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Evaluation of immune checkpoints and tissue residency markers on NK cells from colorectal cancer patients

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1 INTRODUCTION

1.1 NATURAL KILLER CELLS

Natural killer (NK) cells are key elements of innate immunity capable of mediating potent antitumor and antiviral responses, both directly killing tumor or infected cells and indirectly enhancing responses mediated by antibodies and T cells. Indeed, NK-mediated cytotoxicity and cytokine release can influence the activity of additional innate immune cells (dendritic cells, macrophages and granulocytes) and confer regulatory functions to NK cells, capable of influencing subsequent T and B cell responses. From the beginning, NK cells have been described as "naturally" cytotoxic cells, since, unlike cytotoxic T cells, they do not require prior antigen exposure to mediate effector functions. However, more recently this concept has been partially revisited when it was demonstrated that NK cells require priming by various factors (e.g. soluble factors released by other innate immune cells) to reach their full effector potential [1].

NK cells are essential components of innate immunity belonging to group 1 Innate Lymphoid cells (ILCs) and play an important role in antiviral and anti-tumor defense [2]. ILCs represent a heterogeneous group of immune cells located primarily on epithelial surfaces, where they maintain tissue homeostasis, respond rapidly to pathogen invasion by mediating appropriate immune responses, and are also involved in tissue repair. ILCs develop from a common lymphoid progenitor but, unlike T and B lymphocytes, lack the expression of antigen receptors encoded by rearranged genes. ILCs can be considered the innate counterparts of T cell subsets. In particular, NK cells represent the "cytotoxic ILCs", whereas ILC1, ILC2, ILC3 and Lymphoid Tissue inducer (LTi) are considered as "helper ILCs" because they are not cytolytic and produce unique cytokines sets for each subset. Recent advances of our knowledge underline a certain degree of plasticity among the various ILC subsets, mainly due to the influence of the tissue microenvironment.

Human NK cells originate from multipotent hematopoietic stem cells (HSC) in the bone marrow and then continue their maturation in secondary lymphoid organs. NK cells mature through different steps characterized by the expression of specific surface markers [3]. During the development NK cells acquire several receptors and in the last stages of maturation NK cells get the cytotoxic capacity through the formation of lytic granules. This "linear" mechanism of differentiation/maturation is questioned since it has been observed that environmental signals can also modify the NK cells development [4]. In *vitro* studies show that in the presence of certain NK-supporting cytokines, NK cells do not derive only from lymphoid precursors but also from myeloid cells, as well as from pathways that influence the ILC-poiesis from multipotent ILCPS[5]. Environmental signals are indeed able to shape differentiation trajectories in order to drive the proper response and ensure a rapid and localized generation of NK cells in the tissues and in

circulation. Thus, at the moment, it is evident that NK cell development is not “linear” but rather “branched”[4].

During their maturation NK cells acquire activating and inhibitory receptors that regulate their effector function. Indeed, NK cell activity is finely regulated by a balance of signals transduced by activating and inhibitory receptors expressed on NK cells and capable of interacting with their ligands on target cells [1,6]. NK cells can regulate both innate and adaptive immune responses interacting with many immune cell types, including dendritic cells, macrophages, neutrophils and T lymphocytes [7]. These interactions are mediated by cytokine release and cell-to-cell contacts, and play an important role in the regulation of antimicrobial and anti-tumor NK cell functions [8]. NK cells can also interact with different non-immune cells types, such as cancer cells.

Most of our current knowledge about human NK cells comes from studies on cells derived from peripheral blood, also known as “conventional” NK cells (c-NK), but recently, interest in the characterization of NK cells within tissues has increased, and besides recirculating cells, different tissue-resident NK cells (tr-NK), each possessing distinct phenotypic profiles, have been described [6,9].

Based on the cell surface expression of CD56 and CD16, peripheral blood NK cells can be divided into two main populations:

- $CD56^{dim}$ $CD16^{+}$ NK cells: major subset of the peripheral blood NK cells. They are described as mostly cytotoxic, mature NK cells present in the peripheral blood. Indeed, they are well equipped with lytic granules containing perforin and granzymes. Moreover, this NK cell subset expresses high levels of CD16 (FcγRIIIa), a potent activating receptor that mediates antibody-dependent cellular cytotoxicity (ADCC)
- $CD56^{bright}$ $CD16^{neg/dim}$ NK cells: minor subset in the peripheral blood (only 10%), but abundant in lymphoid organs. These cells are described as a mostly immunomodulatory and immature subset, and are responsible for producing cytokines, such as IFN-γ and TNF-α, as well as other soluble factors that attract immune cells in inflammatory sites [9].

The NK cells enact their cytotoxic capacity through two different mechanism: the antibody-dependent cell-mediated cytotoxicity (ADCC) and the natural cytotoxicity [10]. ADCC is a mechanism mediated by NK cells through the CD16 (FcγRIIIa) that binds the Fc portion of IgG antibodies triggering the lysis of targeted cells [11]. Natural cytotoxicity is mediated by activating receptors expressed on the surface of NK cells and it is regulated by NK cell inhibitory receptors [12]. NK cells recognize and kill autologous cells lacking the expression of self HLA-I molecules that are generally expressed on almost all human cells but often down-regulated by tumor or virus-infected cells [13]. This is known as “missing self-recognition”, according to which the absence of inhibitory signals transduced by NK receptors recognizing HLA-I molecules on target cell results in the induction of target killing [10]. The main effector molecules involved in NK cell-mediated target

cell killing are perforin and granzymes B, contained into lytic granules. When NK cells meet the target cell and form the immune synapsis, they release the granules content, and thus perforin creates pores in the membrane of target cell, thus favoring entry of granzymes that mediate caspase cleavage and activation, and the induction of target cell death by apoptosis [8,10]

NK cells are able to sense the up-regulation or down-modulation of the surface expression of several molecules on tumor cells, for this reason they play an important role in immunosurveillance against tumor growth.

1.2 NK RECEPTORS

NK cells have two types of receptors, germline-encoded, type I proteins of Ig-like family or type II proteins of C-type lectin-like receptor family. The NK cell receptors mediate inhibitory or activating signals upon recognition of specific ligands on target cells, thus allowing the NK-mediated control on the surrounding cells. The balance between inhibitory and activating signals transmitted by these receptors finely regulates NK cell function and determines if NK cells kill the target cells or remain inactive (Fig. 1). In particular, autoreactivity is controlled by inhibitory receptors specific for self HLA-I molecules. These receptors are clonally expressed or co-expressed in NK cell subsets, thus creating repertoires of NK cells with different phenotypic and functional characteristics that are capable of responding to different types of virally infected and tumor-transformed cells. Under non-pathological conditions, the interactions between these inhibitory receptors and their specific HLA-I ligands inactivate NK cells, thus preventing killing of healthy cells. During cancer progression, the transformed cells decrease or even lose the expression of HLA-I on their surface, and express ligands for activating NK receptors: two events necessary for the induction of antitumor NK cell responses. Indeed, ligands for the activating NK receptors are generally absent or expressed in small amounts in normal cells, while they are expressed de novo or upregulated at the cell surface in stressed cells and, in particular, in virus-infected or tumor cells. Therefore, signaling through activating receptors can overcome the signaling mediated by inhibitory receptors on diseased cells.

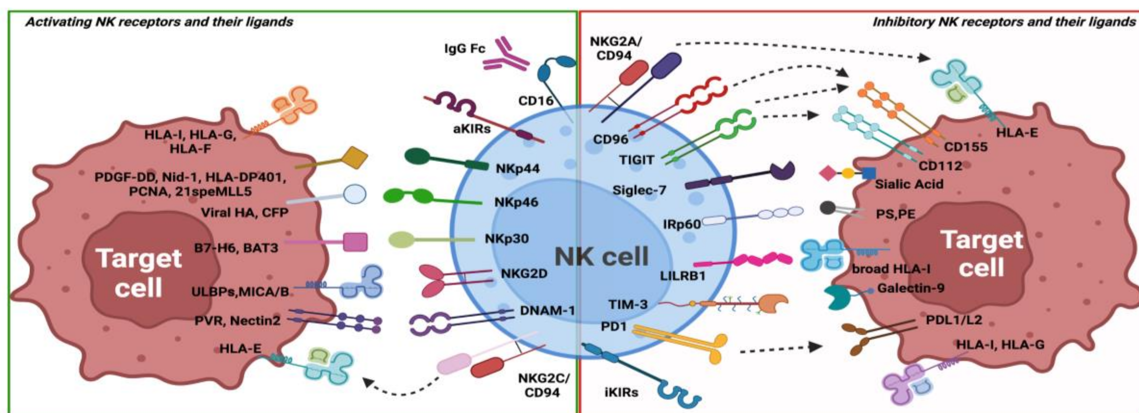

Figure 1: ACTIVATING AND INHIBITORY NK CELLS RECEPTORS AND THEIR LIGANDS. The image is created by using Biorender.com

1.2.1 ACTIVATING RECEPTORs

NK cells express several activating receptors that promote NK cell-mediated killing of pathological cells overexpressing cell-inducible ligands and are important to induce their physiologic crosstalk with other cell types. The activating signals are transmitted through the phosphorylation of the ITAM motifs present in the intracytoplasmic tail of these receptors, or frequently contained into receptor-associated adaptor proteins.

Activating receptors are numerous: CD16 that mediates ADCC, NCRs (including NKp46, NKp30, and NKp44) that play a crucial role in mediating the natural cytotoxicity against tumor cells, often in collaboration with other activating receptors, including DNAM-1 and NKG2D.

1.2.1.1 NCRs

NCR expression was initially thought to be confined to NK cells, and NKp46 is still being considered a reliable NK cell-associated marker, both in humans and in mice. Soon thereafter it became clear that these receptors could also be expressed in other types of immune cells, extending their role to further biological processes.

- NKp46: one of the main activating receptors expressed by resting and activated NK cells. It has been recently detected also on ILC1 and on a small subset of T lymphocytes. NKp46 is a type I transmembrane molecule that belongs to Ig superfamily and is able to induce NK activation, IFN-γ production and degranulation [14]. After binding its ligands, this receptor induces activating signals through the ITAM-bearing molecules CD3-ζ and/or FcεRI-γ [10].

  Even if numerous experimental evidences suggest the existence of multiple NKp46 ligands, at the moment no NKp46-specific cellular ligand on tumor cells has been identified [10]. Recently, a soluble NKp46 ligand has been identified, the Complement Factor P (CFP). CFP is a plasma glycoprotein produced by T cells, neutrophils and monocytes and involved in the regulation of the complement pathway. The recognition of CFP by NKp46 receptor does no result in the modulation of IFN-γ secretion nor in the inhibition of NK cell degranulation, but it seems to facilitate NKp46 interaction with bacterial ligands and the subsequential NK cell activation [15]. NKp46 can be down-modulated in the tumor microenvironment by the presence of L-kynurenine, while it is up-regulated following stimulation by IFN-γ or IL-2 [16]. Other NCR ligands are represented by some molecules of viral origin (e.g., influenza virus-derived hemagglutinin) that are capable of triggering NK cell function against infected cells [17].
- NKp30: activating receptor expressed in both resting and activated NK cells, but it can also be found in γδT cells and other ILCs. NKp30 is a type I transmembrane protein containing a single V-type Ig extracellular membrane that transduces the signal through CD3-ζ adaptor molecule [18]. The cellular ligand of NKp30 is B7H6, a surface protein belonging to B7 family. B7-H6 is not expressed

on health cells, but it is frequently present on the cell surface of many tumor types through a Myc-mediated mechanism [19]. Interestingly, its expression may also be acquired by normal cells upon TLR-mediated stimulation and in the presence of pro-inflammatory cytokines [20]. B7H6 has been shown to be relevant for NKp30-mediated tumor cell recognition and its expression on tumor cells is considered as a poor prognostic factor for various cancer types [21]. B7H6 can be shed as a soluble molecule (sB7H6), and NKp30/sB7H6 interaction can induce a reduced NKp30-dependent tumor cell killing [21,22]. Another NKp30 ligand is represented by BAT3/BAG6, an intracellular protein that may be expressed at the cell surface of tumor or stressed cells. Indeed, BAT3/BAG6 is typically located in the nucleus; however, in cells exposed to heat shock, it can move to the plasma membrane and be secreted in exosomes by tumors and cells. Then, BAT3/BAG6-expressing exosomes can stimulate cytokine release by NK cells upon interaction with NKp30 [23]. NKp30 can be down-modulated by the immunomodulatory cytokine TGF-β, cortisol and methylprednisolone, whereas it can be up-regulated in the presence of IFN-γ and IL-2 [24,25].

- NKp44: an activating receptor expressed in activated NK cells that transduces activating signals through the ITAM-containing adaptor molecule DAP-12 which allows intracellular signaling [26]. It can be found also in ILC1, ILC3 and DC [18]. Indeed, NKp44 is expressed by IFN-γ-producing intraepithelial ILC1 and by a subset of ILC3 present at the epithelial/mucosal surfaces, in tonsils, and in decidua tissue. Notably, $NKp44^+$ ILC3 display a unique cytokine pattern, being able to produce IL-22 upon cytokine stimulation. In these cells, NKp44 stimulation induces TNF-α production and activates a pro-inflammatory program, suggesting that NKp44 could play a role in the pathogenesis of different immune-mediated disease. In addition, NCR^+ ($NKp44^+$) ILC3 have also been detected in the lymphoid infiltrate of non-small cell lung cancer, and have been found to release pro-inflammatory cytokines upon interaction with tumor cells and tumor-associated fibroblasts. The first NKp44 ligand described was the Platelet-Derived Growth Facto (PDGD)-DD that was identified as a soluble ligand of NKp44, and capable of inducing the release of IFN-γ, TNF-α and other pro-inflammatory cytokines and chemokines [27]. The production of PDGF-DD by tumor cells can stimulate angiogenesis and tumor growth through his receptor (PDGF-receptor β) [28], but the interaction between PDGF-DD and NKp44 allows NK cells to recognize and kill cancer cells [27,29]. Recently, another extracellular ligand for NKp44 has been described, Nidogen-1 (NID-1). It has been shown that the interaction between soluble NID-1 and NKp44 leads to the inhibition of NKp44-mediated IFN-γ release and a slight reduction of NK cell cytotoxicity [30]. NID-1 is a glycoprotein essential for the adhesion between cells and the extracellular matrix [31]. Soluble NID-1 can be released by several types of tumors in the extracellular environment so that it can be tied by NKp44. Thus, this ability represents one of the possible suppressive mechanisms that

can prevent NK cell-mediated killing of tumor cells [30]. A splice variant of mixed-lineage 5 (21spe-MLL5) [32] and HLA-DP401 [33] have been described as membrane-bound NKp44-ligands. Another NKp44 ligand is represented by PCNA, an intracellular protein that may be expressed at the cell surface of tumor or stressed cells. Indeed, in normal cells, PCNA is involved in the processes of DNA replication/repair and cell cycle control, but in tumor cells, it can be shuttled to the tumor cell surface and expressed on tumor-derived exosomes, functioning as an NKp44 ligand [34].

1.2.1.2 NKG2D

Natural Killer group 2 member D (NKG2D) is expressed on the surface of NK cells, $CD8^+$ T cells and γδT cells. This receptor is a type II and C-lectin-like molecule that transduces the activating signal through DAP10 adaptor protein [35]. NKG2D cell surface expression can be regulated by several mechanisms. IL-2 and IL-15 increase NKG2D expression, while TGF-β is able to down-modulate the surface expression of this receptor [25,36].

Multiple NKG2D ligands have been identified, the most relevant ones belong to MHC class I chain-related protein A/B (MICA/B) and UL16 binding proteins (ULBP)1-6 [37]. These proteins are usually upregulated on infected, stressed or transformed cells. In particular, MICA and MICB expression is increased in many cancers, being almost absent in healthy tissues. Membrane-bound MICA is able to activate NK cells and their anti-tumor activity, while MICA shedding from tumor cell surface or from cancer-release exosomes impairs NKG2D-dependent tumor cell killing, representing a possible immune evasion mechanism. Moreover, sMICA interaction with NKG2D on NK cells results in receptor down-modulation from the cell surface, with a consequent reduced receptor availability for tumor recognition [35,36].

Other relevant NKG2D ligands belong to UL16-binding proteins (ULBP) family, that are expressed on tumor cells but also on a wide range of healthy tissues [35]. Membrane-bound ULBP2 is able to trigger NK-mediated killing, and when released by tumor, high soluble ULBP2 levels acquire a poor prognostic value, due to ULBP2 ability to down-regulate surface expression of NKG2D and to impair tumor cell lysis [36,37].

1.2.1.3 DNAM-1

Dnax accessory molecule-1 (DNAM-1) is a costimulatory receptor that collaborate with other activating receptors to enhance NK cell function [38]. DNAM-1 is a transmembrane glycoprotein, triggering T- and NK cell-mediated cytotoxicity. DNAM-1 is an important regulator of NK cell-mediated function against tumor transformation [36,38]. It recognize PVR and Nectin-2, two members of the nectin-family that are highly expressed on several tumor cell types but also on normal cells, such as epithelial cells, endothelial cells and fibroblasts, which are protected against NK cell cytotoxicity by the expression of self HLA-I molecules [39]. Cancer cells can develop escape mechanisms to bypass DNAM-1 anti-tumor potential. Indeed, soluble factors (such as IDO or TGFβ) produced by malignant cells or the chronic DNAM-1 exposure to its ligands, PVR and Nectin-2, interfere with DNAM-1 expression [40].

1.2.1.4 CD16

Also known as FcγRIIIA, it is the low-affinity receptor for immunoglobulin Fc fragment, a potent NK cell receptor that mediates ADCC. CD16 is associated to the signal transduction proteins CD3ζ and FcRγ that contain ITAM motifs [6,11]. Upon CD16 activation, NK cells can kill opsonized target cells, as in immunotherapy approaches using IgG antibodies recognizing tumor-associated antigens [41].

1.2.2 HLA CLASS-I SPECIFIC INHIBITORY AND ACTIVATING RECEPTORS

HLA class I molecules can be recognized by three groups of NK receptors: Killer Ig-like Receptors (KIRs), CD94/NKG2 heterodimers, and LILRB1 (also called LIR-1 or ILT2). All of these receptors recognize different Human Leukocyte Antigen-class I (HLA-I) molecules. In particular, while NKG2A is not polymorphic and recognizes the well conserved and non-classical HLA-E molecule, KIRs are represented by a discrete number of polymorphisms that are the consequence of a coevolution with the equally wide range of classical HLA-I molecules [42].

1.2.2.1 CD94/NKG2A

The CD94 glycoprotein may associate with the NKG2A or NKG2C C-type lectin molecules, generating receptors with opposite functions. The first HLA-specific NK cell receptor expressed during differentiation is represented by CD94/NKG2A. This heterodimer is an inhibitory receptor that recognizes non-classical HLA-E, presenting nanomeric peptides cleaved from HLA-A, HLA-B, HLA-C. Thus the bond between NKG2A and HLA-E inhibits the NK activity towards $HLA\text{-}I^{+}$ cells [4]. CD94/NKG2C is the activating counterpart.

1.2.2.2 KIRs

KIRs are the most important HLA class I specific receptors able to recognize HLA-A, HLA-B and HLA-C molecules and are expressed by mature NK cells. KIRs include both inhibitory (iKIR) and activating (aKIR) receptors [13]. These receptors are characterized by 2 or 3 Ig-like extracellular domains, and for this reason the KIRs are called 2D or 3D. Their intracytoplasmic tail is long (L) for iKIRs, or short (S) for aKIRs [13,43]. The long intracytoplasmic tail of iKIRs contains immunoreceptor tyrosine-based inhibitory motifs (ITIM), that upon phosphorylation transduce an inhibitory signal by recruiting Src homology region 2-containing protein tyrosine phosphates, SHP-1 and SHP-2. Differently, aKIR are characterized by a short cytoplasmatic tail without ITIM motifs that transduces activating signals through KARAP/DAP12, an accessory molecule containing ITAM motifs [44].

KIR genes are characterized by a high level of polymorphism. The gene family is located on chromosome 19p13.4 and consists of 13 genes and 2 pseudogenes. Two groups of KIR haplotypes have been defined: KIR A and KIR B. KIR A haplotypes are characterized by a fixed number of genes encoding iKIR (KIR2DL1, KIR2DL3, KIR3DL1 and KIR2DL2) and only one aKIR (KIR2DS4), and by high allelic polymorphism. Differently, KIR B haplotypes display a higher gene content diversity, low allelic polymorphism and a variable number of aKIR (KIR2DS1, KIR2DS2, KIR2DS4, KIR2DS5 and KIR3DS1). The variability in gene

content and allelic polymorphism contribute to the great diversity in KIR genotype between individuals (Parham, 2005). Among the circulating NK cells of different individuals, a highly varied receptor repertoire can be detected. This diversity is primarily due to the high polymorphism of KIR and HLA class I genes, which segregate independently, leading to diverse compound genotypes [45]. KIR expression pattern is a results of a stochastic event but it is highly influenzed by self-HLA class I molecules [13].

The main iKIRs recognize specific epitopes of distincts groups of HLA-A, HLA-B, and HLA-C allotypes, named KIR-ligand (KIR-L).

1.2.2.3 LIR-1 (or LILRB1 or ILT2)

LIR-1 Leucocyte Ig-like Receptor, also called ILT2 (immunoglobulin-like transcripts 2), is another receptor able to recognize HLA-I molecules and transduce inhibitory signals. It is characterized by a long tail including 4 ITIM-like motifs and it is expressed not only on the cell surface of an NK cell subset but also on a fraction of T (both α/β and γ/δ) and B lymphocytes, monocytes, macrophages, and DCs. The characterization of ILT2 ligands indicated that this receptor binds both classical and non-classical HLA-I molecules. Remarkably, ILT2 binds to HLA-G with a three-to-four higher affinity than classical HLA-I allotypes. Since HLA-G is frequently expressed on solid tumors, the inhibitory signals transduced by ILT2 upon ligand recognition can suppress NK-mediated recognition and support tumor escape. More recently, peptide-loaded β2m-HLA-F molecules have been added to the ILT2 ligands. Notably, ILT2 also binds to the human CMV HLA-I homolog UL18 [46].

1.2.3 NON-HLA-I SPECIFIC INHIBITORY RECEPTORS

In addition to HLA-I specific inhibitory receptors that regulate NK cell function and prevent NK-mediated damage to healthy tissues, additional inhibitory checkpoints, responsible for maintaining immune cell homeostasis, have been described in NK cells (including PD-1, TIM-3, TIGIT, LAG-3). Under pathological conditions, some of these checkpoints, which are absent on resting NK cells, can be induced *de novo* and affect antitumor NK cell function upon interaction with their specific ligands that are frequently expressed on the tumor cell surface and facilitate tumor immune escape [47(p201)].

1.2.3.1 PD-1

Programmed Death-Ligand-1 (PD-1) is an inhibitory receptor originally discovered on T cells and playing an important role in maintaining peripheral tolerance and T-cell homeostasis. However, its interaction with PD-1 ligands, PDL-1 and PDL-2, that may be expressed on tumor cells, can inhibit T cell and NK cell functions, contributing to immune escape. For this reason, PD-1 has become one of the most investigated targets for cancer immunotherapy. Although most research has focused on inhibiting PD-1 on T-cells, the interest in understanding its role also in NK cells is emerging [48]. Indeed, PD-1 is expressed on a discrete subset of circulating and fully mature NK cells belonging to CD56^{dim} cell subset from one-fourth of

healthy individuals, but higher proportions of PD-1$^+$ CD56^{dim} NK cells can be detected in patients affected by different tumors [49].

Of note, since the size of PD-1$^+$ NK cell subset is enriched in the tumor microenvironment as compared to peripheral blood of the same patient, it is conceivable that, soluble factors and/or cells in the tumor microenvironment can induce PD-1 expression, such as glucocorticoids and high levels of pro-inflammatory cytokines (IL-12, IL-15, IL-18) [48,50].

1.2.3.2 TIM-3

T-cell immunoglobulin and mucin domain (TIM-3) is an inhibitory receptor expressed on many immune cells such as CD4$^+$, CD8$^+$ and regulatory T cells, B cells, NKT cells and myeloid cells. In NK cells TIM-3 is expressed in resting NK cells, mainly restricted to the CD56^{dim} cell subset, but it can be upregulated in CD56^{bright} subset upon cytokine stimulation [51]. TIM-3 receptor is able to recognize several ligands, in particular it binds galectin-9 that is upregulated in various cancers and chronic infections, causing the apoptosis of Th1 cells [52]. The TIM-3 variable IgV domain binds group protein B1 and this interaction compromises the activation of immune response. Moreover, TIM-3 recognizes phosphatidylserine (PtdSer) and carcinoembryonic antigen cell adhenosin molecule 1 (Ceacam-1) [48,53] . The binding of TIM-3 with such different ligands can modulate the activity of the immune cells.

High frequencies of circulating and/or tumor infiltrating TIM-3$^+$ NK cells have been found in different types of malignant tumors including gastric cancer, lung adenocarcinoma, advanced melanoma and bladder cancer [48]. The increased surface levels of TIM-3 on NK cells in cancers induce NK cell impairment [54]. Some in *vitro* studies show that TIM-3 blockade increases the NK-cell cytotoxicity [55], while other studies have also reported stimulatory functions of TIM-3 [56]. These divergent functions are likely associated with the existence of multiple and different TIM-3 ligands. Co-expression of TIM-3 and PD1 has been shown to mediate the exhaustion of CD8$^+$ T cells in various cancers and chronic viral infections. On the contrary, clear data about a possible co-expression of TIM-3 and PD-1 on NK cells are not yet available [48,57,58].

1.2.3.3 TIGIT

T-cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) is an immune inhibitory receptor of T and activated NK cells. TIGIT competes with the activating receptor DNAM-1 for binding PVR and Nectin-2. Upregulation of both TIGIT and its ligands have been described in multiple cancer types. TIGIT expression on NK cells, and the consequent exhaustion of these cells, has recently been described in colon cancer patients. Moreover, the expression of PVR was shown to correlate with increased VEGF expression and increased angiogenesis. TIGIT expression was proven to suppress both NK and CD8$^+$ T cell function in CRC growth [48,59].

1.2.3.4 LAG-3

Lymphocyte activation gene-3 (LAG-3) is a member of the immunoglobulin superfamily receptors with inhibitory properties [60]. LAG-3 is a co-inhibitory receptor mainly expressed on T and NK cells, but also on other immune cells, such as TILs (tumor infiltrating lymphocytes), Treg, NKT cells, B cells, and DCs. LAG-3 recognizes several molecules such as MHC class II (MHC-II), C-type lectin receptor LSECtin and fibrinogen-like protein-1 (FGL1) on targets cells [48,61]. LAG-3 has been shown to suppress immune responses in several tumors and block T-cell function. On other hand, its specific role in regulating NK-cell function is still not fully clarified and recent finding suggest that LAG-3 is expressed on "adaptive" NK cells and on activated NK cells [62].

LAG-3 is currently considered a good target for immunotherapy in order to strengthen not only T-cell anti-tumor activity, but also the NK cells once probably through ADCC [48].

1.3 NK CELL DIFFERENTIATION

As already described, in peripheral blood there are two subsets of NK cells ($CD56^{bright}$ and $CD56^{dim}$) and $CD56^{bright}$ are considered as the precursors of $CD56^{dim}$ NK cells. The NK cell maturation process occurs through different stages based on phenotypic and functional characteristics. This transition is unidirectional and characterized by the gradual acquisition of CD16, KIRs, perforin, cytotoxic capacity, decreased surface expression of CD56 (from bright to dim), and the progressive downregulation of the CD94/NKG2A surface expression [63].

Notably, NK cells at birth show a limited "state of diversity", but through the branched developmental pathway, they mature and acquire a highly differentiated phenotype. These phenotypes are characterized by the repertoire and function, thus reaching a wide spectrum of diversity determined both by genetic factors and by environmental stimuli such as pathogen exposures [4].

An evident example of highly differentiated NK cells is a particular subset called "adaptive NK cells", a striking example of the effect induced by CMV [64]. CMV is a key driver of NK cell differentiation and specialization by inducing a long-lasting imprinting in NK cell receptor and enhanced effector function, suggesting that innate lymphocytes can share some memory properties with adaptive lymphocytes [1]. This phenomenon is called "trained immunity" or innate memory. CMV induces profound changes in NK cells and their immune response. Indeed, upon CMV infection, NK cells increase the expression of the HLA-E specific activating receptor CD94/NKG2C and also exhibit a highly differentiated phenotype $KIR^{+}NKG2A^{-}LIR1^{+}CD57^{+}Siglec\text{-}7^{-}$ [65]. The adaptive NK cells are characterized by stable epigenetic modifications that regulate expression of both surface receptors and signaling adaptor proteins, thus tuning NK cells responsiveness [66,67].

Adaptive NK cells respond very well to NKG2C triggering releasing cytokines and cytotoxic molecules, and proliferating despite their highly differentiate state. In addition, they are characterized by an increased capacity of ADCC, partly due to the downmodulation of FcεRI-γ [68]. However, adaptive NK cells have been shown to express checkpoint receptors such as PD-1 and LAG-3, upon chronic stimulation through activating receptor engagement; this co-expression can make adaptive NK cells hypofunctional [4].

1.4 NK CELL EDUCATION

During the NK cell differentiation/maturation process individual NK cells downmodulate the expression of CD94/NKG2A and stochastically express the different KIRs, engaging specific KIR-L expressed on the cells. Thus, NK cells have to undergo through a process called "education", by which the inhibitory interactions between KIRs/NKG2A and self-HLA class I ligands will guide the functional capacity of NK cells. NK cell effector function is calibrated by the strength of these inhibitory interactions through a tunable rheostat [69]. This applies not only to the polymorphic iKIR/KIR-L interactions but also to the conserved CD94/NKG2A recognition of HLA-E. Thus, NK cells that express at least one inhibitory receptor (KIR or NKG2A) specific for self-HLA class I molecules become functionally competent. In particular, NK cells are tolerant towards the surrounding healthy cells, expressing normal levels of NK receptors ligands (i.e., high HLA class I molecules and low activating ligands), and the inhibitory signals through HLA-specific inhibitory receptors block NK cell activation. Conversely, NK cells can kill pathological cells, that have down-regulated HLA class I, reducing the inhibition mediated by HLA-specific inhibitory receptors ("Missing self-recognition") [70], and/or that have over-expressed ligands of activating receptors, increasing the strength of activating interactions ("Induced self-recognition") [71].

1.5 TISSUE-RESIDENT NK CELLS AND ILCS

Innate lymphoid cells (ILCs) family includes NK, ILC1, ILC2 and ILC3. Since the discovery of ILC1s, many studies have tried to determine the specific role of ILC1s relative to NK cells in view of their plasticity, but the high degree of similarity between ILCs and NK cells and their plastic relationships in some contexts as well as the lack of specific markers render difficult their distinction. In mice, a key distinction between ILC1s and NK cells is the description of ILC1s as resident cells expressing the α1β1 integrin CD49a, whereas NK cells were described as circulating cells not expressing this integrin. However, in humans, only a small subset of ILC1s in the liver express this integrin, and this definition has since been shown to be insufficient to discriminate between ILC1s and NK cells [72].

A gating strategy currently used for human conventional NK cells is "Lin- CD56+CD127-CD94+NKp80+". The gating strategies used for human ILC1s (excluding the intraepithelial CD127- ILC1 population) are Lin-CD56+/-CD127+CRTH2-CD117-NKp44+/- [72].

A recent study in mice claimed to characterize new markers for distinguishing ILC1s from NK cells, such as the inhibitory receptor CD200R1. ILC1s isolated from mouse liver, small intestine and adipose tissue expressed this marker, whereas NK cells did not. In humans, CD200R1 has been detected in all peripheral blood ILCs ($CD56^{-}CD127^{+}$) but not on NK cells ($CD56^{+}CD127^{-}$) .

NK cells are capable of mediating killing upon direct target cell recognition and degranulation. Unlike NK cells, ILC1s appear to be only weakly cytotoxic, because they express low levels of granzyme B and perforin, the two major cytotoxic molecules. However, it has recently been shown that ILC1s may have a lytic capacity. Indeed, murine ILC1s from the liver or salivary glands constitutively express TRAIL on their surface [72–75].

Members of ILCs family are also able to acquire phenotypes and functional identities based on the tissue and the disease state in which they are found, but in tumor context it is still unclear [76]. In fact, in the tumor ILCs can have a pro- or anti-inflammatory role, but the mechanisms that determine these effects are still unclear. They seem to be due to the interaction between the particular subset of ILCs and the surrounding microenvironment [77]. In humans, ILC1s have been detected in colorectal carcinoma and non-squamous cell lung carcinoma biopsies [78,79], but their role remains unknown. In addition, these cells were more abundant in cancers of the esophagus, stomach, colon and rectum than in adjacent healthy tissues in humans [80].

It has always been thought that immune cells reside mainly in the lymphoid organs and then move to the site of infection or tumor transformation where they initiate their response. In contrast to this dogma, recent studies have revealed localized development and tissue-residency functions for immune cells [81]. NK cells, for example, are circulating cells that continuously move through the blood between lymph nodes and peripheral tissues to recognize and eliminate altered cells. However NK cells are not only circulating cells in the blood, but also tissue-resident cells, acquiring specific phenotype and functions that allow them to better supervise the surrounding environment [4].

1.5.1 cNK vs trNK

Conventional NK (cNK) cells develop in the bone marrow (BM) from HSC. Cytokines play a crucial role in their development, indeed they allow them to express or lose peculiar lineage receptors. Most of the cNK cells trafficking from the BM into the peripheral blood are $CD127^{-}$ $CD11b^{+}$ [82]. In contrast to cNK cells, the developmental stages of trNK cells are poorly defined. Whether there are distinct precursors for trNK cells is yet to be elucidated. It is unknown at what stage the commitment to trNK cells occurs. Moreover, whether specific cytokines drive NK cell progenitors (NKPs) toward tissue residency is an open question [81]. Cytokine-mediated metabolic reprogramming plays an essential role in the developmental progression of cNK cells. In this context, the qualitative roles of cytokines on trNK cells are yet to be established. Cytokines like IL-12 and IL-15 induce different levels of the expression of nutrient transporters in cNK cells. Interestingly, the $CD56^{bright}CXCR6^{+}$ trNK cells express lower levels of Glut1, and higher levels of

the amino acid transporter CD98 compared to peripheral blood CD56^{bright} NK cells upon stimulation, but the functional relevance of such differences requires further investigation [81]. Cytokines profile of trNK cells is quite different when compared to that of cNK cells. Effector functions of cNK cells include the capacity of mediating natural cytotoxicity and producing inflammatory cytokines (such as TNF-α, IFN-γ, IL-8, IL-10) and chemokines (including XCL1, XCL2, CCL1, CCL3, CCL4, CCL5, CCL22, and CXCL8) that generate and sustain the inflammatory environment [83].

Moreover, mice models have shown that trNK cells are lineage distinct from cNK cells with different requirements of transcription factors. Thus, cNK and trNK cells significantly differ in their requirement of the developmental niche and transcriptional networks. The topological organization of trNK cells within the microenvironment in organs such as lung or uterus hold clues about their functions, interacting partners, and cellular signal [84,85].

The cNK and trNK cells share common effector functions, but recent studies indicate that the tissue site can dictate the potential function of the trNK cell subset. In fact, recent studies have shown that the trNK cells in the thymus, liver, lymph nodes and uterus follow developmentally distinct pathways and have specific functions that are exclusively related to organ-specific niches [81,86].

1.5.2 LIVER-RESIDENT NK CELLS

NK cells in liver represent up to 30-50% of all hepatic lymphocytes in contrast to 5-20% in PBMC [87]. In the liver trNK are found in sinusoidal lumen and are associated with sinusoidal endothelial and Kupffer cells, the liver-resident macrophages [88]. In liver NK population is heterogeneous. In humans, intrahepatic resident NK cells (ihNK) include a high proportion of CD56^{bright} NK cells. First, CD56^{bright} CXCR6^{+} NK cells were defined as liver tissue-resident cells, but later among this population, only the Eomes^{hi} Tbet^{lo} cell subset has been considered as trNK cells, because it is absent in blood. This CD56^{bright}CXCR6^{+} trNK cell population is characterized by high expression of CD69 and CXCR6 but low expression of DNAM-1, perforin, and granzyme B, thus attributing a non-cytotoxic function for this trNK cell subset [88]. trNK cells are characterized by specific transcription factors, including Hobit that is highly expressed in CD56^{bright} NK cells, along with the chemokine receptor CXCR6, and the molecules CD69 and CD49a. The effector function of the liver-resident NK cells may not be cytotoxicity, but the production of high levels of inflammatory cytokines [81].

1.5.3 LUNG-RESIDENT NK CELLS

In humans NK cells are about 10-20% of the lymphocytes in the lungs. More than 80% of the lung NK cells are terminally-differentiated CD56^{dim}CD16^{+} cells. The remaining 20% of the NK cells are composed of immature CD56^{bright}CD16^{-} and less-differentiated CD56^{dim}CD16^{-} cells. The majority of lung NK cells are circulating from the blood and express high levels of KIRs [89]. Differently, lung-resident NK cells are primarily characterized by the expression of CD49a, CD69, and CD103 [90]. In lungs there are three distinct trNK cell

subsets: $CD16^- CD69^+CD49a^+CD103^+$, $CD16^-CD69^+CD49a^+CD103^-$ and $CD16^-CD69^+$. The first two subsets express high level of CD49a, Hobit, RGS1, RGS2 and lower levels of CD62L, S1PR5 and KLF3 transcripts compared to the $CD69^-$ subset (these are the hallmark genes of $CD8^+$ tissue-resident memory T-cells). $CD16^-$ $CD69^+$subset is considered similar to cNK [91]. Lung trNK cells play an important role in respiratory diseases, including infections disease, allergy and cancer [81].

1.5.4 LYMPH NODE-RESIDENT NK CELLS

Lymph nodes (LNs) provide niches with diverse and concerted interactions among immune cells, which facilitate robust responses. NK cells in LNs mediate the interaction between innate and adaptive immune cells. LN is the major draining site where tumor- or pathogen-derived antigens are encountered by immune cells. NK cells in the LN are known to interact with antigen-bearing DCs and drive the differentiation of CD4+ T cells. NK cells in LNs consist of multiple subsets that include cNK cells, NK cells possessing thymic origin, and a unique lymphoid-tissue resident NK population (Ltr-NK) that is shown to develop within this organ [92]. More than 75% of NK cells in LNs are $CD56^{bright}$; the Ltr-NK cells are 60% of total NK cells and cover the majority of the $CD56^{bright}$ compartment. The Ltr-NK cells are characterized by the co-expression of CD69 and CXCR6, express NKp46 but not DNAM-1, and most of them are $CD16^-$, $CD49a^-$, $CD127^+$. $CD56^{dim}$ $CD16^+$ Ltr-NK cells express higher levels of FCεRIγ compared to PB $CD56^{dim}$ $CD16^+$ cNK cells.

Recently, a new $CD34^{dim}$ $CD45RA^+$ immature NK subset, which may develop into Ltr-NK cells, has been identified in the LNs [81,93(p200)].

1.5.5 UTERINE-RESIDENT NK CELLS

Potentially, the most relevant immune cells during pregnancy are NK cells, in fact, their number increases significantly during pregnancy and decidualization [94]. Almost all the NK cells in the non-pregnant uterus (endometrium) and pregnant uterus (decidua) are $CD56^{bright}$. Uterine NK cells (uNK) express CD94/NKG2A, lack expression of CD16, and mostly express CD49a and CD103. Interestingly, KIR receptors on uNK cells are different from those expressed on cNK cells. In fact, decidual NK cells are educated by maternal HLA-C molecules, which influences the expression of KIR receptors. Due to this unique “licensing” program, an increased expression of $KIR2DL1^+$ and $KIR2DL2/L3/S2^+$ can be observed on decidua NK cells compared to cNK cells. In addition, uNK cells are transcriptionally distinct from cNK and their microRNA profiles are quite different [95]. In decidua there are three subsets of uNK cells (dNK1, dNK2, dNK3). These subsets co-express the tissue-residency markers CD49a and CD9. dNK1 cells express CD39, CYP26A1 and B4GALNT1, whereas dNK2 cells express ANXA1 and ITGB2, and dNK3 cells express CD160, KLRB1 and CD103, but not CD127. uNK cells play an essential role in the feto-maternal interface [81,96].

1.5.6 CLINICAL RELEVANCE OF trNK CELLS

Even if the cNK cells have been studied for more than 40 years, only recently researchers are focusing on trNK cells. The role of cNK cells in tumor clearance is well established, however the anti-tumor functions of trNK cells are far from being defined. trNK cells express tissue residency markers, less mature phenotype and different development compared to cNK cells. cNK cells are circulating and widely distributed cells, whereas trNK cells are distributed mainly in non-lymphoid tissues. trNK cells have unique and specific characteristics in the different tissues in which they are found. In fact, important differences in their phenotype and functions probably reflect the impact of the local microenvironment.

For example, in the liver half of all NK cells are trNK cells and in this organ their role is the maintenance of hepatic tolerance and homeostasis. Interactions between trNK cells and hepatocytes result in the production of TGFβ that expands Treg cells. Differently, viral infections augment the expression of NKp46 and altered trNK cells are detectable in hepatocellular carcinoma subsequent to a viral infection.
In the lung more than 80% of NK cells are terminally differentiated and are cNK cells, and trNK cells play an essential role in clearing influenza-infected epithelial cells.

In LNs NK cells mediate the interaction between other innate immune cells and adaptive immune cells.

During pregnancy uNK cells play several roles, including the formation of the fetal-maternal interface and placental vascular remodeling, and also the promotion of the fetal development and growth.

These observations emphasize the need to characterize and identify methods to enhance the effector functions of trNK cells in order to propose specific therapies to best exploit them *in situ* [81,97].

1.6 NK CELLS AND TUMOR MICROENVIRONMENT

NK cells have been found in many types of primary and metastatic tumors. The recognition and killing of cancer cells by NK cells depends on an integrated signal resulting from the engagement of both activating and inhibitory NK cell surface receptors. The importance of NK cells in controlling tumor growth and metastatic spreading has been demonstrated and usually the high density of tumor-infiltrating NK cells has been associated with a good prognosis in multiple solid tumors [98] .

However, some tumors are refractory to NK cell-mediated killing for the presence of an immunosuppressive microenvironment that affects NK cell function. Tumor microenvironment (TME) is a complex system, in which cancer cells, fibroblasts, endothelial cells and immune cells create the conditions to promote tumor progression. Numerous evidences indicate that TME produces soluble factors that negatively regulate maturation, proliferation and effector function of NK cells [98].

In several tumors NK cells present an altered phenotype, in particular they have a decreased expression of activating receptors or an increased expression of inhibitory receptors. The most commonly

described alteration is the down-regulation of NCR expression that correlates with histological grade, tumor invasion, lymph nodes metastases, adverse clinical outcome and resistance to treatment or surgery [99]. Also reduced expression of other major activating NK receptors, such as DNAM-1 and NKG2D, can be observed both on peripheral blood NK cells and tumor-associated NK cells in cancer patients [100]. The mechanisms involved in the down-regulation of activating NK receptors have not yet been fully described. At the moment it is known that high production of TGF-β by cancer cells causes a reduction in the expression of NKG2D, thus reducing the ability of NK cells to recognize and kill cancer cells [101]. The inhibition of TGF-β signaling in preclinical studies applied to solid tumors allows the NK cells accumulation in the tumor tissue, the increased release of IFN-γ and the restoration of NKG2D expression [101]. The expression of CD16 is down-regulated in most of tumor-associated NK cells. Furthermore, the increased expression of inhibitory receptors such as NKG2A is associated to a poor prognosis, whereas the expression of PD-1, TIGIT and TIM-3 is associated to a reduced antitumor activity of NK cells. All these phenotypical alterations in TME lead to have few functional NK cells with reduced degranulation capacity and IFN-γ production, as well as reduced content of perforin and granzyme B [99].

Besides the altered NK cell receptor repertoire, also NK maturation can be modified by TME. In fact, an enrichment in $CD56^{bright}$ NK cells was observed in numerous tumors [78]. Moreover, the immunosuppressive cytokine TGF-β is able to inhibit NK cell function, dampening $CD56^{dim}$ NK cell recruitment and favoring that of $CD56^{bright}$ less cytotoxic NK cells [102,103]. Based on these observations TGF-β may be considered a target for enhancing NK cell-mediated antitumor immunity.

Another TME-induced alteration of NK cells is the impaired cellular metabolism, that causes an important NK cell disfunction [104]. Since tumor cells consume a large amount of energy, the TME is very poor in nutrients such as glucose and glutamine, crucial for NK cell survival. Indeed, glycolysis is an important metabolic process for NK cell function, and in general it is increased in activated NK cells. A recent study showed that tumor upregulates the expression of the gluconeogenesis enzyme fructose bisphosphatase 1 (FBP1) and inhibits the glycolysis in tumor-infiltrating NK cells during tumor promotion through a mechanism involving TGF-β. However, when FBP1 is inhibited, NK cells glycolysis is restored. Lactic acid accumulation in the TME is a potent inhibitor of NK cell effector function and viability. Intracellular acidification and decreased ATP synthesis caused by lactic acid may be related to impaired IFN-γ production by NK cells [105]. Another mechanism adopted by tumors in the TME to selectively suppress the effector function of NK cells is the production of prostaglandin E2 (PGE2). PGE2 produced by tumors cells, tumor-associated macrophages and stromal cells represents a key regulator of the NK cell activity [106–108]. In some tumors PGE2 downregulates the expression of activating NK receptors resulting in the inhibition of cytotoxicity [109], and also reduces the NK capability to produce cytokines, such as CCL5 and CXCL1, that recruit preferentially $CD56^{bright}$ NK cells. Preclinical study showed that the PGE2 blocking restores antitumor NK cell function [110,111]. Moreover, another intracellular enzyme indoleamine 2,3-dioxygenase (IDO) is a

critical regulator of the TME converting tryptophan into a number of metabolites with immunosuppressive functions. IDO overexpression is associated with tumor progression and arrest of tumor-infiltrating NK cells. Moreover, also acidosis caused by lactate secretion by tumor cells contributes to NK cell impairment [99,112–114].

In short, the principal role of NK cells is killing cancer cells but the release of immunosuppressive modulators by the TME creates a detrimental condition preventing the NK cell function. Probably the usage of inhibitors for these modulators in a specific immunotherapy could repristinate the NK cell functions [98,99,115].

1.7 ANTI-TUMOR RESPONSE OF NK CELLS AND STRATEGIES TO POTENTIATE NK CELL ANTITUMOR ACTIVITY

NK cells play an important role in tumor surveillance, in fact their main task is to kill cancer cells. However, TME creates conditions that make NK cells less or completely non-functional. Given the innumerable tumor escape strategies, new and more functional approaches are being sought to make the NK cells functional again. At the moment the most used approaches in the clinic for tumor therapy are surgical excision, chemotherapy and radiotherapy often accompanied by immunotherapy. Boosting NK cells through different methods would help in treatment. For this reason, different therapeutic approaches are being developed.

- Cytokines: several preclinical and clinical studies exploit a therapeutic approach based on the use of cytokines able to stimulate and promote NK cell activation persistence and expansion. IL-2 was the first cytokine used to improve antitumor response of NK cells. IL-2 showed important tumor treatment potentials due to its ability to increase activity of NK and T cells. However, the use of this cytokine reported some problems. In fact, high doses of IL-2, that are necessary to allow an efficient anti-tumor response, can cause severe adverse reactions (life-threatening and vascular leak syndrome) [116]. On the contrary, therapy with low doses of IL-2 induces an efficient expansion of NK cells but a limited anti-tumor activity [117,118]. Moreover, IL-2 acting on Treg can induce production of TGF-β and consequently immune suppression.

  Another cytokine used for NK activation without inducing Treg-mediate immune suppression is IL-15. Several studies demonstrated that IL-15 induces expansion and activation of NK cells and $CD8^+$ memory T cells, resulting in tumor regression and decreased metastasis.
- Targeting immune checkpoints: in TME NK cells are not fully functional because express multiple immune checkpoints (IC) such as KIRs, NKG2A, PD1, TIGIT, TIM-3 and LAG-3. High expression of ICs correlates with poor prognosis. Human mAbs that block KIRs on NK cells to restore their functions was the first to enter in clinical practice. In clinical trials lirilumab, used alone to target KIR2DL1, KIR2DL2 and KIR2DL3, showed limited side effect but unfortunately also limited anti-

tumor efficacy[116]. A major progress towards the clinical use of ICs inhibitors has been achieved blocking NKG2A by Monalizumab, alone or in combination with other therapeutic mAbs [119]. Monalizumab promotes NKG2A$^+$ NK cells effector functions against HLA-E$^+$ target cells, and also improves the efficacy of anti-PD-L1 blocking mAbs by increasing the reactivity of NKG2A$^+$ PD-1$^+$ NK cells against HLA-E$^+$ PD-L$^+$ tumor cells. Monalizumab is currently tested in clinical trials as monotherapy or in combination with other antibodies [120].

NK cells from cancer patients that express PD-1 present lower anti-tumor activity [121]. Recently it has been described that the therapeutic effects of PD-1 and PD-L1 blockade may rely also on the anti-tumor activity of NK cells. TIGIT is an immune checkpoint constitutively expressed on NK cells and usually up-regulated on trNK cells. TIGIT blockade is capable of reversing the exhausted status of trNK in CRC patients, thus representing a potential new strategy to explore in immunotherapy[122]. Moreover, in CRC patients TIM-3 is up-regulated on circulating NK cells and correlates with stage of the disease and poor prognostic factors [56,120]. These data support the development of therapies targeting TIM-3 to restore anti-tumor immunity [56]. LAG-3 upon binding its ligand FGL1 facilitates the immune escape of the tumor. In mice it has been shown that LAG-3 reduces the NK cell-mediated anti-metastasis activity. Thus, mAbs blocking LAG-3 is starting to be investigated [123].

- BiKEs, TriKEs, and engagers: Bi- and Tri- specific killer engagers have been designed to create an efficient immunological synapse between NK cells and tumor cells. These molecules are small construct composed by two or three single chain variable fragments (scFv) made up of variable heavy and light chains of an antibody. First, CD16 was chosen to increase NK cell response. In fact, BiKE and TriKE designed with anti-CD16 (scFv) can trigger NK cells more efficiently than the natural mAbs interacting with the FcR CD16 and mediating ADCC. BiKEs and TriKEs have numerous advantages over the use of mAbs, including increased distribution for their small size, lower immunogenicity and great flexibility [51]. In view of the high flexibility of the BiKE/TriKE platform, it can be also envisaged the use of blocking scFv targeting inhibitory checkpoint receptors or TGF-β to reduce the negative signaling in the TME.

- CAR-NK cells: A promising approach to treat advanced cancers that are refractory to conventional therapies is the genetic modification of immune system effector cells. Use of a chimeric antigen receptor (CAR) targeting a tumor-associated antigen provides a valuable approach to increase the efficacy of effector cells. In fact, CAR confers to an immune cell high affinity binding to the tumor cell and induction of a potent effector function. CAR is a genetically engineered protein, composed by an extracellular domain specific for the target antigen, a transmembrane domain and an intracellular signaling domain, responsible for the transduction of the activating signal [98]. Only autologous T cells can be employed due to the risk of inducing severe GvHD. Thus, the major

limitations on the use of CAR-T concern the production costs, the time necessary for the generation and for the expansion of a certain number of cells in times compatible with the risk of tumor progression. In patients treated with CAR-T it is possible to observe adverse reactions, such as cytokine release syndrome or neurotoxicity [124]. For these reasons, the application of CAR technology to the NK cells could be advantageous as compared to CAR-T cells. For example, allogenic NK cells can be used, because they don't cause GvHD. Moreover, clinical trials using CAR-NK cells did not show cytokine release syndrome [125]. The downside of using CAR-NK cells is the difficulty of isolating NK cells from donor PBMC, expanding and transducing them. This has greatly limited the number of clinical trials using CAR-NK cells [120]. Several attempts have been made to obtain a large number of NK cells from PB and increasingly optimized protocols are being developed to increase the ability to expand these NK cells[126].

1.8 ANATOMY OF CRC

Colorectal cancer (CRC) is one of the most frequent malignancies worldwide, is the third most deadly and fourth most commonly diagnosed in the world (Fig. 2).

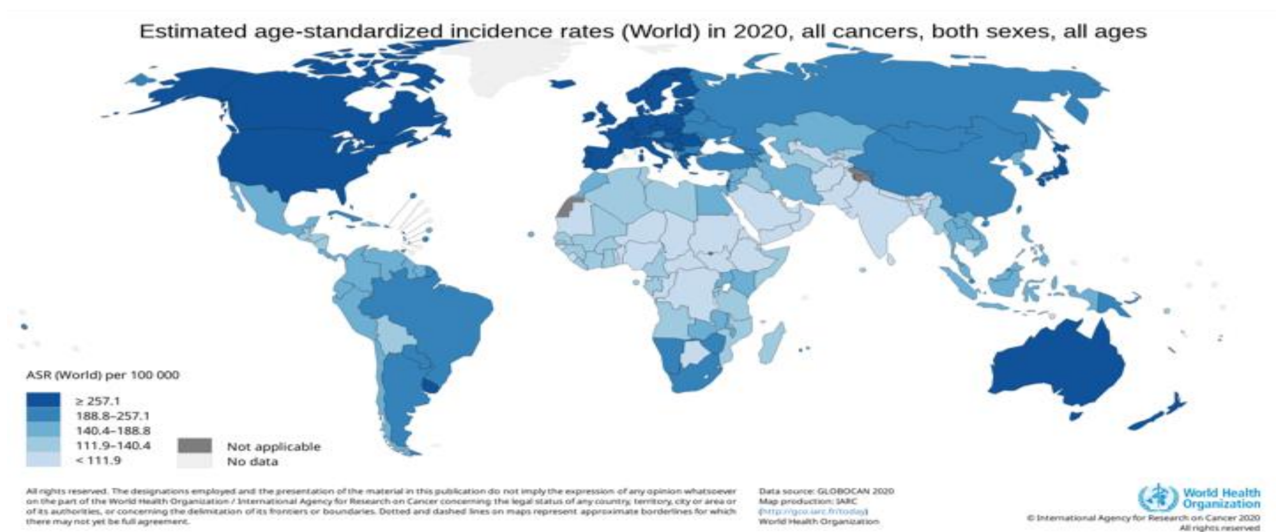

*Figure 2: **EPIDEMIOLOGY OF CRC** data from GLOBOCAN 2020*

CRC is the second most frequent malignancy in males and third one in females. It has been estimated that more than one million individuals develop worldwide colorectal cancer each year and the disease-related mortality correspond to about 33% in the developed world [127,128].

The main function of the colon is to reabsorb water, minerals and nutrients. The gastrointestinal epithelium, to facilitate absorption, is organized to cover crypts and villi. At the bottom of the crypt self-renewing pluripotent stem cells [129] are localized. Progenitor cells differentiate into specialized cells, such as Paneth cells, goblet cells, and enterocytes, that migrate out of the crypt and up the villus. Here they

perform their function and after 14 days they undergo apoptosis. This process is highly regulated by signaling proteins such as Wnt, BMP, TGF-β [130].

The cancer arises when some cells of the epithelium acquire a series of genetic or epigenetic mutations which give them selective advantages [131]. These cells with abnormally intensified replication are hyperproliferative and give rise to a benign adenoma, which then over the years can transform into carcinoma and metastasize [132]. CRC currently comprises a heterogeneous group of diseases caused by numerous mutations or mutagens, making it difficult to design a "catch-all" molecular therapy [133]. However, the CRC development is preceded by a benign neoplastic lesion such as an adenomatous polyp or a serrated polyp, which can growth gradually for 10-20 years before evolves into carcinoma. Moreover, the CRC tumorigenesis is a multi-step pathway involving several factors, such as age, hereditary predisposing factors, diet lifestyle and exposure to carcinogens [128].

Histologically CRC can be divided into several types, including adenocarcinoma, mucinous adenocarcinoma and signet ring cell cancer. CRC is classified by the tumor-lymph node-metastasis (TNM) staging system, that is used for many types of cancer. This system provides information about the localization of tumor, the size of tumor, whether the cancer has spread to nearby lymph nodes and also whether it has spread to a different part of the body (metastasis). After each letter of TNM system there are letter or number that give more details and indicates the seriousness of the tumor:

- T: refers to the size and extent of primary tumor (+ X,0,1,2,3,4)
- N: refers to the number of nearby lymph nodes that have cancer and their location (+ X,0,1,2)
- M: refers to whether the cancer has metastasized (+ X,0,1)

The TNM system helps describe cancer in great detail, but often, it is used in combination with a system grouped into five less-detailed stages:

- Stage 0: abnormal cells are present, they are not cancer but they may become cancer
- Stage 1-2: cancer is present, not spread to nearby lymph node
- Stage 3: cancer is present and it has spread into nearby lymph node
- Stage 4: cancer presents metastasis distant from the primary tumor

The TNM staging system provides broad prognosis information and facilitates decision-making in therapy. To predict the likelihood of detecting metastasis, at least 12 lymph nodes need to be examined. The distance between tumor and transverse margin is considered optimal over 5cm in order to avoid recurrence in the anastomis [134,135].

The CRC can have sporadic or hereditary forms. About 15% of all CRC cases has a genetic origin and hereditary history: the most common forms are Lynch syndrome, representing approximately 5% of the cases, and familial adenomatous polyposis, both of which are caused by autosomal dominant mutations [128].

The Lynch syndrome is caused by mutation in mismatch repair (MMR) genes MLH1, MSH2, MSH6 and PMS2, leading to the inability to repair errors occurring during DNA replication, in particular in the repeat sequences, microsatellites. This leads to an accumulation of mutations in coding and non-coding microsatellites, the so called microsatellite instability (MSI), while the absence of these mutations is called microsatellites stability (MSS)[136]. It is important to identify patients with Lynch syndrome as they have increased risk of developing cancer. The Lynch syndrome tumors are characterized by proximal colonic location, mucinous or signet ring cell type, poor differentiation, and the presence of infiltrating lymphocytes[136] .

Familial adenomatous polyposis (FAP) is caused by mutation in the tumor suppressor gene of APC, leading to numerous adenomatous colorectal polyps. FAP represent approximately 1% of all CRC cases, in fact, in the absence of treatment, the risk of developing cancer within 40 years is 100% [137].

The sporadic form of CRC arises due to accumulation of genetic and epigenetic alterations from normal colonic epithelium. Tumor development goes through various steps leading to the accumulation of mutations in suppressor genes and oncogenes from normal cell to carcinoma. This process can last a few years, in fact four or five genetic alterations are necessary [138]. It has been described that tumorigenesis in the sporadic CRC arises from different pathway, in particular chromosomal instability (CIN) and microsatellite instability (MSI) pathway are the most known, and recently it has been described a third pathway that consists in CpG island methylator phenotype (CIMP) [139,140] .

1.8.1 CHROMOSOMAL INSTABILITY PATHWAY

Chromosomal instability (CIN) is the most common pathway of genomic instability and represents approximately 60-70% of all CRC cases. CIN is characterized by accumulation of mutations in tumor suppressor genes and oncogenes, such as APC, TP53, SMAD2, SMAD4, KRAS, PK3CA and others [141]. These mutations lead to transformation of normal epithelium to colon adenocarcinoma [142]. Most MSS tumors have mutations in the CIN pathway. These tumors are aneuploid or polyploid, highly differentiated, rarely mucinous, have no lymphocytic infiltrate, poor prognosis and no tumor site predominance, all characteristics that distinguish them [141,142].

1.8.2 MICROSATELLITE INSTABILITY PATHWAY

Microsatellite instability (MSI) accounts for approximately 15% of sporadic CRC cases and more than 95% of Lynch syndrome cases. MSI tumors arise from inactivation of 1 of the 4 mismatch repair (MMR) genes: MLH1, MSH2,MSH6 and PMS2 [143]. In the human genome there are >100.000 areas of short tandem repeat sequences, microsatellites, that are susceptible to replication “slippage” and strongly dependent on the MMR system for repair. During normal DNA replication, efficient MMR detects the DNA mismatch errors and MLH1 and/or PMS2 help in the removal of the mismatch errors and in the formation of correct DNA strands[144]. Instead, deficient MMR leads to a rapid accumulation of genetic errors in the

microsatellites causing subsequent MSI. Damage to MMR leads to numerous mutations leading to a “hypermutato” phenotype or development of MSI tumors [144,145].

In sporadic cases MSI is mainly caused by hypermethylation of the promoter region of MLH1, resulting in transcriptional silencing. Instead, in Lynch syndrome, MSI develops from a germline mutation of one of the DNA MMR genes [146]. MSI is more common in stage II than stage III (20% and 12% respectively) and is even less common in stage IV (4%) [147].

MSI tumors usually have peculiar characteristics: preferred location in the right colon, poor differentiation, abundance of tumor infiltrating lymphocytes, diploid DNA content, fewer KRAS and p53 mutations, and less aggressive clinical behavior [146]. Moreover, MSI tumors present a frameshift mutation in specific gene such as β-catenin, transforming growth factor β receptor II (TGF-βRII), epidermal growth factor receptor (EGFR) or Bcl-2-associated X protein (BAX) [148–150]. In CRC patients the microsatellite status is an important prognostic marker, and it is analyzed by testing at least a MMR protein by immunohistochemistry (IHC) or by PCR[151,152]. The diagnosis of MSI based on MMR evaluation by IHC identifies the gene involved by detecting loss of its protein product. This technique is highly concordant with DNA-based MSI testing, and it has been shown to have good sensitivity and excellent specificity [151]. In CRCs with loss of MLH1 protein expression, testing for a mutation in the BRAF oncogene is used to confirm a sporadic case and exclude Lynch syndrome. If patients do not have mutated BRAF is the best to perform germiline mutation testing to check for a probable alteration on the MLH1 gene [153].

The immune system plays an important role in the development and progression of CRC. MSI tumor presents abundant infiltrating lymphocytes (TILs). The abundance of CD3+ CD8+ cytotoxic TILs triggers an immune response resulting in "immunoediting” that leads to immune tolerance where tumor antigen escape the appropriate immune response [144]. The immunoediting is regulated by some immune checkpoints such as PD-1, CTLA4 and LAG3. MSI tumors have much higher mutational burden or neoantigenes (>20 fold) leading to greater responsiveness to immunotherapy than MSS tumors. MSI and MSS tumors differ in both PD-1 expression on TILs and PDL-1 on tumor cells. In fact, the TIL cells of MSI tumors express twice as much PD-1 compared to MSS, and the same for PDL-1 expression on tumor cells [144].

Microsatellite Stability Status can be used as a prognostic marker in CRC patients, especially in cases of localized advanced tumor stage II and III [152]. Numerous studies have observed a clinical correlation between MSI and survival, in fact sporadic cases of CRC with MMR deficiency generally present a favorable prognosis, especially in the early stage, in stage II more than in stage III [154]. Moreover it has been observed that among CRC patients with MSI, those with Lynch syndrome have a better prognosis than those with sporadic CRC [155]. At the moment, surgery remains the primary treatment in case of early diagnosis, however chemotherapy is recommended in high-risk stage II and stage III patients, but recent studies have

shown that CRC patients with MSI do not benefit from chemotherapy [156]. The treatment of MSI CRC patients with immunotherapy seems to be efficient for the abundance of infiltrating lymphocytes [157].

At the time of CRC diagnosis it is important to evaluate the microsatellite status. In fact, this information is not only a predictive marker for the response to chemotherapy and immunotherapy, but it is also a prognostic marker for the clinical outcome [145].

1.8.3 KRAS

KRAS is a protooncogene and a member of the BRAF gene family. In CRC tumorigenesis, KRAS mutations are early events and occurs in 30-50% of all CRC cases [158,159]. Mutations occur more frequently in codons 12 and 13 of exon 2, and less frequently in codon 61 of codon 3 [141], primarily in MSS tumors than in MSI tumors. KRAS mutations lead to constitutive EGFR independent activation and increased tumor cell proliferation, protection against apoptosis, increased metastasis spreading and angiogenesis [160].

The prognostic relevance of KRAS mutations is controversial but the majority of studies associate it with poor prognosis [161,162]. Recently mutated KRAS is used as a predictive marker in patients with metastatic CRC treated with anti-EGFR therapy [162]. In fact, mutated KRAS is associated with a negative response to anti-EGFR therapy. For these reasons the evaluation of KRAS mutations is recommended in CRC patients before starting therapies [163].

1.8.4 BRAF

BRAF is an oncogene, a member of the RAS family, and a mediator of the EGFR pathway. BRAF mutation occurs early in CRC tumorigenesis. BRAF mutation results in a constitutive activation and deregulation of signaling pathway [164]. BRAF mutation occurs in 4-15% of all sporadic CRC cases, most of which are MSI, and it is more frequent in right-sided tumors and in poorly differentiated adenocarcinoma or mucinous carcinoma[165].

BRAF mutation is associated with poor clinical outcome, indeed patients with MSS CRC tumors and mutated BRAF have shorter progression-free survival and overall survival [166].

1.9 NK CELLS IN CRC

In CRC patients, tumor-infiltrating NK cells show important differences with respect to peripheral blood NK cells in terms of both receptor repertoire expression and effector functions. Indeed they show a decreased expression of activating receptors such as NKG2D, NKp30, NKp46 and DNAM, and also in perforin [167–169,170(p201)]. These alterations lead to a reduced IFN-γ secretion, degranulation and cytotoxicity. Moreover, tumor-infiltrating NK cells show an increased expression of various immune checkpoints including KIRs, NKG2A, TIGIT and PD-1, that blocks the NK capacity of killing tumor cells [170,171].

2 AIM OF THE STUDY

CRC is known to be the third most common cancer diagnosed wordwide. A mixture of genetic and epigenetic modifications contributes to CRC etiology. Currently, surgery represents the principal treatment for complete removal of the tumor. However, many cases of CRC are diagnosed at an advanced stage and, despite surgery, develop recurrences or disseminations that can even end in death. For this reason it is very important to identify new biomarkers in the early stages of tumor progression.

In these three years of PhD, I've focused my studies on the analysis of NK cells in PB, tumor and tumor-free mucosa samples derived from CRC patients at different tumor stages with the aim of highlighting the presence of possible phenotypic variations and of their correlation to the compartment of origin or disease stage. It has been evaluated with particular attention the expression of different inhibitory immune-checkpoint receptors such as KIRs, NKG2A, PD-1, TIM-3, TIGIT, and of other markers, such as NKG2C and CD57, which can allow the identification of particular NK cell subsets. An additional goal of this study has been to identify the presence of tissue-resident NK cells (trNK) by specific marker (CD103 and CD49a) and phenotypically characterize them to verify differences with the circulating NK cells. In fact, verifying the presence of trNK cells in these pathologic tissues and understanding which receptors they express could pave the way for more specific therapies to enhance the antitumor NK cell function *in situ.*

In conclusion, these analyses would be important to better understand why NK cells display a reduced anti-tumoral activity and to propose new innovative immunotherapic approaches for CRC patients.

3 MATERIALS AND METHODS

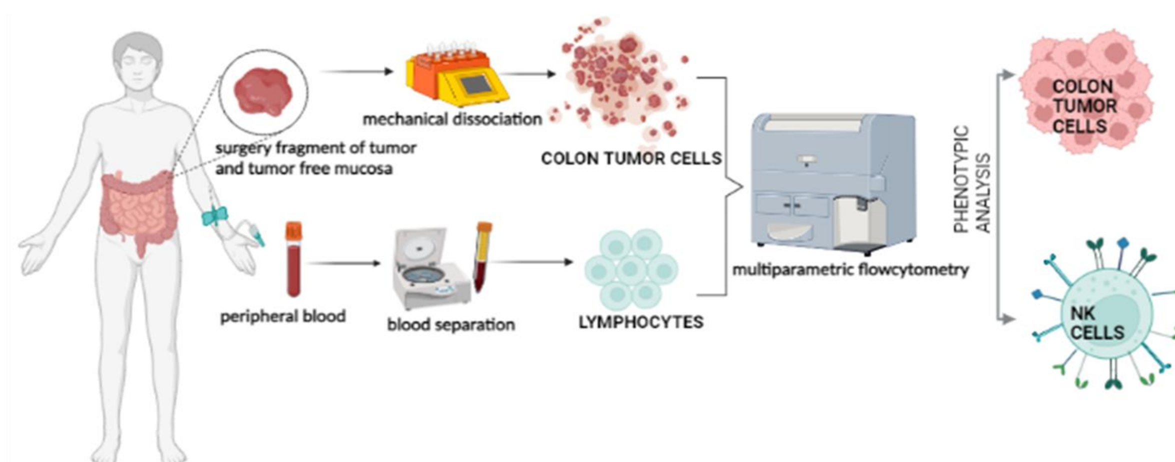

Figure 3: WORKFLOW. *The work involves the dissociation of surgical fragment tissue of tumor and tumor free mucosa of the CRC patients and the isolation of the PBMC from the patient's blood by ficoll. Tissue dissociation is performed by a mechanical dissociation which allows to obtain tumor cells and probable lymphocytic infiltrate. Cells obtained from both ficoll and tissue dissociation are analyzed by multiparametric flow cytometry. The study focuses on the phenotypic analysis of NK cells.*

3.1 PATIENTS

The population analyzed in this study includes 41 patients diagnosed with CRC at different tumor stage. Pathologic staging of all patients was classified according to TNM staging system. Patients underwent surgical resection of the colorectal tumor at the Department of Abdominal Surgery-General Pancreatic and Hepatobiliary Surgery Unit, E.O. Galliera Hospital (Genoa, Italy). All patients enrolled in this study had not received chemotherapy before tumor resection. For each patient, primary tissue (tumor) and tumor-free mucosa were taken by the pathologist and immediately immersed in a MACS Tissue Storage Solution (Milteny Biotec) in order to preserve tissue homeostasis. In addition to the surgical fragments, peripheral blood was also obtained from each patient. Patient cohort analyzed in the study is described in Table 1.

Table 1: ***Patients characteristics***

Samples	Number	% of total
CRC patients	41	
stages		
I	4	9,8%
II	24	58,5%
III	11	26,8%
IV	4	9,8%
Miscrosatellite status		
MSI	10	24,4
MSS	31	75,9%
Age		
>77	18	43,9%
<77	23	56,1%

3.2 ETHICAL STATEMENTS

This study was carried out under the recommendations of the ethical standards of the institutional and/or national research committee. The protocol was approved by the ethics committee of the Liguria Region, Genova, Italy (Galliera codex: 64UCS2018 for CRC patients; n. 39/2012 for healthy donors). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

3.3 TISSUE DISSOCIATION

Tumor and tumor free mucosa preserved in tissue storage were transferred and processed in our laboratory to obtain cells of interest. The tumor and tumor-free mucosa were dissociated using the Tumor Dissociation Kit Human (Miltenyi Biotec) and the gentleMACS Octo Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) accordingly to manufacturer’s instructions. Firstly, the surgical fragments were immersed and washed in a Petri dish with RPMI-1640 and then weighted. The surgical fragments were cut in very small pieces (<0,5cm) and transferred into a gentleMACS C Tube (MiltenyBiotec) (≤1 g/tube) with 4.7mL of RPMI-1640. In tubes containing the tissue pieces, a specific enzyme cocktail (200 µL of Enzyme H, 25 µL of Enzyme R, and 20 µL of Enzyme A) from the Human Tumor Dissociation Kit was added to enzymatically digest the tissue fragments. The C tubes were positioned in the gentleMacs Octo Dissociator with Heater (Milteny Biotec, Bergisch Gladbach, Germany), and the 37°C_ h_TDK2 program was run. At the end of the program the tissues were dissociated and a cell-suspension was obtained. This cell-suspension was filtered in a 50mL falcon through 100-µm cell strainer (Milteny Biotec, Bergisch Gladbach, Germany) to remove aggregates and debris, and after that RPMI-1640 was added up to 50 mL and centrifugated at 1500rpm for 7min. The supernatant was discarded while the pellet was washed with RPMI-1640 and centrifugated at 1500rpm for 7 min and mantained in suspension in a complete culture medium (RPMI 1640 supplemented with 2 mmol/L glutamine, 50 µg/mL penicillin, 50 µg/mL streptomycin, and 10% heat-inactivated FCS (Società Prodotti Antibiotici, Milano, Italy). Finally, live cells were counted by trypan blue dye.

3.4 ISOLATION OF HUMAN LEUKOCYTES

Peripheral Blood Mononuclear cells (PBMCs) were isolated by Ficoll-Paque (density 1.077g/ml, Sigma, St Louis, MO, USA) according to the manufacturer’s instructions. Fresh patients peripheral blood was diluted one-to-one with RPMI-1640 and carefully layered on Ficoll Hypaque (4:3). PBMC was separated from this solution by centrifugation at 2000rpm for 30 min. PBMC-containing plasma-Ficoll interface was collected and washed twice with RPMI-1640 1500rpm for 7min. Finally, cells were suspended in complete culture medium. PBMC isolated from Buffy coat of healthy voluntary blood donor (HD) obtained at Transfusion Center of the IRCCS Ospedale Policlinico San Martino (Genoa, Italy) were used as staining

control in flow cytometry experiments and as reference of normal expression of the different receptors analyzed.

3.5 FLOW CYTOMETRY AND MONOCLONAL ANTIBODIES

Phenotypic analysis of surface markers on PBMC and cell suspension of tumor and tumor-free mucosa was performed by multiparametric flow cytometry on the LSR-Fortessa flow cytometer (BD Biosciences).

Since this instrument can measure up to 18 fluorescence parameters and the main goal was to deep characterize NK cell tumor infiltration, 18-colors mAb cytofluorimetric panels were designed and tested for their efficiency in detecting molecules of interest on PBMCs derived from healthy donors. Once validated, the best performing panel was applied to CRC patient-derived PBMC and tissue samples. This panel includes antibodies indirectly and directly conjugated with fluorochrome (see Table 2) and Brilliant Stain Buffer (BD Biosciences) used to prevent unspecific cross-reactions between fluorescence labeled mAbs. A second multiparametric mAb panel was designed and used for analysis of the expression of some surface markers on cell suspension of tumor and tumor-free mucosa. mAbs used for this analysis were reported in Table 3.

Briefly, for NK cells assay, samples were incubated 30min at 4°C C three times with a) appropriate primary mAbs followed by b) PE- or FITC -conjugated isotype-specific goat anti-mouse secondary reagents (Southern Biotechnology Associated, Birmingham, AL) and c) with fluorochrome-conjugated mAbs. Washings with PBS 5% FCS were performed between one incubation and the next. For tumor cell analysis, samples were incubated 30min at 4°C with fluorochrome-conjugated mAbs.
Finally, all samples were incubated with Fixable Viability Stain 780 (BD Biosciences) to exclude dead cells, according to the manufacturer's instructions.

Data were acquired with FACSDiva™software (BD, Biosences, San Jose, CA, USA) and analyzed with FlowJo software (Tree Star In., Ashland, OR,USA).

Table 2. ***Un-conjugated and fluorochrome-conjugated monoclonal antibodies for NK staining***

Marker	Fluorochrome	Company
CD45	BUV496	BD
CD3	PE-CF594	BD
CD19	PE-CF594	BD
CD14	PE-CF594	BD
CD33	PE-CF594	BD
CD127	BV650	BD
CD56	BV480	BD
CD16	APC-CY7	BD
KIR2DL1/S1 (CD158a/h)	FITC	BD
CD158b(CHLEO)	FITC	BD

KIR3DL1/L2/S1	FITC	our production
NKG2A	PE-CY7	Beckman
PD-1		Kindly provided by prof. D. Olive
TIM-3	PERCP.Cy5.5	BD
LAG-3	APCR-700	BD
TIGIT	BV605	BD
LIR-1	APC	BD
NKG2C	BV421	BD
CD57	BUV395	BD
CD103	BV711	BD
CD49a	BV786	BD
CD69	BUV737	BD

Table 3. ***Fluorochrome-conjugated monoclonal antibodies for tumor cell staining.***

Marker	Fluorochrome	Company
CD90	BV480	BD
SYTO16	BB515	Molecular probes
CD45	BUV496	BD
PDL-1	APC-R700	BD
EPCAM	BV786	BD
CD133	APC	BD
CD24	BV395	BD
PDL-2	APC-R700	BD
HLA-ABC	BV650	BD
CD44	PE	BD

3.6 STATISTICAL ANALYSIS

Statistical analysis has been performed using Mann-Whitney tests to compare the various samples analyzed. Analyses were performed comparing both different compartments (PB vs T vs CTR) within the same tumor stage, and stage I-II and stage III-IV CRC patients in the same compartment. GraphPad Prism version (GraphPad Software, CA, USA) was used to perform all statistical analysis. Results were shown as median and min and max value. p-value ≤ 0.05 was considered statistically significant (*), and p < 0.01 (**), p < 0.001 (***) and p < 0.0001 (****) as highly significant.

4 RESULTS

4.1 EVALUATION OF NK CELLS FREQUENCIES IN PERIPHERAL BLOOD, TUMOR TISSUE AND TUMOR-FREE MUCOSA DERIVED FROM CRC PATIENTS

Cytofluorimetric analyses on single-cell suspensions were performed to evaluate the frequency of NK cells in peripheral blood (PB), tumor-tissue (T) and tumor-free mucosa (CTR) derived from 41 CRC patients. NK cells were identified by gating on viable $CD45^+Lin^-(CD3^-CD19^-CD14^-CD33^-)$, $CD127^-$ (to exclude ILCs), $CD56^+CD16^{+/-}$ cells (Fig. 4a). As shown in Fig. 4b, the frequency of NK cells, evaluated on $CD45^+$ leukocytes, was significantly higher in PB than in T and CTR, while no significant differences existed between tumor and tumor-free mucosa.

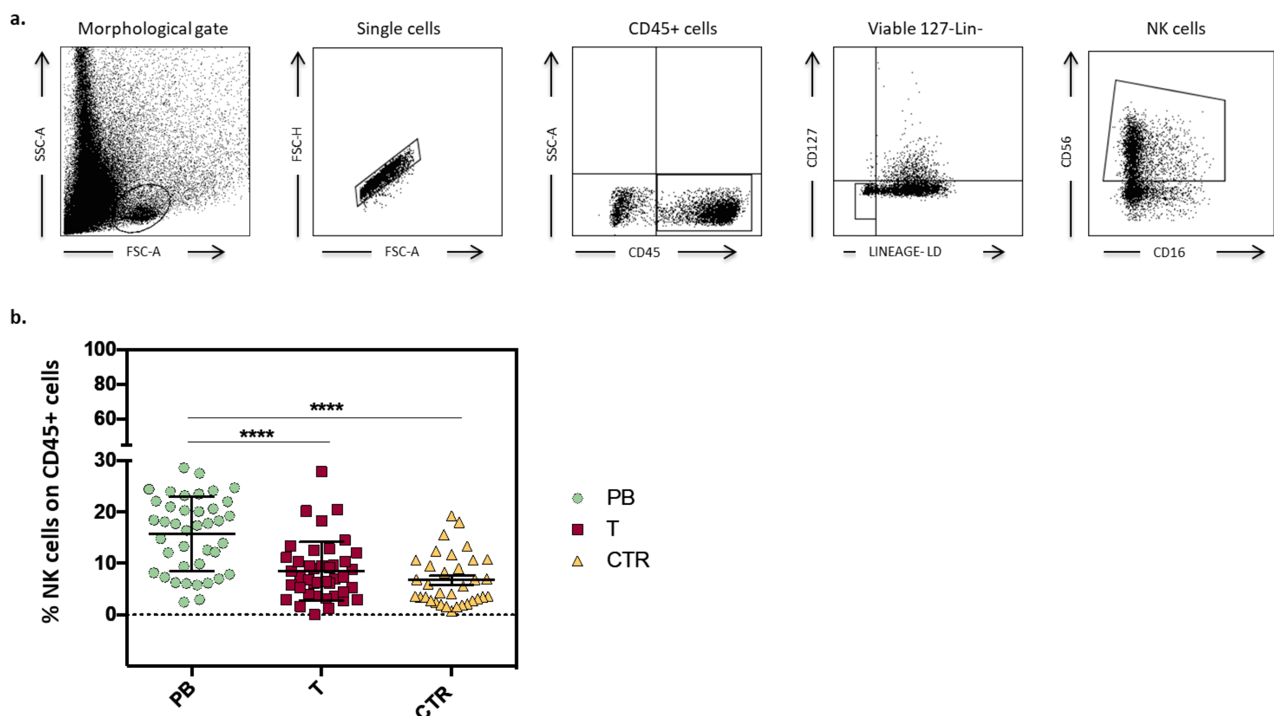

Figure 4: ***EVALUATION OF NK CELLS FREQUENCIES IN PERIPHERAL BLOOD (PB), TUMOR (T) AND TUMOR-FREE TISSUE (CTR), DERIVED FROM CRC PATIENTS.***

a. Gating strategy to identify NK cells: morphological gate on lymphocytes, single cells, $CD45^+$, $CD127^-$, Lin^- (CD3-CD19-CD14- CD33-), viable $CD56^+CD16^{+/-}$ cells.

b. Percentage of NK cells among $CD45^+$ cells in the three compartments analyzed (PB, T and CTR) derived from CRC patients (n=41). NK cells frequency was significantly higher in PB than in T and in CTR p(****). Statistical significance is indicated: $p \leq 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****)

4.2 SURFACE RECEPTORS EXPRESSION ON NK CELLS DERIVED FROM PERPHERAL BLOOD, TUMOR AND TUMOR-FREE MUCOSA OF CRC PATIENTS STRATIFIED BY TUMOR STAGE

To deeply characterize NK cells phenotypically, multiparametric flow cytometry analyses were performed on NK cells derived from PB, T and CTR, stratifying the CRC patients according to their tumor stage (non-metastatic stages I-II vs metastatic stages III-IV). In our analyses, the surface expression of various immune checkpoints (ICs) was evaluated and it included both ICs typically expressed on NK cells such as KIRs and NKG2A, but also other inhibitory receptors such as PD-1, TIM-3, LAG-3 and TIGIT. Further receptors relevant for NK cell activity and subsets identification such as CD16, NKG2C and CD57 have also been analyzed.

First, we focused on the analysis of PB-NK cells derived from CRC patients, stratified by tumor stage (I-II and III-IV), comparing these data with those obtained in age-matched HD PB-NK cells.

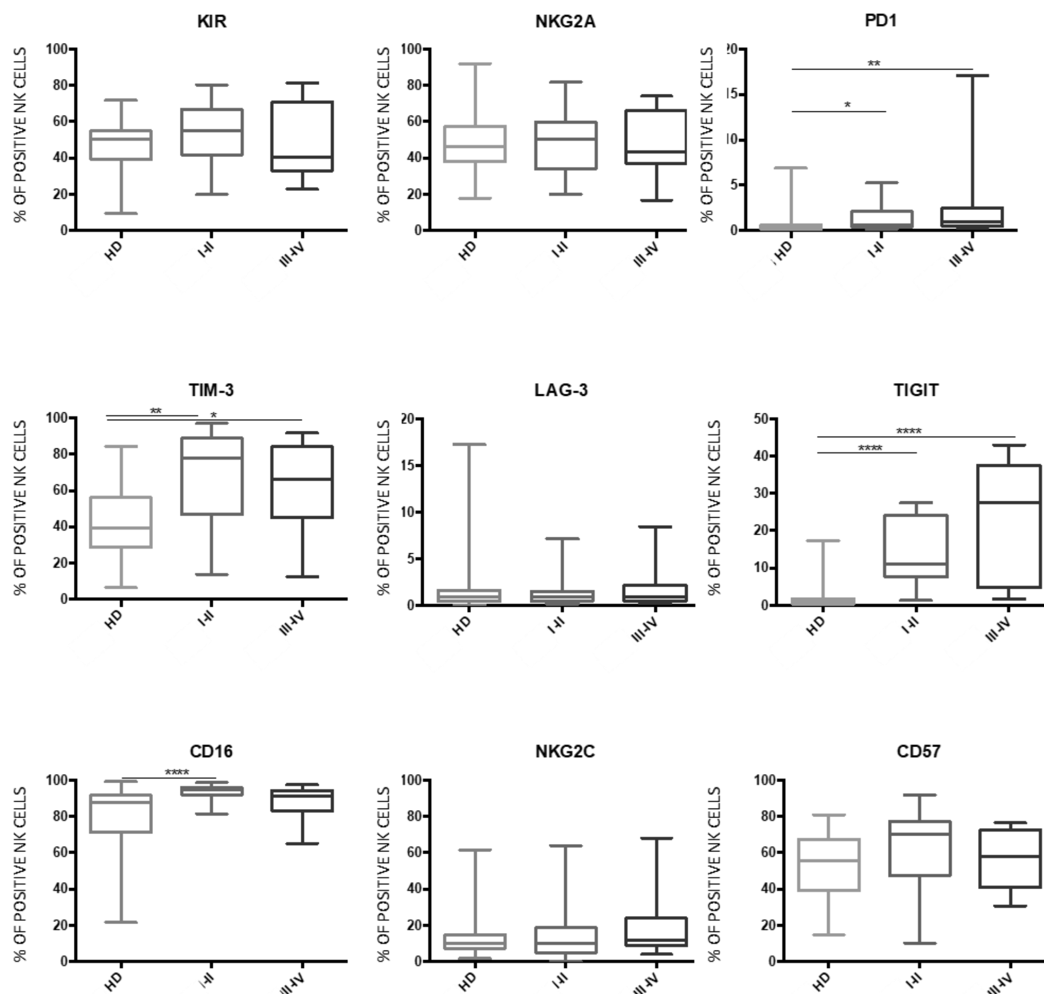

Figure 5: ***PHENOTYPIC CHARACTERIZATION OF PB-NK CELLS FROM CRC PATIENTS STRATIFIED BY TUMOR STAGE***

The percentage of PB-NK cells from HDs (n=39), stage I-II CRC patients (n=26) and stage III-IV CRC patients (n=15), expressing the indicated surface markers, is shown as box plots. Statistical significance among groups was calculated using Kruscall-Wallis p-value ≤ 0.05 (*), p < 0.01 (**), p < 0.001 (***) and p < 0.0001 (****).

As shown in Fig.5, the percentage of NK cells expressing PD-1, TIM-3 and TIGIT is significantly higher in CRC patients than in HDs. Interestingly, TIGIT and, to a lesser extent, PD1 expression show a tendency to increase with tumor progression. Instead, LAG-3 presents very low values, similar in the three groups of PB NK cells analyzed. Moreover, no significant differences among the three groups were observed for NKG2A, KIRs, CD16, NKG2C and CD57 expression.

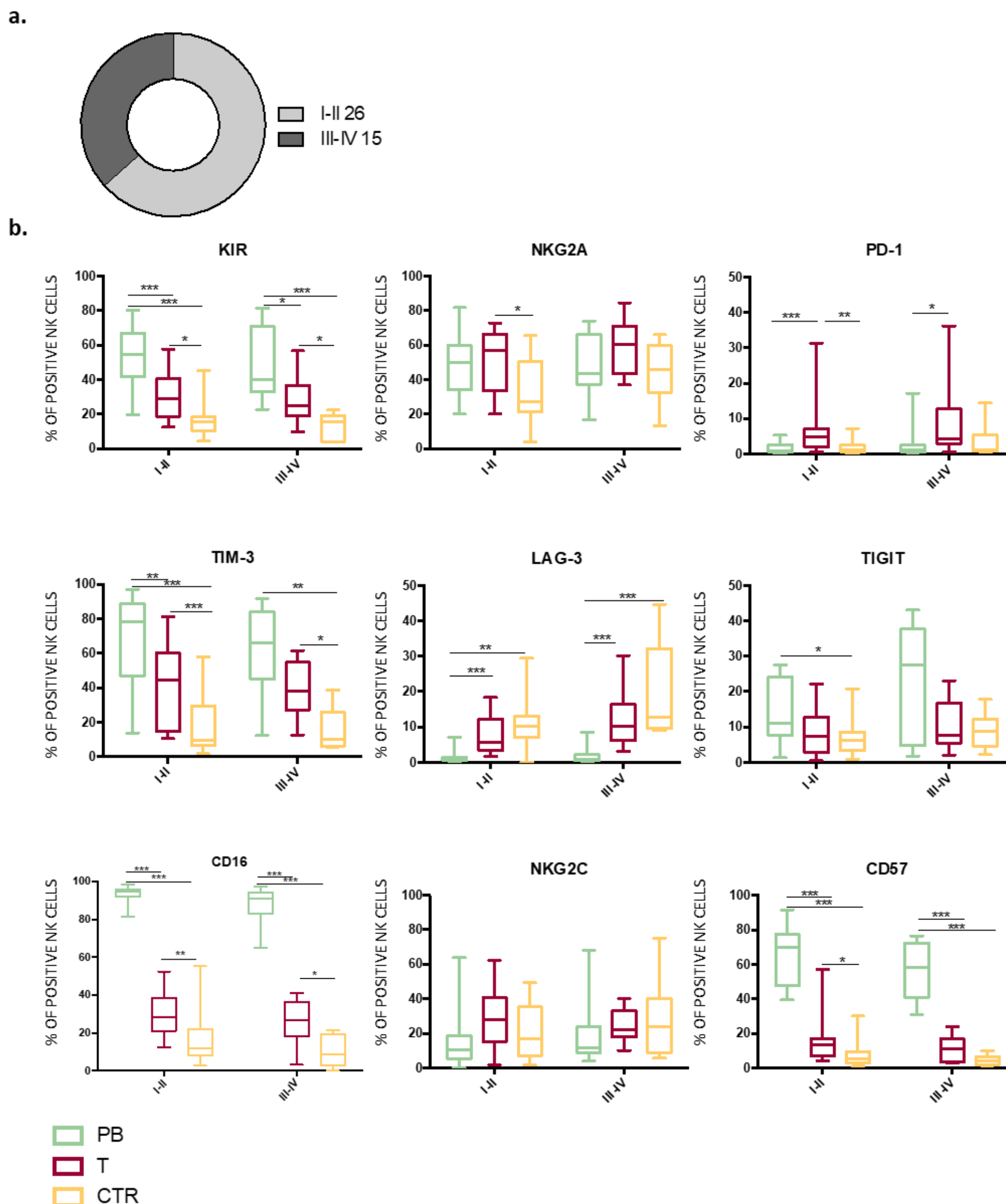

*Figure 6: **PHENOTYPIC CHARACTERIZATION OF NK CELLS DERIVED FROM PB, T AND CTR OF CRC PATIENTS STRATIFIED BY TUMOR STAGE**.*

a. *Number of CRC patients analyzed in each stage: I-II=26 and III-IV= 15*
b. *The percentage of PB, T and CTR NK cells expressing the indicated surface markers is shown as box plots stratifying CRC patients by tumor stage. Comparisons were performed between different compartments (PB vs T vs CTR) within the same tumor stage, and between stage I-II and stage III-IV CRC patients in the same compartment. Statistical significance was calculated with Mann-Whitney p-value ≤ 0.05 (*), p < 0.01 (**), p < 0.001 (***) and p < 0.0001 (****).*

Next we compared the expression of the different surface molecules on NK cells derived from the various compartments (PB, T and CTR) of the CRC patients stratified by tumor stage (Fig.6a). As shown in Fig.6b, KIR expression was significantly higher in PB than in T and CTR, but it could be detected on a significant larger fraction of tumor-associated NK cells (T) as compared to tumor-free mucosa (CTR), independently of the tumor stage. The same trend was also observed for TIM-3 and TIGIT, although statistical significance was not reached for the latter. NKG2A was slightly more expressed in T than in PB and in CTR in both stages but the difference was significant only in non-metastatic CRC patients. Interestingly, the immune checkpoint PD-1 resulted more expressed in tumor-associated NK cells than in tumor-free mucosa and PB. Instead, LAG-3 was significantly more expressed on tissue-derived NK cells (both from T and CTR) than on PB-NK cells, with a slight tendency to increase in patients with advanced tumors. At variance with PB-NK cells, only a minor fraction of T and CTR NK cells expressed CD16. Moreover, CD57, a marker used to identify terminally differentiated cells in PB, was expressed only on a very small fraction of tissue-associated NK cells. NKG2C did not show any significant difference at any stages analyzed.

Overall these analyses show that tumor-associated NK cells show a significant increase in immune checkpoints expression such as TIM-3, PD-1, KIRs and NKG2A as compared to tumor-free mucosa.

4.3 PHENOTYPIC CHARACTERIZATION OF NK CELLS DERIVED FROM PERIPHERAL BLOOD, TUMOR TISSUES AND TUMOR-FREE MUCOSA OF CRC PATIENTS STRATIFIED BY MSI/MSS CONDITION

It's well known that MSI CRC is characterized by an enriched infiltrate of immune cells and that blockade of the PD-1/PDL-1 axis proved to be effective in the treatment of these patients. For this reason the expression of PD-1 was carefully evaluated on NK cells together with other molecules, stratifying the patients by MSI/MSS condition (Fig.7a).

The results depicted in Fig. 7b show a remarkably higher expression of PD-1 on tumor-associated NK cells derived from MSI CRC patients (T MSI) as compared to MSS CRC patients (T MSS). Notably, PD-1 expression was higher in T than in PB and CTR in both MSI and MSS CRC patients. The surface expression of no other NK cell inhibitory receptors significantly correlated with the MSI condition, although it can be observed that TIM-3 was expressed on a slightly higher fraction of tumor-associated NK cells from MSI than from MSS CRC patients. As already observed in the whole cohort (Fig.6), TIM-3 expression is lower in tumor and in tumor-free mucosa than in PB also upon MSI/MSS stratification. In addition, NKG2C expression is slightly higher on T MSI than T MSS although not statistically significant. On the contrary, CD57 is significantly more expressed in MSS T NK cells than MSI T NK cells. These observations suggest that the

efficacy of immunotherapeutic treatments in MSI CRC patients based on PD-1/PDL-1 blockade (e.g. anti-PD-1 or anti-PD-L mAbs) could rely also on a superior anti-tumor potential exerted by PD1^{+} NK cells upon mAb-mediated masking of the PD-1 inhibitory receptor.

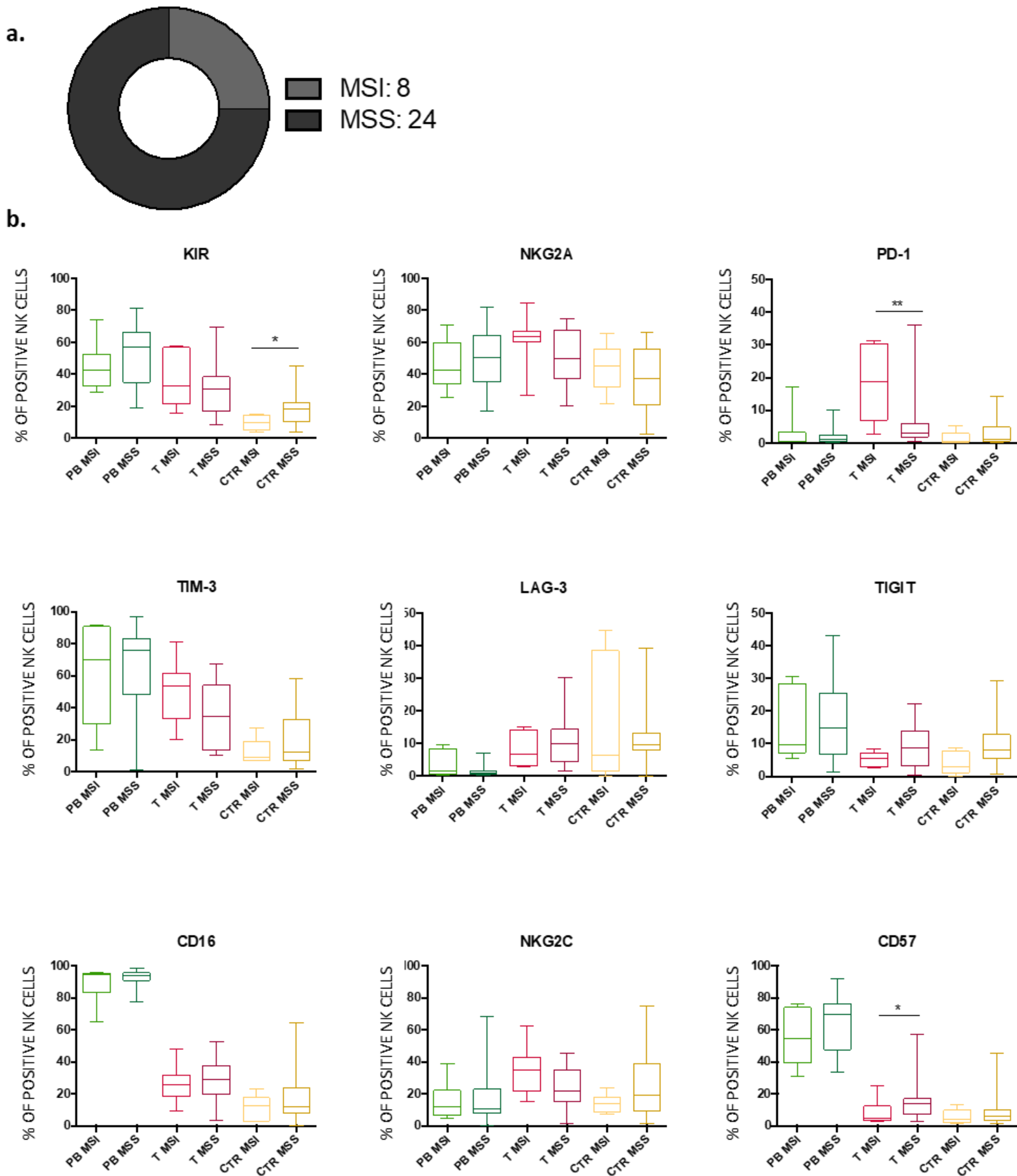

*Figure 7: : **SURFACE RECEPTORS EXPRESSION ON NK CELLS DERIVED FROM PB, T AND CTR OF CRC PATIENTS STRATIFIED BY MSI/MSS CONDITION***

a. *Number of CRC patients analyzed: MSI=8 and MSS=24.*
b. *The percentage of PB, T and CTR NK cells expressing the indicated surface markers is shown as box plots stratifying CRC patients by MSI and MSS condition. Comparisons were made within the same compartments (PB, T, CTR) between MSI and MSS patients. Statistical significance was calculated with Mann-Whitney p-value ≤ 0.05 (*), p < 0.01 (**), p < 0.001 (***) and p < 0.0001 (****)*

4.4 IDENTIFICATION AND CHARACTERIZATION OF TISSUE-RESIDENT NK CELLS DERIVED FROM TUMOR TISSUES AND TUMOR-FREE MUCOSA OF CRC PATIENTS STRATIFIED BY MSI/MSS CONDITION

NK cells have been found in both tumor and normal colonic tissues from the analyzed CRC patients, but it remains to be clarified whether these cells could be resident NK cells or represent recirculating PB-derived NK cells, homing temporarily to these tissues. To investigate this issue, the expression of the tissue-residency markers CD103 and CD49a have been analyzed on NK cells derived from tumor and tumor-free mucosa of CRC patients stratified by MSI/MSS condition. Indeed, these surface receptors have been suggested to facilitate the retention of lymphocytes in peripheral tissues including the gut, thus confining immune responses adapted to local needs. Thus, in our analyses NK cells expressing one or both markers (i.e. $CD103^{+}CD49a^{-}$, $CD103^{+}CD49a^{+}$ and $CD103^{-}CD49a^{+}$ NK cells) were considered tissue-resident NK cells, whereas NK cells lacking both markers (i.e. $CD103^{-}$ $CD49a^{-}$ NK cells) were indicated as non-tissue-resident NK cells (Fig.8).

First, we observed that in tumor tissues derived from MSI CRC patients there was a predominance of tissue-resident NK cells (Fig.8b). On the contrary, a greater frequency of non-resident NK cells was present in MSS CRC tumors (Fig.8b). We next analyzed and compared the surface signature of these two tumor-associated NK cell subsets (tissue-resident vs non-tissue-resident) to get insights in the heterogeneity of tissue-derived NK cell populations, primarily in terms of immune checkpoint expression.

As previously shown (Fig.7), $PD1^{+}$ NK cells were enriched in tumor tissues from MSI compared to MSS CRC. Interestingly, by dissecting tumor-associated NK cells based on CD103 and CD49a expression we could observe that in MSI CRC patients, $PD\text{-}1^{+}$ NK cells were mainly tissue-resident (Fig.8c).

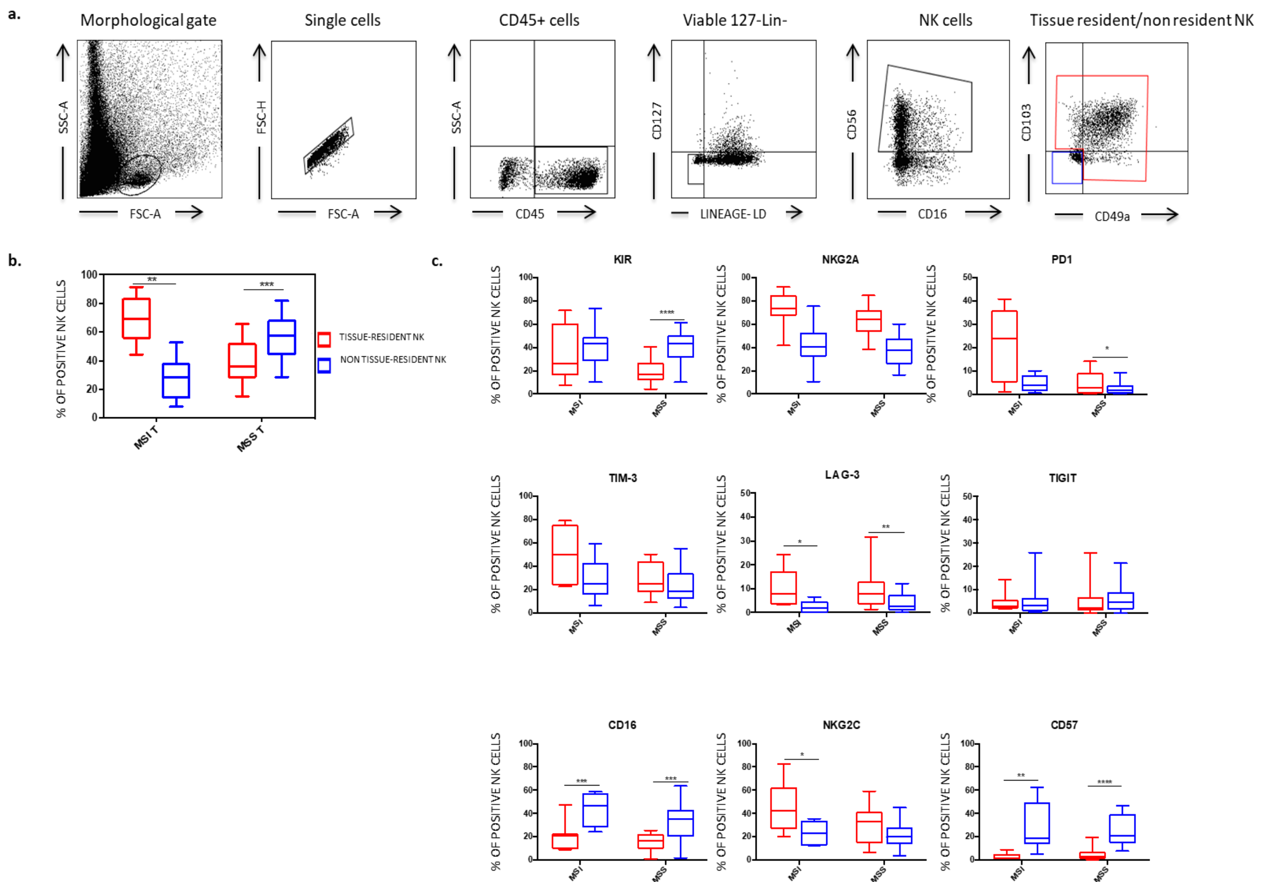

*Figure 8: **IDENTIFICATION AND CHARACTERIZATION OF TISSUE-RESIDENT NK CELLS DERIVED FROM T AND CTR OF CRC PATIENTS STRATIFIED BY MSI/MSS CONDITION***

a. *Gating strategy to select tissue-resident NK cells from tumor tissues: morphological gate on lymphocytes, single cells, CD45+ CD127- Lin- (CD3-CD19-CD14-CD33-) CD56+CD16+/-, CD103+CD49a-/CD103+CD49a+/CD103-CD49a+ (in red). Gating strategy to select non-tissue-resident NK cells from tumor tissues: morphological gate on lymphocytes, single cells, CD45+ CD127- Lin- (CD3-CD19-CD14-CD33-) CD56+CD16+/-, CD103-CD49a- (in blue).*

b. *Frequencies of tissue-resident and non-tissue-resident NK cells derived from tumor tissues of MSI and MSS CRC patients.*

c. *The surface receptors expression has been analyzed on tissue-resident and non-tissue-resident NK cells derived from tumor tissues of MSI and MSS CRC patients and depicted as box plots indicating the percentage of NK cells expressing the indicated surface marker. Comparisons between tissue-resident and non-tissue-resident NK cells were made within each tumor condition. Statistical significance was calculated with Mann-Whitney p-value ≤ 0.05 (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****)*

Similarly, NKG2A$^+$, NKG2C$^+$, LAG-3$^+$ and TIM-3$^+$ NK cells were more represented in tissue-resident NK cells in both MSI and MSS CRC patients. However, due to the limited sample size of MSI CRC patients, statistical significance was not reached for all markers. On the contrary, CD16$^+$ and CD57$^+$ NK cells were mainly non-tissue-resident in both MSI and MSS CRC patients. In addition, although a substantial fraction of tissue-resident NK cells expressed KIRs, the frequency of KIR$^+$ NK cells tended to be higher in non-resident NK cells from both MSI and MSS CRC where it reached statistical significance. Thus, a lower expression of markers typically expressed on mature circulating NK cells, such as CD16, CD57 and KIRs, characterized CD49a$^-$CD103$^-$ non-tissue-resident NK cells.

Taken together these results support the hypothesys that tissue-associated NK cells lacking the tissue residency markers CD49a and CD103 represent recirculating PB-derived NK cells.

4.5 HETEROGENEITY OF TISSUE-ASSOCIATED NK CELL SUBSETS IN CRC PATIENTS STRATIFIED BY MSI/MSS CONDITION

In order to better investigate the heterogeneity of tumor-associated NK cells and possibly identify specific NK cell subets co-expressing different immune checkpoints and tissue residency markers in the two groups of MSI/MSS CRC patients, we analyzed the previously shown mutiparametric flow cytometry data by dimensional reduction using t-SNE algorithm. In particular, we could confirm that the expression of PD-1 and the presence of tissue-residence markers, CD103 and CD49a, are higher in MSI than MSS patients. Moreover, in MSI CRC patients, four different PD-1$^+$ NK cell subsets can be identified that mainly co-express tissue residency markers (CD103, CD49a, CD69), are characterized by a distinct expression of the inhibitory receptors NKG2A, KIR, TIM-3, TIGIT, consistently lack LAG-3 and CD57 and can express NKG2C in combination with KIR and/or NKG2A (Fig.9 colored circles).

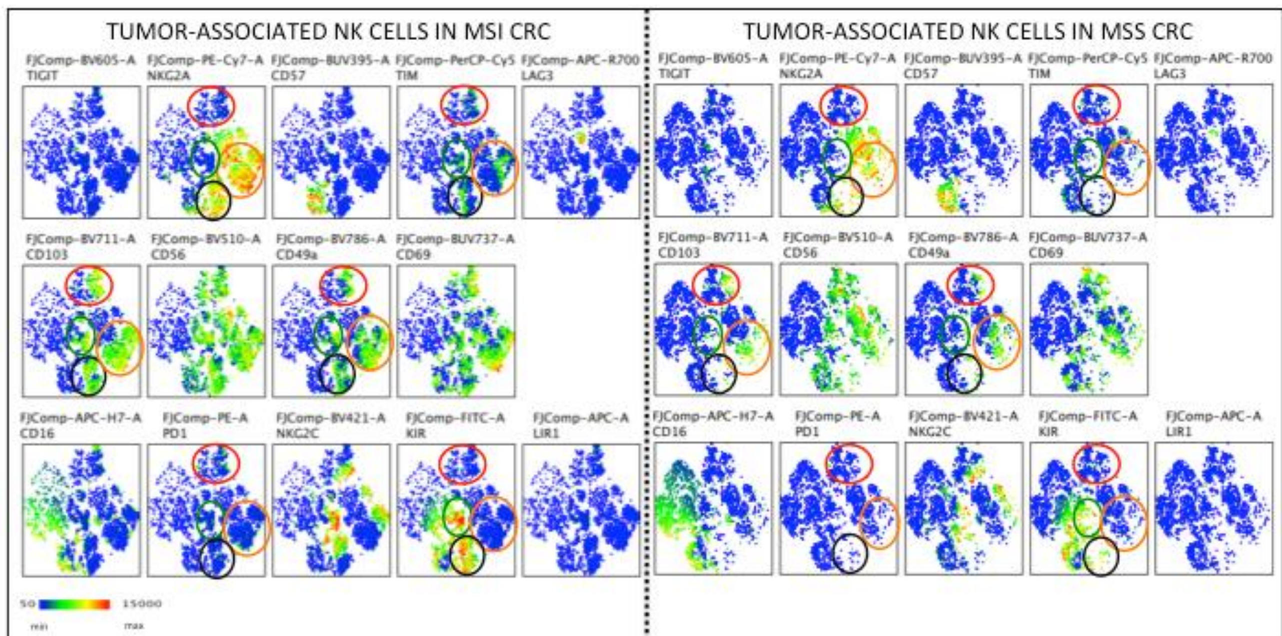

***Figure 9: t-SNE REPRESENTATION OF DIFFERENT IMMUNE CHECKPOINTS AND TISSUE-RESIDENCY MARKERS ON TUMOR-ASSOCIATED NK CELLS FROM MSI/MSS CRC PATIENTS**. t-SNE representation of the coexpression of TIGIT, NKG2A, CD57, TIM-3, LAG-3, CD103, CD56, CD49a, CD69, CD127, CD16, PD-1, NKG2C, KIRs, and LIR-1 on tumor-associated NK cells from three representative MSI CRC patients, and three representative MSS CRC patients, identifying four PD-1+ populations in MSI CRC patients:*

a. *characterized by the CD56+CD127-**NKG2A+KIR-NKG2C-**CD57-CD103+CD49a+CD69+LAG-3-TIM-3+/- phenotype (**orange circle**),*
b. *characterized by the CD56+CD127-**NKG2A+KIR+NKG2C+/-**CD57-CD103+CD49a+CD69+LAG-3-TIM-3+/- phenotype (**black circle***
c. *characterized by the CD56+CD127-**NKG2A-KIR+NKG2C+/-**CD57-CD103+/-CD49a+/-CD69+/-LAG-3-TIM-3+/- phenotype (**green circle**)*
d. *characterized by the CD56+CD127-**NKG2A-KIR-NKG2C+/-**CD57-CD103+/-CD49a+/-CD69+/-LAG-3-TIM-3+/- phenotype (**red circle**)*

Gate strategy: *morphological gate on lymphocytes, single cells, CD45+ CD127- Lin- (CD3-CD19-CD14-CD33-) CD56+CD16+/-.*

4.6 ANALYSIS OF TUMOR CELLS DERIVED FROM CRC PATIENTS

In tumor samples derived by the same CRC patients used for NK cell analysis and characterized by MSI and MSS condition, $CD45^-$ cells have been phenotypically characterized. Inside this population, tumor cells can be identified as cells expressing EpCAM at high densities and not expressing CD90. $CD45^-$ $EPCAM^+$ $CD90^-$ cells were detectable in large amounts in all MSI and MSS patients and without significant differences in terms of percentages. However, this population has been further characterized for the surface expression of cancer stem cells markers, such as CD133, CD24 and CD44, and for the surface expression of the main immune checkpoint ligands, such as PDL1-2 and HLA-class I molecules (Fig.10).

CD133 and CD44 showed a trend of larger expression on MSI than MSS CRC tumor cells, whereas CD24 and HLA-class I molecules seemed substantially expressed at the same frequency in the two conditions. Interestingly, a significant difference of PD-Ls expression on tumor cells was observed by comparing MSI or MSS CRC. In particular PDL1-2 was more expressed in MSI than in MSS CRC patients (Fig.10).

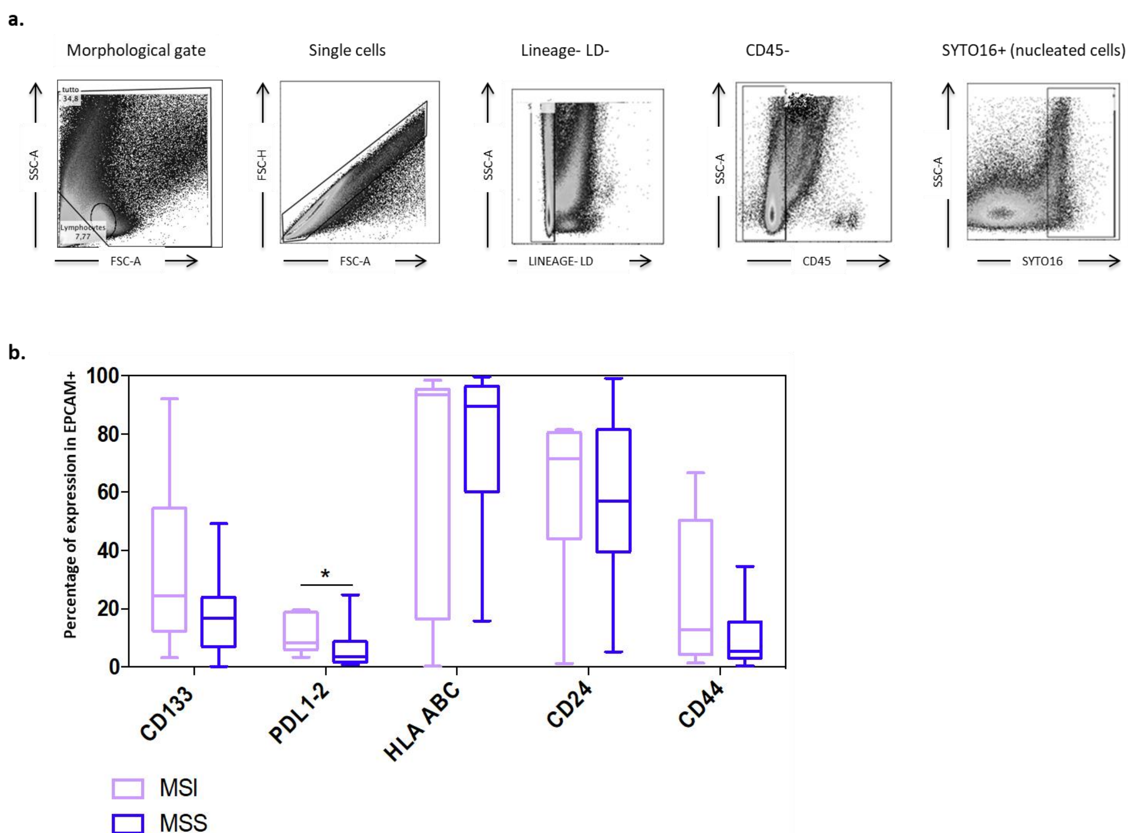

Figure 10: ***PHENOTYPIC CHARACTERIZATION OF TUMOR CELLS IN MSI AND MSS CRC PATIENTS.***

- ***a.*** *Gating strategy of tumor cells derived from CRC patients: morphological gate, single cells, lineage- viable , CD45- SYTO16+*
- *b. Surface expression of cancer stem cells markers and immune checkpoint ligands has been evaluated on cancer cells derived from tumor fragment of CRC patients (MSI=8 and MSS=24).*

4.7 DISCUSSION

Immunotherapy can represent an additional strategy to complement surgery, radiotherapy and chemotherapy to increase survival of patients with CRC, especially when the disease is diagnosed in later stages. However, given the high heterogeneity of CRCs, the efficacy of immunotherapeutic approaches is variable [156]. In particular, ICIs therapy is effective only in a small group of CRC patients characterized by microsatellite instability (MSI) and mismatch-repair deficiency (dMMR), which accounts for less than 20% of patients [144,145]. This group of CRCs is usually characterized by enriched infiltration of immune cells, a feature that has been related to the containment of metastases [146], a good clinical outcome and a positive response to ICI immunotherapies [154,157]. In particular, high numbers of CD8^{+} and CD4^{+} T cells, with Th1 profile, and of NK cells have been correlated with better prognosis in CRC patients [144]. However, due the complex interplay between tumour-associated immune cells in the context of cancer immunotherapy, MSI status alone is often not enough to precisely predict response to ICI [156] . Thus, the features of the immune cells in the TME could influence the outcome of immunotherapy and survival outcome in patients.

Moreover, tumour-infiltrating lymphocytes (TILs) can undergo distinct programs of differentiation, acquiring features of tissue-residency or exhaustion, a process during which T cells upregulate inhibitory receptors such as PD-1 and lose functionality. While residency and exhaustion programs of CD8^{+} T cells are relatively well-studied, these programs have only recently been appreciated in CD4^{+} T cells and remain largely unknown in tumour-associated NK cells [144].

Based on these preclinical and clinical data, it’s conceivable that a more detailed characterization of tumor-associated NK cells and novel immune-mediated therapies aimed at increasing the number and/or function of NK cells in tumor lesions could be useful strategies in CRC containment [157].

For these reasons, one of the main aims of the present study has been the characterization of tumor-associated NK cells, mainly in terms of immune checkpoints expression, and their comparison with NK cells present in peripheral blood or tumor-free samples derived from the same patient, in order to obtain more information that could be useful for suggesting more specific therapies capable of improving antitumor NK cell function *in situ*.

Another important aim has been the definition of new biomarkers related to NK cell population that can help monitor CRC patients at different tumor stages during the clinical follow-up. At this regard we have observed a significant upregulation of TIGIT expression on PB NK cells in CRC patients when compared with healthy donors, and this upregulation showed an interesting tendency to increase with tumor progression. Thus, the evaluation of TIGIT expression on PB NK cells could represent a possible biomarker to monitor the clinical follow-up of CRC patients at different tumor stage. However, further analyses are

needed to confirm this promising result, for example the evaluation of TIGIT expression on PB NK cells after surgery and/or after immunotherapy treatment of CRC patients and its correlation with prognosis.

Our results are in line with what was previously described for T cells. Indeed, the proportion of CD3^{+}TIGIT^{+} T cells had been shown to be increased in peripheral blood and cancer tissue of CRC patients when compared with the healthy donors, and a strong association was also observed between the elevated TIGIT expression and poor prognosis in the same patients [172]. Previous studies had also displayed that TIGIT and CD155 (a TIGIT ligand) are elevated in CRC compared to normal tissue [173]. Furthermore, TIGIT overexpression was also found on TILs in additional tumors, including lung [174], kidney [175], liver [176], glioma[177], melanoma [178], gastric cancer [172], and neuroblastomas [179], suggesting a possible role of TIGIT in the progression of different types of solid tumors.

Moreover, in the present study, we have observed that the percentage of TIGIT^{+} NK cells was higher in peripheral blood than in tumor tissues of CRC patients but, differently from previous studies [180], our data show a similar percentage of TIGIT^{+} NK cells in tumor and tumor-free tissues. Interestingly, tumor-associated TIGIT^{+} NK cells were mostly represented in MSS (although with a small percentage) than in MSI CRC, and they included both trNK cells and non-trNK cells, without specific preferences. Considering these data, the TIGIT blockade could enhance the anti-tumor function of both TIGIT^{+} PB NK cells infiltrating the tumor and tumor-associated TIGIT^{+} tissue-resident NK cells. However, since the TIGIT^{+} NK cell subset was poorly represented in the tumor (primarily in MSI CRC), it is likely that TIGIT blockade could act predominantly on TIGIT^{+} NK cells circulating from the blood to the tumor.

Even the percentage of PD-1^{+} NK cells (although it covered a small NK cell subset) was significantly higher in the PB of CRC patients than in the PB of HDs, and it showed a tendency to increase with tumor progression. However, unlike TIGIT^{+} NK cells, PD-1^{+} NK cells were significantly enriched in tumor tissues compared to PB and tumor-free tissues in MSI CRC patients. Interestingly, these tumor-associated PD-1^{+} NK cells expressed low levels of CD57 (a marker used to identify terminally differentiated NK cells in PB) and high levels of tissue-residency markers, thus suggesting that these cells may not circulate from the blood to the tumor, but rather differentiate in the tumor tissue. Notably, the enrichment of PD-1^{+} NK cells and of PD-Ls^{+} tumor cells in the tumor tissue of MSI CRC patients suggest that the efficacy of immunotherapeutic treatments in MSI CRC patients based on PD-1/PDL-1 blockade (e.g. anti-PD-1 or anti-PD-L mAbs) could rely also on a superior anti-tumor potential exerted by PD1^{+} NK cells upon mAb-mediated masking of the PD-1 inhibitory receptor.

Moreover, in agreement with a previous study [181], also TIM-3 expression was significantly higher on PB NK cells in CRC patients than in HDs. In addition, we observed that TIM-3$^+$ NK cells were significantly higher in tumor than in tumor-free tissues, but with no preference between non-metastatic (stages I-II) and metastatic (stages III-IV) CRC. Interestingly, tumor-associated TIM-3$^+$ NK cells were mostly present (even if with no statistically significant difference) in MSI CRC compared to MSS CRC. In addition, in MSI CRC, tumor-associated TIM-3$^+$ NK cells were predominantly composed by tissue-resident NK cells, whereas in MSS CRC they were equally composed by tissue-resident NK cells and non-tissue-resident NK cells.

Thus, the enrichment of PD-1$^+$ and TIM-3$^+$ tissue-resident NK cells observed in MSI CRC supports the development of therapies targeting both TIM-3 and PD-1 to restore anti-tumor responses of tumor-associated NK cells in MSI CRC patients. In this regard, our data show that tumor-associated TIM-3$^+$ NK cells can also express additional ICs that are typically expressed by NK cells (such as NKG2A and inhibitory KIRs). For this reason, a more detailed analysis to evaluate the expression of the ligands for NKG2A and KIRs on tumor cells could be useful to understand if additional ICI may be needed to fully enhance the NK cell-mediated antitumor response in the tumor tissue of MSI CRC patients. This aspect will be the subject of further studies currently undergoing in our laboratory.

Differently from TIGIT and TIM-3, LAG-3 was expressed on a very small fraction of PB NK cells both in CRC patients and HDs, but on a discrete NK cell subset both in tumor and tumor-free tissues of CRC patients, regardless of tumor stage. Notably, a large fraction of tissue-associated LAG-3$^+$ NK cells expressed tissue-residency markers. This result deserves to be further investigated in more detail, especially if we consider that the expression of LAG-3 seems to be increased on NK cells in the tumor-free tissue than in the tumor tissue.

All these data demonstrate that tumor-associated NK cells are a heterogeneous population composed by both NK cells circulating from the blood to the tumor and tissue-resident NK cells, expressing different combinations of immune checkpoints according to the type of CRC (for example MSI vs MSS CRC). It is likely that these aspects can influence the efficacy of the immunotherapeutic approaches actually used for the treatment of the heterogeneous group of CRCs. Thus, combined immunotherapies diversified on the basis of NK cell features related to the CRC type could be more effective as compared to the currently used therapies and could better exploit the antitumor activity of the blood-to-tumor circulating NK cells and of tumor-resident NK cell subsets.

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6 PHD PORTFOLIO

6.1 PUBLICATIONS

- M. Greppi, G. Tabellini, O. Patrizi, **V. Obino**, M. Bozzo, M.Rutigliani, F. Gorlero, M. Di Luca, L.Paleari, P. Castagnola, V. Vellone, P. André, D. Mavilio, S. Candiani, C .Jandus, L. Moretta, A. De Censi, D. Olive, S. Sivori, E. Vivier, F. Rampinelli, S. Parolini, S. Pesce, E. Marcenaro Novel ovarian cancer PD1+ NK cell subsets, targetable by combined immune-checkpoint blockade. Submitted
- M. Della Chiesa, C. Setti, C. Giordano, **V. Obino**, M. Greppi, S. Pesce, E. Marcenaro, M. Rutigliani, N. Provinciali, L. Paleari, A. DeCensi, S. Sivori, S. Carlomagno. "NK Cell-Based Immunotherapy in Colorectal Cancer" Vaccines 10 (7), 1033, July 2022. I.F 4.961 Category: Immunology
- L. Paleari, S. Pesce, M. Rutigliani, M. Greppi, **V. Obino**, F. Gorlero, V. G. Vellone and E. Marcenaro "New Insights into Endometrial Cancer" Cancers, 13 (7), 1496, Mar 2022 I.F 6. 921 Category: Immunology
- T. Bachetti, F. Rosamilia, M. Bartolucci, G. Santamaria, M. Mosconi, S. Sartori, M. R. De Filippo, M. Di Duca, **V. Obino**, S. Avanzin, D. Mavilio, S. Candiani, A. Petretto, A. Pini Prato, I. Ceccherini and F. Lantieri "The OSMR" Gene is Involved in Hirschsprung Associated Enterocolitis Susceptibility through an Altered Downstream Signaling" Molecular Sciences (8), 3831, Apr 2021 I.F 6.208 Category: Genetic Disease
- M. Bozzo, S. Costa, **V. Obino**, T.Bachetti, E. Marcenaro, M.Pestarino, M.Schubert and S. Candiani "Functional Conservation and Genetic Divergence of Chordate Glycinergic Neurotransmission: Insights from Amphioxus Glycine Transporters" Cells, 10 (12), 3392, Dec 2021 I.F 7.67 Category: Developmental biology
- Bozzo M, Lacalli TC, **Obino V**, Caicci F, Marcenaro E, Bachetti T, Manni L, Pestarino M, Schubert M, Candiani S."Amphioxus neuroglia: Molecular characterization and evidence for early compartmentalization of the developing nerve cord" Glia 69, E580-E581, Feb 2021 I.F 8.073 Category: Developmental biology
- Ferretti E, CarlomagnoS, Pesce S, Muccio L**, Obino V**, Greppi M, Solari A, Setti C, Marcenaro E, Della Chiesa M, Sivori S."Role of the main non-HLA-specific activating NK receptors in pancreatic, colorectal and gastric tumors surveillance" Cancer 12, 3705, Dec 2020 I.F 6.921 Category: Immunology
- Pesce S, Trabanelli S, Di Vito C, Greppi M, **Obino V**, Guolo F, Minetto P, Bozzo M, Calvi M, Zaghi E, "Cancer Immunotherapy by Blocking Immune Checkpoints on Innate Lymphocytes" Cancer, Oct 2020 I.F 6. 921 Category: Immunology

- Pesce S, Greppi M, Ferretti E, **Obino V**, Carlomagno S, Rutigliani M, Thoren FB, Sivori S, Castagnola P, Candiani S, Marcenaro E "miRNAs in NK Cell-Based Immune Responses and Cancer Immunotherapy" Front Cell Dev Biol. 8,119, Feb 2020 I.F 6.081 Category: Immunology
- Candiani S, Carestiato S, Mack AF, Bani D, Bozzo M, **Obino V**, Ori M, Rosamilia F, De Sarlo M, Pestarino M, Ceccherini I, Bachetti T "Alexander Disease Modelling in Zebrafish: An in Vivo System Suitable to Perform Drug screening" Genes, Dec 2020 I.F 4.41 Category: Genetic Disease
- P. Malaspina, E. Catellani, B. Burlando, D. Brignole, L. Cornara, M. Bazzicalupo, S. Candiani, **V.Obino**, V. De Feo, L.Caputo, P. Giordani "Depigmenting potential of lichen extracts evaluated by in vitro and in vivo tests" PeerJ. 8, e9150, Apr 2020 I.F: 3,06 Category: Botany
- Minetto P, Guolo F, Pesce S, Greppi M, **Obino V**, Ferretti E, Sivori S, Genova C, Lemoli RM, Marcenaro E. "Harnessing NK Cells for Cancer Treatment" Front Immunol. 10, 2836, Dec 2019 I.F. 8.786 Category: Immunology

6.2 PARTICIPATION IN CONGRESS

- XIII National Congress SIICA. Napoli, 2022. **V. Obino**, Chiara Setti, Chiara Giordano, Simona Carlomagno, Elisa Ferretti, Marco Greppi, Silvia Pesce, Agnese Solari, Letizia Muccio, Tania Buttiron Webber, Laura Paleari, Matteo Clavarezza , Andrea Barberis, Marco Filauro, Nicoletta Provinciali, Mariangela Rutigliani, Emanuela Marcenaro, Mariella Della Chiesa, Andrea De Censi, Simona Sivori. Titolo: Dissection of heterogeneity in tumor-infiltrating NK cells from CRC patients
  Selected for Poster Presentation

6.3 COURSES

- ACCMED: Summer school on Immuno-Oncology: emerging targets and combination therapies- Sep 2021
- English course Level 11- Sep 2022
- GIC SCHOOL: L'ONCOLOGIA NELLA CITOMETRIA. DETERMINAZIONE CITOMETRICA DI EVENTI RARI: Isolamento e fenotipo di cancer stem cells (CSCs), cellule tumorali circolanti (CTCI), vescicole extracellulari (EVS), proliferazione e morte cellulare- Nov 2020
- GIC SCHOOL: citometria multiparametrica in immunologia: dal fenotipo ai test funzionali- Nov 2020

6.4 WEBINAR

- Immuno-oncology insights with the latest flow cytometry and novel imaging techniques: Biopharmaceuticals webinars series
- Understanding the biological complexity of tissue with integrated imaging and analysis

- Quantifying T cell exhaustion: an insight into BITE antibodies and CAR-T cells for improved therapeutics

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