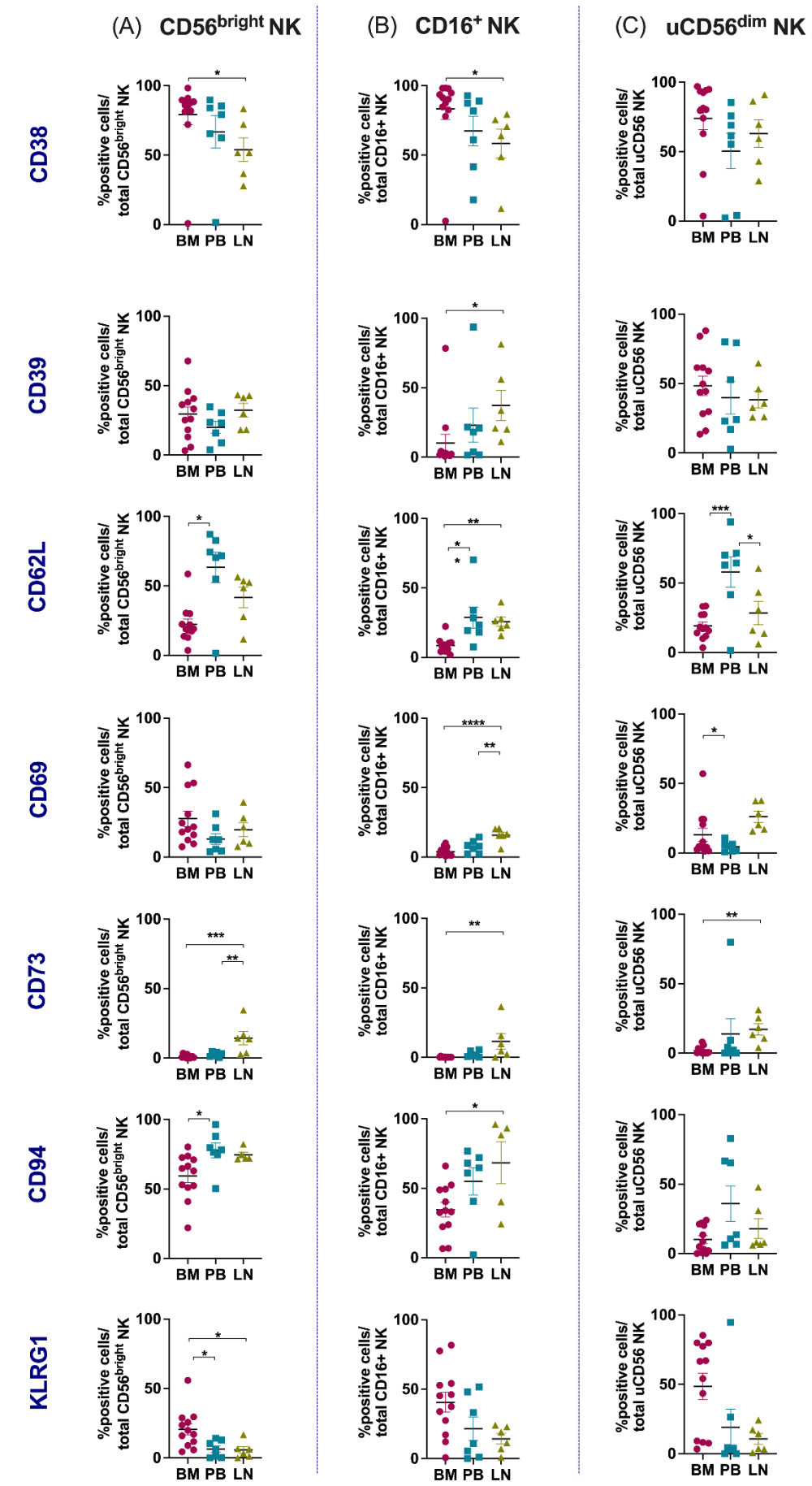
(A) CD56bright NK
(B) CD16+ NK
(C) uCD56dim NK
CD38
CD39
CD62L
CD69
CD73
CD94
KLRG1
%positive cells/ total CD56bright NK
%positive cells/ total CD16+ NK
%positive cells/ total uCD56 NK
BM PB LN
0
50
100
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Figure 14.NK cells may have a pro-tumour role in NHL

(A, B, C) NK cell phenotype comparisons among NHL tissues were investigated using monoclonal antibodies to detect cell activation and immune checkpoint marker expression. KLRG1 show a trend of decreased expression, however the differences were not statistically significant. For this analysis, data set normality was tested by Shapiro-Wilk test; if the distribution was considered normal multiple ordinary one-way Anova was applied, if the data were not normally distributed Kruskal-Wallis analysis was applied.

4.5. NHL affects NK cell activation through cell-cell contact mediated by PBMCs *in vitro*

Our hypothesis was that tumour cells could alter NK functionality in patients. To test this, we performed different *in vitro* experiments. We used two different NHL cell lines: SU-DHL-4, an aggressive NHL cell line expressing PDL-1 and SU-DHL-10, a more indolent lymphoma cells with no PDL-1 or PDL-2 expression[266]. We put in MLTC PBMCs from healthy donors with these two cell lines using as controls both a co-culture with B cells purified from healthy donors (CD19) and PBMCs from healthy donors cultured alone. The addition of two controls allowed us to monitor possible phenotypical changes, at PBMC level, due to the addition of a B cell type belonging to a different individual thus, the addition of PBMCs+CD19 control allowed to better comprehend the effect on helper ILCs and NK cells specifically due to the tumour.

After 48 MLTC we monitored the cytokine produced in the supernatant to control if the addiction of an allogenic cell could alter the environment and the state of cells in culture (**Figure 15**), however no significant changes in cytokine production was recorded.

After 48 hours of MLTCs, we checked NK phenotype (**Figure 16**). At variance, NK cells were more phenotypically active when cells were cultured with the tumour cells lines. In particular CD69 and perforin expression were higher in $CD56^{bright}$ NK when total PBMCs were cultured with the two NHL cell lines. However, $CD16^{+}$ NK and uCD56 NK reduced granzyme B expression in presence of tumours.

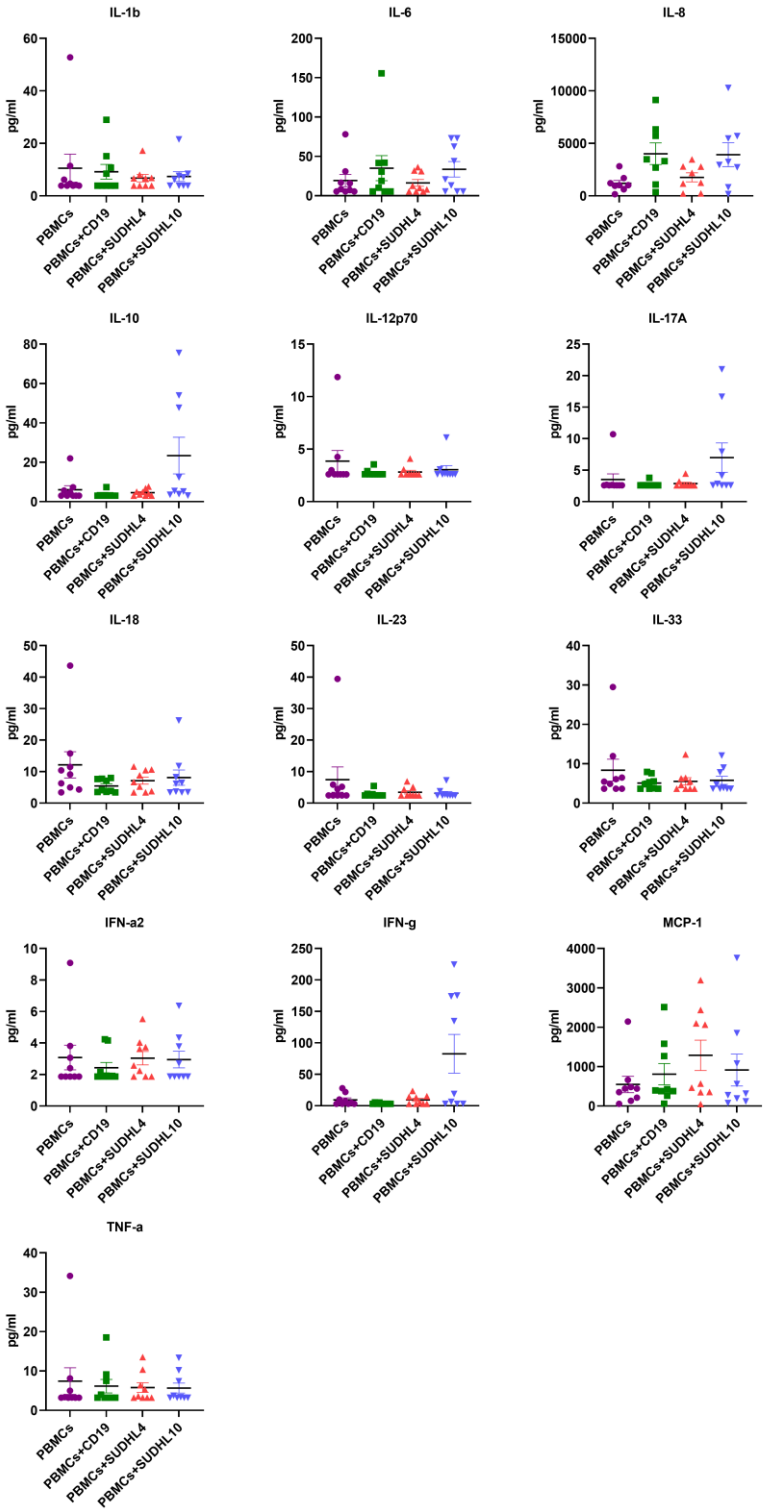
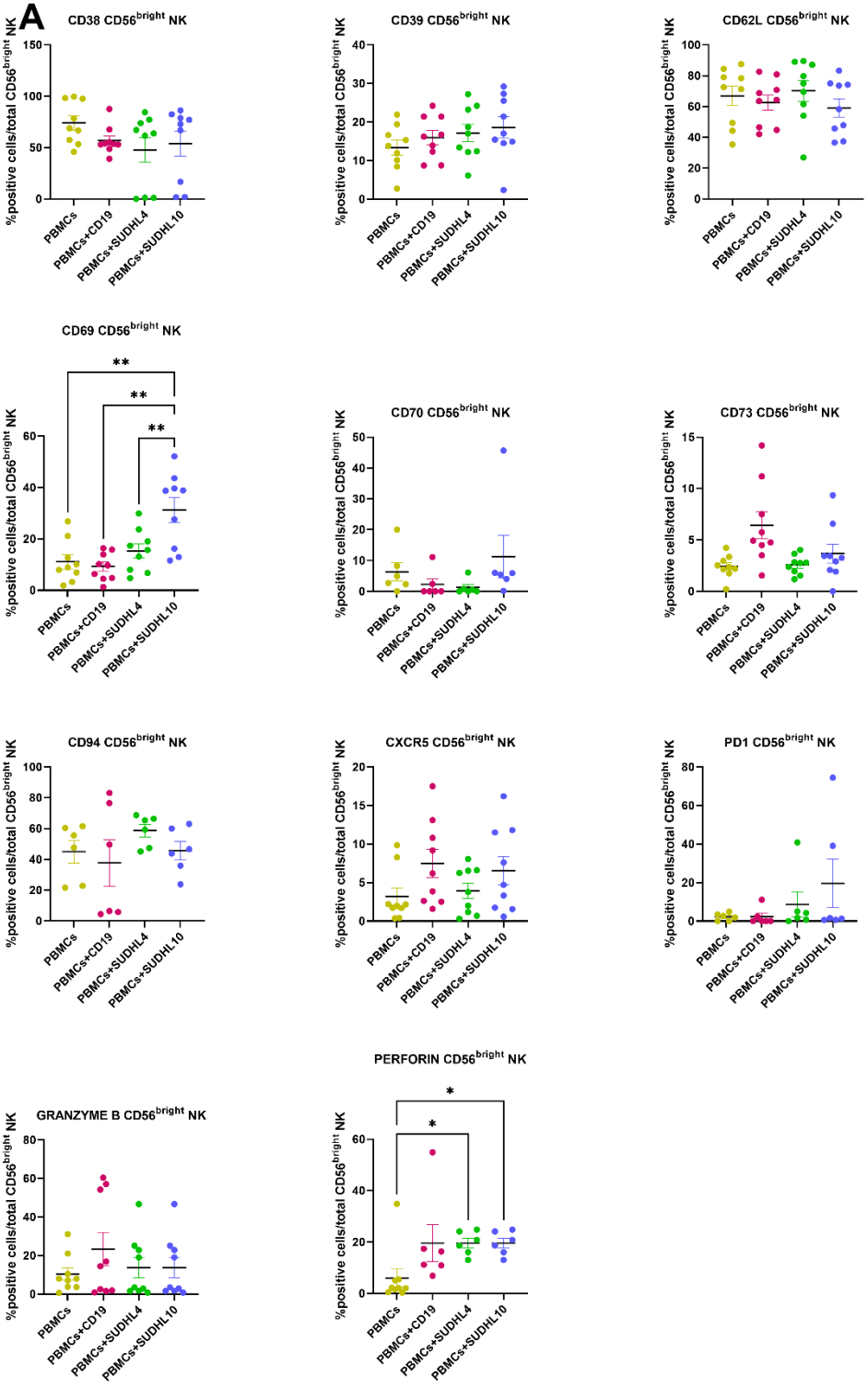


Figure 15. Supernatant analysis after 48 hours MLTC revealed no significant changes in cytokine production

In vitro MLTCs with total PBMCs reveal no significant changes in terms of cytokine release in the supernatants. The results are expressed in pg/ml.


A
CD38 CD56bright NK
CD39 CD56bright NK
CD62L CD56bright NK
CD69 CD56bright NK
CD70 CD56bright NK
CD73 CD56bright NK
CD94 CD56bright NK
CXCR5 CD56bright NK
PD1 CD56bright NK
PERFORIN CD56bright NK
GRANZYME B CD56bright NK
%positive cells/total CD56bright NK
PBMCs
PBMCs+CD19
PBMCs+SUDHL4
PBMCs+SUDHL10
**
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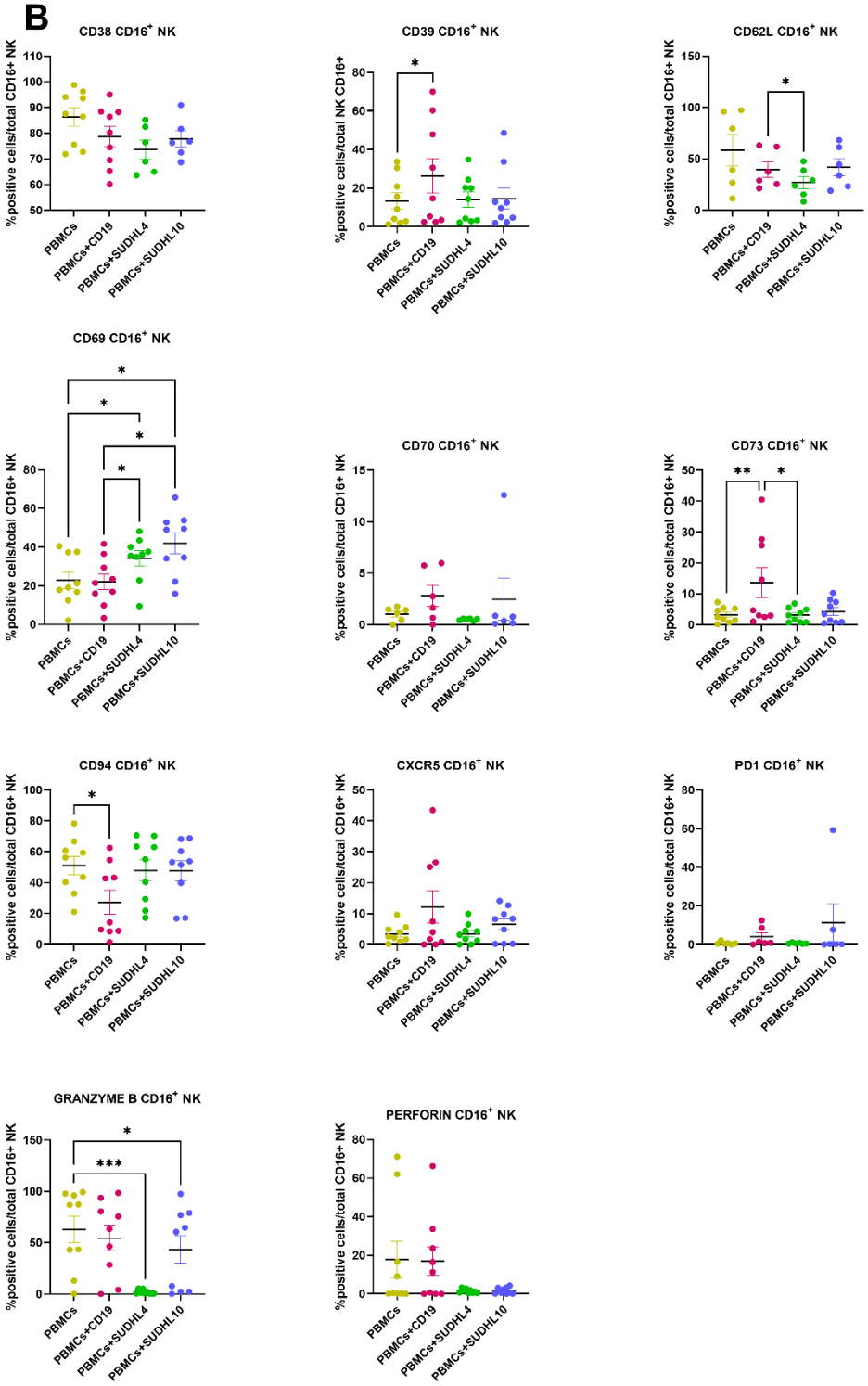
B
CD38 CD16+ NK
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CD62L CD16+ NK
CD69 CD16+ NK
CD70 CD16+ NK
CD73 CD16+ NK
CD94 CD16+ NK
CXCR5 CD16+ NK
PD1 CD16+ NK
GRANZYME B CD16+ NK
PERFORIN CD16+ NK
%positive cells/total CD16+ NK
%positive cells/total NK CD16+
PBMCs
PBMCs+CD19
PBMCs+SUDHL4
PBMCs+SUDHL10

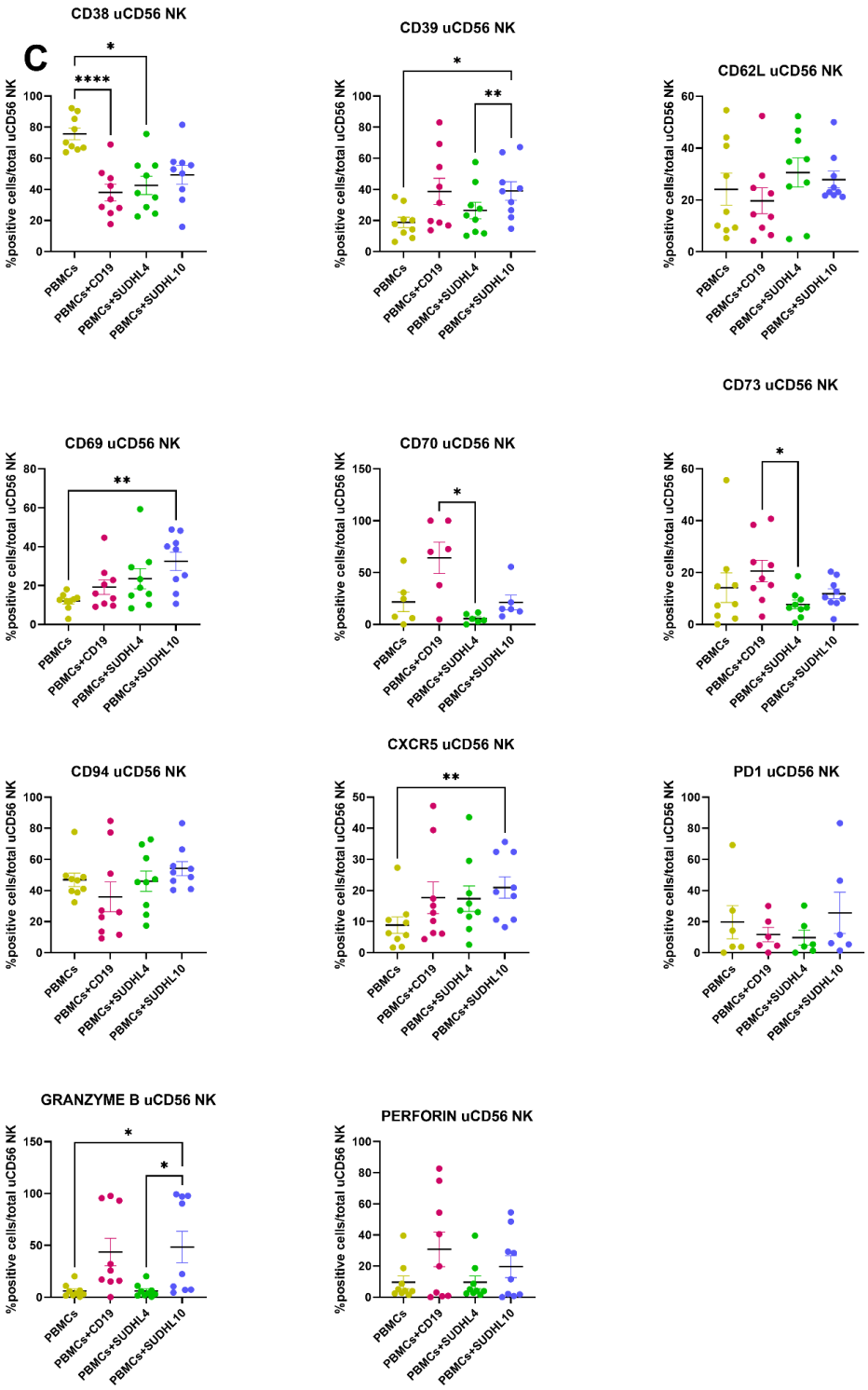


Figure 16. MLTCs experiments show that the presence of tumour cell lines alters NK state

In vitro MLTC with total PBMCs reveal tumour cell lines are responsible for phenotype modulation in NK cells. Percentages were expressed in terms of positivity for each cell marker on total (A) $CD56^{bright}$ NK, (B) $CD16^{+}$ NK or (C) $uCD56^{dim}$ NK cells. Statistical

significance was tested using paired one-way ANOVA in case of normal distribution of the data set or multiple paired non-parametric Friedman.

Going deeper into details, we were aware that the two cell lines we were using for the *in vitro* experiments show expression of the molecule HLA-E which have the ability to bind NKG2A expressed by NK cells with a consequent inhibitory signal. To test if the immune checkpoint molecule NKG2A was involved in NK regulation in presence of tumour cell lines, we checked the expression of the marker NKG2A on NK cells in concomitance with CD94. As result, we did not find any difference in the expression of these markers in NK cells when put in MLTC with tumour cell lines, this suggests that NK activity was not modulated by the immune checkpoint signal NKG2A/CD94 with HLA-E (**Figure 17**).

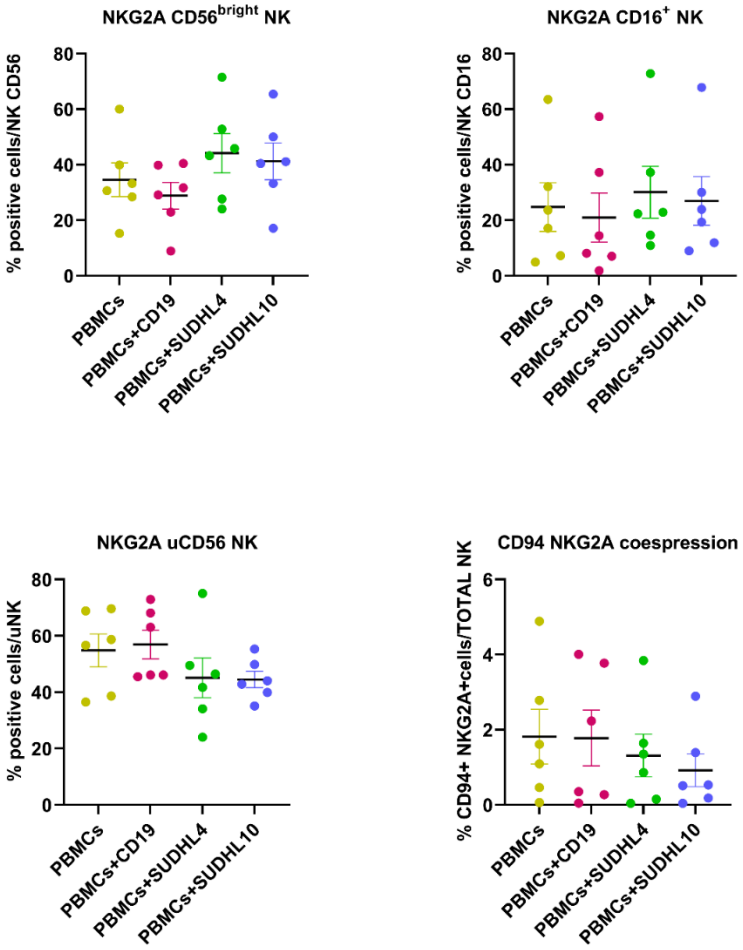
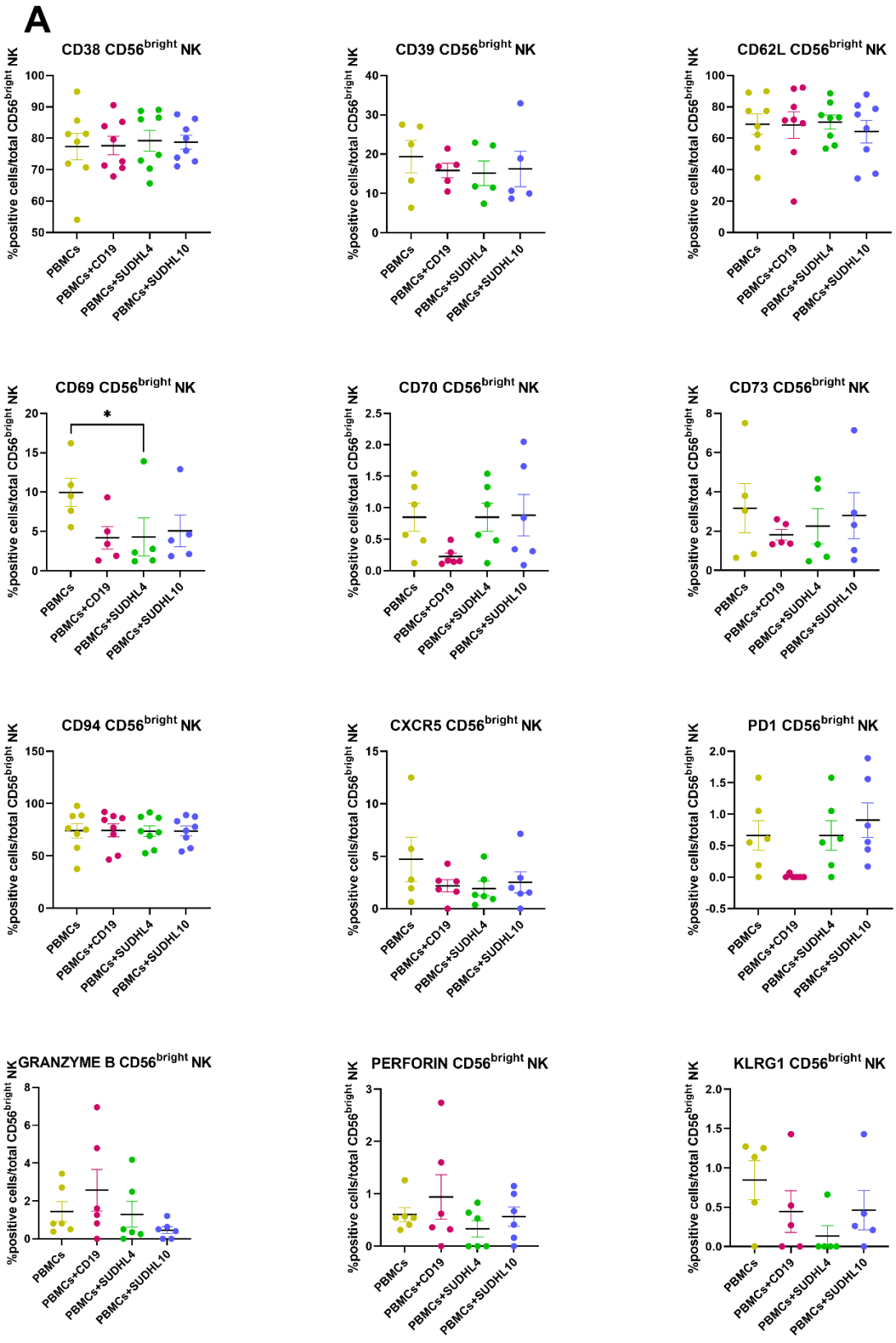


Figure 17. Expression of NKG2A and CD94 on NK cells in *in vitro* MLTC

The expression of the inhibitory receptor NKG2A was evaluated through flow cytometry. After 48h of *in vitro* culture the expression of NKG2A was measured in each NK population

and CD94/NKG2A co-expression revealed no significant changes between cell lines and controls.

The *in vitro* experiments were repeated using PBMCs and tumor cell lines. In this case we blocked all the possible physical contacts between PBMCs and co-cultured cells, either the healthy B cells or the NHL cell lines, leaving the possibility to the supernatant to be shared in the culture. After 48 hours, we explored NK phenotype, which did not show any changes as reported in the previous experiments, thus suggesting the importance of the PBMCs- NHL cell contact to determine the effect on NK phenotype (**Figure 18**).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PD1 CD56bright NK
GRANZYME B CD56bright NK
PERFORIN CD56bright NK
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PBMCs+CD19
PBMCs+SUDHL4
PBMCs+SUDHL10
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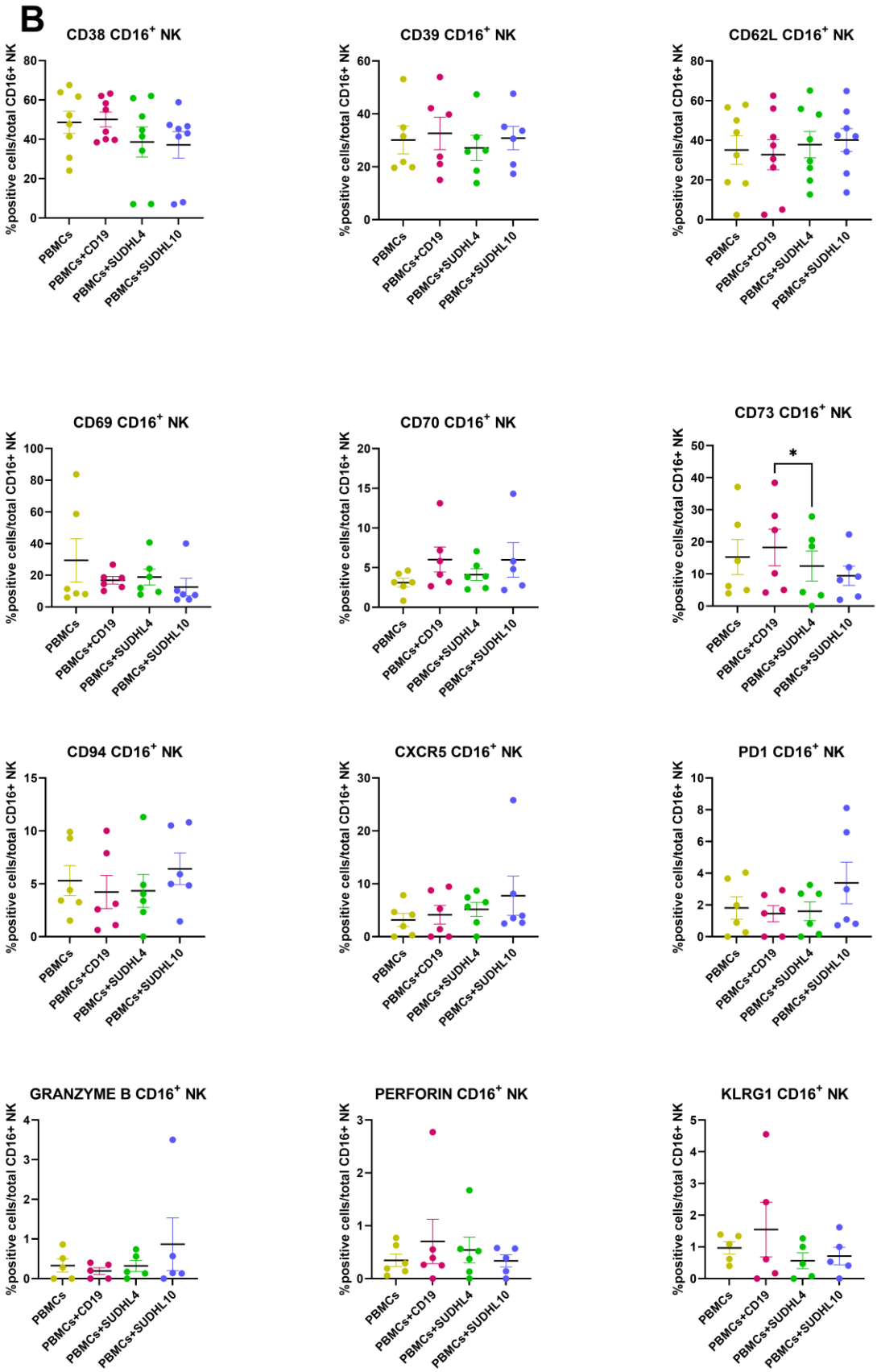
B
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CD94 CD16+ NK
CXCR5 CD16+ NK
PD1 CD16+ NK
GRANZYME B CD16+ NK
PERFORIN CD16+ NK
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PBMCs
PBMCs+CD19
PBMCs+SUDHL4
PBMCs+SUDHL10
*

C

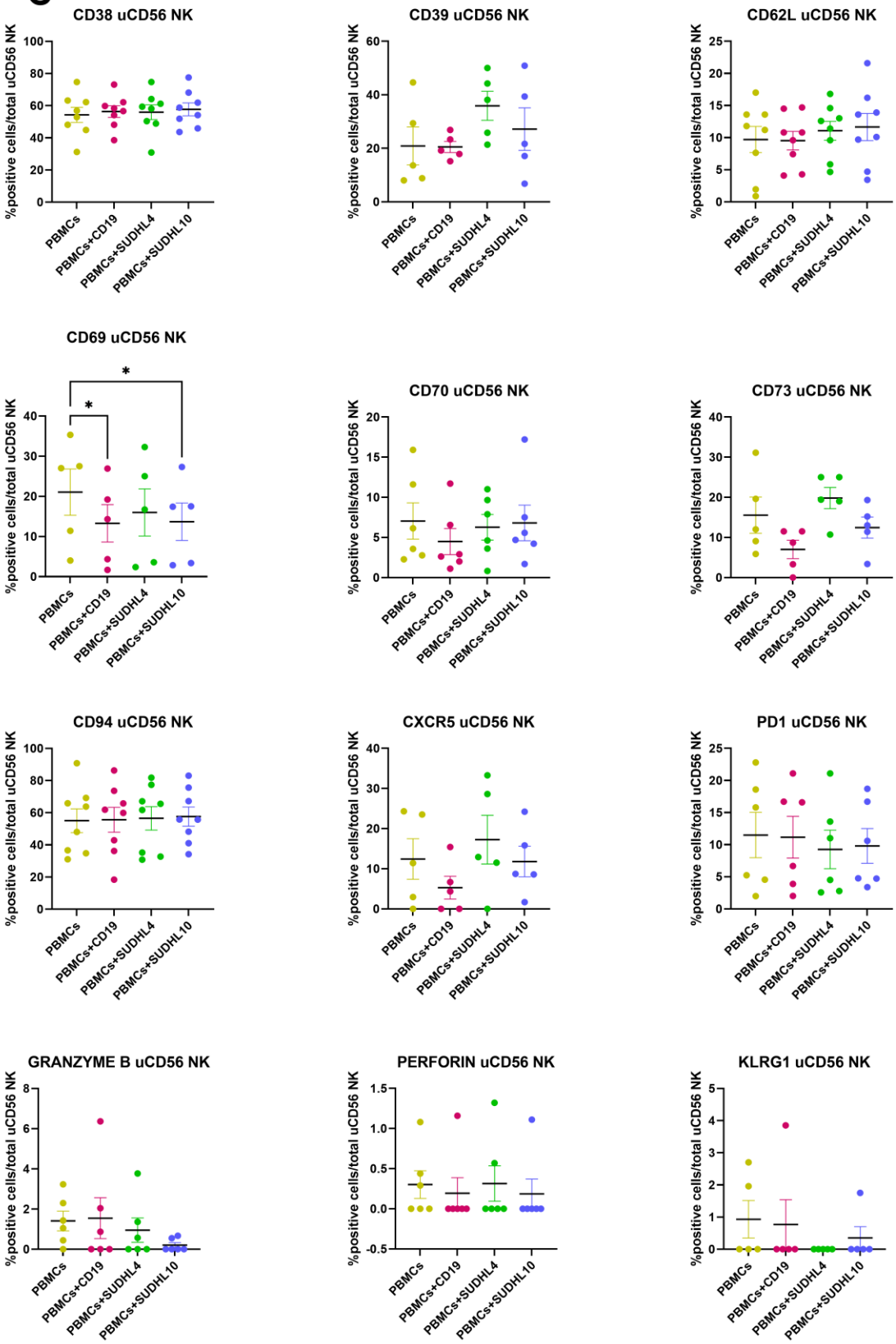

CD38 uCD56 NK
CD39 uCD56 NK
CD62L uCD56 NK
CD69 uCD56 NK
CD70 uCD56 NK
CD73 uCD56 NK
CD94 uCD56 NK
CXCR5 uCD56 NK
PD1 uCD56 NK
GRANZYME B uCD56 NK
PERFORIN uCD56 NK
KLRG1 uCD56 NK
%positive cells/total uCD56 NK
PBMCs
PBMCs+CD19
PBMCs+SUDHL4
PBMCs+SUDHL10
*
*

Figure 18. Tumor-NK cell contact is fundamental for NK phenotype changes

The experiment was performed excluding cell-cell contact, using total PBMCs derived from healthy donors. Statistical significance was tested using paired one-way ANOVA in case of normal distribution of the data set or multiple paired non-parametric Friedman test.

The experiments of MLTC was repeated using sorted purified NK cells. Each NK cell population from healthy donors was cultured either with one tumor cell line or with healthy B cells as a control. In this experiment we chose one cell line due to the low cell number obtained after cell sorting purification. The only control chosen was healthy B cells, because we wanted to monitor what was the effect of the addition of a tumour cell compared to a normal one and in this case the NK cells alone did not have the possibility to provide the appropriate control.

In terms of pheontype, NK cells show a slight trend of increased expression for CD39 concerning $CD56^{bright}$ NK (**Figure 19**), but we did not record any other specific phenotype changes. However, monitoring the cytokine release in the supernatant after 48h MLTC, revealed that in absence of total PBMCs each NK cell subset studied, show higher production of IFN-γ, granulysin, granzyme A and B in presence of the tumour cells compared to the control (**Figure 20 A, B, C**).

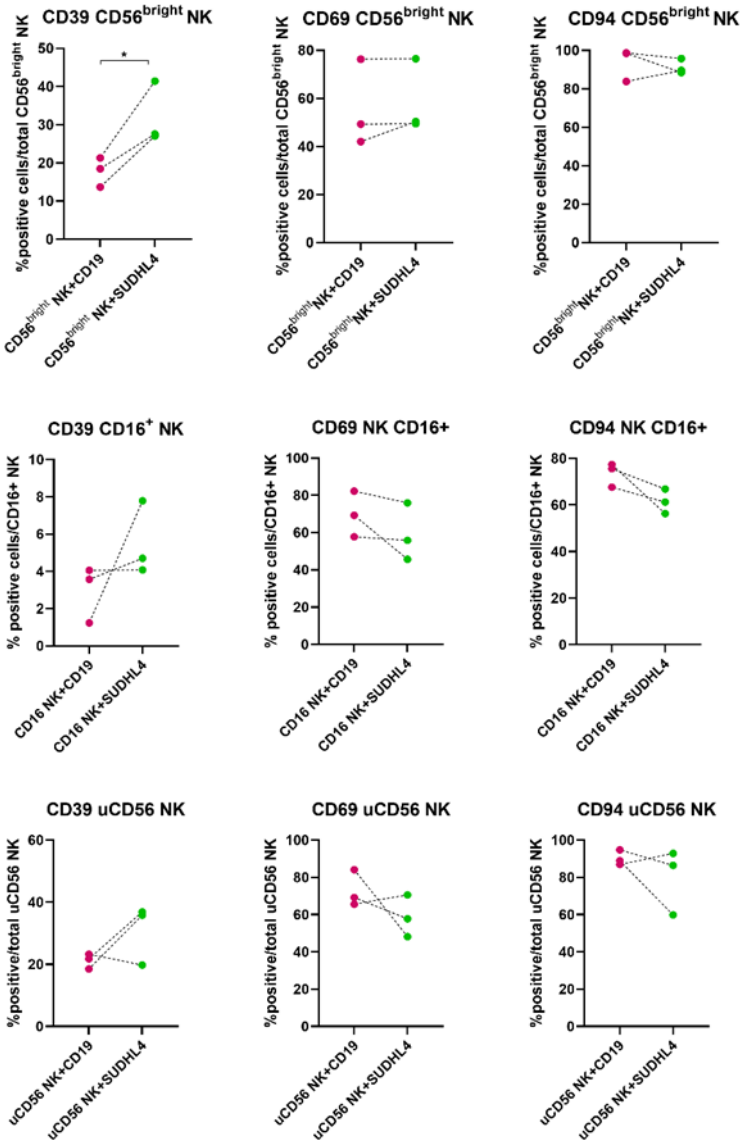


Figure 19. MLTCs experiments with NK cells revealed only slight changes in NK phenotypes in presence of tumour cell line SU-DHL-4

The experiment was performed using 3 independent HD sorted purified NK cells ($CD56^{bright}$ NK, CD16+ NK, $uCD56^{dim}$ NK). Cell phenotype evaluation after 48 hours MLTC reveal that NK cells did not show significant variations in terms of phenotype in absence of other PBMCs, thus indicating that their modulation could be driven by other cells of the immune system and not by the tumour cell line alone. Percentages refer positivity of each cell family for a precise marker. Statistical analysis was conducted using paired t-test or Wilcoxon test, depending on data distribution.

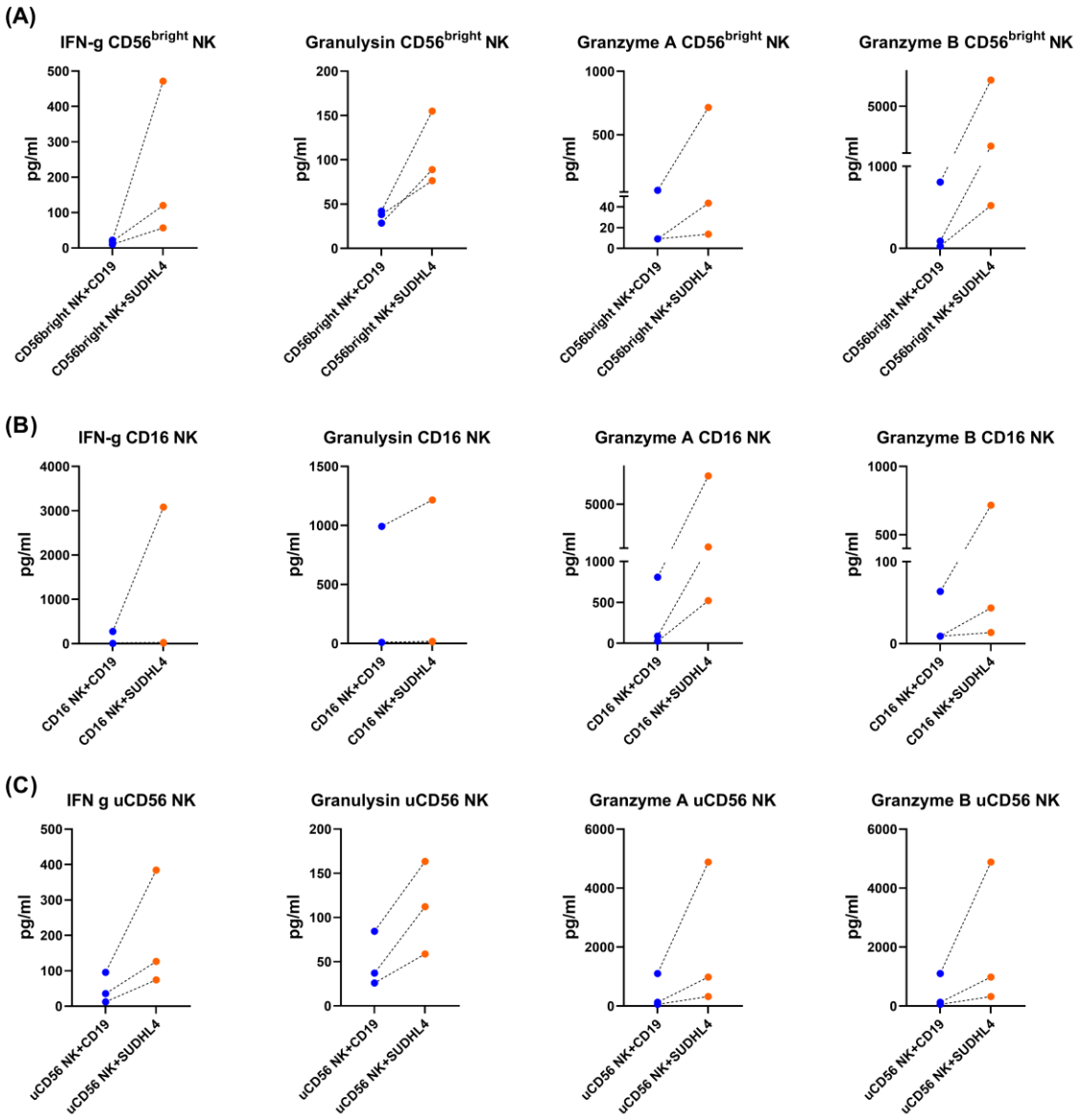


Figure 20. MLTCs experiments with NK cells reveal cytotoxic potential of NK in presence of tumour cell line SU-DHL-4

The experiment was performed using 3 independent HD sorted purified NK cells (CD56^{bright} NK, CD16+ NK, uCD56^{dim} NK). Through BiolegendPlex we revealed the cytokine released by NK cells (**A, B, C**) in presence or absence of tumour cell line. Statistical analysis was conducted using paired t-test or Wilcoxon test, depending on data distribution.

4.6. Helper ILC phenotype is altered in NHL patients compared to HD

Considering ILCs, we had seen that there were no differences in terms of helper ILC distributions by comparing NHL patients and healthy donors; therefore, we wanted to go

into the details and investigate whether helper ILC phenotype and activation status were altered in NHL. For this aim, we designed flow cytometry panels that included different markers for cell activation or cell exhaustion and markers of cell recruitment to tissues (**table 7** & **table 8**). Samples of whole blood derived from HD and NHL patients were analysed by flow cytometry. We found that, among the markers studied, helper ILCs in NHL patients showed a slightly decrease of PD-1 and CD62L expression and a concomitant increase of functional activation markers, such as CD69, granzyme B and perforin (**Figure 21**). CD69 upregulation suggests that helper ILCs could be recruited to the tissues affected by NHL and activated. The greater production of granzyme B and perforin by patients' helper ILCs suggests a potential active involvement in the anti-tumour activity. CD62L is mutually exclusive with activation markers such as CD69[125] therefore this result strengthens the evidence that helper ILCs are in activated state.

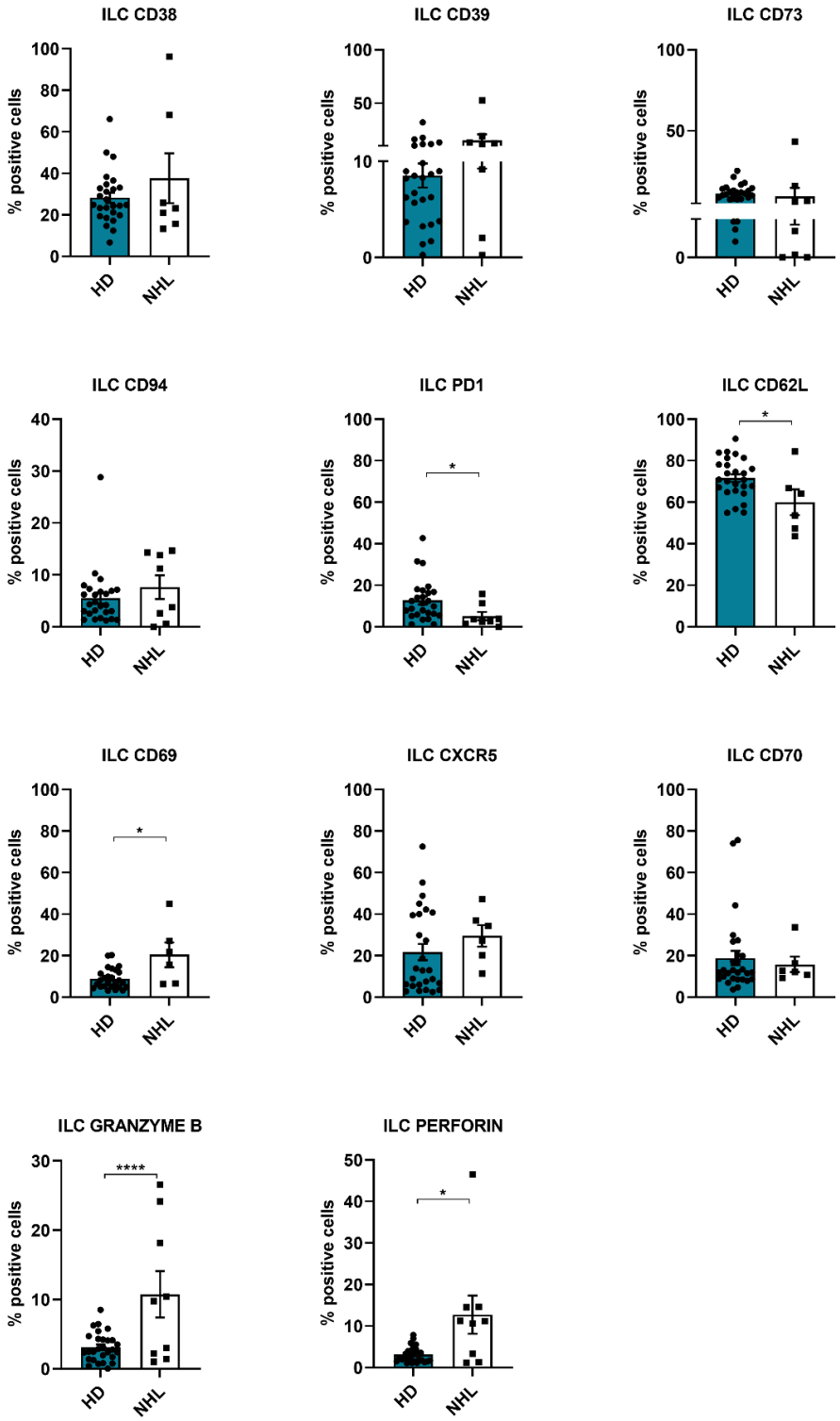


Figure 21. ILCs phenotyping in patients and healthy donors reveals helper ILC activation in NHL

Whole peripheral blood from patients and healthy donors was stained with monoclonal antibody to detect helper ILCs and exploit their phenotype Each marker was considered

independent. For CD38 NHL n=7, for CD39, PD1, CD73 NHL n=8 and for CD94, granzyme B and perforin NHL n=9, for CD62L, CD69 and CXCR5 NHL n=6. Percentages were expressed considering the positivity for each marker referred to the gate of total helper ILCs (Lin^- /$CD56^{dim/-}$ /$CD127^+$). Statistical significance was tested 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.

4.7. ILCs are primed in NHL patients and helper ILCs reach lymph nodes in an activated state

As performed for NK cells, we analysed and compared the frequency and the phenotype of ILCs in BM, PB and LN of NHL patients. Comparing the three different compartments we found that in the LNs the percentage of total ILCs was increased compared to PB (**Figure 22**). Moreover, considering some of the activation markers investigated, helper ILCs had a peculiar pattern of activation in the three different tissues; CD62L together with CD70, granzyme B and perforin showed their highest expression in PB. Expression level of CXCR5 was unchanged in ILCs from PB and LN, while lower in BM. CD39, CD69 and PD1 were more expressed in ILCs from LN, while CD38 show the lowest expression in LN and the highest in the BM. Instead, CD73 and CD94 were equally expressed. Thus, ILCs accumulate in the LN, but they seem to be more functionally active in the periphery than at tumour site.

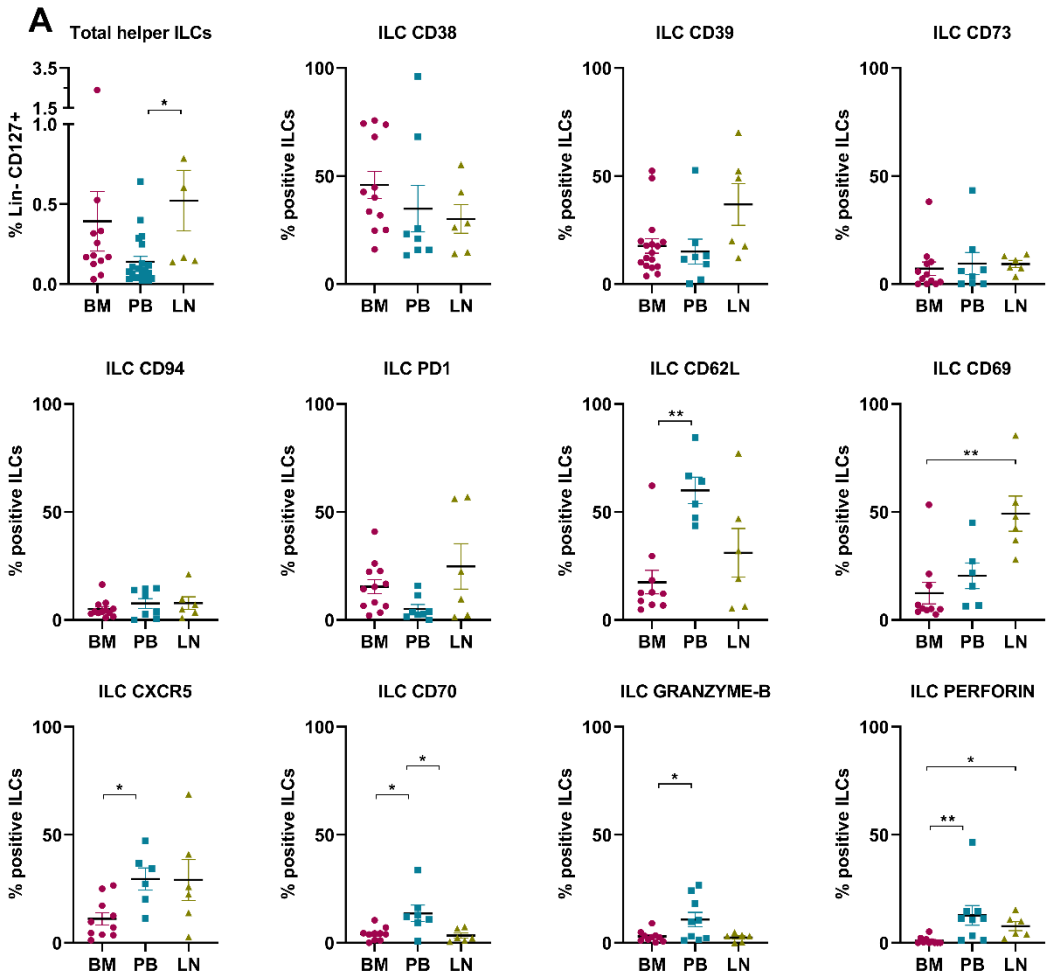


Figure 22. ILCs phenotyping in patients and healthy donors reveals NHL helper ILC activation

Percentage was expressed considering the positivity for each marker in the gate of helper ILCs ($Lin^{-}/CD56^{dim/-}/CD127^{+}$). Statistical analysis revealed that CD62L changed dramatically its expression ($p<0.01$) in PB compared to BM. CD69 ($p<0.01$) and CXCR5 ($p<0.05$) enhanced their expression in PB compared to BM. Variable expression among the tissues was detected also for CD70 which rose its expression in PB compared to BM ($p<0.01$) and LN ($p<0.01$). Perforin was restricted to PB and LN enhancing its expression compared to BM. For this analysis, data set normality was tested by Shapiro-Wilk test; if the distribution was considered normal multiple ordinary one-way Anova was applied, if the data were not normally distributed Kruskal-Wallis analysis was applied.

As next step, we designed an *in vitro* experiment to test if the cytokines upregulated in the plasma of NHL patients were able to modulate ILCs. Fist we sort patient ILC subsets and put in culture with upregulated cytokines upregulated in NHL plasma. Our aim was to verify if the above-mentioned cytokines were able to confer a survival advantage to a specific subset. In our hands no one of the subsets formed clones, thus meaning that cytokines in NHL do not participate in ILC survival advantage. We therefore performed another experiment through which we wanted to assess whether the cytokines upregulated in NHL were responsible for ILC activation observed in NHL compared to HD.

To this aim, we sorted ILC1s, ILC2s and ILCPs from peripheral blood of HD and NHL patients; then we cultured them for 48 hours in a medium supplemented with all the cytokines we found to be upregulated in patients' plasma (TSLP, IL1-2p40, IL-27, and IL-18) plus IL-2, using culture with IL-2 alone as a control (**Figure 23**).

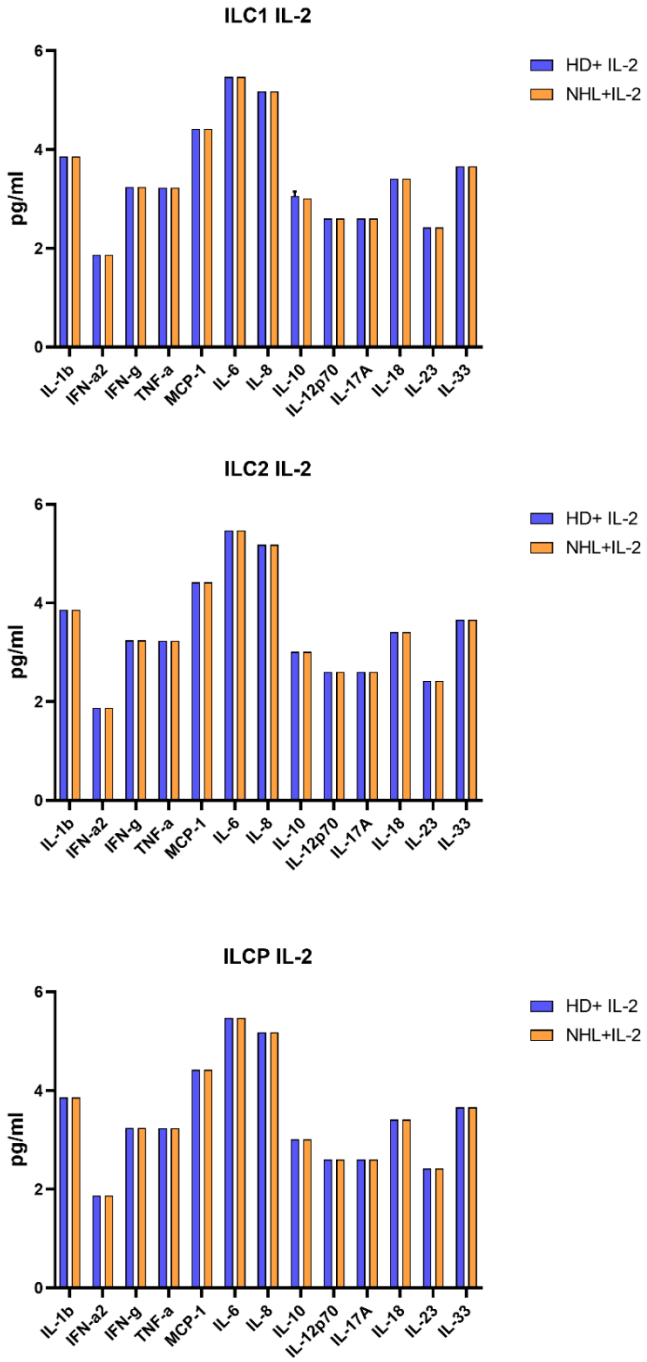


Figure 23. Helper ILC stimulation with the only IL-2 did not show any effect on cytokine production

The three ILC subsets did not show any detectable cytokine production when stimulated with IL-2 alone. Cytokines in the supernatant were measure thorough LegendPlex™. The experiment includes three independent biological replicates for both NHL and HD. The pg/ml obtained in this experiment were near to the minimum concentration measurable with the method applied.

As a result, we found that patients' ILCs were less functionally active compared to that of healthy controls; indeed, they released a lower amount of downstream cytokines when stimulated with the cytokine mix. To be sure that the viability of the cells was the same for HD and NHL patients we performed a vitality assay through which we could confirm that the cells were viable (**Figure 24**).

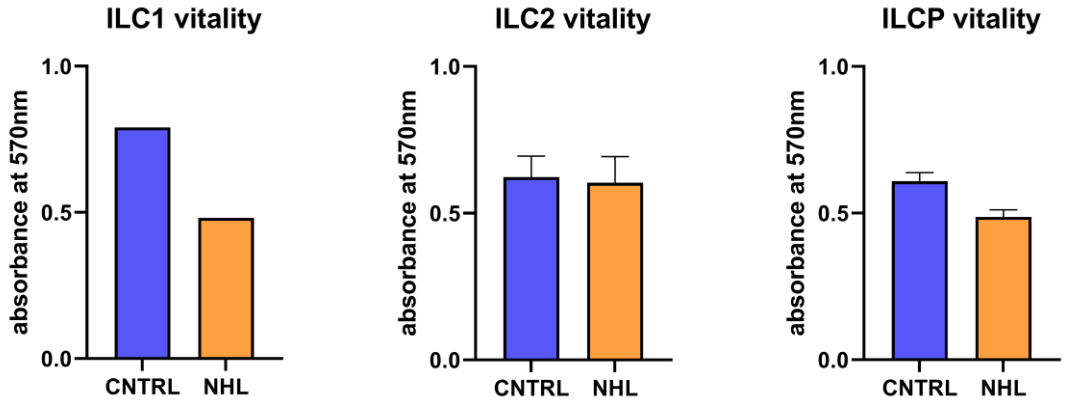


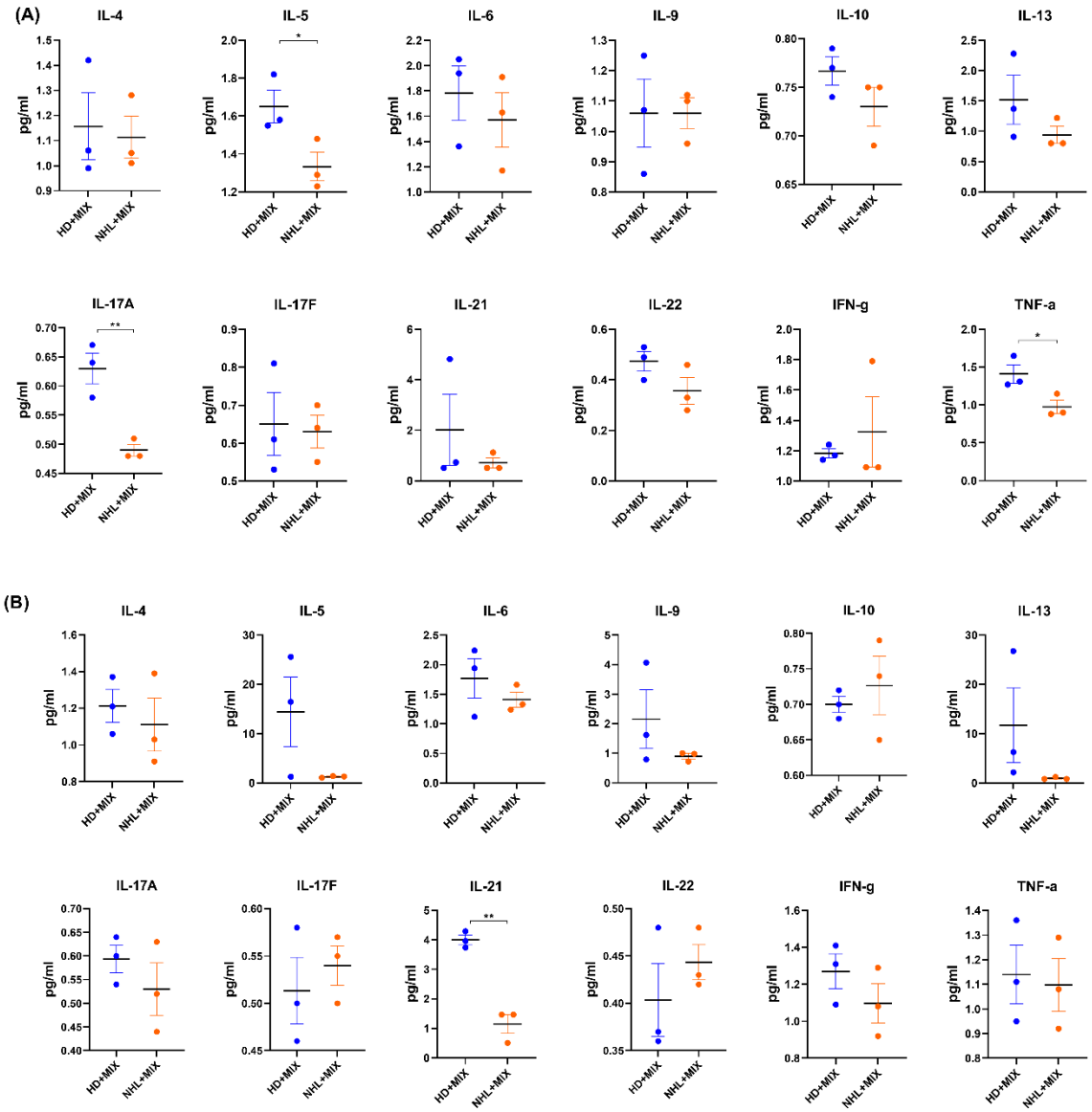
Figure 24. Helper ILC vitality check after 48 hours culture

ILCs after 48h stimulation with cytokine mix were tested for a vitality check with MTT assay which measures the metabolism of the cells giving information on their vitality. MTT after its transformation into insoluble formazan was detected by measuring the absorbance at 590nm.

Specifically, ILC1s sorted from patients produced less IL-5, IL-17A and TNF-a compared to the ILC1s in healthy donors. ILC2s show a lower amount of IL-21 compared to controls as the case of patients' ILCP which also IFNγ production even if this effect was not statistically significant. This experiment was important to measure the effect of a re-stimulation of helper ILCs from NHL and to understand their possible involvement on ILC regulation (**Figure 25**).

Taking these data together with the data of *ex vivo* cell phenotyping, ILCs in NHL patients show a more activated phenotype compared to healthy donors but suggested that a perpetuated stimulus with inflammation-related cytokines could result either in an overstimulation of the cells or an induced cell exhaustion.

Therefore, despite being active and endowed with antitumor function, ILCs become unable to display their role against the tumour. However, the cytokines explored had an activator effect on helper ILCs in HD. This experiment despite having a low replicate number, was important to monitor the effects of NHL upregulated cytokines on ILCs and to describe their potential role as inhibitors.



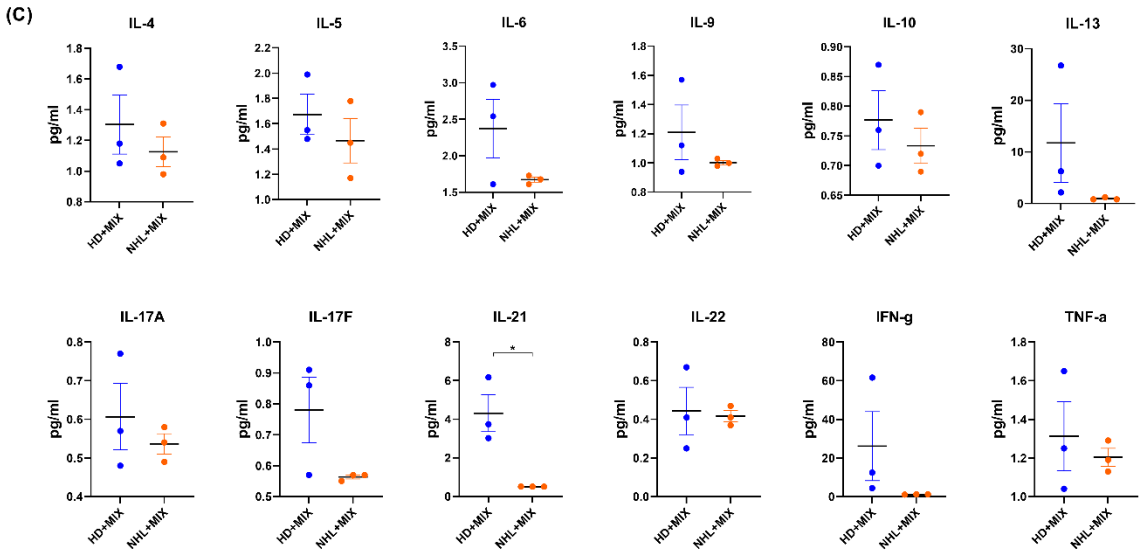


Figure 25. Cytokines upregulated in NHL are responsible of helper ILC overstimulation and exhaustion in patients

Experiment was conducted on three independent healthy donors and three NHL patients. ILCs were sorted purified from buffy coats or whole peripheral blood. After sorting they were cultured with IL-2 (10 U/ml) to sustain their growth and the mix of cytokines found to be upregulated in NHL plasma, IL-12p40 (50 ng/ml), IL-18 (50 ng/ml), IL-27 (50 ng/ml). Cell seeding density was 100 cells/µl. After 48 hours, supernatants were collected and frozen. LegenPlex™ analysis for downstream cytokines was applied. (A) ILC1, (B) ILC2 and (C) ILCP.

Considering the previous results reported and the effects of cytokine overstimulation we tried to perform experiments of cytokine and chemokine stimulation. Buffy coat samples derived from healthy donors were used for cell-sorting purification of helper ILCs and NK cells. Cells were cultured alone with IL-2 and the different cytokines and chemokines found upregulated in NHL patients given alone or in combination. This experiment was performed to detect possible phenotype modifications due to cytokines or chemokines addiction. The experiment was too laborious and variable, moreover the cells obtained in particular concerning helper ILCs which were too low amount of cells

4.8. NHL affects helper ILC cell activation through cell-cell contact mediated by PBMCs *in vitro*

As we previously performed to explore helper ILC functions and modulation in NHL we carried out *in vitro* experiments. Using the cell lines, SU-DHL-4, and SU-DHL-10. We cultured PBMCs from healthy donors with these two cell lines using as controls a co-culture with B cells purified from HD (CD19) and PBMCs from healthy donors cultured alone. After 48 hours of MLTC, we checked helper ILC and NK phenotypes. Helper ILCs showed a significant lower expression of the activation marker CD38 when co-cultured with the SU-DHL-4 cell line compared to the two controls. CD39 and CD73 were both significantly down modulated in presence of the two tumour cell lines, while CD62L reduced expression was found after SU-DHL-10 MLTC. CXCR5 was the only marker, which was found to be upregulated in presence of tumour cell lines. In contrast, expression of granzyme B was abrogated compared to the controls, thus indicating that ILC cytolysis potential and activity were inhibited by the two tumours cell lines (**Figure 26**). We also check if the expression of granzyme B was due prominently to ILC1 and ILCP. Results show that the expression of granzyme varied especially in ILC1, while we did not find a consistent trend when considering ILCP; moreover, we were not able to gate ILC2s in these experiments since they were too low number of cells. (**Figure 27**).

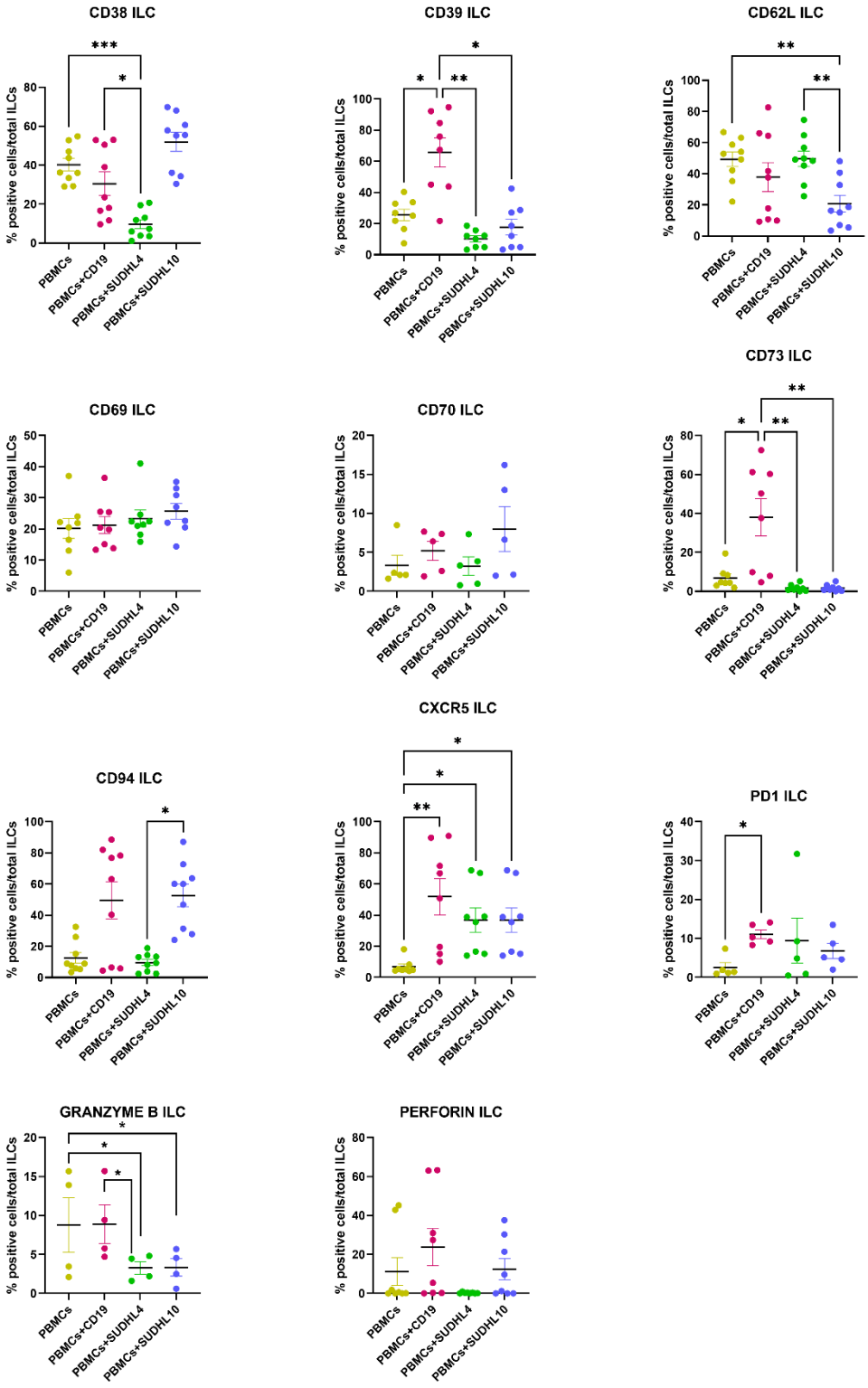


Figure 26 A. ILC phenotyping after 48h of MLTC with tumour cell lines

MLTC experiments with total PBMCs were conducted for 48h in presence or absence of NHL tumour cell lines. 9 replicates were performed and 4 replicates for ILC granzyme B evaluation. Statistical significance was tested using paired one-way ANOVA in case of

normal distribution of the data set or multiple paired non-parametric Friedman. Granzyme B was evaluated with multiple paired test Holm-Šídák for ratios; p values were considered significant when $p<0.05$.

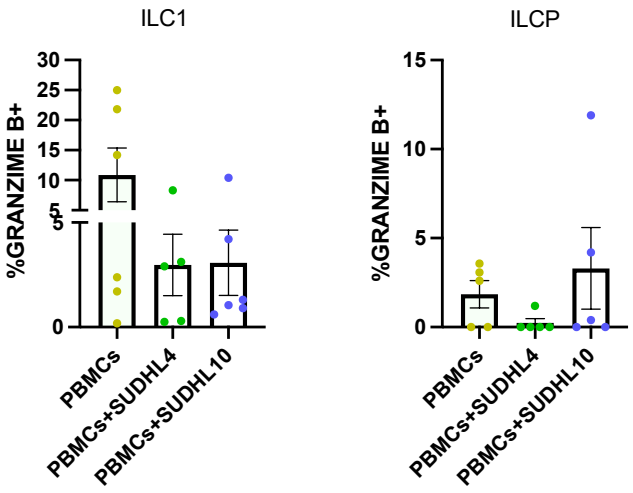


Figure 27. ILC1 and ILCP expression of granzyme-B.

The graphs show the cytoplasmic expression of granzyme in ILC1 and ILCP highlighting that ILC1 were the main responsible of granzyme-B fluctuation across the different sample types.

4.9. Cell contact is fundamental for tumour derived phenotypical changes in ILCs

We wanted to investigate if the effects on helper ILC and NK phenotype were mediated by cell-cell contact. To this aim, we repeated the same *in vitro* MLTC experiments as done before, however in this case excluding PBMCs-tumour cell contact by using 0.3 µm pore transwell plates. After 48 hours, we checked the phenotypes of ILCs as described before. In this case, no consistent changes were recorded, suggesting that the capability of tumour cells to modulate helper ILC activation, as for NK, was cell-cell contact dependent (**Figure 28**).

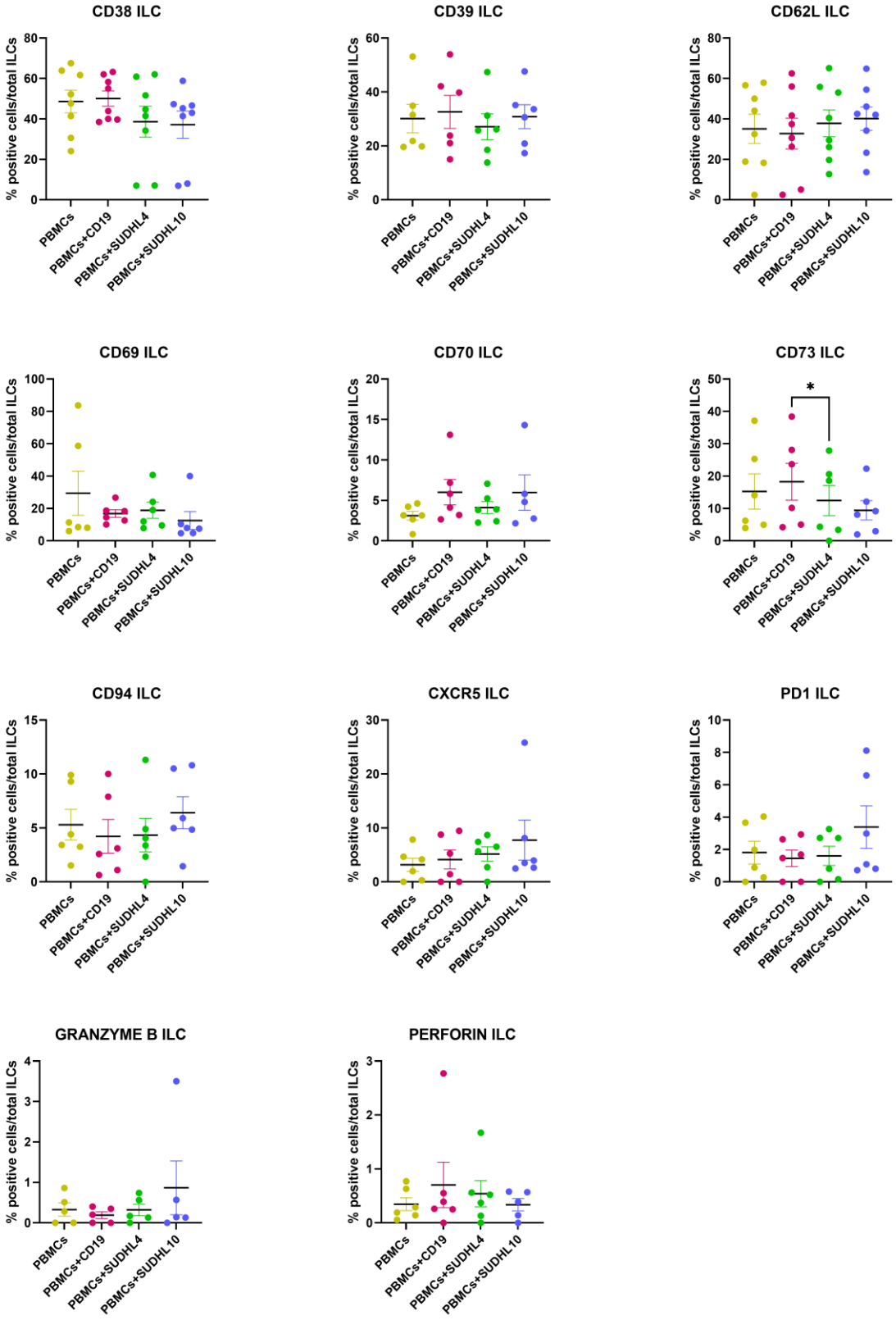


Figure 28. Excluding cell-tumour contact, ILCs do not show phenotype changes

The experiment was repeated 7 times excluding cell-cell contact between tumour and PBMCs. Data were analysed using paired one-way ANOVA in case of normal distribution of the data set or multiple paired non-parametric Friedman.

Moreover, we tested also if the contact between tumour cells and helper ILCs was sufficient to induce the observed phenotype modifications, or if other cells among the PBMCs played a key role. For this reason, we purified helper ILCs cells by FACS sorting and put them in MLTC with SU-DHL-4 cell line and in culture with B cells purified from HD as a control. Again, the choice of only one control and one cell line was due to the limiting cell number obtained. Results show that, excluding the other cells of the immune system contained in PBMCs, the phenotype of helper ILCs turned likely activated in presence of tumour cells compared to the control. Specifically, B cells co-culture did not show to generate the same activation of ILCs as the tumour, which was instead able to increase the expression of unmuted markers in MLTC experiments with total PBMCs, as observed *ex vivo* (**Figure 29**). This experiment even if not conclusive alone, was important to understand that ILCs were modulated by other cells of immune system in presence of tumour cells *in vitro*.

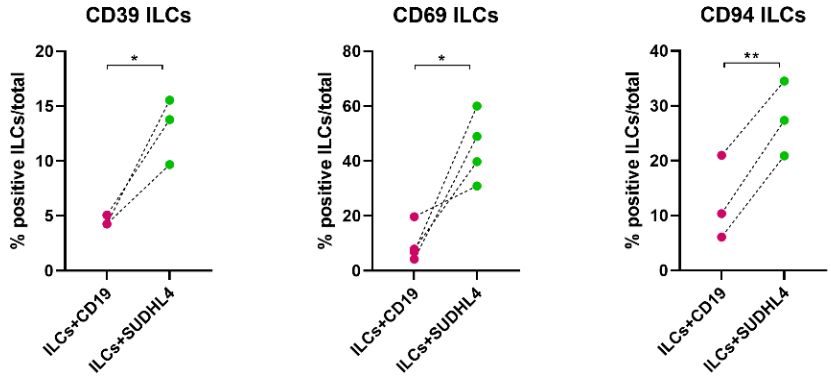


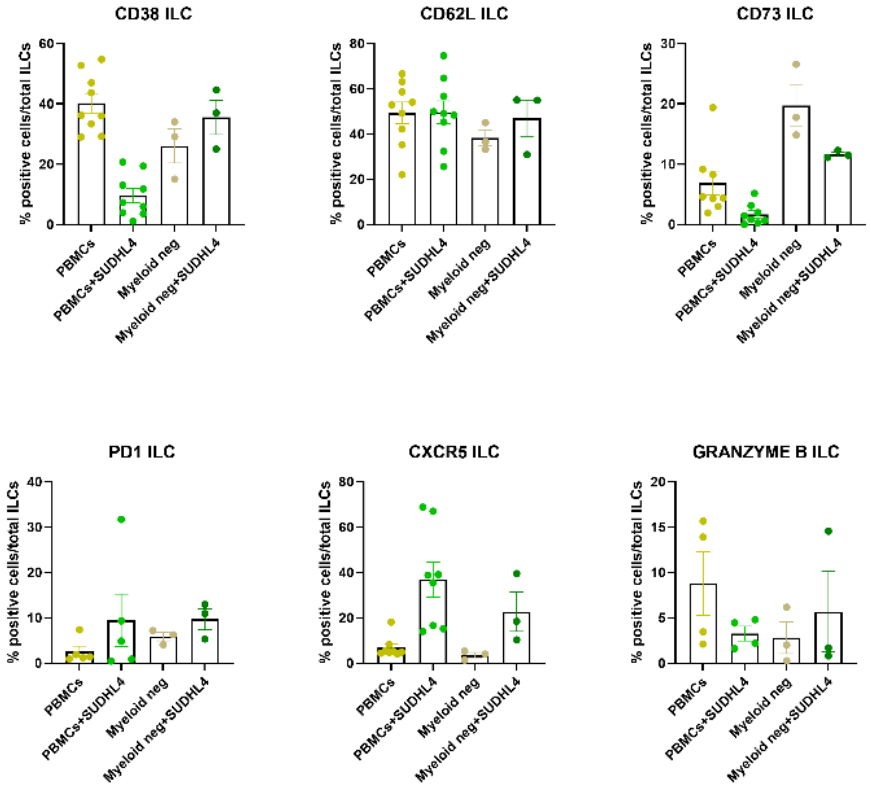
Figure 29. MLTCs experiments with sorted purified ILCs and NK cells reveal ILC activation in presence of tumour cell line SU-DHL-4

The experiment was performed using 3 independent HD sorted purified ILCs. Cell phenotype evaluation after 48 hours culture reveals helper ILCs increment of activation marker expression, in contrast to what observed in the *in vitro* experiments with total PBMCs. Percentages refer positivity of each cell family for a precise marker. Statistical

analysis was conducted using paired t-test or Wilcoxon test, depending on data distribution.

4.10. T regulatory cells play a key role on helper ILC modulation in NHL

Up to this point, we were aware that phenotypic changes due to NHL on ILCs were recorded only in presence of all the PBMCs *in vitro*. Our aim was to unveil what immune cell population among the PBMCs can counteract directly or indirectly ILCs *in vitro*. Therefore, we repeated the *in vitro* experiments excluding single families of immune cells. First, myeloid cells were excluded; three samples of PB of three independent healthy donors were purified from myeloid cells and then put in culture with alone as a control or in MLTC with the two tumour cell lines. Results show that the markers explored follow a very similar trend to what observed in *in vitro* experiments with total PBMCs after 48h (**Figure 30**).



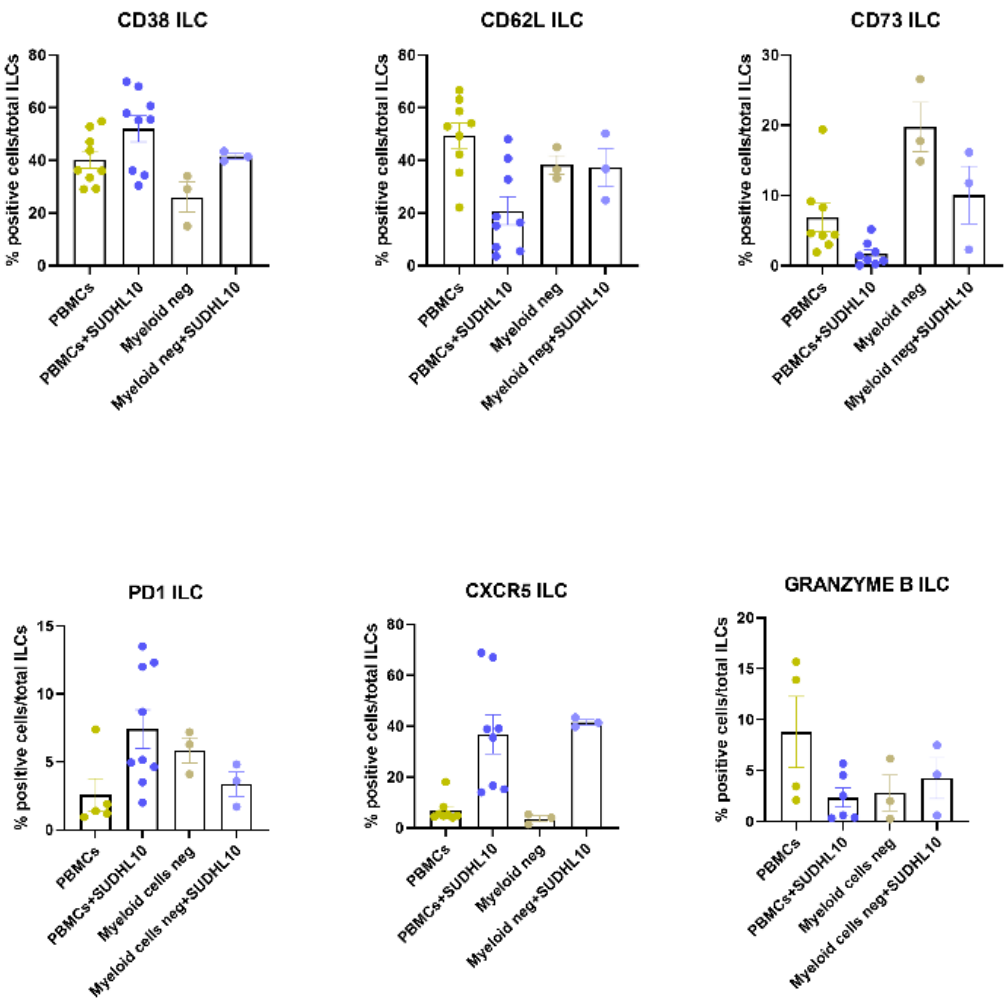


Figure 30. ILC phenotype modulation after myeloid cell exclusion

Myeloid cells were excluded by sorting purification from PBMCs. Cells were put in MLTC with tumor cell lines for 48h and then ILC phenotype was explored through flow cytometry. In the graph shown above, we reported the percentages of positive ILCs for each marker and compare it with the data found in the previous *in vitro* experiment using total PBMCs. The two columns on the left of each graph report the same data shown in figure 26.

We repeated the same experiment by excluding $CD3^+$ cells by sorting purification. After 48 hours, results show that CD38, which was down modulated in MLTCs with total PBMCs and tumour cell lines, displaying a trend of rescuing its expression, especially in presence of SU-DHL-4. Moreover, also CD62L was upregulated in presence of SU-DHL-4 cell lines

and did not show any modulation when CD3 negative PBMCs were cultured with SU-DHL-10 (**Figure 31**).

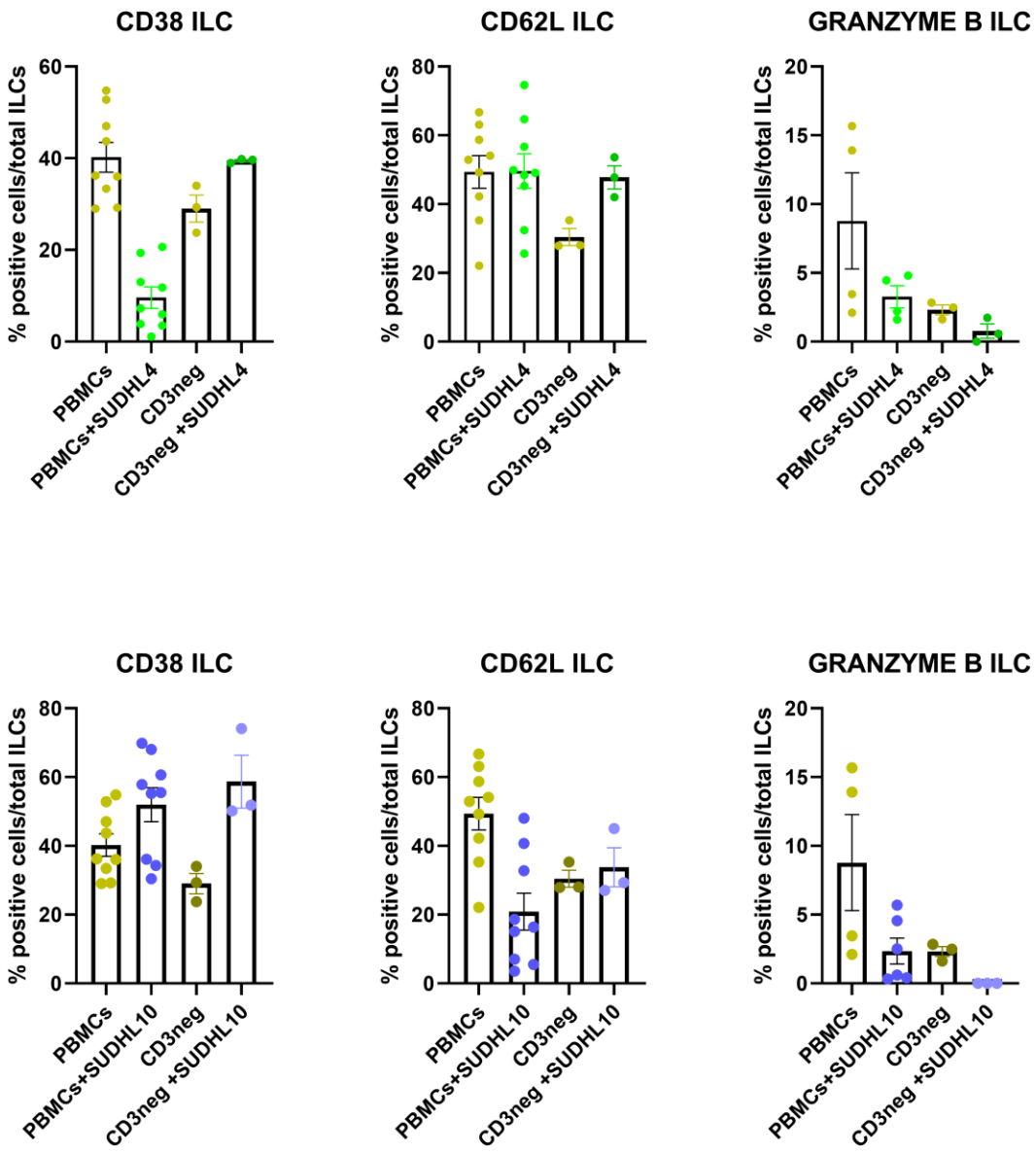


Figure 31. ILC phenotype modulation after total T cell exclusion

$CD3^+$ T cells were excluded by sorting purification from PBMCs. Cells were put in MLTC with tumor cell lines for 48h and then ILC phenotype was studied through flow cytometry. In the graph shown above, we reported the percentage of positive ILCs for each marker explored and compare it with the data found in the previous *in vitro* experiment with total PBMCs. The two columns on the left of each graph report the same data shown in figure 26.

These findings support the hypothesis that the responsible of helper ILC phenotype modulation belonged to $CD3^+$ cells.

Taking together all the results obtained we focused on T regulatory cells, especially because in NHL plasma IL-27, which is important for T regulatory functions, was increased in concentration compared to healthy controls. Therefore, T regulatory cells were removed from PBMCs by sorting purification. T regulatory cell depleted PBMCs were put in MLTC for 48 h with the two tumour cell lines and alone as a control. Phenotype analysis revealed that CD38 activation marker and granzyme B expression was increased in presence of tumour cell lines, thus indicating that helper ILCs rescue their activity in absence of T regulatory cells (**figure 32**). These findings suggest that T regulatory cells can play a crucial role in the inhibition of ILC function, thus contributing to the down regulation of their potential anti-tumour role.

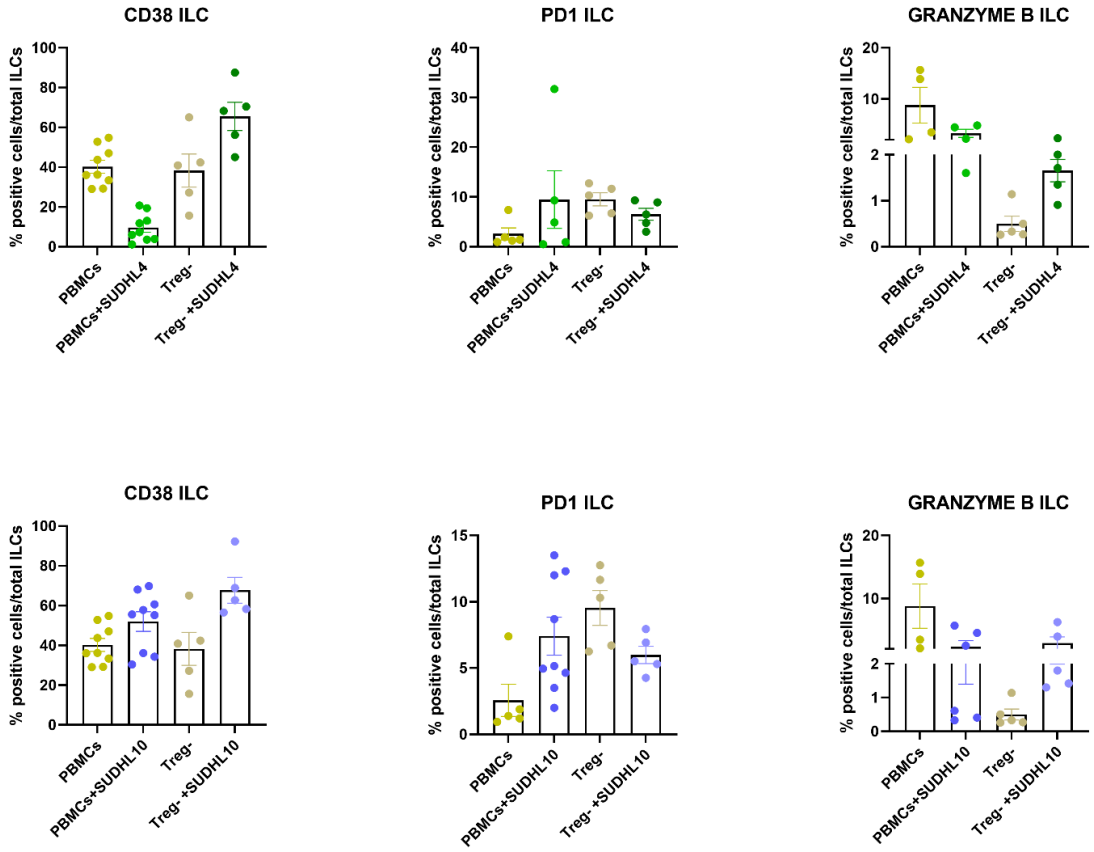


Figure 32. MLTCs experiments with Treg exclusion reveal their key role in inhibiting ILC function

The experiment was performed using 5 independent HD whose PBMCs were sorted purified to exclude T reg cells. Cell phenotype evaluation after 48 hours MLTC reveals that helper ILCs increase CD38 marker expression, which was down modulated in the *in vitro* experiments with total PBMCs. Moreover, also granzyme B turned to be expressed inside cell cytoplasm. Even if not statistically significant, it was possible to observe also a PD-1 down modulation in presence of the tumour cell liens. Percentages refer positivity of each cell family for a precise marker. Statistical analysis was conducted using paired t-test or Wilcoxon test, depending on data distribution. The two columns on the left of each graph report the same data shown in figure 26.

4.11. Hodgkin lymphomas

In literature, data on ILCs in HL disease are nowadays absent. We therefore decided to explore the subset distribution and the phenotype of both NK cells and ILCs in the peripheral blood of patients affected by HL. We used the same of flow cytometry panels applied for NHL. First, we found that in patients affected by HL the percentage of total helper ILCs calculated considering the $CD45^+$ gate of lymphocytes was significantly increased compared to HD. We compared 12 HL patients with 50 HD. Exploring the three different ILC subsets, we found that in patients there was an increment of ILC1 concomitantly with a drop of ILC2 percentage. Concerning NK cells none of the subpopulation considered was significantly changed in terms of percentage in HL compared to HD (**Figure 33**).

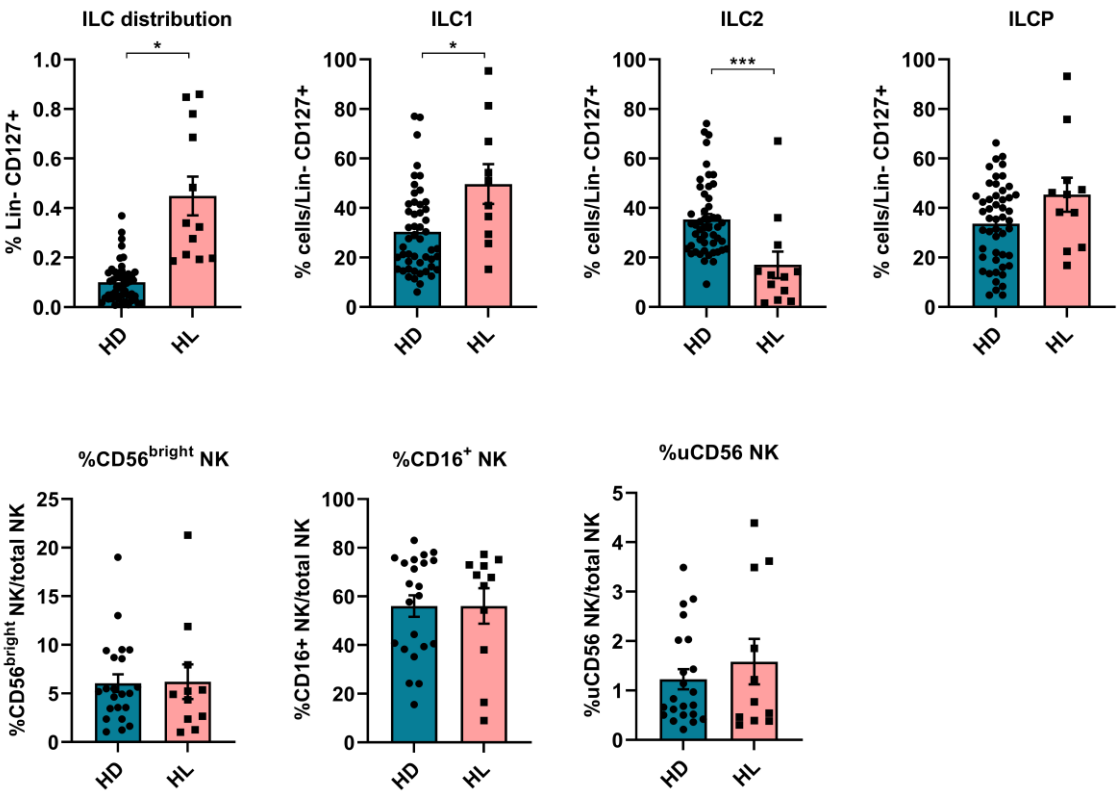


Figure 33. ILC subset distribution changes between HD and HL

The comparison was made considering 50 HDs and 12 HL patients, concerning NK cells controls used were 22. Graphs report the percentage of total ILCs expressed considering the gate of Lin- CD127+ cells. ILC subsets were calculated considering the total ILC gate. NK cell populations were calculated considering the $CD45^+$ $CD3^-$ $CD19^-$ $CD20^-$ gate.

Similarly to what performed for NHL, we explored the phenotype of ILCs and NK cells in HL, through flow cytometry analysis. Analysing ILCs, we found that HL patients show a lower expression of CXCR5 and higher levels of granzyme B and perforin (**Figure 34**). NK cells show different phenotypes (**Figure 35)**. $CD56^{bright}$ NK, despite having a reduced KLRG1 expression, downmodulate CD62L and CD94 concomitantly with a high expression of CD73. $CD16^+$ NK showed a significant increment only one of the activation markers, CD38, and a downmodulation of CD94 and KLRG1. Regarding $uCD56^{dim}$ NK, they show a decreased level of all the activation markers.

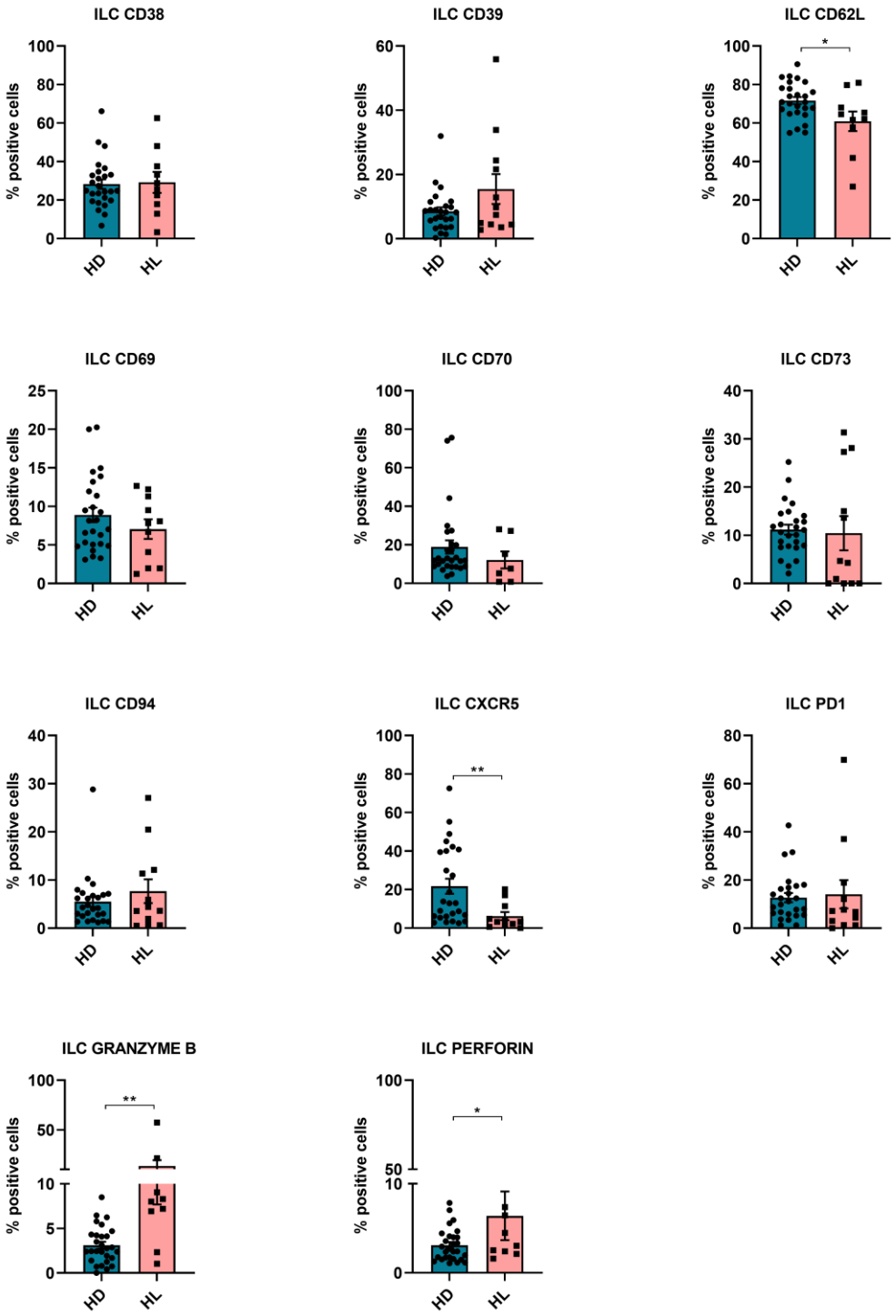


Figure 34. ILC phenotype in HL and HD

The comparison was made considering 25 HD and 12 HL patients. Graphs report the percentage of positive ILCs for each cell marker studied expressed considering the gate of Lin- CD127+ total cells. C subsets were calculated considering the total ILC gate.

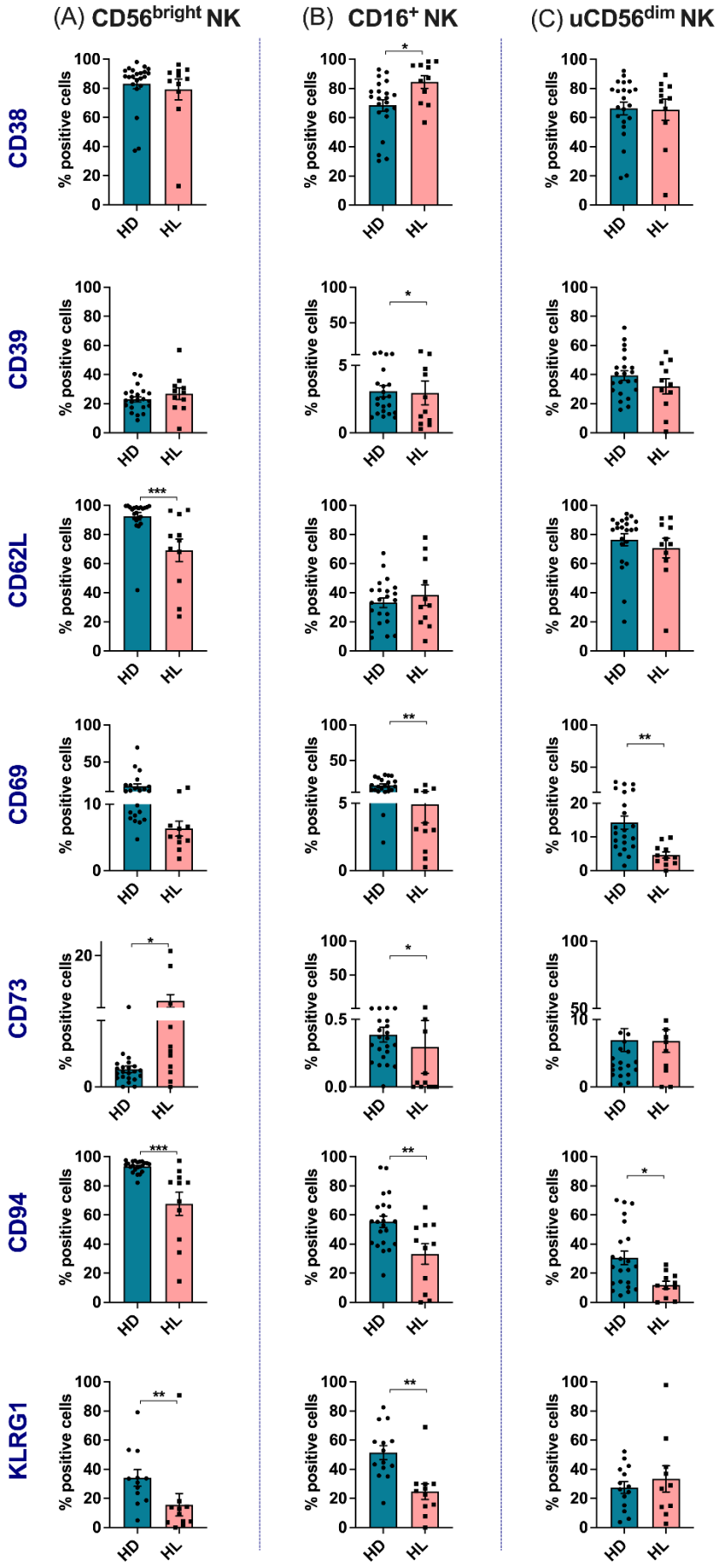

(A) CD56bright NK
(B) CD16+ NK
(C) uCD56dim NK
CD38
CD39
CD62L
CD69
CD73
CD94
KLRG1
% positive cells
HD
HL

Figure 35. NK phenotype in HL and HD

The comparison was made considering 25 HDs and 12 HL patients. Graphs report the percentage of positive ILCs for each cell marker studied expressed considering the gate of Lin- CD127+ total cells. C subsets were calculated considering the total ILC gate. Statistical significance was tested using Wilcoxon (Mann-Whitney) non-parametric test after testing the not-normal distribution of data through Shapiro-Wilk test. For normal distributed data sets, parametric t test was applied.

With the aim of exploring if some cytokines accumulated in the patients' plasma could affect the frequency and the phenotype of patients' ILCs and NK, we measured and compared the plasma cytokines of HL patients and HD. Figure 35 shows all the cytokines analysed. None of them resulted significantly different in patients compared to HD (**Figure 36**).

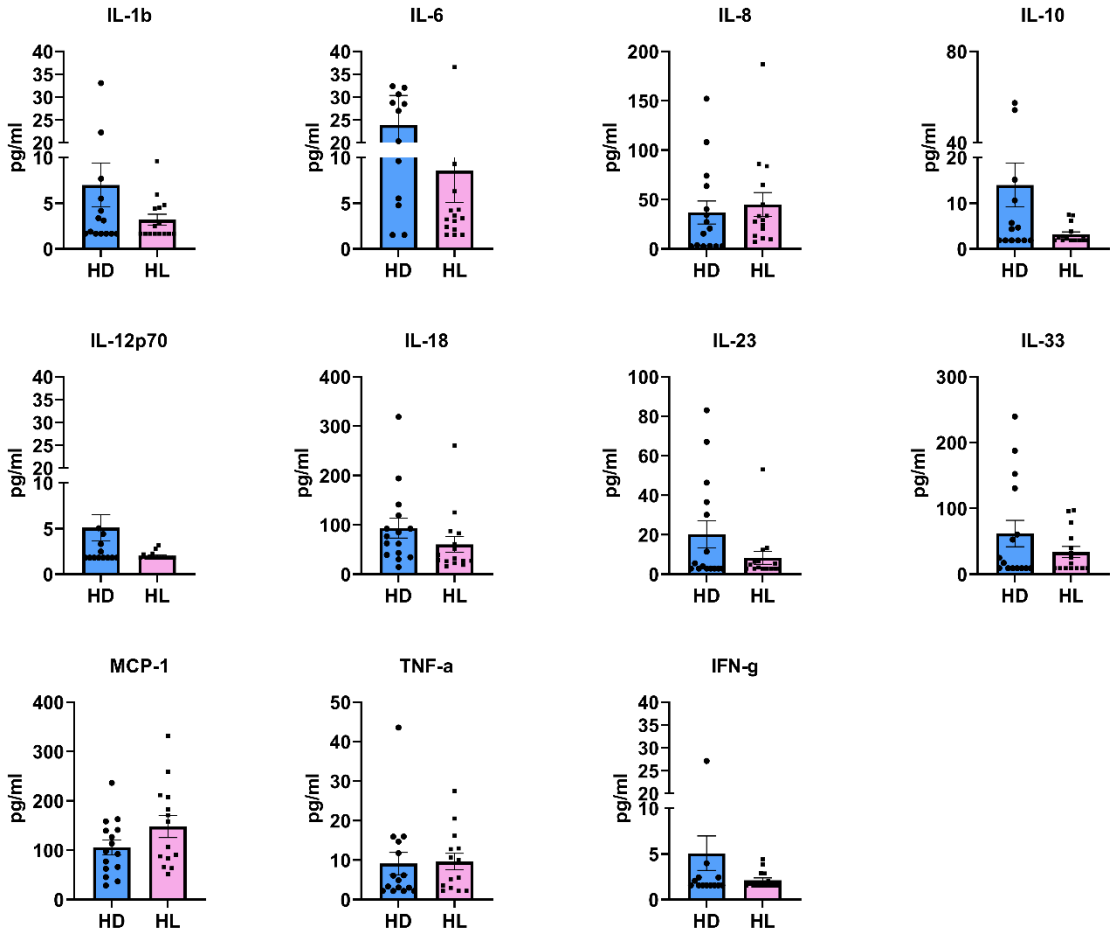


Figure 36. Cytokines in HL patients reveal no changes compared to healthy controls

The comparison was made considering 15 HD and 15 HL patients. Graphs report measurement of cytokine concentration in plasma expressed as pg/ml.

5. Discussion

5.1. Innate lymphoid cells in NHL

Helper ILCs are recently discovered cells in the innate immune system which lacks lineage specific receptors ad originate from a common lymphoid progenitor[44,267]. The role of helper ILCs in cancer is on debate and almost unexplored in lymphomas.
In our study, our aim was to describe the unexplored role and the behaviour of helper ILCs and NK cells in two B cell lymphomas: B-NHL and B-HL.

Our exploration started considering patients affected by NHL and healthy donors. Since the lack of data from literature concerning ILCs in this pathology, we first started by looking for any potential differences in terms of NK cell and ILC distribution in peripheral blood. Comparing healthy donors to NHL patients we did not find any difference in terms of helper ILC distribution, neither considering the total ILCs, defined as number of cells gated on the $CD45^+$ events, nor in terms of subset distribution, taking into consideration ILC1, ILC2 and ILCP. We considered ILCP since, as described by Lim A. et al.[126], ILC3 are absent in peripheral blood, instead there is a subset phenotypically similar to ILC3, named ILCP, able to give rise, according to the stimuli perceived in tissues, to NK cells and ILCs. Additionally, considering NK cells, in peripheral blood there were no changes in NHL patients compared to HD concerning $CD56^{bright}$ NK and $uCD56^{dim}$ NK distribution, however a significant drop of $CD16^+$ NK has been detected in patients. Peripheral $CD16^+$ NK are described to be the responsible of cell cytotoxicity and to a less extend to cytokine production[61]. Taking this consideration, our results suggested that the down modulation of this NK cell population might be associated with an immune escape mechanism potentially promoted by the tumour. This mechanism of immune-escape has been proposed also in head and neck cancer, in which $CD16^+$ NK decreased in patients throughout all the tumour stages compared to HD[268]. $CD16^+$ cells are also potent players in antibody dependent cell cytotoxicity (ADCC), since they recognize antibody-coated target cells[269]. Indeed, one of the most administered therapies for NHL is rituximab, which acts by binding B cell CD20 marker exposing its Fc receptor to $CD16^+$ NK, thus mediating ADCC [270]. The low number of $CD16^+$ NK in NHL shown by our finding may also suggest a possible way of immune escaping.

Then, we decided to explore the phenotype of NK cells and ILCs with the aim to unveil possible differences between HD and NHL. ILC and NK state of activation and expression of markers linked to immune-checkpoints or to cell exhaustion were explored. The choice of the markers for cell phenotyping was done matching data from literature and our hypothesis about NHL modulation. Among many markers explored, only some were significantly changed comparing HD and NHL.

Our results showed that ILCs were phenotypically activated. Indeed, they show an increased expression of activation markers in NHL compared to healthy controls, such as CD69. It has been published that CD69 is mainly expressed in mucosal tissue rather than non-mucosal one (included PB)[100], however the raised frequency of $CD69^+$ ILCs suggested a cell activation determined by NHL. Moreover, in NHL, the higher expression of granzyme B in ILCs suggested their potential cytotoxic activity in patients. Concomitantly PD-1, an immune checkpoint marker, was downregulated. In literature it has been reported that high expression of PD-1 in ILC2 blocks STAT-5 phosphorylation diminishing the amount of IL-5 and IL-13 produced[183]. The role of these two cytokines in tumour is contrasting; for example in *in vivo* models of metastatic melanoma, the release of IL-5 by ILC2 recruits eosinophils, determining anti-tumour activity [152]; a similar situation happens also in CRC [271]. Oppositely, in lung cancer the secretion of IL-5 and IL-33 actuated by ILC2s limit NK IFN-γ production and the cytotoxic potential. The role of IL-13 has been described as detrimental for cancer [160]. PD-1 expression is also a marker to identify ILC committed progenitor[272,273] and it is lost during cell differentiation. However, PD-1 is upregulated during inflammation, as observed for ILC2s in lung[258]. As described above, in inflammatory condition the expression of PD-1 affects the cytokine release, in contrast in cancer it has been suggested that using PD-1 checkpoint blockade could restore type-2 responses leading to cancer growth and progression[274]. Expression of PD-1 in ILC3 was instead found in malignant pleural effusions and associated with hampering of anti-tumour effect[275].

All together, these results suggest that ILCs in NHL peripheral blood were potentially endowed by anti-tumour activity, especially for their expression of granzyme and perforin.

Exploring NK cell phenotype, we found that $CD56^{bright}$ NK and $uCD56^{dim}$ NK show a lower expression of activation markers. $CD16^+$ NK cells were the only ones displaying a pro-tumour activity, by means of CD39 and CD73 increase, however concomitant with downregulation of the inhibitory molecule KLRG1. KLRG1 is expressed by about the 50-

80%[276] of NK cells and especially by mature CD56^{dim} NKs[277]. The high expression of this receptor associates with a decreased IFN-γ production, a low cell proliferation and an increment of NK cell apoptosis[278–280]. Therefore, the down modulation of KLRG1, detected in our cell phenotyping, suggested that NK cells could be potentially in an activated state in NHL.

CD39 and CD73 are mostly expressed by T cells, but also by tumour cells and other cells of the immune system[183]. CD39 and CD73 are important sequential ectoenzymes with the ability to transform extracellular ATP/ADP into immunosuppressive adenosine (Ado). The balance between ATP/ADP and adenosine is crucial to determine the immune responses, since adenosine acts as a potent immune suppressor, while ATP as a stimulator[281]. ATP is usually released after cell death, leading to phagocyte recruitment, moreover it favours antitumor T cell priming and DC maturation[282]. In contrast, Ado supresses the immune cells, such as Th1 and Th2, NK cells and enhances the Treg suppressive functions[283], additionally, cancer immune evasion often involves Ado in the tumour microenvironment[282]. CD39 and CD73 have been defined as immune checkpoints for tumour infiltrating NK cells, with an active role in the establishment of an immunosuppressive environment, as demonstrated for different tumours such as breast cancer, ovarian cancer and melanoma[284–286]. Importantly, NK cells expressing CD73 upregulate the IL-10 that in turn suppresses CD4^{+} T cells. Data from literature show that CD73 expression is induced by the tumour, therefore this suggests a mechanism of immune-evasion in which NK cells are deviated toward a regulatory property[262].

Based on the results obtained from the phenotype evaluation, our hypothesis was that NK cells were inhibited or endowed with pro-tumour activity by NHL. In many tumours, TME influences NK cells, altering number and functions of these cells[287]. Moreover, tumour cells may directly prompt downregulation of NK activating receptors and NK functions in many different mechanisms[288]. Tumour cells can chronically engage the activation receptor NKG2D through ligands exposed, thus determining NK cell function impairment. A similar effect has been shown through the tumour expression of soluble ligands for NKp30, that determines impairment of NK cell functions[288]; for example, in B-CLL the soluble factor BAG6 released by tumour cells binds to NKp30, causing suppression of NK cell cytotoxicity and favouring cancer immune evasion [289].

Thus, we performed experiments with the aim to better explore NHL-NK interactions.

Since many chemokines and cytokines play a role in immune cell functional modulation and could be involved in determining phenotype changes of ILCs and NK cells, we decided to compare the expression levels of a panel of cytokines and chemokines in the plasma of NHL patients and healthy donors.

The three chemokines we found to be upregulated in patients (MIG, IP-10, MIP-1b) are responsible for cell recruitment and cell retention to tissues. In details, MIG and IP-10 belong to CXC chemokine family, induced by IFN-γ. They are responsible of stimulating $CD4^+$ T cells by inducing the expression of Rorγ-t and T-bet, which contribute to T cell polarization toward Th17 or Th1, respectively[263]. Rorγ-t and T-bet are fundamental transcription factors for ILC3 and ILC1 polarization. These data suggest that not only the chemokines but also the cytokines found to be upregulated in patients' plasma could be responsible for innate lymphoid cell modulation.

Among the cytokines we found to be upregulated in NHL plasma, data from literature show that IL-12p40 and IL-18 can induce cell cytotoxicity[35]. In particular it has been described that ILC cytotoxicity is dependent on IL-12, and could be likely displayed when NK cell functions are silenced or reduced[290]. This finding supports our evidence that ILCs in patients show higher expression of granzyme B and perforin compared to healthy controls. TSLP is known to have the ability to induce ILC2 giving them a survival advantage[291]. IL-27 mediates Th1 development from naïve $CD4^+$ T cells[264]. Its role in terms of Treg functions is contrasting, since it is able to induce Treg expansion and proliferation, but is also implicated in anti-inflammatory function thus inhibiting Tregs [265]. Tregs can block anti-tumour responses by creating an immunosuppressive environment.

In literature, it has been described that in TME-associated cytokines can build immune suppressive milieu[292]. The action of these cytokines is not only restricted to site of tumour localization, however they can exert systemic effects, for example promoting methastatis[293]. On the other side, also systemic inflammation and of course the cytokines related to it can foster an immune suppressive environment in TME[294]. Taking these considerations together, we could suppose that systemic cytokines could either derive from TME or influence the tumour site, although are important for immune cell behaviour in NHL.

We then performed *in vitro* studies to assess the direct effects of these upregulated cytokines on helper ILCs isolated from of NHL patients and healthy donors. Our results show that the presence of a repeated stimulus with the above-mentioned cytokines had a suppressive effect on patients' ILCs that in turn produced reduced amount of IL-5, IL-17A, IL-21, IFN-γ.

IL-5 is a Th2 cytokine, produced by different immune cells among which $CD4^+$ Th2 cells, NK cells and importantly ILC2s. This cytokine supports activation, survival and adhesion of eosinophils[35,295]. IL-17A drives the attraction of neutrophils, responsible of defending the organism against pathogens. Cell sources of IL-17A are NK cells, $CD8^+$ T cells, and $CD4^+$ Th17 cells[35], but also ILC3s. TNF-a is known as double face cytokine, able to act either as proinflammatory or as an immunosuppressor [35]. IL-21 sources are T cells (Th17 and Th9) and NK T cells; this cytokine activates JAK-STAT pathway [296] indeed inducing RORγt expression which in turn activates Th17 and neutrophils. IL-21 promotes also $CD8^+$ T cell functions and anti-tumour activity[297], moreover it is known to inhibit $CD4^+$ T cell suppression acted by Treg cells[298]. Taking these findings together, we highlighted that despite ILCs show activated phenotype, they may be dysregulated in their functions by the negative priming received in peripheral blood. In particular, they could become exhausted after repeated stimuli of cytokines received at the periphery of NHL patients.

Exhaustion of ILCs has been defined as a process in which the cells are not able to produce cytokines anymore, rather than showing a phenotype with increased expression of exhaustion markers. The existence of exhausted-like ILCs has been described in allergic conditions by Miyamoto C. et al. who show the presence of hyporesponsive IL-10^+ $TIGIT^+$ ILC2s with low capacity of cytokine production[299].

Focusing on ILCs and NKs in NHL patients, we also investigate ILC/NK distribution and phenotype comparing the BM, PB and LNs of NHL. We were aware about the importance to compare BM and LN of NHL with healthy donors, as performed with PB, however, due to the unavailability of these sample types we decided to see the overall changes among the NHL tissues.

By exploring ILCs in three different compartments in patients, we found that the main site of ILC enrichment was the LN, where NHL cells are located. Moreover, we found that, in LN, ILCs increased CD69 and perforin expression compared to PB and BM, thus suggesting that ILCs in NHL site are in activated state and endowed with cytotoxic potential.

We also evaluate the expression of CD62L in ILC. CD62L is an adhesion molecule usually expressed by naïve T cells, however it has been described in literature that this marker is important for ILCs to enter the lymph nodes, and that in mice, tissue resident ILCs lack CD62L expression thus suggesting that this marker is restricted by ILC precursor population[300]. Our results showed that ILCs have the highest expression of this molecule in PB, but not in LN. This suggests that in NHL tumour site ILCs are mature and not more

precursors, therefore they can terminally differentiate and potentially exploit their activity in the tissue.

Exploring NK cells, we found different modulations, NK cells upregulated CD39/CD73 expression in case of NK $CD16^{+}$ or only CD73 when considering $uCD56^{dim}$ NK and $CD56^{bright}$ NK in LNs. Consistently with data from literature, we found that CD62L was mainly expressed by NK cells in peripheral blood and not in BM, since tissue resident NK cells have been described to not express this marker[61].

Moreover, we found that CD38 expression in all NK populations tended to decrease in PB and LN compared to BM. CD38 is transmembrane glycoprotein with multiple functions, and which is expressed by activated immune cells. Among its roles there are the control of cell proliferation and differentiation, apoptosis, cell migration[301], the enhancement of response to chemokines; moreover, CD38 expressing NK cells show strengthening concerning IFN-γ production and responses to tumour cells[302,303]. The lower expression of CD38 in LNs compared to the other tissues suggested a lower activation of NK cells at tumour site.

Through our *in vitro* experiments, we tried to simulate the situation in which not primed ILCs derived from PB of healthy donors encounter NHL cells, to explore their behaviour and regulation. We were aware about the limitations of this approach, for example, we excluded tumour microenvironment and the complexity of the immune milieu proper of NHL tumour site. Interestingly, our results showed that ILC activation was turned off by tumour cells in a contact-dependent manner in presence of other PBMC immune cells. ILC phenotype could be different depending on the NHL cell line used (SUDHL 4 and 10); in particular, the different effect could be caused also by the differential expression of PDL-1 and PDL-2. SU-DHL-10, a more indolent-like lymphoma cells do not show PDL-1 or PDL-2 expression[266].

Importantly, in these experiments we included two types of controls: the first were the PBMCs grown alone, the other were the PBMCs grown with healthy B cells. This experimental strategy was applied to monitor possible effects of generating an allogenic cell-PBMCs culture. We therefore compared MLTC with PBMCs and culture with HD B cells. Of course, we were aware that we could monitor a phenotypic change in the co-culture with healthy B cells due to allogenic MLTCs, however we wanted to be sure that the effect was different from that caused by the tumour. Therefore, for our final considerations, we decided to use only the PBMCs alone as a control, since we had established that the effects on ILCs

and NK cells was not due to the addition of an allogenic cell, but only to the NHL cell lines of our interest.

Results of these experiments show that $CD56^{bright}$ NK cells increase the amount of intracytoplasmic perforin and the expression of CD69 on the cell surface. This suggests a potential state of activation regarding this NK cell subset, reached when in contact with NHL cell lines.

Oppositely, $CD16^{+}$ NK show a lower level of granzyme B and perforin, suggesting a low potential cytotoxicity. $uCD56^{dim}$ NK cells show increment of granzyme B, however concomitant to higher expression of CD39 in presence of SU-DHL-10. Overall, the data concerning NK cells, highlighted that NHL modulates their phenotype suggesting a potential tumour immune evasion exerted on $CD16^{+}$ NK and $uCD56^{dim}$ NK.

We did not find differences in terms of CD70 expression on NK cells, a marker that is known to be triggered by CD27, expressed on NHL tumour cells. CD70/CD27 binding results in NK cell activation, IFN-γ production and therefore NHL cells elimination[96].

CD94 represents another marker typical of T cells and NK cells, this receptor forms heterodimers with NKG2 molecules in order to exploit either an inhibitory or an activation function of NK cells[304].

These results suggest a very complex mechanism of NK phenotype control exerted by tumour cell lines, however not determined by NKG2A/CD94 bound to HLA-E on NHL cell lines.

Considering ILCs, the overall phenotype described a low cell activation in presence of tumour, also suggesting an immune suppression acted directly or indirectly by the tumour cell lines. Moreover, excluding cell-cell contact between the tumour and the total PBMCs, we determined that cell contact was necessary to induce changes on ILC and NK phenotype, and that molecules produced by tumour cells, therefore cytokines, were not sufficient to determine this effect, without excluding their possible cooperation with other factors in determining this effect.

NK cells cultured alone with the tumour cell line SU-DHL-4 release higher quantity of lytic molecules in the supernatants compared to the control concomitant to no changes in their phenotype. These data suggest that NK cells in the absence of other immune cells can rescue anti-tumour functions and they do not have phenotypic modulation.

Purified ILCs expressed high percentage of markers of activation in the presence of NHL cells. Thus, compared to MLTC results, this evidence unveiled that some immune cell subsets counteract the activity of ILCs, by acting as negative regulator.

The data shown in the *in vitro* experiments were consistent with the *ex vivo* phenotyping. Indeed, in the LN we found a trend of reduced activation markers and cytolytic potential in ILCs.

Overall, we concluded that cytokines upregulated in NHL plasma could induce cell activation, confirmed also with cytokine release after stimulation observed in HD sorted ILCs; however, a perpetuated stimulus acted negatively onto peripheral blood ILCs. In addition, NHL can exert an immunosuppressive role, considering ILCs at the tumour site. This regulation was exploited in a cell-cell contact dependent manner, mediated by another cell of the immune system. Tregs are $CD4^+$ T cells with the ability to supress effector T cells and to maintain immune homeostasis, this prevent the excessive responses to antigens[305]. Tregs also favour the mechanisms of immune escape , indeed once expanded they inhibit the activation of T cells by secreting IL-10 and TGF-β and hampering tumour suppression[306].

Tregs require direct cell-cell contact and the production of TGF-β and IL-10 to display their suppressive activity, such as reducing cytotoxic activity and type Th1 cytokine secretion. Not only T cell, but also B cells and DCs[307] can be suppressed by Tregs, but it has also been demonstrated that they can act on NK cells, thus inhibiting their functions[308]. Recently it has been published that in NHL an higher portion of Tregs is present in the tissues involved by the disease compared to the normal one[309], however the number of Tregs in peripheral blood are not relevant to define the status of disease [310]. Thus, we hypothesized an inhibitory role of Tregs also onto ILCs in NHL. For these reasons, we depleted Tregs from healthy donor PBMCs prior to co-culture them with NHL cell lines. Our data indicated that Tregs are involved in negative regulation of ILCs in NHL. It remains unclear if the effect is direct into ILCs or there is the involvement of other immune cells. We developed a model, which describes and summarizes our findings (**Figure 37**).

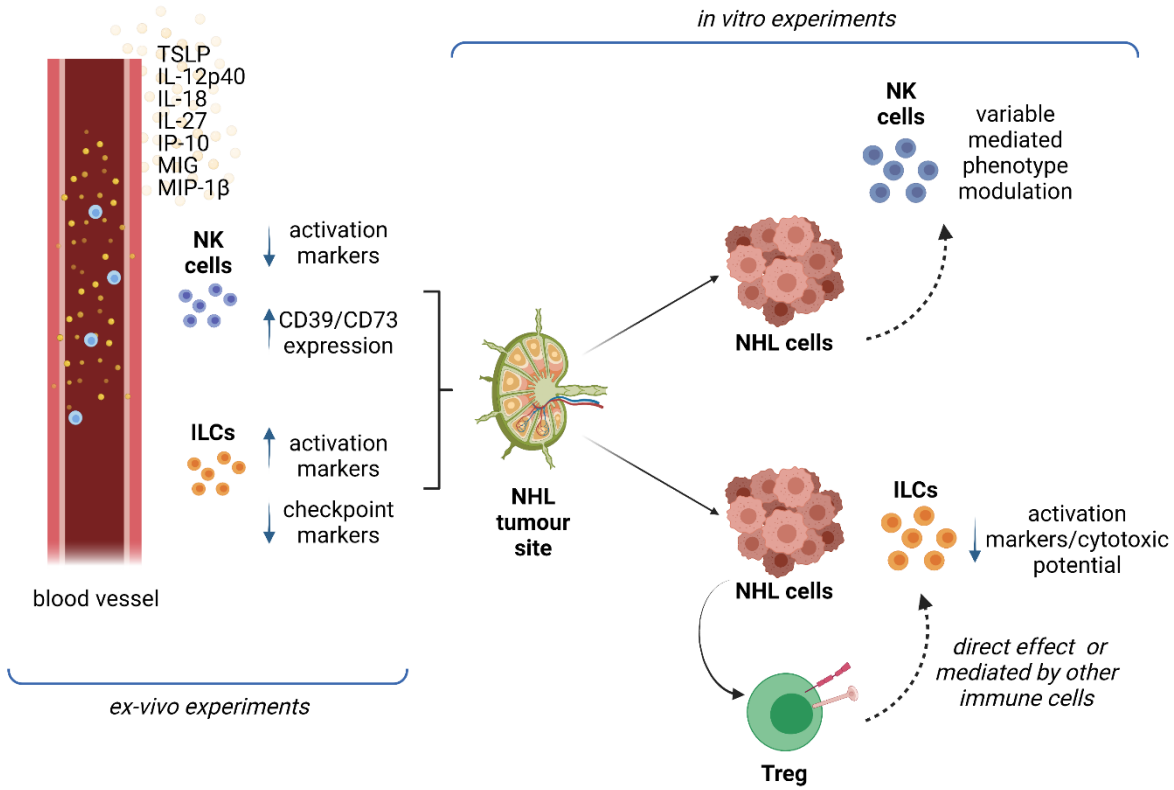


Figure 37. Proposal for a model of ILC and NK cell behaviour in NHL

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Our results could be important to better understand how to deal with NHL and develop more synergic therapies that avoid tumour progression or relapse.

For example, in order to possibly block induced Treg immunosuppressive functions, different strategies have been investigated, such as the inhibition of the immune checkpoints that regulate the activity of these cells. Cytotoxic T lymphocyte antigen 4 (CTLA-4) is an immune checkpoint molecule constitutively expressed by Treg cells, it is able to inhibit T cell proliferation and activation[311] by bounding costimulatory ligands (CD80 and CD86) on APCs, thus antagonizing CD28 or promoting the recruitment of inhibitory effectors[312]. Blocking CTLA-4 in cancer has been demonstrated to enhance anti-tumour immunity. Anti-CTLA-4 monotherapy has been shown to increase the long term survival of patients affected by advanced-stage melanoma[313], since the effect of inhibition is to reduce Foxp3 and in turn the activity of Tregs[314]. However, despite the promising results obtained in mice and in the *in vivo* experiments, anti-CTLA-4 therapy resulted inefficient or less effective considering renal cell carcinoma, non-small-cell lung cancer, and prostate cancer[315].

Another proposed way to inhibit Treg suppressive function is to target PD-1/PDL-1 axis. PDL-1 has been demonstrated to enhance the conversion of naïve CD4+ T cells into Tregs, moreover it increases their immunosuppressive functions[316]. However, some studies reported that treatment with anti-PD1 reduces the activity of Tregs, others that it increased Tregs immunosuppression[317,318]. Among the immune checkpoints of Tregs there are also anti-CD25 monoclonal antibodies which target CD25 expressed by Tregs, however different studies have shown that targeting this surface molecule may also induce a decrement of effector T cells, thus limiting the efficacy of this kind of therapy [319]. Promising targets are CD39 and CD73 since Tregs highly express these ectoenzymes which favour their immune suppressive activity[319]. The treatment has not be prolonged for long time, otherwise also effector T cells may become downmodulated reaching a pro-tumour effect[315]. Concerning NK cells, we had seen through our experiments that they show high level of CD39 and CD73 when cultured in presence of other immune cells and NHL cells. This suggested that in order to release the complete function of NK cells a possible therapy should target this axis, however we did not clarify what cells of the immune system determine this effect on NK cells. A recent opened clinical trial NCT05075564 is evaluating the clinical activity of ES002023, an anti-CD39 antibody in patients with advanced or metastatic solid tumours. Another important clinical trial, NCT04572152 is testing the antitumor activity of AK119 (an anti-CD73) in combination with AK104 (a bispecific antibody targeting PD1/CTLA-4).

Our study has some limitations. First, the experiments were manly done *in vitro*; therefore, in order to have a clear description of Treg role further study should be needed. Deeper investigation should also be done to determine if Tregs are involved in direct or indirect mechanism of ILC/NK immune modulation. We focused more on ILCs instead of NK cells, since they were mostly unexplored, however further experiments on NK cells should be done to clarify better their mechanism of regulation acted by NHL cells. However, despite these limitations, this study indicates the possibility to modulate ILCs indirectly or directly in NHL acting on Treg and immune checkpoints, suggesting new therapeutic targets for lymphoma therapies.

5.2. ILCs in HL and future perspective

Considering HL, we described the phenotype and frequencies of NK and ILCs in HD and HL peripheral blood. The differences recorded were suggestive of the tumour microenvironment, since this tumour determine important effects also considering

peripheral blood. Differently to what found for NHL, we did not see any significant differences of cytokine levels in plasma.

In order to unveil the possible interaction of ILCs with Treg or other immune cells present in the tumour microenvironment (LN) and with the tumour cells we planned to analyse a single cell RNA seq data set published in 2020[320]. In this work, authors found that in lymph nodes affected by HL there was a high enrichment of induced Tregs. Independently from EBV infection $FOXP3^+$ Tregs seem to be enriched in MHC-II positive HSR cell, while LAG3+ Tregs were majorly present in MHC-II negative HSR. $LAG3^+$ T cells produce TGF-β and IL-10, which contribute to immune-suppressive environment and HSR cells negative for MHC class II expression secrete cytokines able to induce $CD4^+$ T cells expressing LAG-3, while MHC class II^+ HSRs attract Th17 cells and $Foxp3^+$ Tregs through cytokines production. We will investigate in particular the interplay of ILCs and Tregs in inducing immunosuppression.

At present time, therapies with immune-checkpoint inhibitors such as nivolumab show to be effective in refractory/relapsed classic HL leading to durable remission[321]. However, $CD4^+$ T cells expressing LAG-3 do not co-express PD-1. Recent clinical trials (NCI 02061761) showed that removing $LAG3^+$ T cells turned in T cell reactivation avoiding the unuseful PD-1 blockade. Yttrium-90-labeled anti-CD25 is a newly proposed therapy for refractory HL, which is now under investigation through a clinical trial (NCT04871607) combined with chemotherapy. This therapeutic strategy could be promising also by its contribution to Treg blocking in TME.

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