UNIVERSITY OF GENOA
DEPARTMENT OF EXPERIMENTAL MEDICINE
(DIMES)

PhD in Clinical and Experimental Immunology
XXXIV cycle

"Identification of common lymphoid precursors cells in tumor tissues and peripheral blood of cancer patients"

Supervisor: Prof. Andrea De Maria

PhD candidate: Carola Perrone

CONTENTS

1.INTRODUCTION

1.1 NK cells development

NK cells were originally described as cytolytic effector lymphocytes, which, unlike cytotoxic T cells, can directly induce the death of tumor cells and virus-infected cells in the absence of specific immunization (Vivier E et al, 2011), due to their constitutive expression of perforin and granzyme and ready ability to produce high amounts of IFNγ and other proinflammatory cytokines. The NK cell involvement in the regulation of downstream responses was recognized with continuous coordination of and support to downstream adaptive immune responses through crosstalk with dendritic cells (Moretta A, 2002) and with T cells (Peppa D et al, 2013).

The predominant sites of the human body in which NK cells are found include secondary lymphoid organs, bone marrow, liver, lungs, and decidua while an overall minority of body NK cells (<2%) circulates in peripheral blood where they represent 5–15% of blood lymphocytes (Bozzano et al 2019).

Regarding the origin and development of NK cells, we know that NK cells are generated from hematopoietic stem cells (HSCs) residing in the bone marrow (BM) which has been identified as the main source of HSCs in the body (Jacobson LO et al; Till JE) and the main site where progressive NK cell development takes place (Colucci et al, 2008). HSCs are multipotent cells and are able to generate more differentiated precursors along the pathways toward production of erythrocytes, leucocytes and platelets.

The first fate decision toward NK cells downstream of HSCs is represented by the divergence of lymphoid and myeloid lineages. This step is followed by myeloid–lymphoid divergence in which common lymphoid progenitors (CLPs), and common myeloid progenitors are generated (Weissman et al, 2008). Neither the thymus nor the spleen appear to be essential for the growth of NK from CLPs, as shown by the persistence of NK cells and the function

preserved in cases of thymectomy or splenectomy (Ramos et al, 1996; Passlick et al, 1991).

The stepwise process of lymphoid differentiation from multipotent HSC to the earliest lymphoid-primed multipotent progenitor (LMPP) occurs in BM (Roy et al, 2012). Also recently, has been identified a NK cell-restricted progenitor (NKP), downstream of LMPP and CLP-like cells in human bone marrow, umbilical cord blood, tonsils, and fetal tissues, representing the unipotent NK cell precursor devoid of potential toward other lymphoid lineages. NKPs generated fully mature and functional NK cells both *in vitro* and *in vivo* and lacked the potential to produce T cells, B cells, innate lymphoid cells (ILCs), or myeloid cells at the single-cell level *in vitro*. Renoux et al. identification of this earliest steps of human NK cell commitment, conserved throughout ontogeny, provided new insights into human hematopoiesis with implications for the development of NK cell-based therapies. (Renoux et al, 2015).

Investigating on secondary lymphoid tissue (SLT) NK cell composition was emerged that the BM could not be the predominant site of NK cell development. Indeed, reports showed that lymph nodes were harboring predominantly large numbers of $CD56^{bright}$ NK cells adjacent to T-cell-rich areas (Fehniger et al, 2003) and that NK cells with a $CD56^{dim}$ phenotype developed from $CD56^{bright}$ NK cells in SLTs (Ferlazzo et al, 2004). In addition, Freud et al. described and characterized CD34+ CLP in lymph nodes that generate $CD56^{bright}$ NK cells *in vitro* (Freud et al, 2005).

CLP have been recovered from PB and from UCB and are believed to transit from BM through PB toward peripheral tissues for further development. and this supports that the vast majority of NK cells may be generated in peripheral tissues (e.g., SLT) as progenies from CLP traveling from the BM.

1.2 T cell development

T cells are an important adaptive immune response arm that mediates cell-mediated immunity (Hu et al, 2018). Moreover, these cells are also implicated as major drivers of many inflammatory and autoimmune diseases. T cells express a receptor with the potential to recognize diverse antigens from pathogens, tumors, and the environment and also maintain immunological memory and self-tolerance. They have to come into contact with rare antigen-carrying cells and this is not an easy task. This feat is achieved in a timely manner by targeted migration (homing), a process that is coordinately regulated with T cell differentiation state. In addition, naive T cells recirculate through the paracortical regions of secondary lymphoid organs (SLOs), a strategy that maximizes their opportunity for antigen detection. (Masopust et al, 2013).

T lymphocytes originate from BM progenitors that migrate to the thymus for maturation, selection, and subsequent export to the periphery.

Peripheral T cells comprise different subsets, including naive T cells, which have the capacity to respond to new antigens, memory T cells, which derive from previous antigen activation and maintain long-term immunity, and regulatory T (Treg) cells, which keep immune responses in check. Immune responses commence when naive T cells encounter antigen and costimulatory ligands presented by dendritic cells (DCs), resulting in interleukin 2 (IL-2) production, proliferation, and differentiation to effector cells that then migrate to diverse sites to promote pathogen clearance through production of effector cytokines and cytotoxic mediators. Activated effector cells are short-lived, although a proportion survive as memory T cells that persist as heterogeneous subsets based on migration, tissue localization, and self-renewal capacities (Kumar et al, 2018).

It is essential to explain the role of Notch signaling, which plays a critical role during T cell development from CLP cells that seed the thymus from the BM.

In 1999 Radtke suggested that inducible deletion of Notch-1 in newborn mice or bone marrow stem cells resulted in a severe block in thymocyte development. As a result of Notch's deletion, in the thymus most cells expressed B cell line commitment markers and phenotypically resembled immature B cells in the bone marrow (Radtke et al, 1999).

In addition, when bone marrow stem cells expressing constitutively active Notch-1 were transferred into irradiated hosts, they gave rise to a thymus-independent population of cells in the BM that expressed markers of T cell lineage commitment, including CD4, CD8 (Pui et al, 1999) (Deftos and Bevan, 2000).

Always in studies on mouse models, it was found that Notch-1 has also been implicated in later stages of thymocyte development, consistent with its expression on thymocytes throughout their maturation. Following commitment to the T cell lineage, developing T cells differentiate into either the αβ or γδ lineage. Furthermore, developing thymocytes can differentiate into either the CD4+ or CD8+ single-positive (SP) lineages. CD4+CD8+ double-positive (DP) thymocytes with αβ TCRs that interact with MHC class I ligands differentiate into the CD8+ SP lineage whereas DP thymocytes with αβ TCRs that interact with MHC class II ligands differentiate into the CD4+ SP lineage. Notch signaling has also been implicated at this stage in T cell development (Deftos and Bevan, 2000). CD4+ or CD8+ SP thymocytes ultimately emerge into the periphery as naive T cells exhibiting CD45RA+CCR7+ phenotypes (Kumar et al, 2018). 9%–10% of human CD4+ SP thymocytes is represented by T regulatory (Treg) cells (Watanabe et al, 2005), defined as CD4+CD25+ cells expressing the Foxp3 transcription factor (Hori et al, 2003) with naive CD45RA+CCR7+ phenotype and that play a particularly important role in maintaining immune homeostasis (Owen et al, 2019).

Naive blood-borne T cells enter SLOs, where they scan antigen-presenting cells (APCs) for cognate antigen before egressing via the efferent lymphatics and then pass through the thoracic duct before returning to the blood. These migrations take place thanks to rolling on high endothelial venules (HEVs), a

specialized type of post-capillary vascular endothelium present within lymph node paracortical regions. This action is typically mediated via short-term interactions between CD62L (L-selectin) on T cells.

Furthermore, CC-chemokine ligand 21 (CCL21), which is immobilized on HEVs via binding to heparin glycosaminoglycans, is recognized by CCR7 (expressed on lymphocyte surface) and mediates signaling and subsequent activation of lymphocyte function- associated antigen 1 (LFA1) (Masopust et al, 2013).

Some T cells may remain within the SLO such as CD4+ T follicular helper (T_{FH}) cells that upregulate CXCR5 and migrate to lymphoid follicles to provide B lymphocyte help (Ma et al, 2012) (León et al, 2021) but non-lymphoid tissues represent sites of infections for most pathogen, indeed most T cells exit the SLO to seek infected cells. Following the resolution of an infection, most effector T cells die, but a fraction of responding T cells differentiate into long-lived memory T cells. Memory T cells, unlike naive T cells (which have SLO-restricted homing patterns), also exhibit a much broader anatomical distribution, being present in both SLOs and non-lymphoid tissues. There are two memory T cell subsets, the central memory T cell (T_{CM}) and the effector memory T cell (T_{EM}).

T_{CM} cells, defined by the expression of lymph-node-homing molecules, recirculate through SLOs with no effector function. T_{EM} cells were defined in blood by the absence of SLO-homing molecules and migrate to inflamed peripheral tissues displaying immediate effector function. (Sallusto et al, 2004).

At the peripheral tissue level there is a heterogeneous subset of T cells called tissue-resident memory T (T_{rm}) cells with variable frequency of expression of surface molecules based on tissue localization. In humans, in T_{rm} cells from skin was found that the CD49a+CD103+ subset of CD8+ T_{rm} cells represented a subset with superior cytotoxic abilities compared with those of their CD49a– counterparts (Cheuk et al, 2017). Also, memory T cells with a T_{rm} cell phenotype CD69+CD49a+CD103+ can be found in human lymphoid tissue

(Kumar et al, 2017). CD69 is a membrane-bound, type II C-lectin receptor and it is a classical early marker of lymphocyte activation. CD69 is expressed by several subsets of tissue resident immune cells, and is therefore considered a marker of tissue retention (Cibrián D et al, 2017). CD103 forms a heterodimer with β7 integrin and binds to E-cadherin (also known as cadherin 1) on epithelial cells (Hadley GA. and Higgins JMG, 2014).

Human T_{rm} cells most likely have key roles in mediating protective responses and maintaining long-term immunity with T cell clones specific to the pathogens encountered at their site of residence. Human T cell development, differentiation, and maintenance are all dynamic processes that occur in our organism.

1.3 Lin-CD34+DNAM-1^{bright}CXCR4+ cell population: a new common lymphoid precursor

Recently our group has identified new CLP which represent a new paradigm of NK and T development. The novel CLP has been identified in patients with HIV, HCV, TB, COPD (chronic obstructive pulmonary disease), and with PAPA syndrome (sterile pyogenic arthritis, pyoderma gangrenous and acne) who share a chronic state of inflammation. These cells appear to be absent or present in a reduced percentage in the peripheral blood of healthy donors (HD). The precursors are characterized by the expression of Lin-CD34+DNAM-1^{bright}CXCR4+ markers (Bozzano et al, 2015) (Fig 1).

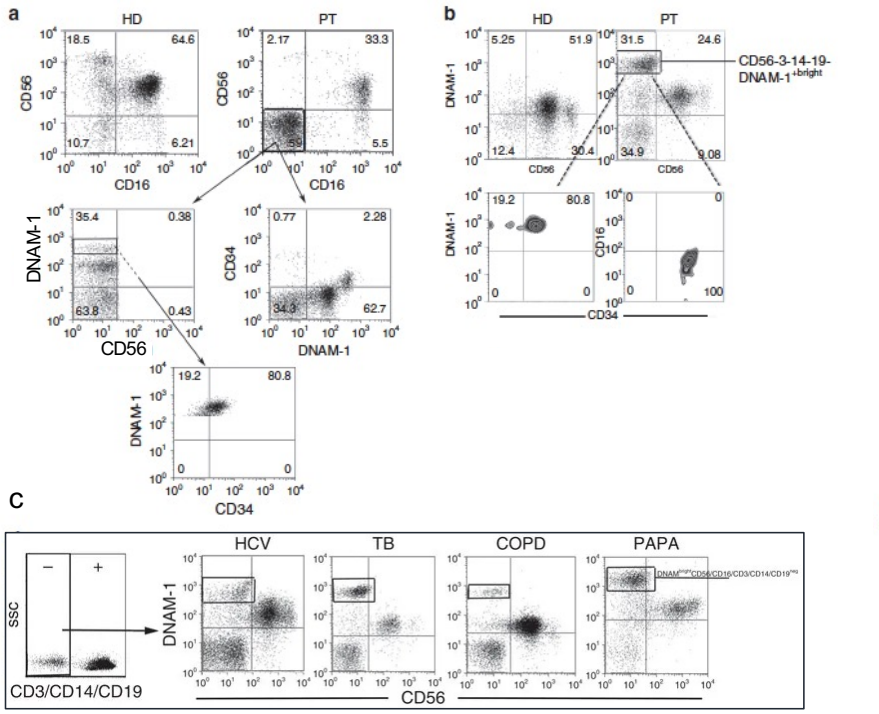


Figure 1. Identification of CLP Lin-CD34+DNAM-1^{bright}CXCR4+ in HIV, HCV, TB, COPD, PAPA patients with chronic state of inflammation. (**a**) Flow cytometric analysis of CD56−CD16− cells proportion in fresh PBMCs from HIV-infected patients and HD donors. In CD3−CD14−CD19−-gated PBMCs different proportions of CD56−CD16− were observed in HIV-infected patients (PT) compared with HDs. In HIV patients, CD56−CD16−-gated cells expressed DNAM-1 and CD34. (**b**) Flow cytometric analysis of DNAM-1 expression on fresh PBMCs from HIV-infected patients and HD donors. DNAM-1brightCD56− cells are observed in HIV-1 infected patient CD3−CD14−CD19−-gated PBMCs (upper row). DNAM-1brightCD56− cells express CD34 but do not express CD16 (FcγRIII) (lower row). (**c**) Flow cytometric analysis of DNAM-1 expression on fresh Lin-PBMCs from chronic HCV, TB, COPD patients and PAPA-syndrome patients. Lin-DNAM-1^{bright}CD34+ cells (gates shown) were observed in HCV, TB-infected patient, in COPD and PAPA patients (in CD3−CD14−CD19−-gated PBMCs).
Modified from Bozzano F. et al, 'Emergency exit' of bone-marrow-resident CD34^{+}DNAM-1^{bright}CXCR4^{+} committed lymphoid precursors during chronic infection and inflammation. *Nat. Commun.* 6:8109 doi: 10.1038/ncomms9109 (2015)

The relevance of the discovery of Lin-CD34+DNAM-1^{bright}CXCR4+ cells in the peripheral blood of patients with a chronic state of inflammation and/or affected by infectious diseases, lies in their *in vitro* cultured progenies.

These precursors, cultured *in vitro* in specific medium enriched with rhIL7, rhIL15, rhFLT3, rhSCF, give rise to NK (CD56+CD3-), T (CD56-CD3+; T CD4 +, T CD8 +) and NKT (CD56+CD3+) progeny.

NK progenies derived from "classical" CD34+ precursors (DNAM-1^{neg}) are usually immature, CD56^{bright} and KIR^{neg}. In NK progenies derived from Lin-CD34+DNAM-1^{bright}CXCR4+ precursor cells, both the CD56^{bright} and the CD56^{dim} population are present. This NK progeny is phenotypically mature on the basis of the expression of inhibitory receptors such as KIRs and NKG2A and activators such as NCRs (natural cytotoxicity receptors; NKp30, NKp44, NKp46), DNAM-1 and NKG2D. Furthermore, the NK progeny is functional as evidenced by its ability of releasing IFN-γ and its cytotoxic activity against target cells such as K562.

Recent reports would indicate that T and NK cells are generated from T/NK common progenitors (Klein Wolterink RG et al, 2010) (Izon DJ, 2008). Indeed, in humans, bipotent T/NK progenitors could be identified in PB of HDs, although at very low frequencies (Kyoizumi S et al, 2013). In agreement with these reports, and different from cord blood CD34^{+}cells (Grzywacz B et al, 2006) (Sivori et al, 2003), CD34^{+}DNAM-1^{bright} cells could give rise to mature NK and T cells but not to myelomonocytes, and expressed transcription factors associated with T- and NK-cell maturation. Microarray analysis further confirmed that transcripts for myeloid differentiation (for example, BCL6 and RASGRP4) were shut off in CD34^{+}DNAM-1^{bright} cells.

Lin-CD34+DNAM-1^{bright}CXCR4+ cells also express CX3CR1 and CXCR1 chemokine receptors that bind to fractalchine (CX3CL1) and IL-8 (CXCL8), respectively, both of which are inflammatory chemotactic molecules usually produced in inflamed peripheral tissues (Korbecki J et al, 2020) (Russo RC et al, 2014) This suggests that these precursors are potentially able to migrate to inflamed tissues following the gradient of their ligands (Bozzano et al, 2015).

In human NK cells, DNAM-1 (CD226) functions primarily as an activating receptor involved in killing of different tumour targets and in NK-DC crosstalk. However, an additional functional activity of DNAM-1 is represented by its involvement in transendothelial cell migration on interaction with its ligand PVR (CD155) (Reymond N et al, 2004).

The finding of high levels of DNAM-1 expression on Lin-$CD34^{+}$DNAM-1^{bright} cells supports the notion that DNAM-1 may play a role in the migration of these precursors from BM. The BM origin of Lin^{-}DNAM-$1^{bright}CD34^{+}$ cells is suggested by their surface expression of CXCR4 (Bozzano et al, 2015), a G-protein coupled receptor expressed on hematopoietic stem cell (HSC) (Foudi A. et al, 2006) that can regulate their quiescence, maintenance, and retention through binding to its stromal cell-derived factor-1 (SDF-1) ligand (Singh P et al, 2020). Bozzano et al (2015) showed that CXCR4 is expressed on all BM $CD34^{+}$ cells in HD, irrespective of DNAM-1 expression. Interestingly, $CD34^{+}$DNAM-1^{-} cells from HD PBMC do not express CXCR4, thus suggesting that under normal conditions $Lin^{-}CD34^{+}$DNAM-$1^{bright}CXCR4^{+}$ precursors are barely detectable in PB, and may be released from BM in case of need such as during chronic inflammation (as found in HIV, HCV, TB, COPD and PAPA PBMC) (Bozzano et al, 2015)

There are no data so far in the scientific literature about the presence of Lin-CD34+DNAM-1^{bright}CXCR4+ precursor cells, in peripheral blood and in tissues of cancer patients. Accordingly, to address this point we set up this work to search for Lin-CD34+DNAM-1^{bright}CXCR4+ cells in tumor samples, characterizing the co-expression of DNAM-1 and CXCR4 on CD34+ cells.

1.4 Identification and characterization of novel Lin-CD56-CD16+Perf-CXCR4+ lymphoid precursors

Furthermore, while looking for additional inflammatory precursors in HIV and HCV patients, our lab has highlighted the presence of CD3-CD14-CD19-CD56-CD16+ cells, which give rise to progenies of the lymphoid lineage (CD56-CD3+; CD56+CD3+; CD56+CD3-) when cultured *in vitro* with the same medium (FLT3, SCF, rhIL-7, rhIL-151 feeder PBMC+ 721.221AEH) used for Lin-CD34+ DNAM-1^{bright}CXCR4 + cell cultures. In these cell cultures, the CD16+CD56+ (NK) cell fraction is predominant. These cells also express NKG2C, NKp30, NKG2D, DNAM-1, and CD57. In particular, a mature CD56+CD16+NKG2C+ NK progeny predominates in the presence of HLA-E expressing feeder cells (Bozzano et al, 2021).

To be remembered is that cells with CD3-CD56-CD16+ phenotype were previously identified as "exhausted NK cells" (Bjorkstrom NK et al, 2010) (Mavilio D et al, 2005). The growth of mature NK cells together with T-cell progenies confirmed the stemness nature of at least some cells included within the Lin-CD56-CD16+ PB subset and the data therefore support the view that there are two different cell populations within CD3-CD56-CD16+ PBMC cell fraction: CD56-CD16+ exhausted NK cells and common lymphocyte precursors.

In support of this possibility, this study provided cytofluorimetric evidence that Lin-CD56-CD16+ PBMCs could be divided into two subpopulations on the basis of presence or absence of Perforin expression that are represented by CD16+CD56-Perf+NKp30+NKG2C+CD94+CD57+CD85j+CD7+ and by CD16+CD56-Perf-NKp30-NKG2C-CD94-CD57-CD85j^{low}CD7- circulating common lymphocyte precursors, which express CXCR4 (Bozzano et al, 2021) (Fig 2).

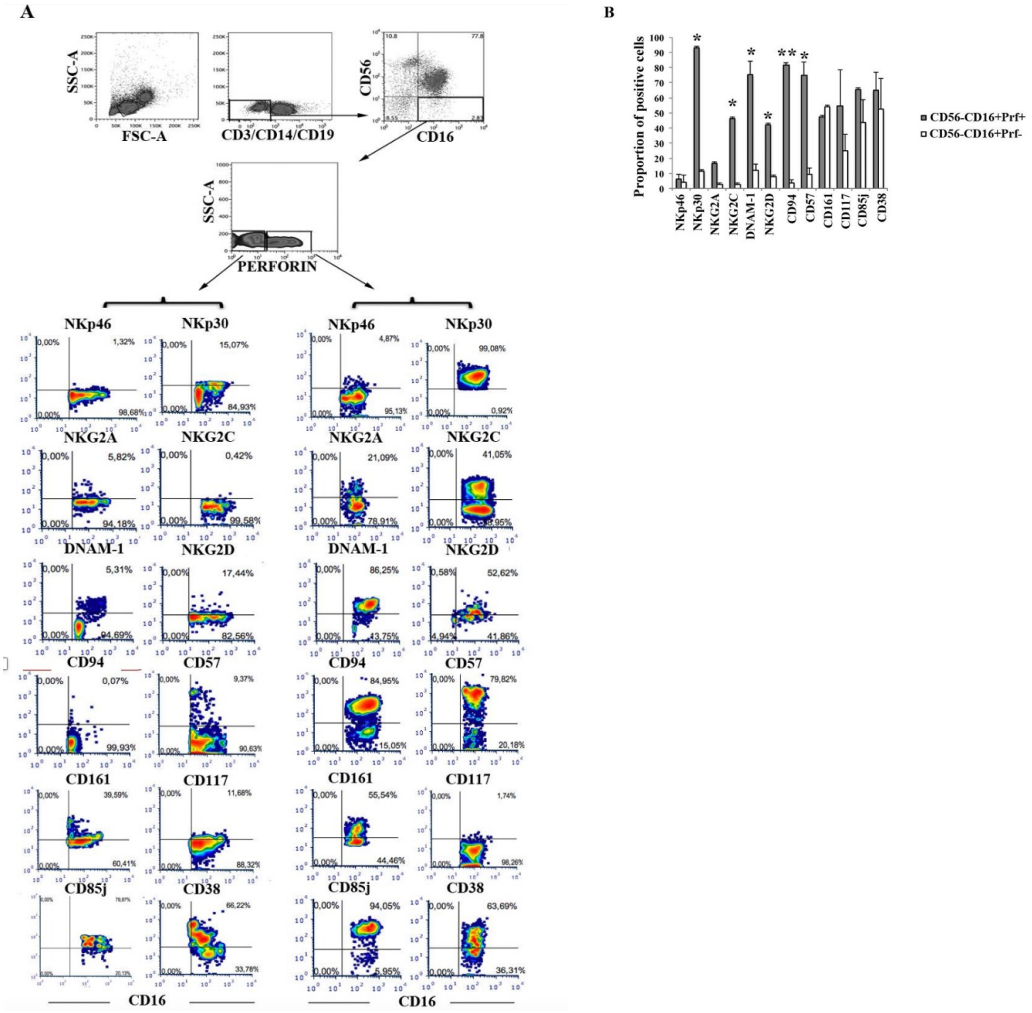


Figure 2. Characterization of precursor cells in PB Lin-CD16+CD56- cells by flow cytometry. (**A**) PB Lin-CD16+CD56- PBMCs are composed of 2 overlaying populations: (upper row) gating strategy; (lower row) gating split according to Perf expression. (**B**) Comparison of surface antigen expression of Lin2CD161CD562CD942/dimPerf2CD572 PBMC progenitors versus Lin2CD161CD562CD941Perf1CD571 NK cells. Histograms (mean±SD) Mann-Whitney U test.
Modified from Bozzano F. et al, HCMV-controlling NKG2C $^{+}$ NK cells originate from novel circulating inflammatory precursors.J Allergy Clin Immunol. 2021 Jun;147(6):2343-2357.

1.5 Tumor microenviroment

Natural Killer (NK) cells play a major role in the immune response against viruses and tumors due to their ability to kill transformed or virus-infected cells (Yokoyama WM et al, 2004). Human tumors, in addition to NK cells, are generally infiltrated by inflammatory cells (Balkwill and Mantovani, 2001). Although these infiltrates of inflammatory cells can vary in size and composition from tumor to tumor (Whiteside, 2008).

Immune cells present in the tumor include those mediating adaptive immunity, T lymphocytes, dendritic cells (DC) and occasional B cells, as well as effectors of innate immunity, macrophages, polymorphonuclear leukocytes and NK cells (Whiteside, 2007). The extravasation of Tumor-infiltrating lymphocytes (TILs) into the tissue is facilitated by selectins, integrins, and chemokines. Chemokines released by tumor cells and its stroma influence the immune cell infiltration, tumor cell proliferation, and metastasis (Ben-Baruch A, 2008).

NK cells can target and eliminate cancer cells through secretion of cytolytic granules, and trigger an immune response via secretion of immunomodulatory cytokines (Vivier E. et al, 2008). In contrast to T- and B-cells, NK cells express a multitude of intrinsic germline-encoded activating and inhibiting membrane receptors, and therefore do not require antigen specificity (Lanier LL, 2003; 1998). Activating and co-activating NK cell receptors include the natural cytotoxicity receptors (NCRs) NKp46, NKp30, and NKp44, CD16, NKG2D, NKG2C and DNAX Accessory Molecule-1 (DNAM-1) (Moretta L and Moretta A., 2004) (Vivier E et al, 2011). But also the important inhibitory receptors on NK cells engage with MHC-I ligands to down modulate the NK cell response, and these include the Killer-cell immunoglobulin-like receptors (KIRs), and the CD94/NKG2A heterodimer (Moretta L and Moretta A., 2004). Activation of NK cells is based on the equilibrium between activating and inhibitory signals derived from binding to their ligands on target cells (Long EO et al, 2013).

NK cell function is partially complementary to T-cells, as they can target and lyse MHC-I deficient cells, in a process known as “missing-self recognition” (Ben-Shmuel A. et al, 2020).

Activation of cytotoxic T cells is an antigen-specific process requiring the interaction of the TCR–CD3 complex with a processed tumor antigen–derived peptide bound to MHC class I molecule as well as costimulatory signals (CD8 and CD28) (Martínez-Lostao L. et al, 2015).

Despite the different mechanisms to kill cancer target cells, inside the tumor immune cells are confronted with a suppressive milieu (Ben-Shmuel A. et al, 2020).

Tumor cells evade the immune cells by various mechanisms. For example the downregulation of tumor antigens, releasing immunosuppressive extracellular vesicles including exosomes, releasing immunosuppressive molecules including IL-10 and transforming growth factor β (TGF-β), shedding soluble major histocompatibility complex (MHC)-I, loss of adhesion molecules such as ICAMI, developing resistance to apoptosis by upregulation of BCL-2 and other anti-apoptosis molecules, and overexpressing programmed death ligand 1 (PD-L1) as well as Fas ligand and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). Tumor-released molecules shape the tumor microenvironment (TME) and induce immunosuppression that debilitate robust antitumor immune responses (Labani-Motlagh A. et al, 2020).

1.6 AIMS

In view of a complete absence of information in the literature about the presence of Lin-CD34+DNAM-1^{bright}CXCR4+ or Lin-CD56-CD16+CD7-CXCR4+ precursor cells in peripheral blood of cancer patients, we performed experiments with the aim of :

1. Identifying and characterizing Lin-CD34+DNAM-1^{bright}CXCR4+ and Lin-CD56-CD16+CD7-CXCR4+ precursor cells in the peripheral blood of cancer patients with Non-small cell lung cancer (NSCLC), Kaposi's sarcoma (KS) lymphoma.
2. Identifying CLPs in inflamed target tissue namely tumor tissue.
3. Characterizing phenotypically and functionally NK cell and T cell progenies generated by Lin-CD34+DNAM^{bright} and Lin-CD56-CD16+ precursors isolated from cancer-tissue cultured in vitro in specific medium enriched with rhIL7, rhIL15, rhFLT3, rhSCF and their comparison with those obtained from uninvolved tissue and CLPs from peripheral blood.
4. characterizing the trend of Lin-CD34+DNAM-1^{bright}CXCR4+ and Lin-CD56-CD16+CD7-CXCR4+ precursors in the peripheral blood of NSCLC patients following therapeutic treatment.

2.MATERIALS AND METHODS

2.1 Tissue and Peripheral blood samples

Cancer tissues were obtained from 15 patients (recruited thanks to the collaboration with Dr. E. Munari - Pathology Unit, Sacro Cuore Hospital, Negrar, Verona and with Prof. Giancarlo Pariscenti, Thoracic Surgery Unit, San Martino Hospital, Genoa) who underwent surgical resection of tumor (NSCLC, KS, bladder cancer). Non-tumor tissue called “uninvolved tissue” counterpart was also collected.

Peripheral blood samples of 18 cancer patients (affected by lymphoma, NSCLC, KS, breast cancer) with progressive disease were collected.

Peripheral blood samples of 18 Non-Small-Cells Lung Cancer (NSCLC) patients at different time points before and during NSCLC treatment were collected.

2.2 Cell isolation

After pathology preparation for diagnostic purposes, samples of involved and uninvolved organ samples were selected at the department of Pathology, San Martino Hospital, Genoa and Pathology Unit, Sacro Cuore Hospital, Negrar, Verona. The samples of neoplastic and non-neoplastic tissue were mechanically dissociated and cell suspensions obtained were filtered through a 40μm cell strainer (Jet Biofil, Guangzhou, China). In order to isolate TIL cells (tumor infiltrating lymphocytes), cell suspensions were isolated by a density gradient centrifugation (Ficoll-Hypaque).

Peripheral blood mononuclear cells (PBMCs) of cancer patients were obtained by density gradient centrifugation (Ficoll-Hypaque) and processed immediately or cryopreserved at -86 °C for further processing.

2.3 Immunofluorescence analysis

Cells were analyzed by 6-8 color multi-parameter flow cytofluorometry. Direct staining was performed incubating cells with fluorochrome-conjugated monoclonal antibodies (mAbs) for 15 minutes at 4°C. Cells were then washed and the flow cytometric analysis was performed (FACSFortessa, BD, Mountain View, CA, USA). Mean fluorescence intensity ratios are calculated as follows: MFI sample/MFI negative control and mean fluorescence intensity absolute are calculated as follows: MFI sample-MFI negative control. Data were analyzed using FlowJo (Tree Star, Inc, BD, Ashland, Ore) and FCS Express 7 (De Novo Software, Pasadena, Calif).

2.4 Cell culture

Highly purified Lin-CD34+DNAM-1^{bright} and CD3-CD4-CD19-CD56-CD16+ populations were obtained using FACSAria (BD Biosciences) cell sorter. Purified cells were cultured in limiting dilution in precursor complete medium with Myelocult medium (StemCell Technologies, Vancouver, British Columbia, Canada) supplemented with 10% human AB serum (ICN Pharmaceuticals Italy, Milano, Italy), 5% FCS and purified recombinant human rhIL-15, rhIL-7, SCF, FLT3-L (PeproThec, London, UK) at the final concentration of 20 ng/ml with irradiated Feeder cells, for 30 days.

2.5 Cytokine Production Assay

CD56-CD3+ progenies *in vitro* derived from tissue and peripheral blood sorted CLPs were plated in 96-well plates (20000 cell/well) in culture medium (RPMI 1620 (BioWhittaker/Lonza) supplemented with 10% FCS, L-glutamine (2 mM), and 1% antibiotic mixture (penicillin–streptomycin 5 mg/mL)) with or without PMA (25 ng/mL) and ionomycin (1 ug/mL) and incubated at 37°C overnight. After centrifuging the plates, the supernatants were collected and stored at -20°C to measure cytokine release subsequently. Cytokines were

measured using a customized MILLIPLEX MAP Human Th17 Magnetic Bead Panel assay (Millipore), and the plate was read by a MAGPIX® with xPONENT® software, following the manufacturer's instructions. In particular, we analyzed the following cytokines: TNF-α, IFN-γ, IL-2, IL-4, IL-5, IL-9, IL-13, IL-17A, IL-17F, IL-10.

2.6 Cytotoxicity assay

In order to study the activity of Lin-CD34+DNAM-1^{bright} and CD3-CD4-CD19-CD56-CD16+ progenies in killing target tumor cells was assayed using $FcγR^{+}$ mouse P815 target cells and A549 human lung carcinoma target cells.

Cytotoxic activity against P815 cells was tested in reverse ADCC at 1:1 E/T ratio in complete medium in the absence or presence of appropriate mAbs (0.1 μg/mL). When using A549 cells as targets, cytotoxicity of target cells was tested without addition of mAbs at 1:1 Effector:Target (E:T) ratio .

P815 and A549 cell lines were stained with PKH26 using PKH26 Red Fluorescent Cell Linker Kits for General Cell Membrane Labeling (Sigma-Aldrich) and incubated with effector cells for 6 hours. Citofix/Citoperm (Fixation and Permeabilization solution, BD Pharmingen) was used for maximal cellular cytotoxicity. Basal condition was represented by the percentage of spontaneous mortality of monoculture target cells.

After incubation cells were stained with TO-PRO 3 Iodide (Invitrogen) at 1uM concentration to test the double-positive PKH+TOPRO+ killed target cells. Positive control for maximal lysis was represented by Citofix/Citoperm and medium alone was used to evaluate basal lysis as negative control.

The sample cytotoxic activity was calculated as: (sample-basal)/(maximum-basal) *100.

2.7 Statistical analysis

Statistical analysis was performed using the Mann-Whitney U test for unpaired datasets for comparisons. Analysis was performed using JMP 10.0 (SAS).

3. RESULTS

3.1 Identification of Lin-CD34+DNAM-1^{bright}CXCR4+ and Lin-CD34-CD16+CD7- cells in peripheral blood of cancer patients.

The presence of "inflammatory" CLP cells that had been previously found in patients with chronic inflammation and/or with infectious diseases, was first investigated in patients with progressive disease (lymphoma, NSCLC, KS). In order to verify whether these precursors are released from the BM in cancer patients with progressive disease.

Using a reverse flow cytometric strategy with Lin$^-$ selection stain (which includes anti-CD3, -CD19, -CD20, -CD14, -CD16, -CD56 mAbs) we found the presence of Lin-CD34+DNAM-1^{bright} precursor cells and by analyzing the CD3, CD14, CD19 negative cells, Lin-CD56-CD16+CD7- precursor cells were detected in peripheral blood of cancer patients. Both precursor cell populations were CXCR4 positive (Fig 3A).

In order to provide a perspective on the frequency of circulating inflammatory precursors in patients with cancer, we next compared their frequency with the frequency of the same cells that are detected in typical inflammatory conditions including HIV infection and acute SARS-CoV-2 infection. A relevant frequency of "inflammatory" Lin-CD34+DNAM-1^{bright}CXCR4+ cells is detected in HIV patients on antiretroviral treatment (Bozzano et al, 2015). Analysis of 23 patients with acute SARS-CoV-2 infection showed a relevant frequency of Lin-CD34+DNAM-1^{bright}CXCR4+ cells (Bozzano et al, PLoS Pathog. 2021). Frequencies in cancer patients at diagnosis and in patients with viral infection were compared with healthy donors (HD) who have very low levels of circulating inflammatory CLP precursors.

As shown in Fig.1B, the frequency of Lin-CD34+DNAM-1^{bright} cells in COVID-19 patients was significantly higher when compared to HIV patients and cancer patients (27,5±24,07 vs. 10,2±1,4 vs. 5,07±1,4; p=0,0036, p=0,0001 respectively; Fig 1B). Overall, only modest increase in the

circulation of Lin-CD34+DNAM-1^{bright}CXCR4+ cells was detected in tumor patients at the time of first diagnosis, and this increase was not significant, when compared to HDs (Fig 3B).

We also studied the frequency of another subset of Lin-CD56-CD16+CD7- precursor cells that is able to give rise to lymphoid progenies when cultured in vitro in specific medium. This subset is phenotypically immature, and differed from the Lin-CD56-CD16+CD7+ subset which is phenotypically mature as shown recently (Bozzano et al, JACI, 2021).

Lin-CD56-CD16+ cells in peripheral blood of cancer patients were 6,6±1,6% and in this subset the relative proportion of Lin-CD56-CD16+CD7-CXCR4+ (called Lin-CD56-CD16+CD7-) CLPs were 49,5±9,1% (mean±sem).

Previous studies showed a unique potential trafficking ability of "inflammatory" Lin-CD34+DNAM-1^{bright}CXCR4+ cells (Bozzano et al, 2015). In order to further characterize circulating inflammatory CLPs in cancer patients, we therefore next assessed their surface expression of chemokine receptors.

Flow cytometric dot plot analysis showed that CD34+DNAM-1^{bright} cells express high levels of CX3CR1 and low levels of CXCR1, CXCR3 and CD62L, CCR7 was also expressed. This suggests the ability of these precursor cells to migrate towards inflamed tissues (Fig 3C).

The same analysis was also performed for the first time on Lin-CD56-CD16+CD7- precursor cells. Lin-CD56-CD16+CD7- precursor cells also expressed high levels of CX3CR1 in peripheral blood of cancer patients but CD62L expression was found to be bimodal, suggesting a partial migration of these cells to the lymph nodes as well as in inflamed tissues (Fig 3C).

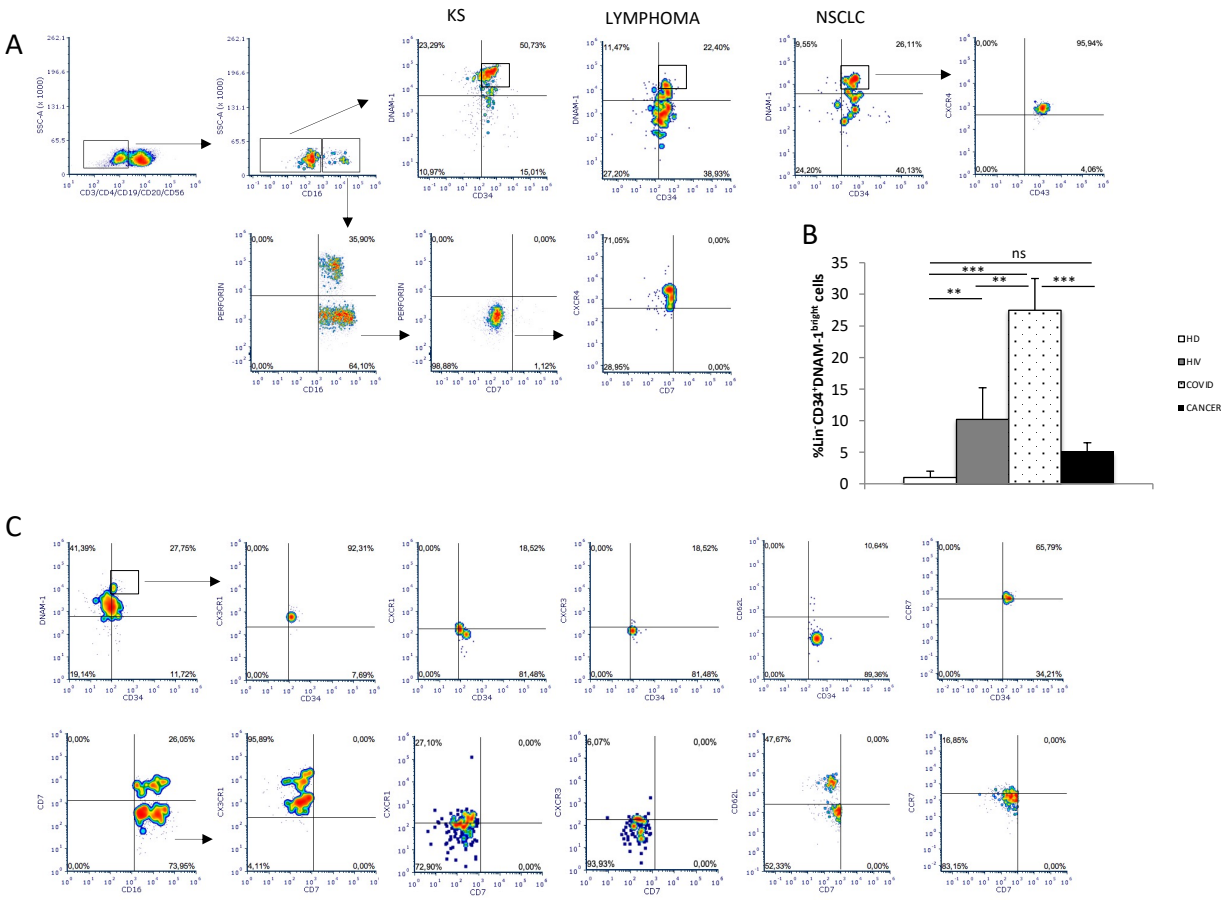


Figure 3: Identification and characterization of Lin−CD34+DNAM-1^{bright}and Lin-CD56-CD16+CD7- cells in peripheral blood of cancer patients by flow cytometry. (**A**) Gating strategy and cytofluorimetric analysis showing the presence of CLPs CXCR4+ in cancer PBMC. Starting from the CD3/CD14/CD19/CD20/CD56 negative gate Lin−CD34+DNAM-1^{bright} precursor cells are shown in the gate of CD16- cells and Lin-CD56-CD16+CD7- precursor cells are shown in the gate of CD16+ cells. Representative of 18 experiments. (**B**) Representation of Lin-CD34+DNAM-1^{bright} precursor cells in different pathological conditions characterized by inflammation compared to HDs. Cumulative circulation of Lin-CD34+DNAM-1^{bright} common lymphocyte precursors in COVID-19 patients (#28) is increased compared to HD (#18), to HIV-1 patients (#15) and to CANCER patients (#18) (COVID vs. HD, ***p<0,0001; COVID vs. HIV, **p=0,0036; COVID vs. CANCER, ***p=0,0001; HIV vs. HD, **p=0,0020; CANCER vs. HD, p=0,1 ns). Histograms shows mean±SD. Mann-Whitney U-test analysis is shown. (**C**) Flow cytometric analysis of CXCR3, CXCR1, CXCR3, CD62L and CCR7 chemokine receptor expression by Lin-CD34+DNAM-1^{bright} and Lin-CD56-CD16+CD7- PBMC. Representative of 2 experiments.

3.2 Increase of Lin-CD34DNAM-1^{bright}CXCR4+ inflammatory lymphoid precursors in peripheral blood of cancer patients following NSCLC therapeutic treatment.

Since our findings showed that the frequency of Lin-CD34+DNAM-1^{bright}CXCR4+ cells in patients with previous disease is not very high when compared with patients with inflammatory and/or infectious conditions (as in the case of HIV and SARS-CoV-2 mentioned before), we wondered whether this is so during the whole course of the disease or therapeutic treatment.

To address this question, we investigated the trend of Lin-CD34+DNAM-1^{bright}CXCR4+ cells in peripheral blood of patients with lung cancer (NSCLC) following therapy (chemotherapy (CT) and/or immunotherapy (IT)) by collecting samples at different time points: before therapy (T0) and after the first cycle of therapy (21 days: T1). As shown by analysis of a representative patient, using the gating strategy shown in Fig. 4A a dramatic increase in Lin-CD34+DNAM-1^{bright} cells is evident 21days after CT (T1) as shown by dot plot representation of DNAM-1 and CD34 expression on Lin- PBMC. (Fig 4A). When considering the entire cohort (18 patients) our analysis revealed a significant increase in percentage of Lin-CD34+DNAM-1^{bright} precursors after the first cycle of therapy compared to before therapy (1,11±1,10 vs. 5,68±2,07; p=0,0072; Fig 4B).

To understand whether this represented an active mobilization of all CD34 cells in general or rather only of inflammatory Lin-CD34+DNAM-1^{bright} cells we further analyzed the two components in the entire cohort.

We could not detect an increase in CD34+ cells (Fig 4C) following CT/IT but an increase in CD34+DNAM-1^{bright} considering total CD34+ cells between T0 and T1 (15,31±10,8 vs. 29,36±19,9; p=0,005; Fig 4D). Data supported by the analysis of Fold Change that demonstrated that CD34+DNAM-1^{bright} cell frequency fold change is significantly higher than the one of classical CD34+ cells (called CD34+DNAM-1^{-}) (p=0,03; Fig 4E).

Overall, therefore, these data indicate that there was a release from the BM in the bloodstream only of inflammatory precursor cells following CT or IT.

Since Lin-CD56-CD16+CD7- cells have been recently characterized to circulate and have developmental potential as inflammatory CLPs (Bozzano et al, JACI, 2021) we also performed the same evaluation for these precursors in peripheral blood of treated patients with NSCLC. No difference in percentage before and after therapy in Lin-CD56-CD16+ and Lin-CD56-CD16+CD7- cell populations were found (Fig 4F, G).

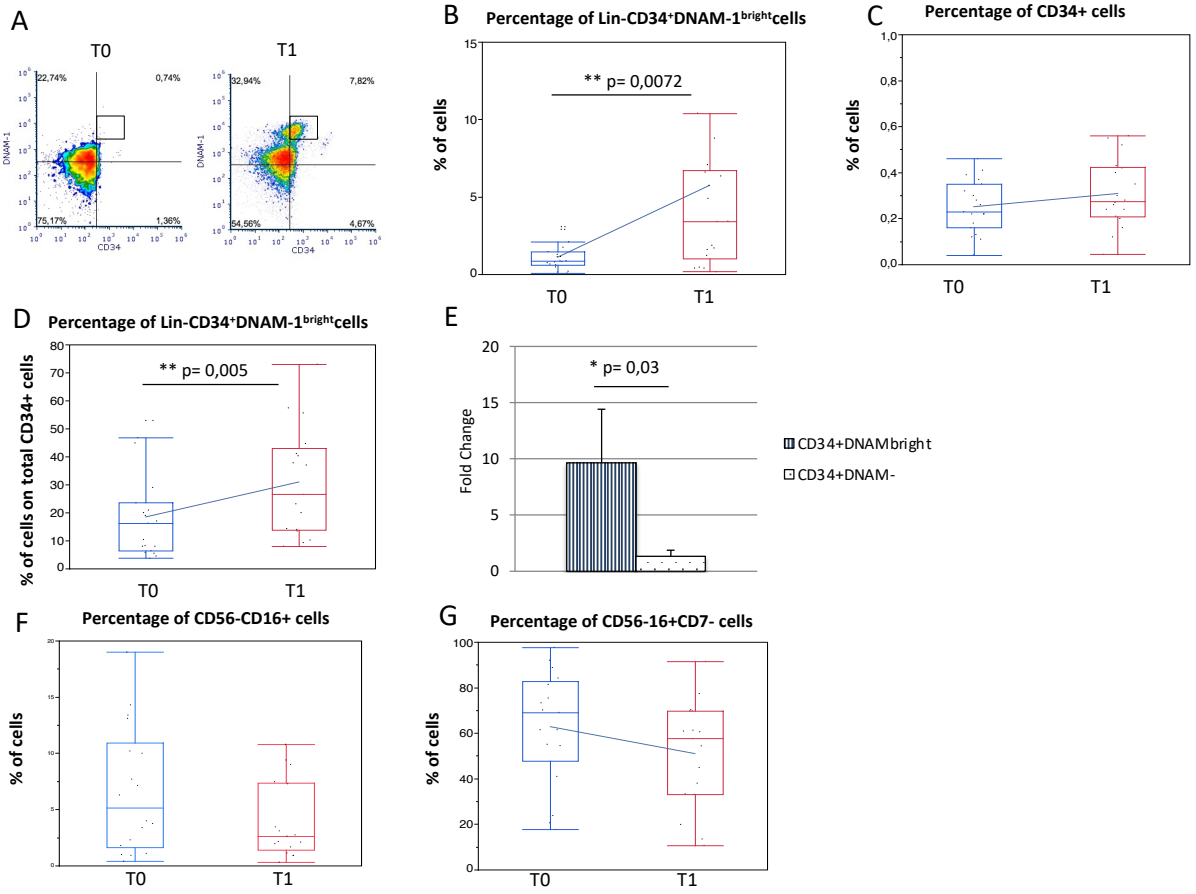


Figure 4: Frequency of Lin-CD34DNAM-1^{bright} and Lin-CD56-CD16+CD7- precursor cells in peripheral blood of cancer patients following NSCLC therapeutic treatment. Experiments represent 2 time points for each 18 cencer patients. (**A**) Dot plots show flow cytometric analysis of Lin-CD34+DNAM-1^{bright} cells in peripheral blood of NSCLC patients before therapy (T0) and after the first cycle of therapy (T1). (**B**) Box-plot representation of the proportion of Lin-CD34+DNAM-1^{bright} in peripheral blood of cancer patients. T1 vs. T0 (**p=0,007). (**C**) Box-plot representation of CD34+ cells percentage in peripheral blood of cancer patients. No differences in percentage of CD34+ cells before and after thepary (T0: 0,23±0,12; T1: 0,31±0,15). Experiments of 2 time points for each of 18 cancer patients. (**D**) Box-plot representation of increase of Lin-CD34+DNAM-1^{bright} cells over total CD34+ cells at T1 (15,3±10,8 vs. 29,3±19,9; **p=0,005). (**E**) Fold change analysis showed increase of Lin-CD34+DNAM-1^{bright} cells compared to virtually no increase of Lin-CD34+DNAM-1- between T0 and T1 (*p=0,03). Histograms indicate the mean±sem. (**F-G**) Box-plot representation of Lin-CD56-CD16+ and Lin-CD56-CD16+CD7- cells frequency. No difference in percentage before vs. after therapy were detected.

3.3 Identification of Lin$^-$CD34+DNAM-1^{bright}CXCR4+ and *Lin-CD34-CD16+CD7- cells in lung tissue samples from NSCLC patients.*

Expression of chemokine receptors on precursor cells in peripheral blood of cancer patients led us study whether they are actually able to enter the tissue involved by cancer growth. Therefore, we searched for these cells in cancer-tissues, and precisely we focused on lung tumor tissues (5 cancer-tissue vs. 5 cancer PB).

DNAM-1 has been initially characterized as an activating receptor and was constitutively expressed by most NK cells, T cells, macrophages, and DCs. (Zingoni A et al, Front. Immunol., 2013). An additional functional activity of DNAM-1 was indeed represented by its involvement in transendothelial cell migration upon engagement by its two ligands, poliovirus receptor (PVR; CD155) and Nectin-2 (CD112) (Reymond N. et al, 2004).

The finding of expression of chemokine receptors on peripheral blood Lin-CD34$^+$DNAM-1^{bright} cells supported the view that these precursor cells can migrate into inflamed tissues and enter the tissues exploiting DNAM-1 expression.

Considering our hypothesis that a DNAM-1 has a role in transendothelial cell migration of Lin-CD34+DNAM-1^{bright}CXCR4+ cells we wondered that it also has a role in migration into inflamed tissues and whether down-expression of DNAM-1 could occur in tissues upon entry.

Comparison of DNAM-1 MFI on Lin-CD34$^+$ cells of peripheral blood and tumor tissue of the same cancer patients revealed MFI with higher intensity in peripheral blood CLPs vs. cancer-tissue CLPs (p=0,03; Fig. 5A). When however comparing MFIr, the difference in DNAM-1 molecule density persisted but was more comparable in blood and in tissue samples. (Fig. 5A)

Since Lin-CD34+DNAM-1^{bright}CXCR4+ cells are also recognized by CXCR4 expression, we additionally verified that the above data corresponded actually to these cells and not to different subsets of CD34+ cells. To this end, CXCR4 expression was studied on cells with different DNAM-1 molecule As shown in

Fig. 5B Lin-CD34+DNAM-1^{bright} cells, Lin-CD34+DNAM-1^{dim}cells, Lin-CD34+DNAM-1^{-} cells were studied. Although Lin-CD34+DNAM-1^{bright} cells have higher CXCR4 intensity, CXCR4 is expressed also on all DNAM-1 positive CD34+ cells while it is not expressed on CD34+DNAM-1- cells (Fig. 5B). These results allowed to conclude that Lin-CD34+DNAM-1^{bright}CXCR4+ cells may enter tumor tissues upon migration from the blood and can be recovered in these tissues.

In view of these data showing that inflammatory precursors actually enter tumor tissue and can be recovered there, we next extensively studied 15 cancer-tissue and 14 uninvolved tissue samples. Accordingly, using the same gating strategy of cytofluorimetric analysis used to identify precursor cell populations in peripheral blood of cancer patients, Lin-CD34+DNAM-1^{bright} cells and Lin-CD56-CD16+CD7- were identified in tumor tissue and nearby tissue called “uninvolved tissue” (Fig 5C).

Both precursor populations expressed CXCR4, a molecule which is expressed on circulating inflammatory precursors exiting the bone marrow.

We next analyzed the frequency of Lin-CD34+DNAM-1^{bright} cells recovered in blood, tumor and uninvolved tissue samples in order to provide an initial assessment of tissue trafficking of these cells. Analysis of their frequencies between involved and uninvolved tissue showed no differences. The percentage of peripheral blood Lin-CD34+DNAM-1^{bright} cells on the contrary was higher compared to tissue Lin-CD34+DNAM-1^{bright} (Fig 5D). In this regard, we hypothesized that a down regulation of DNAM-1 molecule on tissue Lin-CD34+DNAM-1^{bright} cells during cell extravasation and entry may be involved, as shown above. However, this difference could be due at least in part due also to actual differences in tissue entry in addition to differences in surface molecule expression.

To address this point, expression of chemokine receptors was investigated. As shown in Fig.5C we observed a peculiar tissue-enrichment pattern of cells according to chemokine receptor expression, when compared to PB precursors.

Expression of CX3CR1 and low levels of CXCR1, CXCR3, CD62L, and CCR7 in inflammatory precursors emerged from this analysis (Fig 5C).

Lin-CD34+DNAM-1^{bright} cells and Lin-CD56-CD16+CD7- tissue cells express more CX3CR1, when each population is compared with its peripheral blood correspondent. Peripheral blood Lin-CD34+DNAM-1^{bright} cells expressed more CCR7 in addition to CX3CR1. Peripheral blood Lin-CD56-CD16+CD7- cells, on the other hand, mainly express CD62L and CX3CR1 (Fig 5D).

Analysis of surface chemokine receptor expression on CD34+DNAM-1^{bright} and CD56-CD16+CD7- suggested that precursor cells may migrate according to concentration gradients of CXCL9, CXCL10, and CXCL11 which are chemokines known to be present in the tumor microenvironment and are ligands of CXCR3 and CX3CL1, ligand of CX3CR1. Thus a purely quantitative cell trafficking may be excluded by DNAM-1 and chemokine receptor expression analysis.

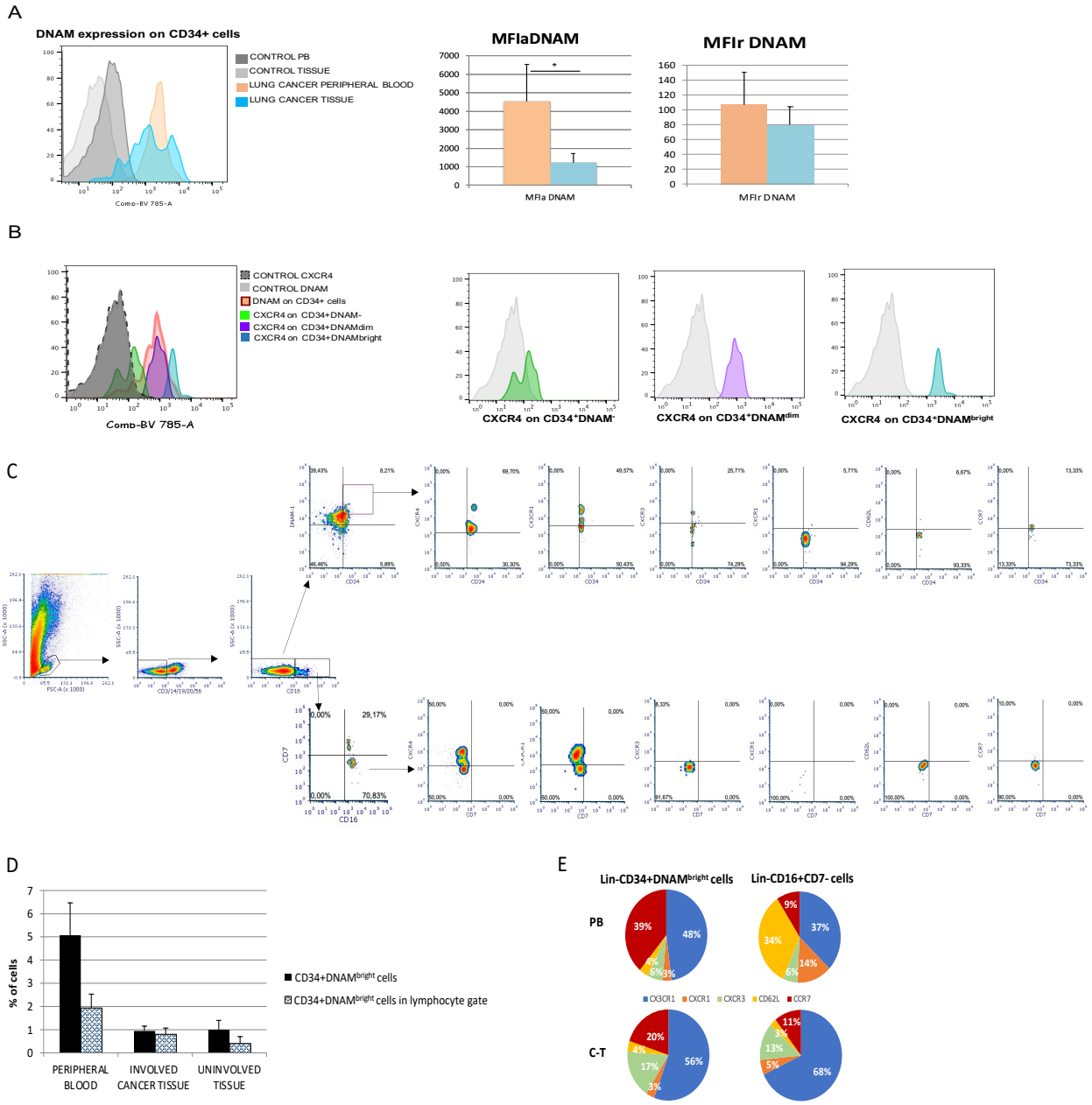


Figure 5: Identification and characterization of Lin−CD34+DNAM-1^{bright} and Lin-CD56-CD16+CD7- cells in lung tissue samples from NSCLC patients by flow cytometry. (**A**) Left: comparison between DNAM-1 mean fluorescence intensity (MFI) of peripheral blood CD34+cells and lung cancer-tissue CD34+ cells. Right: comparison revealed a higher DNAM-1 MFI in peripheral blood CD34+cells (*p=0,0314). No significant differences in MFI ratio. (**B**) MFI of CXCR4 evaluated based on different expression of DNAM-1 molecule in three CD34+ cell populations of cancer-tissue samples: Lin-CD34+DNAM-1^{bright} cells, Lin-CD34+DNAM-1^{dim}cells, Lin-CD34+DNAM-1^{-} cells. (**C**) Gating strategy and cytofluorimetric analysis showing the presence of CXCR4+ CLPs in cancer-tissue of NSCLC patients. Starting from the CD3/CD14/CD19/CD20/CD56 negative gate, Lin−CD34+DNAM-1^{bright} precursor cells are shown in the gate of CD16- cells and Lin-CD56-CD16+CD7- precursor cells are shown in the gate of CD16+ cells. Representative of 15 experiments. (**D**) Percentage of Lin−CD34+DNAM-1^{bright} cells in peripheral blood of cancer patients, lung cancer-tissue and lung uninvolved tissue. Histograms show mean±sem. Representative of 15 cancer-tissue samples, 14 uninvolved tissue, 18 peripheral blood. (**E**) Chemokine receptors pie chart analysis of CLPs derived from peripheral blood and tissue of cancer patients. CX3CR1 expression prevails in tissue CLPs. Peripheral blood Lin−CD34+DNAM-1^{bright} cells equally expressed CX3CR1 and CCR7. Peripheral blood Lin-CD56-CD16+CD7- cells expressed CX3CR1 and CD62L. Representative of two experiments.

3.4 Tumor tissue is enriched in tissue NK cells compared to the healthy counterpart.

The analysis of cancer-tissue samples focused with particular attention on the expression of CD69, CD103 and CD49d (integrin α4) on surface of tissue NK cells infiltrating the tumor given that recent reports suggest that the expression of adhesion molecule markers such as CD69, CD103 and CD49a (integrin α1) are able to discriminate circulating NK cells from tissue-resident NKcell (Björkström et al, 2016). We also know that lung cancer tissue-resident NKcell express high levels of CD69 marker (Carrega et al, 2008, Carrega et al 2017).

A phenotypic analysis based on the expression of the adhesion molecules CD69, CD103, and CD49d was conducted to investigate the tissue nature of NK cells in lung cancerous and involved tissue samples. NK subsets were also explored, specifically $CD56^{bright}$ $CD16^{+/-}$ cells (called $CD56^{bright}$ NK cells), $CD56^{dim}$ $CD16^{+}$ (called $CD56^{dim}$ NK cells) and $CD56^{-}CD16^{+}$ (aka "exhausted") (Fig. 6A).

Analysis of the co-expression of CD69, CD103 and CD49d adhesion molecules showed that the percentage of CD69+CD103+CD49d+ NK cells found in the cancer-tissue was significantly higher than that in the uninvolved tissue counterpart (p=0,0240; Fig 6B). Analysis of NK subsets showed that within $CD56^{dim}$ subset derived from cancer-tissue, the adhesion molecules co-expression was significantly higher when compared with the $CD56^{dim}$ subset found in non-involved tissues from the same donor (p=0.0239; Fig 6C). No differences emerged in terms of CD69, CD103 and CD49d co-expression in $CD56^{bright}$ and CD56-CD16+ NK subsets between tumor- and uninvolved-tissue.

In both tissue types, we found a higher co-expression of integrin and tissue-retention molecules in the $CD56^{bright}$ NK subset, with lower frequencies of CD69, CD103 and CD49d expression in CD56-CD16+ cells (Fig 6D).

According to these findings, CD56-CD16+ cells appeared to be predominantly not tissue-resident cells, but rather recent migrants. This aspect led us to

hypothesize that CD56-CD16+ cells were part of a subset enriched with precursor cells.

The next step was to investigate the presence or absence of adhesion molecules on the cell surface of the CLPs under investigation. CD34+DNAM-1^{bright} and Lin-CD56-CD16+CD7- from peripheral blood and tissue from cancer patients were then compared on the basis of CD69, CD103, and CD49d co-expression.

We found a low rate of co-expression of adhesion molecules on CLPs when compared with Lin-CD56-CD16+CD7+ cells that represented a subset of phenotypically mature cells and not precursor cells (Bozzano et al, JACI, 2021) (Fig 6E).

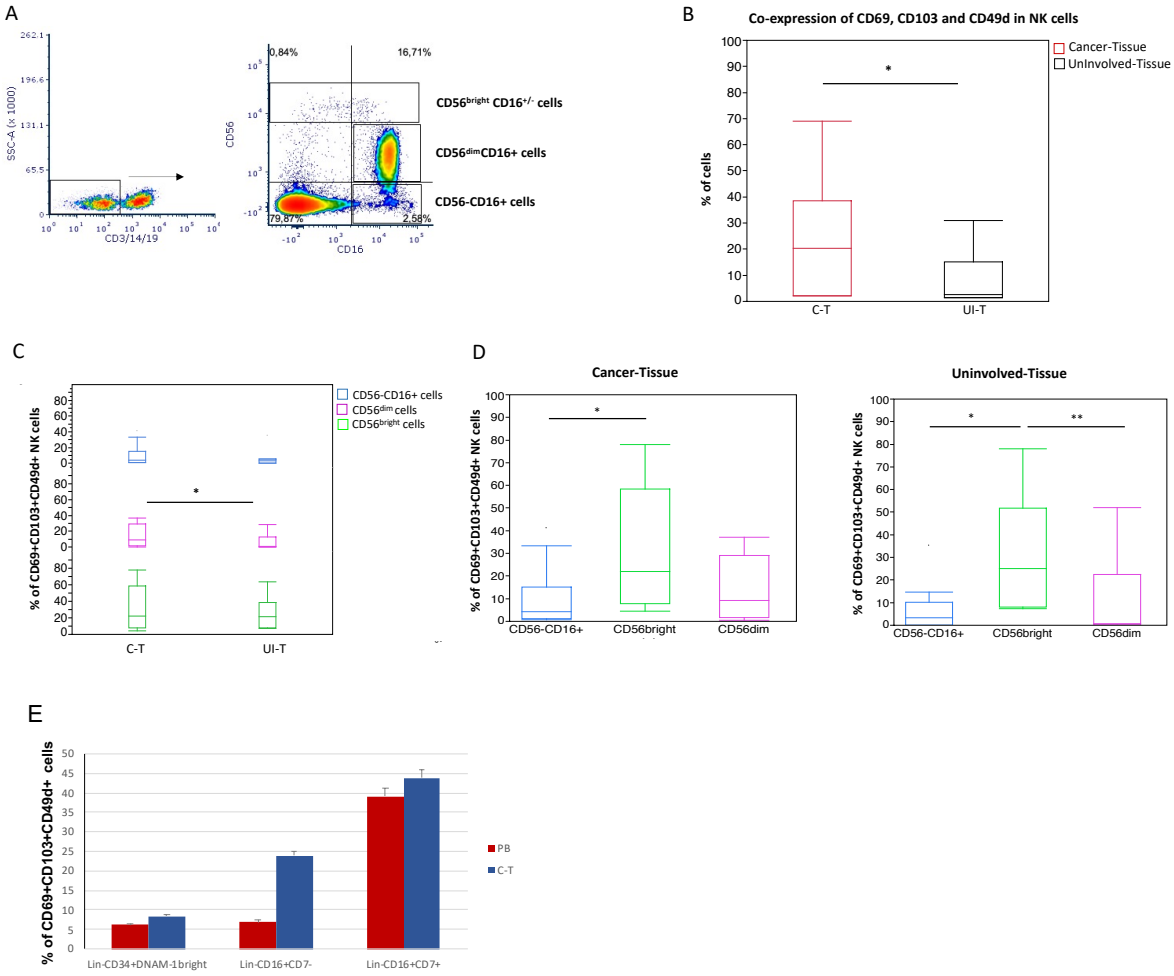


Figure 6: Expression of adhesion molecule on tumor tissue NK cells compared to the uninvolved counterpart. (**A**) Dot plot representation of NK subsets. (**B-D**) Box-plot representation of CD69+CD103+CD49D+ NK cells. Representative of 14 lung cancer-tissue samples and 13 lung uninvolved tissue. Mann-Whitney U test analysis is shown. (B) Co-expression was higher in

cancer-tissue NK vs. uninvolved tissue NK (*p=0,0240). (C) Comparison of co-expressions in NK subsets. In $CD56^{dim}$ cancer-tissue NK cells the co-expression was higher when compared with that of the uninvolved $CD56^{dim}$ subset (*p=0.0239). (D) Cancer-tissue CD56-CD16+ cells presented lower co-expression than $CD56^{bright}$ cells (*p=0,0173). Uninvolved tissue CD56-CD16+ cells had lower co-expression than $CD56^{bright}$ cells (**p=0,0061) and $CD56^{dim}$ cells had lower co-expression than $CD56^{bright}$ cells (*p=0,0273). (**E**) In the histogram is shown a very low co-expression of adhesion molecules of CLPs compared to the co-expression of phenotypically mature subset of Lin-CD56-CD16+CD7+.

3.5 In vitro culture of Lin-CD34+DNAM-1^{bright}CXCR4+ and Lin-CD56-CD16+ precursor cells derived from neoplastic, non-neoplastic tissue samples and peripheral blood of cancer patients.

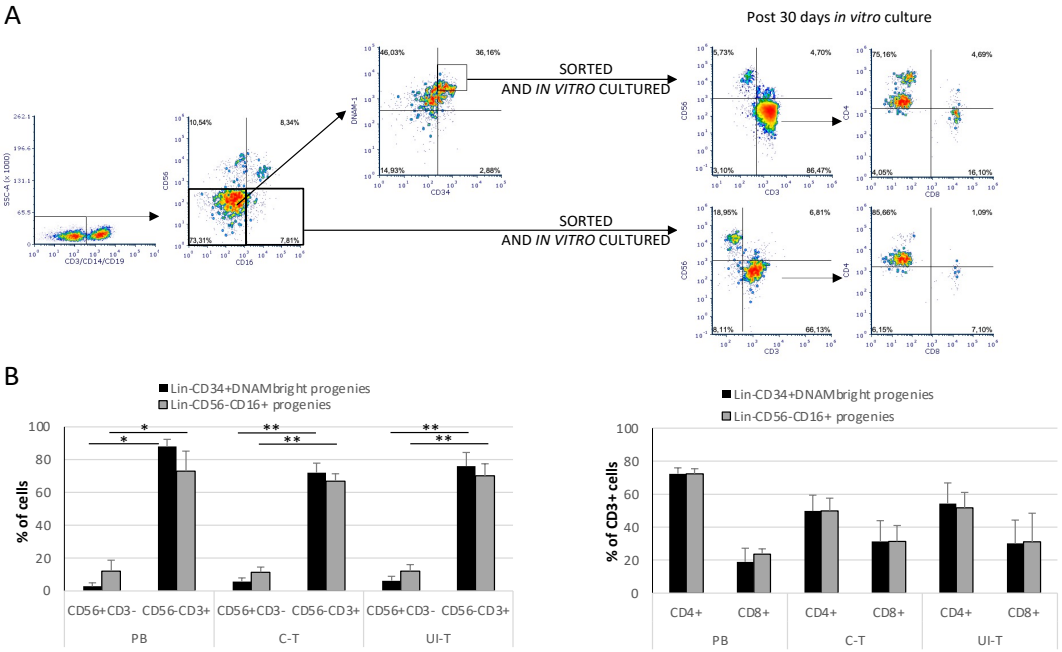
Cell sorting experiments, conducted on cancerous and uninvolved tissue (C-T and UI-T) samples and cancer peripheral blood (PB), allowed the isolation of highly purified Lin-CD34+DNAM-1^{bright}CXCR4+ and Lin-CD56-CD16+ cells. We performed limiting dilution experiments of these cells and clonal growth was detected in 18 experiments (7 lung cancer-tissue, 7 lung uninvolved tissue and 4 cancer peripheral blood (NSCLC patients)). These cells cultured *in vitro* in complete medium enriched with FLT3, SCF, rhIL-7, rhIL-15, feeder PBMC for 30±1,5 days (mean±ds) days gave rise to progenies composed of cells of the lymphoid lineage that were CD56+CD3-(NK progeny), CD56-CD3+ (T progeny) and it was also possible to distinguish the CD3+CD4+ (CD4+) and CD3+CD8+ (CD8+) population within the T progeny (Fig. 7A).

NK progenies derived *in vitro* from Lin-CD34+DNAM-1^{bright} of C-T, UI-T and PB of cancer patients represent a small percentage (5,7±2,2%; 6,3±2,6%;

2,9±2,1% respectively (mean±sem)) when compared to T-cell progenies. Nevertheless, it was possible to detect the expression of inhibitory receptors KIRs, NKG2A (more in PB), LIR (more in PB) and activating receptors such as NCRs with high frequencies of Nkp30 expression and lower frequencies of Nkp46, DNAM-1, highly expression and NKG2D (lesser in PB). NK cell progenies, expressed also HLADR and CD57 (more in PB) molecules and to a lesser extent also NKG2C. these findings on the phenotype of NK cell progenies are in line with the original description of these progenies (Bozzano et al, Nat.Comm 2015, JACI 2021). NK cell progenies derived *in vitro* from purified Lin-CD56-CD16+ cells also represented a minor proportion of progenies in all samples. In these cases the NK cell phenotype showed high expression of Nkp30 and DNAM-1, lower expression of NKG2C and expression of Nkp46, NKG2D, HLADR (very low in UI-T samples), CD57 (low in UI-T and PB samples), KIR and NKG2A (Fig 7C and D). These findings were in line with previous data, and the comparatively lower expression of NKG2C observed here could be ascribed to the absence of HLA-E+ cells within the stimulating feeder in the present culture conditions.
In view of the predominant growth of T cell progenies *in vitro* from inflammatory precursor purified in tumor patients, we concentrated the activity on a characterization of these T progenies.

In vitro cultures from purified Lin-CD34+DNAM-1^{bright} cell precursors genereted predominantly T cells (CD56-CD3+) from cancer-tissue, uninvolved tissue and peripheral blood (72±5,8%; 76,2±5,8%; 88±4,3%; p=0,0022; p=0,0051; p=0,0304 respectively (mean±sem)). CD4+ and CD8+ cell populations were present in all progenies with a prevalence, although not significant, of CD4+ cells compared to CD8+ cells. Analysis of these cells match the data obtained among progenies derived from Lin-CD56-CD16+ cells (Fig 7B).
T progenies derived *in vitro* from Lin-CD34+DNAM-1^{bright} showed high expression of DNAM-1 and Nkp30, Nkp46 to a lesser extent and were also

expressed HLADR, NKG2D, KIR and NKG2A (lesser in PB). T progeny *in vitro* derived from Lin-CD56-CD16+ cells expressed high level of DNAM-1. In these cells were found the expression also of Nkp30, Nkp46, HLADR, NKG2D (slightly less in PB), KIR and NKG2A (lesser in PB) (Fig 7C and D).



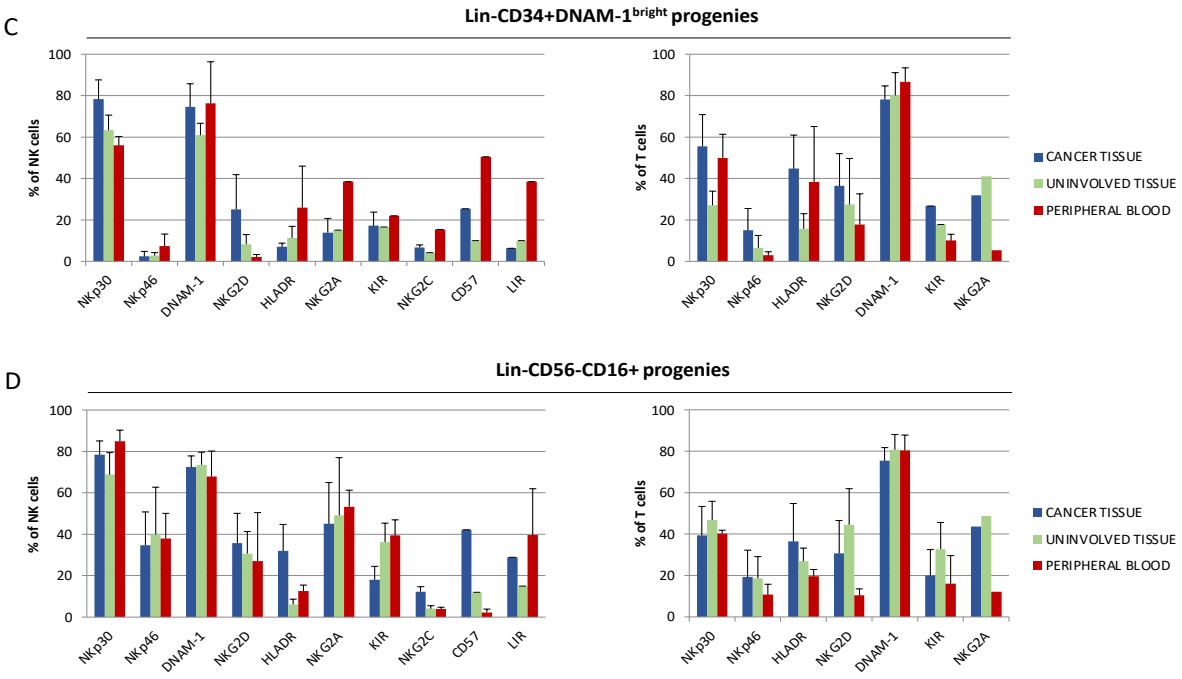


Figure 7: *In vitro* culture of highly purified Lin-CD34+DNAM-1^{bright} and Lin-CD56-CD16+ cells from peripheral blood (PB), cancer tissue (C-T) and uninvolved tissue (UI-T) generates NK and T progenies. (**A**) Flow cytometric purification strategy and culture of Lin-CD34+DNAM-1^{bright} and Lin-CD56-CD16+ cells isolated from PB, C-T and UI-T. (**B**) In vitro cultures from highly purified Lin-CD34+DNAM-1^{bright} and Lin-CD56-CD16+ cells (18 sorts for each of the two population). Histograms indicate the proportion of progeny cells (shown as mean±sem) as evaluated by flow cytometry. (**C**-**D**) Cumulative expression of given markers by NK and T cell progenies from highly purified PB, C-T and UI-T CLPs. Histograms (mean ±sem) show 36 different cultures from 18 different sorts/sample type.

3.6 Characterization of in vitro derived T progenies from CLP cells derived from cancer and uninvolved tissue and peripheral blood of cancer patients.

After analyzing surface molecule expression of T progenies, the next step was to investigate the type of TCR ($\alpha\beta$ or $\gamma\delta$) expressed and to provide a nearer characterization of CD4+ cell population found in progenies *in vitro* grown from Lin-CD34+DNAM-1^{bright}CXCR4+ and Lin-CD56-CD16+ cells.

Cytofluorimetric analysis showed that T progenies expressed TCR $\alpha\beta$ (Fig 8A) both on T progenies *in vitro* derived from Lin-CD34+DNAM-1^{bright} precursor cells and on T progenies *in vitro* derived from Lin-CD56-CD16+ cells, in cancerous, uninvolved tissue and cancer peripheral blood (Fig 8B).

CD4+ T cells were represented the majority of T progenies generated *in vitro* from CLPs. To provide a more in depth evaluation of the T helper subset distribution in these progenies by cytofluorimetric analysis we used a previously validated gating strategy (Sallusto, Annu. Rev. Immunology, 2016) that revealed T helper subsets (Fig 8C).

Analysis of CD4+ T cell progenies showed a bimodal expression of CD45RA. A higher frequency of CD4+CD45RA- cells could be noted, with a smaller but consistent frequency of CD4+CD45RA+ in all progenies analyzed (Fig 8D).

We next focused on the CD4+CD45RA- progenies and evaluated their T helper attribute, into Th1, Th1*, Th2 and Th17 based on the expression of CXCR3, CCR4, CCR6 and CCR10 (Fig 8C).

CD4+ subpopulations of T progenies *in vitro* derived from C-T Lin-CD34+DNAM-1^{bright} cells were Th1, Th1*, Th2, Th17 (5,1±1,1; 8,9±6,8; 14,2±9,9; 10,1±5,3 respectively (mean±sem) and from C-T Lin-CD56-CD16+ cells (10,8±3,2; 10,7±7,6; 9,5±7,3; 1,4±1 respectively (mean±sem)).

Subpopulations of CD4+ progenies *in vitro* derived from UI-T Lin-CD34+DNAM-1^{bright} cells were Th1, Th1*, Th2, Th17 (6,6±1,9; 9,6±5,9; 6,1±2,9; 2,3±2,1 respectively (mean±sem)) and from UI-T Lin-CD56-CD16+ cells were mainly Th1 and Th1* (16,8±11,2; 8,5±7,2 respectively

(mean±sem)) but also Th2 and Th17 (1,5±0,5; 3,1±2,7 respectively (mean±sem)) were detected.

Finally CD4+ T helper subsets of T progenies *in vitro* derived from cancer PB Lin-CD34+DNAM-1^{bright} cells showed phenotypically likely subsets of Th1, Th1*, Th2 and Th17 (7,8±2,3; 10,7±3,2; 4,5±2,7; 3,6±0,2 respectively (mean±sem)) and from cancer PB Lin-CD56-CD16+ cells T helper subsets were Th1, Th1*, Th2 (12,5±3,8; 10,7±5,3; 7±2,2 respectively (mean±sem)) and to a lesser extent Th17 (2,3±1,5) (Fig 8E).

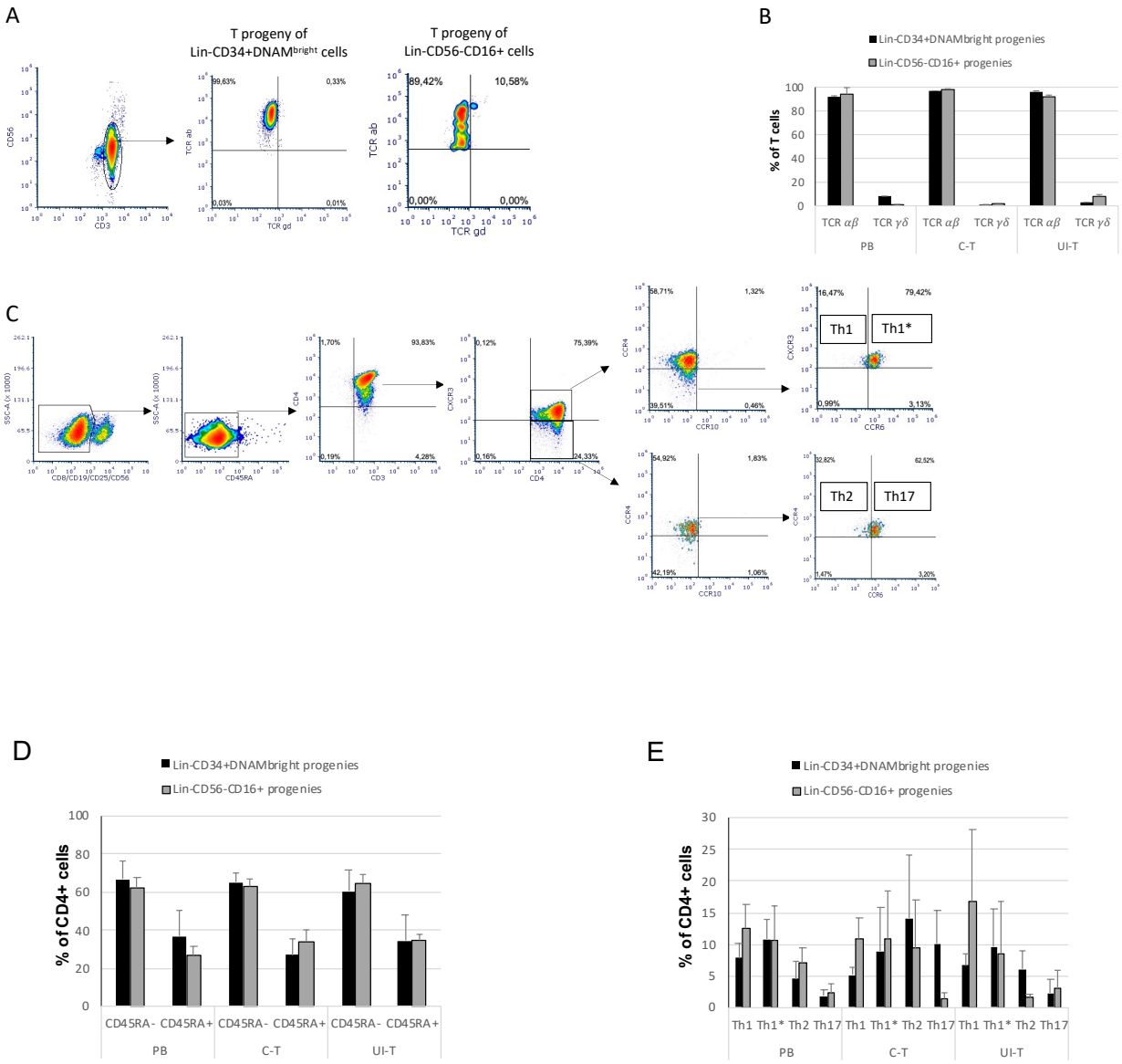


Figure 8: Characterization of *in vitro* derived T progenies from CLP cells derived from tissue samples and peripheral blood of cancer patients. PB (peripheral blood); C-T (cancer tissue); UI-T (uninvolved tissue). (**A**) Flow cytometric analysis of PB, C-T and UI-T Lin-CD34+DNAM-1^{bright} and Lin-CD56-CD16+-derived T cell progeny grown *in vitro* for the expression of TCR molecules. CD3^{+}CD56-gated cells were studied for the expression of TCR $\alpha\beta$ or $\gamma\delta$ after 30 days from purification of precursors. Representative of 2 experiments. (**B**) In histograms shown frequencies of TCR $\alpha\beta$ or $\gamma\delta$ in T-cell progenies *in* derived from CLPs. Histograms indicate mean±sem. (**C**) Flow cytometric analysis of T helper subsets Th1, Th1*, Th2 and Th17. (**D**) CD4+ T-cell progenies derived from Lin-CD34+DNAM-1^{bright} and Lin-CD56-CD16+ cells contain both CD45RA- and CD45RA+ cells. (**E**) Frequancy of Th CD4+ progenies *in vitro* derived from cancer PB, C-T and UI-T Lin-CD34+DNAM-1^{bright} and Lin-CD56-CD16+ cells.

3.7 Functional activity of T progenies in vitro derived progenies from tissue and peripheral blood CLP cells.

To evaluate the functionality of T progenies derived *in vitro* from their precursor cells, a cytotoxicity assay was performed with a lung cancer target cell line A549 and with mouse mastocytoma target cell line P815 with a effector- target ratio of 1:1 for 6 hours. The percentage of mortality of target cells is based on the percentage of PKH+TOPRO+ double positive cells because target cells labeled with PKH and TOPRO+ (which was a nuclear counterstain and dead cell indicator) corresponded to cells killed by effector cells.

Experiments showed that T progenies *in vitro* derived from tissue Lin-CD34+DNAM-1^{bright} cells were functional when the A549 target cell line was used as a target for cytotoxic activity (Fig 9A). They showed more cytotoxic activity against A549 target cell line than T progenies *in vitro* derived from tissue Lin-CD56-CD16+ cells. T progenies *in vitro* derived from peripheral blood CLPs were also functional but in lower percentage (Fig 9B).

In experiments using a redirected killing assay, FcγR^{+} P815 target cell lysis was detected in the presence of aCD3 and aNKp30 mAbs, in the case of T progenies *in vitro* derived from C-T Lin-CD34+DNAM-1^{bright} cells (Fig. 9C and D).

Cytokine Production Assay reveals in the culture medium of in vitro derived T progenies the presence of the cytokines TNF-α, IFN-γ, IL-2, IL-5, IL-9, IL-13, IL-17A, IL-10 when stimulated with PMA and ionomycin. This finding matches with the characterization of the T helper subpopulation described previously (Fig. 9E) and with the spectrum produced by T helper cells described in the work of Sallusto in 2016. Th1 and Th1* cells produce IFN-γ; Th2 cells produce IL-4, IL-5 and IL-13; Th17 cells produce IL-17A, IL- 17F (Sallusto, 2016). Cytokine standards table (Supplementary 1).

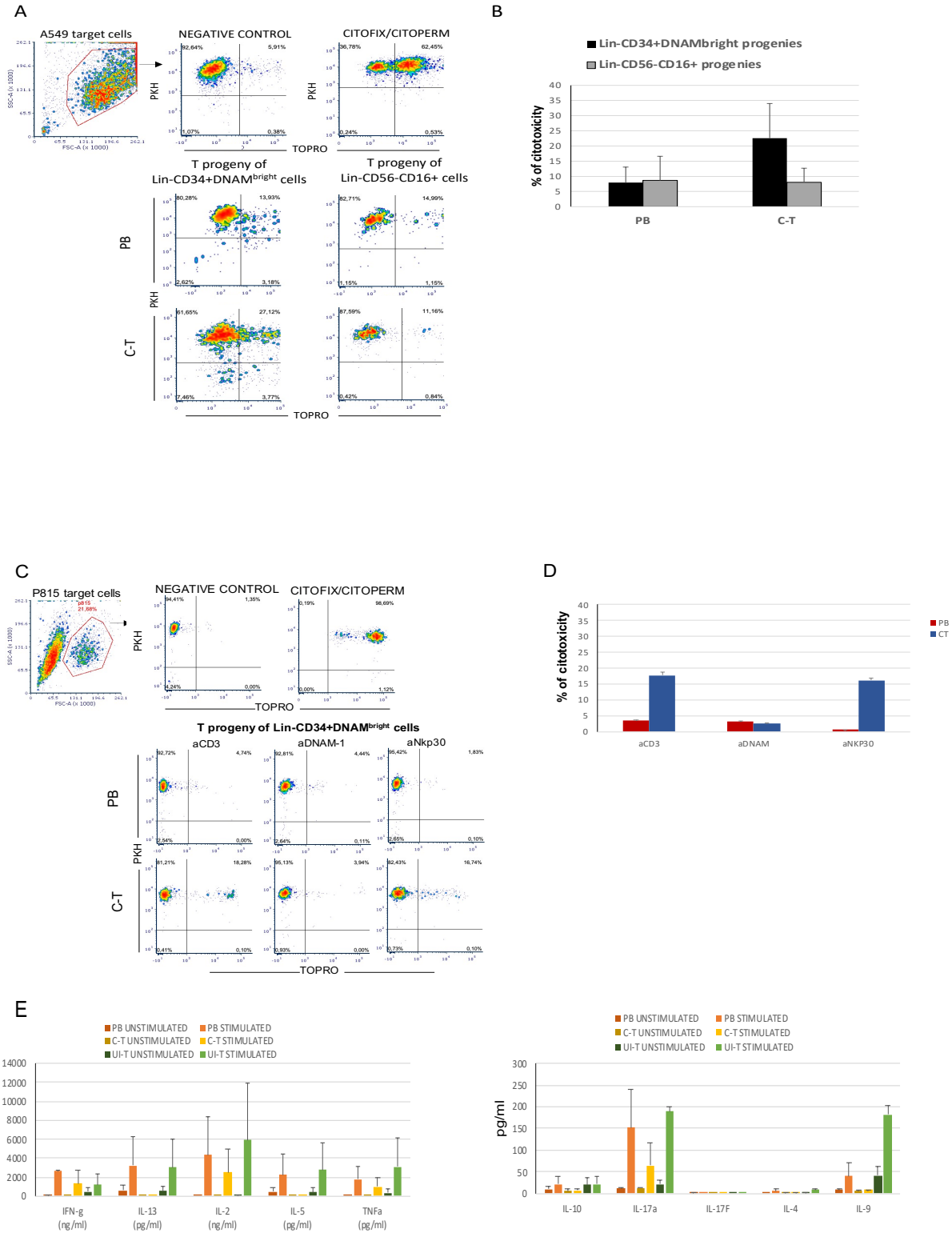


Figure 9: Functional activity of T-cell progenies derived *in vitro* from CLP cells isolated from cancer tissue (C-T), UI-T (uninvolved tissue) and peripheral blood (PB) CLP cells; cytotoxicity assay against A459 and P815 target cells at 6 hours with E:T ratio 1:1 and cytokine production assay (**A**) Flow cytometric cytotoxicity assay of T-cell progenies against A549 cell line targets. (**B**) Histograms indicate the specific cytotoxic activity. (**C**) Flow cytometric cytotoxicity assay of T-cell progenies derived *in vitro* from PB and C-T Lin-CD34+DNAM-1^{bright} against P815 target cell line. Redirected triggering was obtained by addition of aCD3, aDNAM and aNkp30 mAbs. (**D**) Cytotoxic activity of C-T Lin-CD34+DNAM-1^{bright} T progeny

against P815 target cells in the presence of aCD3 and aNkp30 mAbs. PB and C-T Lin-CD3 4+DNAM-1^{bright} T progeny were poorly cytotoxic. Mean+sem. duplicate wells. (**E**) Cytokine Production in the culture medium of in vitro derived T progenies cultured either in medium or with PMA+ionomycin stimulation. Mean+S.D. of 2 different populations from different donors.

4. DISCUSSION

Lin-CD34+DNAM-1^{bright}CXCR4+ and Lin-CD56-CD16+CD7-CXCR4+ precursor cells, known also as “inflammatory precursors”, have been so far identified and characterized in the peripheral blood of patients with chronic inflammation including HIV-1 infections, HCV chronic infections and non-infectious inflammation including COPD and PAPA syndrome. In the present work we provide evidence that they are indeed present also in the peripheral blood of patients being treated for tumor, and most importantly that they can be identified, characterized and grown from tumor tissues of the same patients.

We here observed that Lin-CD34+DNAM-1^{bright}CXCR4+ precursors are not increased in peripheral blood of patients with NSCLC stage 4 or 5 before the start of CT or IT or CT+IT.

The absence of an increase in Lin-CD34+DNAM-1^{bright}CXCR4+ cells frequency in NSCLC patients before therapy compared to HDs is in line with the notion that counter-inflammatory measures are taken by tumors or that a successful tumor growth does not provide systemic inflammatory stimuli. In the vast majority of established tumors, the presence of tumor-infiltrating lymphocytes is insufficient for curtailing tumor growth. This happens because in these conditions the infiltrating lymphocytes trigger a process of immunosurveillance called immunoediting (Dunn et al., 2004, Smyth et al., 2006). During this process, the balance between antitumor and tumor-promoting immunity may be tilted in favor of tumor growth, in which the tumor cell modifies its repertoire of tumor antigens toward lower immunogenicity and also reshapes the tumor microenvironment to become immunosuppressive.

Understanding where and how immune cells are activated and recruited to the tumor site is a critical issue. Dieu-Nosjean et al. found in many of their NSCLC tumors that immune cells were organized in tertiary lymphoid structures (TLS), which are otherwise absent in the non-tumoral lung. Using an indirect marker for these structures, the authors found that adaptive

immunity in lung cancers can be initiated independent of secondary lymphoid organs. Moreover, the authors found that these tertiary lymphoid structures called tumor-induced bronchus-associated lymphoid tissue were highly associated with a favorable outcome (Dieu-Nosjean MC et al, 2008; Bremnes RM et al, 2011). Furthermore, in De Pasquale et al. Report is explained that formation of TLS is a relevant mechanism underlying the progression of the disease progression. It is hypothesized that presentation of autoantigens may favourably occur in TLS and trigger a detrimental T cell response, resulting in central nervous system (CNS) damage (De Pasquale, 2021).

Lin-CD34+DNAM-1^{bright}CXCR4+ increase in the peripheral blood can be clearly monitored in NSCLC patients undergoing treatment and appears after both CT or CT+IT after 21days from treatment initiation. Indeed, a strong tumor-associated inflammatory response can be initiated by cancer therapy (Grivennikov et al, 2010). Radiation and chemotherapy cause massive necrotic death of cancer cells and surrounding tissues, which in turn triggers an inflammatory reaction analogous to a wound-healing response (Zong and Thompson, 2006). Higher levels of inflammatory factors are observed in patients with cancer during chemotherapy. In this regard, for example, it has been reported that during chemotherapy in breast cancer patients, increased plasma levels of inflammatory cytokines IL-6 and IL-8 can be observed (Pusztai L et al, 2004). Inflammation influences the host immune response to tumors and can be exploited in cancer immunotherapy (Dougan and Dranoff, 2009) and to augment the response to chemotherapy (Zitvogel et al., 2008).

Accordingly, the present observation that an increase in circulating inflammatory Lin-CD34+DNAM-1^{bright}CXCR4+ cells can be detected only following chemotherapy is in line with the above observations showing that CT may induce local in the tumor area with subsequent systemic inflammation recruitment from the BM of precursors. In addition, the present demonstration that the increase in CD34+ precursors in PBMC is significantly associated with CD34+DNAM^{bright} cells and not to conventional CD34+DNAM- cells,

indicates that these inflammatory cells may be instrumental in the immune response against the tumor that is triggered by chemotherapy.

As shown previously, inflammatory CLPs (Lin-CD34+DNAM-1^{bright}CXCR4+) express CXCR4 and DNAM-1 to high levels (Bozzano et al, 2015). Inflammatory stimuli are thought to regulate CXCL12 expression (Ueda et al, 2004) and loss of CXCR4 leading to lymphocyte release from BM sinusoids (Beck et al, 2014).

Thus, CXCR4 expression confirms their origin from the BM upon inflammatory stimuli generated here after CT. Expression of DNAM-1 on CLP exiting the BM may have a relevant functional role in the fate of these cells. Indeed, DNAM-1 involvement in transendothelial cell migration is quite relevant, in addition to its more commonly known function as a triggering receptor on NK and Tcells.

Its first characterization as DNAX accessory molecule-1 (DNAM-1) occurred in 1996 by Shibuya et al. in peripheral blood cytotoxic T lymphocytes (CTLs). In humans, DNAM-1 is constitutively expressed on a subset of B lymphocytes and on all T lymphocytes, NK cells, monocytes, and platelets (Shibuya A et al, 1996). Its ligands are represented by poliovirus receptor (PVR; CD155) and Nectin-2 (CD112).

Tumor target cells frequently coexpress two cellular DNAM-1 ligands, PVR and Nectin-2, whereas they display low levels of HLA class I molecules. Upon engagement by these ligands, DNAM-1 induces NK cell–mediated cytotoxicity (Bottino C et al, 2003).

In addition to its cytotoxicity triggering function it has been shown that DNAM-1 interaction with PVR and/or Nectin-2 expressed on endothelial cells may contribute to cellular transmigration through primary vascular endothelial cells (Kojima H et al, 2003). Leukocytes escape the blood circulation by interacting with the endothelial cells of the vessel walls from blood to secondary lymphoid organs or underlying tissues through a multistep adhesion molecule cascade involving DNAM-1 (Reymond N et al, 2004) PECAM (CD31) (Newman et al, 1990) and CD99 (Schenkel et al, 2002) (Muller WA et al,

2015). In this cascade, DNAM-1 represents the “central gate” to cellular transmigration across tight junctions in epithelial cells by forming a molecular complex of adhesion molecules with PVR and Nectin-2.

Accordingly, the high molecule density of DNAM-1expressed on Lin-CD34+DNAM-1^{bright}CXCR4+ cells are in line with its involvement in precursor cell migration from bone marrow during inflammation and their potential for entry into peripheral tissues.

Travel of cells in blood and their trafficking towards tissues is regulated by several factors including local chemokine production. Accordingly, chemokine receptor expression represents a targeted mobility mechanism for cells of the immune system, including CLPs exiting the BM. The type of chemokine receptor expressed on the presently studied precursors which includes CX3CR1, CXCR1, CXCR3, CCR7 and CD62L indicates that after mobilization, they are poised to migrate towards tissues expressing the cognate chemokine(s).

In this respect, previous work showed that peripheral blood inflammatory Lin-CD34+DNAM-1^{bright}CXCR4+ precursors from HIV-1 or HCV patients express CXCR1 and CX3CR1with higher frequency compared to “canonical” DNAM-CD34+ cells (Bozzano et al, 2015).

Here we confirm the also also in tumor patients Lin-CD34+DNAM-1^{bright}CXCR4+ cells circulating in peripheral blood after CT express to a relevant extent CXCR1, CXCR3 and CX3CR1 in addition to CCR7.

Accordingly, it could be assumed that a portion of cells that express CX3CR1, migrates into cancer-tissues driven by its ligand CX3CL1 (or Fractalkine) which is commonly expressed in the tumor microenvironment and is capable of inducing cell migration in both tumor cells and immune cells with anti-tumor activity (Rivas-Fuentes S et al, 2021). Similar to patient with chronic infections, another fraction of PB inflammatory CLP cells in tumor patients appear to be targeted to lymph nodes, where stromal cells highly express CCR7 ligands (CCL19 and CCL21), (Randolph G J et al, 2005) (Sánchez-Sánchez N et al, 2006).

Recent work characterizing Lin-CD56-CD16+CD7-CXCR4+ cells as CLP could not provide evidences of targeted direction of these precursors by chemokine receptor analysis (Bozzano et al, JACI 2021). Here, we provide evidence that Lin-CD56-CD16+CD7-CXCR4+ cells, differ from Lin-CD34+DNAM-1^{bright}CXCR4+ cells, since they express mainly CX3CR1 and CD62L. Interestingly, CD62L plays an essential role in lymphocyte homing to both lymphoid tissues and sites of inflammation (Yang S et al, 2011) and could contribute to tumor tissue trafficking of CLPs. It can be therefore hypothesized that Lin-CD56-CD16+CD7-CXCR4+ CLPs are poised to partly different tissues and areas compared to CD34+DNAM-1^{bright}CXCR4+.

In view of these findings that clearly suggested a targeted trajectory into inflamed tissues, we verified whether inflammatory CLPs detected in PB of tumor patients could be actually recovered from tumor tissues.

As shown by tissue processing/extraction experiments, we were able to identify both Lin-CD34+DNAM-1^{bright}CXCR4+ and Lin-CD56-CD16+CD7-CXCR4+ cells in tumor tissues thus showing conclusively that inflammatory CLPs indeed enter into tumor tissue. To verify whether their frequencies in blood and tissue were compatible with a targeted migration, we next studied the relative frequencies and their chemokine receptor expression.

Frequencies in tissues are in the same order of magnitude compared to PB thus signaling a relevant entry of these cells into inflamed tissues. The percentage of CLPs found in tissues is slightly but not significantly lower and therefore supported the hypothesis that only a part of inflammatory CLPs in peripheral blood enters into tumor tissue. To better understand the reason for the lower frequency of CLPs in tissues, we investigated the surface expression of chemokine receptors.

Indeed, tissue-trafficking Lin-CD34+DNAM-1^{bright}CXCR4+ express considerable amounts of CX3CR1, the receptor for fractalkine, and CXCR3 which binds CXCL9, CXCL10 and CXCL11, while a decreased frequency of CCR7 expression is present, contrary to what is observed in PB. Similarly, tissue-trafficking Lin-CD56-CD16+CD7-CXCR4+ cells are predominantly

expressing CX3CR1 and CXCR3, while a considerable fraction of their PB counterparts also express CD62L. In both instances this represents a considerable relative enrichment when compared to their counterparts circulating in the PB, suggesting that these recently trafficking CLP follow specific trajectories of extravasation into tumor tissue while a different fraction of their circulating counterpart is directed towards different tissues, such as lymph nodes.

Thus, these results contribute to explain how the frequency of inflammatory CLP may be lower in tissues compared to PBMC and the mechanisms regulating tissue entry.

Analysis of integrin expression or of expression of tissue-retention molecules on cells allows to clarify whether trafficking cells are recent migrants to tissues (integrin-neg or integrin-low) or whether they may be considered tissue resident cells. Tissue residency has been defined for NK cells in to a certain extent in some tissues.

Markers such as CD69, CD103 and CD49a, can be useful for the identification and isolation of human tissue-resident (tr) NK cells, in most cases with $CD56^{bright}$ NK cell subset prevalence, and are functionally involved in retaining also other lymphocytes in tissues (Castriconi et al, 2018). In human secondary lymphoid tissues, NK cells are characterized by co-expression of CD69 and CXCR6 and high expression of CD54 (ICAM-1) (Lugthart et al, 2016). Liver trNK cells are $CD56^{bright}$ NK cells characterized by higher level of EOMES transcription factor, expression of CXCR6 and CD69 as well as CCR5 but absence of CD62L and CCR7 (Melsen et al, 2016). In lung tissues about 80% of NK cells populating these organs belongs to the $CD56^{dim}$ fraction and express high levels of CD69 (Carrega et al, 2008). In addition, in a study of our group, a significant circulation of CD69+CD49d+CD103+ NK cells were found in peripheral blood of SARS-CoV-2 patients, signaling an extensive trafficking derangement from lung tissues during symptomatic SARS-CoV-2 infection (Bozzano et al, PLoS Pathog. 2021).

In the spectrum of tissue-resident lymphocytes tissue-resident memory T (T_{RM}) cells have been characterized. In this regard, most findings on T_{RM} cells identify CD8+ T_{RM} cells (Sun et al, 2019) (Melsen et al, 2018) while CD4+ T cells have been poorly characterized and may represent a fraction of resent migrants during acute pathology (Chen et al, 2010). Coexpression of CD69 and CD103 is observed on the majority of TRM cells, however, some T_{RM} CD8+Tcells fall into other phenotypic categories (Mueller et al, 2016). For example, although CD103+ T_{RM} cells are generally found in the small intestine and gut, both CD103+ and CD103− T_{RM} cells can be found in the kidney (Casey et al, 2012; Ma et al, 2017). In addition, CD69+CD103+CXCR6+CXCR3+ T_{RM} cells have been identified in the liver, where CXCR6 seems to represent a pivotal tissue retention receptor for both T and NK cells.

In the present work, analysis of chemokine or tissue-retention molecule expression on both Lin-CD34+DNAM-1^{bright}CXCR4+ and Lin-CD56-CD16+CD7-CXCR4+ inflammatory CLPs showed that these precursors substantially do not express CD69, CD49d and CD103. This confirms the notion that they are recent migrants into the tissues. This is in line also with their chemokine expression pattern discussed above.

The demonstration of a specific trafficking of inflammatory precursors as recent migrants in tumor tissue from PBMC in cancer patients needed to be corroborated by their functional analysis and developmental potential. In order to characterize the developmental trajectories of the two main subsets of inflammatory CLPs in cancer patients, we cultured them after high purity sorting and limiting dilution analysis (LDA) to avoid the bias of carryover of other cells. Culture conditions were standard for CLPs that were previously characterized and included FLT3, SCF, IL7 and IL15 (Bozzano et al, 2015; Bozzano et al, JACI 2021). Parallel cultures were performed from cells purified from tissues and PBMC in order to compare progenies and to verify their comparative phenotype.

We were able to show that Lin-CD34+DNAM-1^{bright}CXCR4+ and Lin-CD56-CD16+CD7-CXCR4+ precursor cells purified from tumor tissues and from peripheral blood of cancer patients, give origin to lymphoid progenies. No differences were observed in progenies from PB and tissue-derived CLPs, thus showing their functional identity.

In line with previous work (Bozzano et al, 2015; Bozzano et al, JACI 2021), inflammatory CLPs did not give rise to myelomonocytic lineage as is usually the case from CD34+DNAM- precursors (Sivori et al, 2003). Interestingly, different from inflammatory CLPs derived from patients with chronic infection (Bozzano et al, 2015; Bozzano et al, JACI 2021) such as HIV-1 or HCV, the T-cell progenies from inflammatory CLPs in patients with cancer predominated in all experiments both from circulating as well as from tissue-derived CLPs. A fraction of NK cell progenies was always detected from LDA and bulk cultures, however their frequency was always $\leq$10% of growing cells, and also a minor fraction of CD56+CD3+ cells (<5%). There is so far no explanation for this apparent disease-associated functional skewing, namely a NK cell prevalence in progenies from inflammatory CLPs in infection-related inflammation vs. T-cell progeny prevalence in CLPs from cancer patients. A hypothesis could be related to fine differences in inflammatory CLPs being released from the BM during different types of inflammation, and additional work is needed to clarify this point.

In view of the minor involvement of NK cell progenies in the present work, the research focus was therefore concentrated on characterizing T-cell progenies.

T-cell progenies derived *in vitro* from CLPs isolated from tissue and PB highly express activating NK cell receptors including DNAM-1, the major NCR NKp30, and NKG2D. In addition they express to a considerable extent inhibitory receptors such as KIRs and NKG2A.

Interestingly, the majority of maturing progenies were represented by CD4+ T cells, rather than CD8+T cells. In view of their prevalence, we therefore

characterized the CD4+ progeny cells according to their T helper (Th) cell subset phenotype.

It is common knowledge that differentiated Th cells leave the lymph nodes, enter circulation to extravasate into peripheral inflamed tissues. Here, upon antigen recognition, they produce different cytokines to help cells of the innate and adaptive immune systems to clear the pathogen. Thus, the heterogeneity of Th cells contributes to specific adaptive immune help depending on specific tissue and pathogenetic requirements defines the type of immune and tissue response that is appropriate for the type of pathogen attack (Sallusto, 2016).

Cytofluorimetric analysis of CD4+ T-cell progenies derived *in vitro* from CLPs isolated from tissue and PB in a non-polarized environment showed the that Th1, Th1*, Th2, and Th17 were represented. However the prevalent Th cell type in this analysis was represented by Th1 and Th1* in T-cell progenies derived *in vitro* from PB and UI-T CLPs and by Th2 and Th17 in T-cell progenies derived *in vitro* from C-T CLPs.

In line with this observation, also the cytokine production assay confirmed a correspondence between the phenotype of Th cells found in our experiments and the description of cytokine production of these cells, described by Sallusto in 2016. Cytokine assays on cell culture supernatants here confirmed the presence of IL-13, IL5, IL-10 and IL-17a while the overall potential for production upon maximal stimulus (PMA+Ionomycin) showed production of TNF-α, IFN-γ, IL-2, IL-5, IL-9, IL-13, IL-17A, IL-10. It is described that Th1 and Th1* cells produce IFN-γ, Th2 cells produce IL-4, IL-5 and IL-13, Th17 cells produce IL-17A, IL- 17F (Sallusto, 2016).

To complete the evaluation of a functional activity for these T-cell progenies we also performed cytotoxicity assays, showing that T-cell progenies displayed direct cytotoxicity against A549 human lung carcinoma target cells, and that under some conditions they could be triggered via CD3 and NKp30 triggering.

Overall these experiments show that tissue-seeding inflammatory CLPs generate predominantly CD4+ maturing T cells that are able to functionally

contribute to local help with cytokine production and could also exert cytotoxic activity. In general cytotoxic activity does not represent one of the main functions of CD4+ T cells, and this is in line with the difficulty in observing redirected killing activity. However, the direct cytotoxic activity against A549 cells shows a NK-like activity that is TCR-independent and that could contribute to local control of tumor tissues. However it should be underlined that these findings are so far preliminary, since none of the cytotoxicity assays could be performed in an autologous MHC setting, and also no clue is available so far for T cell specificity according to TCR rearrangement. Thus additional work is needed to address these points.

5. CONCLUDING REMARKS

The work essentially clarifies some points so far unexplored such as the presence of inflammatory CLPs not only in the peripheral blood of patients with infectious and non-infectious diseases but also in peripheral blood of cancer patients, where they are particularly increased after chemotherapy and/or immunotherapy.

The surface expression analysis of chemokine receptors has shed light on the likely migration and trafficking of CLPs highlighting that circulating precursor cells can follow different fates to different tissues. At the same time, we found in CLPs a low expression of adhesion molecules suggesting recent migration into tissues.

Also it has been assumed that from these CLPs derive NK and T lymphoid progenies expressing activating and inhibitory receptors. We can hypothesize that these lymphoid progenies can enrich the immunological defenses against the tumor and can take part in the killing of tumor cells, given the demonstrated experimental cytotoxic activity with target cells such as A549.

However, there are points still to be clarified such as, for example, the spectrum of co-expression of chemokine receptors and adhesion molecules, to

try to understand the complex trafficking between peripheral blood and tissues. Regarding the progeny derived in vitro from CLPs, in this study we have described the phenotypic characteristics detected by a flow cytometric investigation that cannot explain the mechanisms that occur during *in vitro* development of NK and T cells. Open questions remain about apparently thymus-independent maturation mechanisms that led to *in vitro* generation of these T progenies in a medium with hematopoietic factors and in the absence of Notch signaling pathway as defined by our present culture conditions, that plays a critical role at multiple steps during T cell development and regulates cell fate choices.

We wonder if progeny cells are oligoclonal or polyclonal we don't know if in T cell progenies *in vitro* derived there are other subpopulations such as T central memory (T_{cm}) (CD4+CCR7+/CD62L+) or T effector memory (T_{em}) (CD4+CCR7-/CD62L-).

Further functional assay on lymphoid progenies would be useful, in addition to combining phenotypic characterization of *in vitro* derived progenies, with transcriptomic analysis.

Inflammatory CLPs actually open up novel scenarios of immune functioning and defenses that have been largely left unexplored until now.

This work sheds some new light on the effect of inflammation derived from cancer pathology on the selective mobilization of these precursors from bone marrow to peripheral blood and from peripheral blood to tissues. It also makes us understand how these precursors are present in various diseases and could be used for diagnostic or predictive purposes, for example in immunotherapy or chemotherapy, given the recent findings in which it is illustrated how the frequency of Lin-CD34+DNAM-1^{bright}CXCR4+ in the peripheral blood of patients with SARS-CoV-2 is correlated to disease course severity.

6. REFERENCES

Balkwill F, Mantovani A. *Inflammation and cancer: back to Virchow?* Lancet 2001. 357: 539–545.

Beck T. C., Gomes A. C., Cyster J. G. & Pereira J. P. *CXCR4 and a cell-extrinsic mechanism control immature B lymphocyte egress from bone marrow*. J. Exp. Med. 211, 2567–2581 (2014).

Ben-Shmuel A, Biber G and Barda-Saad M. *Unleashing Natural Killer Cells in the Tumor Microenvironment–The Next Generation of Immunotherapy?* Front. Immunol., 21 February 2020 | https://doi.org/10.3389/fimmu.2020.00275

Ben-Baruch A. *Organ selectivity in metastasis: regulation by chemokines and their receptors*. Clin Exp Metastasis. (2008) 25:345–56. doi: 10.1007/s10585-007-9097-3

Bjorkstrom NK, Ljunggren HG, MichaelssonJ. *Emerging insights into natural killer cells in human peripheral tissues.* NatRev Immunol. 2016;16:310–320.

Bjorkstrom NK, Ljunggren HG, Sandberg JK. *CD56 negative NK cells: origin, function, and role in chronic viral disease*. Trends Immunol 2010;31:401-6.

Bottino C, Castriconi R, Pende D, Rivera P, Nanni M, Carnemolla B, Cantoni C, Grassi J, Marcenaro S, Reymond N, Vitale M, Moretta L, Lopez M, Moretta A. *Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule.* J Exp Med. 2003 Aug 18; 198(4):557-67.

Bozzano F, Marras F, Ascierto ML, Cantoni C, Cenderello G, Dentone C, Di Biagio A, Orofino G, Mantia E, Boni S, De Leo P, Picciotto A, Braido F, Antonini F, Wang E, Marincola F, Moretta L, De Maria A. *'Emergency exit' of bone-marrow-resident CD34(+)DNAM-1(bright)CXCR4(+)-committed lymphoid precursors during chronic infection and inflammation.* Nat Commun. 2015 Oct 5;6:8109.

Bozzano F, Perrone C, Moretta L, De Maria A. *NK Cell Precursors in Human Bone Marrow in Health and Inflammation.* Front Immunol. 2019 Aug 28;10:2045. doi: 10.3389/fimmu.2019.02045. eCollection 2019.

Bozzano F, Della Chiesa M, Pelosi A, Antonini F, Ascierto ML, Del Zotto G, Moretta F, Muccio F, Luganini A, Gribaudo G, Cenderello G, Dentone C, Nicolini L, Moretta A, Moretta L, De Maria A. *HCMV-controlling NKG2C $^{+}$ NK cells originate from novel circulating inflammatory precursors*.J Allergy Clin Immunol. 2021 Jun;147(6):2343-2357

Bremnes RM, Al-Shibli K, Donnem T, Sirera R, Al-Saad S, Andersen S, Stenvold H, Camps C, Busund LT. *The Role of Tumor-Infiltrating Immune Cells and Chronic Inflammation at the Tumor Site on Cancer Development, Progression, and Prognosis: Emphasis on Non-small Cell Lung Cancer.* Journal of Thoracic Oncology, 2011.Volume 6, Issue 4. Pages 824-833.

Carrega P, Morandi B, Costa R, Frumento G, Forte G, Altavilla G, Ratto GB, Mingari MC, Moretta L, Ferlazzo G. *Natural killer cells infiltrating human no small-cell lung cancer are enriched in CD56 bright CD16(-) cells and display an impaired capability to kill tumor cells.* Cancer. 2008 Feb 15;112(4):863-75

Carrega P, Ferlazzo G. Natural Killers Are Made Not Born: *How to Exploit NK Cells in Lung Malignancies*. Front Immunol. 2017 Mar 13;8:277

Casey KA, et al. *Antigen-independent differentiation and maintenance of effector-like resident memory T cells in tissues*. J. Immunol. 2012; 188:4866–4875. doi: 10.4049/jimmunol.1200402.

Castriconi R, Carrega P, Dondero A , Bellora F , Casu B , Regis S , Ferlazzo G, Bottino C. *Molecular Mechanisms Directing Migration and Retention of Natural Killer Cells in Human Tissues.* Front Immunol. 2018 Oct 11;9:2324. doi: 10.3389/fimmu.2018.02324. eCollection 2018.

Chen J, Lau YF, Lamirande EW, Paddock CD, Bartlett JH, Zaki SR, Subbarao K. *Cellular immune responses to severe acute respiratory syndrome coronavirus (SARS-CoV) infection in senescent BALB/c mice: CD4+ T cells are important in control of SARS-CoV infection.* Virol. 2010 Feb;84(3):1289-301. doi: 10.1128/JVI.01281-09. Epub 2009 Nov 11.

Cheuk S, Schlums H, Gallais Sérézal I, Martini E, Chiang SC, Mar- quardt N, Gibbs A, Detlofsson E, Introini A, Forkel M, et al. *CD49a Expression Defines Tissue-Resident CD8+ T Cells Poised for Cytotoxic Function in Human Skin*. Immunity 46, 287–300 (2017).

Cibrián D, Sánchez-Madrid F. *CD69: from activation marker to metabolic gatekeeper.* Eur J Immunol.2017 Jun;47(6):946-953. doi: 10.1002/eji.201646837.

Colucci F, Caligiuri MA, Di Santo JP. *What does it take to make a natural killer?* Nat Rev Immunol. (2003) 3:413–25. doi: 10.1038/nri1088

De Pasquale C, Campana S , Bonaccorsi I , Carrega P , Ferlazzo G. *ILC in chronic inflammation, cancer and targeting with biologicals*. Mol Aspects Med. 2021 Aug;80:100963. doi: 10.1016/j.mam.2021.100963. Epub 2021 Mar 14.

Deftos ML, Bevan MJ. *Notch signaling in T cell development* Curr Opin Immunol. 2000 Apr;12(2):166-72. doi: 10.1016/s0952-7915(99)00067-9.

Dieu-Nosjean MC, Antoine M, Danel C, Heudes D, Wislez M, Poulot V, Rabbe, Laurans NL, Tartour E, de Chaisemartin L, Lebecque S, Fridman WE and Cadranel J. *Long-term survival for patients with non-small-cell lung cancer with intratumoral lymphoid structures.* J Clin Oncol, 26 (2008), pp. 4410-4417

Dougan M, Dranoff G. *Immune therapy for cancer*. Annu Rev Immunol. 2009;27:83–117

Fehniger TA, Cooper MA, Nuovo GJ, Cella M, Facchetti F, Colonna M, et al. *$CD56^{bright}$ natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity*. *Blood.* (2003) 101:3052–7. doi: 10.1182/blood-2002-09-2876

Ferlazzo G, Thomas D, Lin SL, Goodman K, Morandi B, Muller WA, et al. *The abundant NK cells in human secondary lymphoid tissues require activation to express killer cell Ig-like receptors and become cytolytic*. J Immunol. (2004) 172:1455–62. doi: 10.4049/jimmunol.172.3.1455

Foudi A, Jarrier P, Zhang Y, Wittner M, Geay JF, Lecluse Y, Nagasawa T, Vainchenker W, Louache F. *Reduced retention of radioprotective hematopoietic cells within the bone marrow microenvironment in CXCR4−/− chimeric mice*. Blood. 2006;107:2243-2251.

Freud AG, Becknell B, Roychowdhury S, Mao HC, Ferketich AK, Nuovo GJ, et al. A human CD34(+) subset resides in lymph nodes and differentiates into CD56bright natural killer cells. *Immunity.* (2005) 22:295–304. doi: 10.1016/j.immuni.2005.01.013

Grivennikov SI, Greten FR, Karin M. *Immunity, inflammation, and cancer.* Cell. 2010 Mar 19;140(6):883-99. doi: 10.1016/j.cell.2010.01.025.

Grzywacz B, Kataria N, Sikora M, Oostendorp RA, Dzierzak EA, Blazar BR, Miller JS, Verneris MR. *Coordinated acquisition of inhibitory and activating receptors and functional properties by developing human natural killer cells.* Blood 2006 Dec 1;108(12):3824-33.

Hadley GA. and Higgins JMG. *Integrin αEβ7: molecular features and functional significance in the immune system*. Adv. Exp. Med. Biol. 819, 97–110 (2014).

Hori S, Nomura T, Sakaguchi S. *Control of regulatory T cell development by the transcription factor Foxp3*. Science (2003). 299, 1057–1061.

Hu Z, Zou Q, Su B. *Regulation of T cell immunity by cellular metabolism.* Front Med. 2018 Aug;12(4):463-472. doi: 10.1007/s11684-018-0668-2. Epub 2018 Aug 16.

Izon DJ, *T-cell development: thymus-settling progenitors: settled?.* Immunol Cell Biol2008 Oct;86(7):552-3.

Jacobson LO, Simmons EL, Marks EK, Eldredge JH. *Recovery from radiation injury*. Science. (1951) 113:510–1. doi: 10.1126/science.113.2940.510.

Kyoizumi S, Kubo Y, Kajimura J, Yoshida K, Imai K, Hayashi T, Nakachi K, Young LF, Moore MA, van den Brink MRM, Kusunoki Y. *Age-associated changes in the differentiation potentials of human circulating hematopoietic progenitors to T- or NK-lineage cells.* J Immunol 2013 Jun 15;190(12):6164-72.

Klein Wolterink RG, García-Ojeda ME, Vosshenrich CAJ, Hendriks RW, Di Santo JP, *The intrathymic crossroads of T and NK cell differentiation*. Immunol Rev 2010 Nov;238(1):126-37.

Kojima H, Kanada H, Shimizu S, Kasama E, Shibuya K, Nakauchi H, Nagasawa T, Shibuya A. *CD226 mediates platelet and megakaryocytic cell adhesion to vascular endothelial cells.* J Biol Chem. 2003 Sep 19; 278(38):36748-53.

Korbecki J, Simińska D, Kojder K, Grochans S, Gutowska I, Chlubek D, Baranowska-Bosiacka I. *Fractalkine/CX3CL1 in Neoplastic Processes.* Int J Mol Sci. 2020 May 25;21(10):3723. doi: 10.3390/ijms21103723.

Kumar BV, Ma W, Miron M, Granot T, Guyer RS, Carpenter DJ, Senda T, Sun X, Ho SH, Lerner H, et al. *Human Tissue-Resident Memory T Cells Are Defined by Core Transcriptional and Functional Signatures in Lymphoid and Mucosal Sites*. Cell Rep. 20, 2921–2934 (2017).

Kumar BV, Connors TJ, Farber DL. *Human T Cell Development, Localization, and Function throughout Life.* Immunity. 2018 Feb 20;48(2):202-213. doi: 10.1016/j.immuni.2018.01.007.

Labani-Motlagh A, Ashja-Mahdavi M and Loskog A. *The Tumor Microenvironment: A Milieu Hindering and Obstructing Antitumor Immune Responses.* Front. Immunol., 15 May 2020||https://doi.org/10.3389/fimmu.2020.00940

Lanier LL. *Natural killer cell receptor signaling*. Curr Opin Immunol. (2003) 15:308–14. doi: 10.1016/S0952-7915(03)00039-6

Lanier LL. *NK cell receptors*. Annu Rev Immunol. (1998) 16:359–93. doi: 10.1146/annurev.immunol.16.1.359

León B et al. *Regulation of TH2 development by CXCR5+ dendritic cells and lymphotoxin-expressing B cells. Nature Immunol*. (2012) 13, 681–690.

Long EO, Kim HS, Liu D, Peterson ME, Rajagopalan S. *Controlling natural killer cell responses: integration of signals for activation and inhibition*. Annu Rev Immunol. (2013) 31:227–58. doi: 10.1146/annurev-immunol-020711-075005

Lugthart G, Melsen JE, Vervat C, van Ostaijen-Ten Dam MM, Corver WE, Roelen DL, et al. Human lymphoid tissues harbor a distinct CD69+CXCR6+ NK cell population. J Immunol. (2016) 197:78–84. doi: 10.4049/jimmunol.1502603

Ma CS, Deenick EK, Batten M & Tangye SG. *The origins, function, and regulation of T follicular helper cells.* J. Exp. Med. 209, 1241–1253 (2012).

Ma C, Mishra S, Demel EL, Liu Y, Zhang N. *TGF-beta controls the formation of kidney-resident T cells via promoting effector T cell extravasation*. J. Immunol. 2017;198:749–756.

Martínez-Lostao L, Anel A and Pardo J. *How Do Cytotoxic Lymphocytes Kill Cancer Cells?* CCR-15-0685 Published November 2015. DOI: 10.1158/1078-0432.

Masopust D , Schenkel LM. *The integration of T cell migration, differentiation and function.* Nat Rev Immunol. 2013 May;13(5):309-20. doi: 10.1038/nri3442. Epub 2013 Apr 19.

Mavilio D, Lombardo G, Benjamin J, Kim D, Follman D, Marcenaro E, et al. *Characterization of CD56-/CD161 natural killer (NK) cells: a highly dysfunctional NK subset expanded in HIV-infected viremic individuals*. Proc Natl Acad Sci U S A 2005;102:2886-91.

Melsen JE, Lugthart G, Lankester AC, Schilham MW. *Human circulating and tissue-resident CD56(bright) natural killer cell populations.* Front Immunol. (2016) 7:262. doi: 10.3389/fimmu.2016.00262.

Melsen JE, Lugthart G, Vervat C, Kielbasa SM , van der Zeeuw SAJ , Buermans HPJ , van Ostaijen-Ten Dam MM , Lankester AC, Schilham MW. *Human Bone Marrow-Resident Natural Killer Cells Have a Unique Transcriptional Profile and Resemble Resident Memory CD8 + T Cells.* Front Immunol. 2018 Aug 22;9:1829. doi: 10.3389/fimmu.2018.01829. eCollection 2018.

Moretta A. *Natural killer cells and dendritic cells: rendezvous in abused tissues*. Nat Rev Immunol. (2002) 2:957–65. doi: 10.1038/nri956

Moretta L, Moretta A. Unravelling natural killer cell function: triggering and inhibitory human NK receptors. *EMBO J.* (2004) 23:255–9. doi: 10.1038/sj.emboj.7600019

Mueller SN, Mackay LK. *Tissue-resident memory T cells: local specialists in immune defence*. Nat. Rev. Immunol. 2016; 16:79–89. doi: 10.1038/nri.2015.3

Muller WA. *The regulation of transendothelial migration: new knowledge and new questions.* Cardiovasc Res. 2015 Aug 1;107(3):310-20. doi: 10.1093/cvr/cvv145. Epub 2015 May 17.

Newman P J, Berndt M C, Gorski J, White G C, Lyman S, Paddock C, Muller W A. *PECAM-1 (CD31) cloning and relation to adhesion molecules of the immunoglobulin gene superfamily*. Science.1990 Mar 9;247(4947):1219-22

Owen DL, Sjaastad LE , Farrar MA. *Regulatory T Cell Development in the Thymus*. J Immunol. 2019 Oct 15;203(8):2031-2041. doi: 10.4049/jimmunol.1900662.

Passlick B, Izbicki JR, Waydhas C, Nast-Kolb D, Schweiberer L, Ziegler-Heitbrock HW. Posttraumatic splenectomy does not influence human peripheral blood mononuclear cell subsets. *J Clin Lab Immunol.* (1991) 34:157–61.

Peppa D, Gill US, Reynolds G, Easom NJ, Pallett LJ, Schurich A, Micco L, Nebbia G, Singh HD, Adams DH, Kennedy PTF, Maini MK. *Up-regulation of a death receptor renders antiviral T cells susceptible to NK cell-mediated deletion.* J Exp Med. (2013) 210:99–114. doi: 10.1084/jem.20121172

Pui JC, Allman D, Xu L, DeRocco S, Karnell FG, Bakkour S, Lee JY, Kadesch T, Hardy RR, Aster JC, Pear WS. *Notch1 expression in early lymphopoiesis influences B versus T lineage determination.* Immunity 1999, 11:299-308.

Pusztai L, Mendoza TR, Reuben JM, Martinez MM, Willey JS, Lara J, et al. *Changes in plasma levels of inflammatory cytokines in response to paclitaxel chemotherapy*. Cytokine 2004; 25:94–102.

Radtke F, Wilson A, Stark G, Bauer M, van Meerwijk J, MacDonald HR, Aguet M. *Deficient T cell fate specification in mice with an induced inactivation of Notch1*. Immunity 1999, 10:547-558.

Ramos SB, Garcia AB, Viana SR, Voltarelli JC, Falcao RP. *Phenotypic and functional evaluation of natural killer cells in thymectomized children*. *Clin Immunol Immunopathol.* (1996) 81:277–81. doi: 10.1006/clin.1996.0189

Randolph G J, Angeli V, Swartz MA. *Dendritic-cell trafficking to lymph nodes through lymphatic vessels*. Nat. Rev. Immunol. 2005.**5**: 617-628.

Renoux VM, Zriwil A, Peitzsch C, Michaelsson J, Friberg D, Soneji S, et al. *Identification of a human natural killer cell lineage-restricted progenitor in fetal*

and adult tissues. Immunity. (2015) 43:394–407. doi: 10.1016/j.immuni.2015.07.011

Reymond N, Imbert AM, Devilard E, Fabre S, Chabannon C, Xerri L, Farnarier C, Cantoni C, Bottino C, Moretta A, Dubreuil P Lopez M. *DNAM-1 and PVR Regulate Monocyte Migration through Endothelial Junctions.* J Exp Med.2004 May 17; 199(10): 1331–1341.

Rivas-Fuentes S, Salgado-Aguayo A, Arratia-Quijada J and Gorocica-Rosete P. *Regulation and biological functions of the CX3CL1-CX3CR1 axis and its relevance in solid cancer: A mini-review.* J Cancer. 2021; 12(2): 571–583.

Roy A, Cowan G, Mead AJ, Filippi S, Bohn G, Chaidos A, Tunstall O, Y. Chan, Choolani JKM, Bennett P, Kumar S, Atkinson D, Wyatt-Ashmead J, Hu M, Stumpf MPH, Goudevenou K, O'Connor D, Chou ST, Weiss MJ, Karadimitris S, Jacobsen SE, Vyas P and Roberts I. *Perturbation of fetal liver hematopoietic stem and progenitor cell development by trisomy 21*. Proc. Natl. Acad. Sci. USA, PNAS 109 (2012), (43) pp. 17579-17584

Russo RC, Garcia C, Teixeira MM, Amaral FA. *The CXCL8/IL-8 chemokine family and its receptors in inflammatory diseases* Expert Rev Clin Immunol.2014 May;10(5):593-619. doi: 10.1586/1744666X.2014.894886. Epub 2014 Mar 29.

Sallusto F, Geginat J & Lanzavecchia A. *Central memory and effector memory T cell subsets: function, generation, and maintenance*. Annu. Rev. Immunol. 22, 745–763 (2004).

Sallusto F., *Heterogeneity of Human CD4+ T Cells Against Microbes.* Annu. Rev. Immunol. 2016. 34:317–34

Sánchez-Sánchez N, Riol-Blanco L and Rodríguez-Fernández JL. *The Multiple Personalities of the Chemokine Receptor CCR7 in Dendritic Cells.* J Immunol May 1, 2006, 176 (9) 5153-5159

Shibuya A, Campbell D, Hannum C, Yssel H, Franz-Bacon K, McClanahan T, Kitamura T, Nicholl J, Sutherland GR, Lanier LL, Phillips JH. *DNAM-1, a novel adhesion molecule involved in the cytolytic function of T lymphocytes.* Immunity. 1996 Jun; 4(6):573-81.

Singh P, Mohammad KS, Pelus LM. *CXCR4 expression in the bone marrow microenvironment is required for hematopoietic stem and progenitor cell maintenance and early hematopoietic regeneration after myeloablation.* Stem Cells 2020 Jul;38(7):849-859. doi: 10.1002/stem.3174. Epub 2020 Mar 19.

Sivori S, Cantoni C, Parolini S, Marcenaro E, Conte R, Moretta L, Moretta A. *IL-21 induces both rapid maturation of human CD34+ cell precursors towards NK cells and acquisition of surface killer Ig-like receptors.* Eur. J. Immunol. 33, 3439–3447 (2003).

Schenkel AR, Mamdouh Z, Chen X, Liebman RM, Muller WA. *CD99 plays a major role in the migration of monocytes through endothelial junctions.* Nat. Immunol. 2002 Feb;3(2):143-50. doi: 10.1038/ni749. Epub 2002 Jan 14.

Sun H, Sun C, Xiao W and Sun R. *Tissue-resident lymphocytes: from adaptive to innate immunity*. Cell Mol Immunol. 2019 Mar; 16(3): 205–215.

Till JE, Mc CE. *A direct measurement of the radiation sensitivity of normal mouse bone marrow cells*. Radiat Res. (1961) 14:213–22. doi: 10.2307/3570892

Ueda Y, Yang K, Foster SJ, Kondo M & Kelsoe G. *Inflammation controls B lymphopoiesis by regulating chemokine CXCL12 expression*. J. Exp. Med. 199, 47–58 (2004).

Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. *Functions of natural killer cells*. Nat Immunol. (2008) 9:503–10. doi: 10.1038/ni1582

Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, et al. Innate or adaptive immunity? The example of natural killer cells. *Science.* (2011) 331:44–9. doi: 10.1126/science.1198687

Watanabe R, Gehad A, Yang C, Scott LL, Teague JE, Schlapbach C, Elco CP, Huang V, Matos TR, Kupper TS, and Clark RA. *Human skin is protected by four*

functionally and phenotypically discrete populations of resident and recirculating memory T cells. Sci. Transl. Med (2015). 7, 279ra39.

Wei Liao, Jian-Xin Lin, nd Warren J. Leonard. *Interleukin-2 at the Crossroads of Effector Responses, Tolerance, and Immunotherapy*. Immunity. 2013 Jan 24; 38(1): 13–25.

Weissman IL, Shizuru JA. *The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases*. *Blood.* (2008) 112:3543–53. doi: 10.1182/blood-2008-08-078220. (Weissman et al, 2008)

Whiteside TL. *The tumor microenvironment and its role in promoting tumor growth*. Oncogene volume 27, pages 5904–5912 (2008)

Whiteside TL. (2007). *The Local Tumor Microenvironment*. In: Kaufmann H, Wolchok JD (eds). *General Principles of Tumor Immunotherapy: Basic and Clinical Applications of Tumor Immunology*. Springer, pp 145–167.

Yang S, Liu F, Wang QJ, Rosenberg SA, Morgan RA. *The shedding of CD62L (L-selectin) regulates the acquisition of lytic activity in human tumor reactive T lymphocytes.* PLoS One 2011;6(7):e22560. doi: 10.1371/journal.pone.0022560.

Yokoyama WM, Kim S, French AR.T*he dynamic life of natural killer cells.* AnnuRev Immunol. 2004;22:405-29.

Zitvogel L, Apetoh L, Ghiringhelli F, Kroemer G. *Immunological aspects of cancer chemotherapy*. Nat Rev Immunol. 2008;8:59–73

7. Supplementary Table.

Sample		IFN-g	IL-10	IL-13	IL-17a	IL-17F	IL-2	IL-22	IL-4	IL-5	IL-9	TNFa
Standard1		9,997	1,231	6,782	12,338	0,026	12,906	0,040	0,013	6,423	9,190	2,696
Standard2		36,896	5,234	28,989	48,875	0,100	49,342	0,151	0,084	23,882	35,477	10,282
Standard3		176,683	22,740	134,177	207,038	0,395	223,658	0,725	0,428	107,419	174,045	41,357
Standard4		600,989	76,497	462,537	778,772	1,674	736,658	2,278	1,369	373,447	516,477	153,709
Standard5		2478,398	291,110	1817,971	3088,591	5,698	3128,786	8,583	5,791	1616,364	2229,648	650,195
Standard6		N/A	1344,629	7749,177	20647,138	34,985	16114,617	49,151	26,668	6172,817	10969,194	2434,139
Standard7		N/A	5147,336	30601,577	N/A	148,521	64569,618	152,504	112,470	N/A	N/A	N/A

Supplementary 1: Cytokine standards table.