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The role of NK cells in Breast Cancer

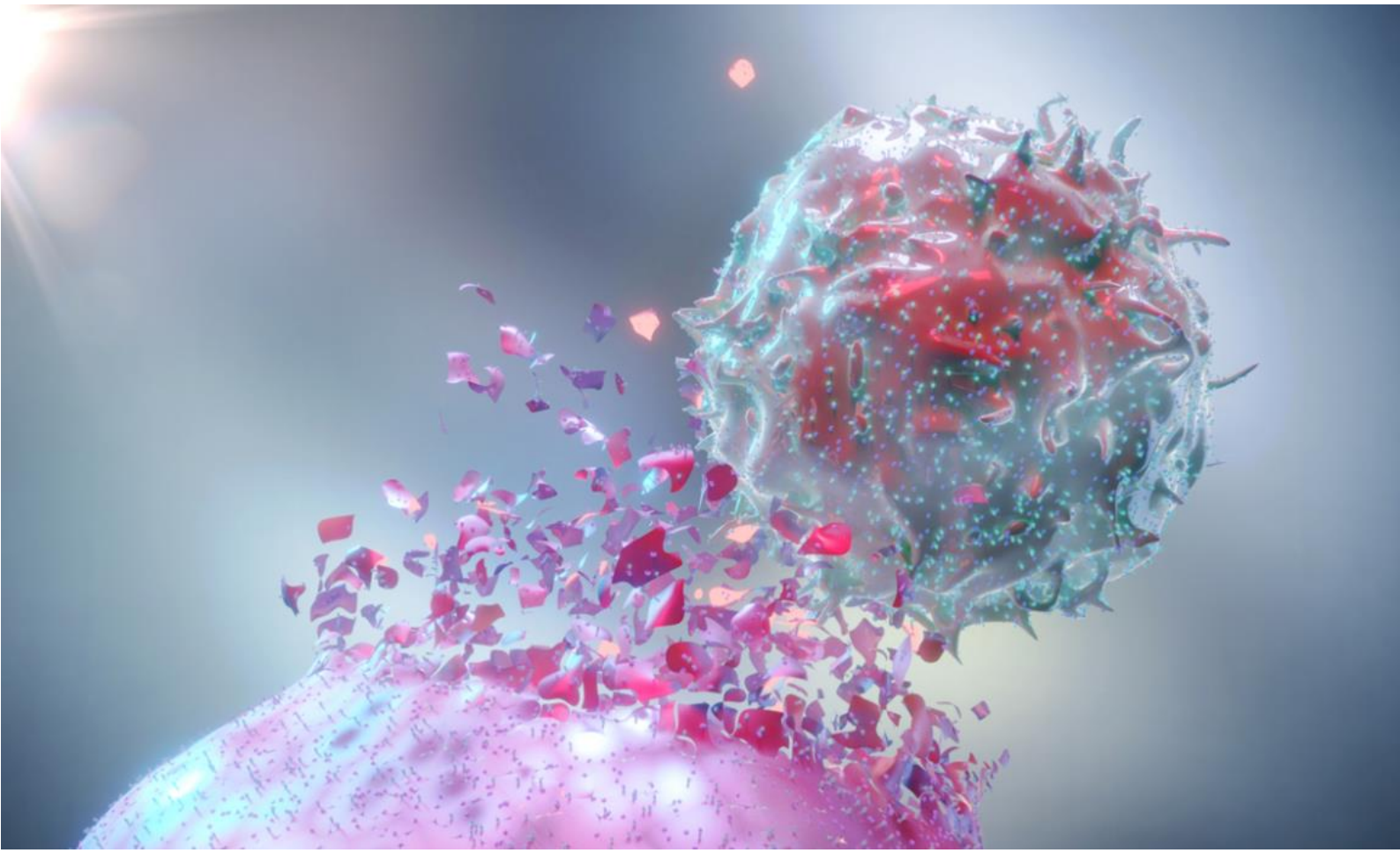

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Illustration: NK cell (blue) attacking tumour cell (pink) (Lucar, 2019)

Abstract

Breast cancer belongs to one of the most frequent malignancies in women, whereby metastasis is the major cause of death among these patients. Immune cells play an important role both in defence against cancer and disease progression. In breast cancer, NK cells eliminate tumour cells by their cytotoxic capacity as well as enhancing cross-priming of cytotoxic lymphocytes and T helper cell type 1 (Th1) polarization of $CD4^+$ T cells. However, upon disease progression, tumour cells modulate NK cells leading to decreased expression of activation receptors and an increase in inhibitory receptors, resulting in impaired detection and targeting of tumour cells.

NK cells can be divided into main subsets $CD56^{bright}CD16^-$ and $CD56^{dim}CD16^+$, whereby subset $CD56^{bright}$ covers most of the NK cell population in breast cancer. Since the presence of this subset showed either an improved or deteriorated overall survival, more insight is necessary regarding NK cell subsets in breast cancer. Despite the different subsets, NK cells should be considered for immunotherapy focusing on improvement of the mediated immune response. Inhibitors can be used to dampen the inhibitory signals, and stimulatory agonists for activation receptors stimulate NK cell functionality. Also, increased expression of TGFβ and soluble NKG2D, detrimental for NK cells, could be neutralized. For developing optimal therapies, personalized treatments, using chimere antigen receptor (CAR) NK cells, would be interesting due to the heterogeneity of NK cells within the tumours and between patients. Importantly, when developing treatments, the interaction with other immune cells should also be considered.

Abbreviations

ADCC	antibody-dependent cell cytotoxicity
CAR	chimere antigen receptor
CCL	c-c- motif chemokine ligand
DC	Dendritic cells
DNAM-1	DNAX accessory molecule 1
GARP	TGFβ receptor glycoprotein-A repetitions predominant
GM-CSF	granulocyte-macrophage colony stimulating factor
HER2	human epidermal growth factor receptor 2
IDO	indoleamine-pyrrole 2,3-dioxygenase
IL	interleukin
INF-γ	Interferon γ
NKG2A	natural killer group 2 member A
NKp	Natural killer protein
MHC1	major histocompatibility complex 1
PGE_2	prostaglandin E2
TGF	transforming growth factor
Th1	T-helper cell type 1
TME	tumour microenvironment
TNBC	triple-negative breast cancer
TNF	tumour necrosis factor
TRAIL	tumour necrosis factor-related apoptosis-inducing ligand

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1. Introduction

Breast cancer, colon cancer and lung cancer are the three most frequent cancers in women worldwide (Harbeck & Gnant, 2017; Jafari et al., 2018). Breast cancer can be caused by genetic factors, such as mutations in, among others, tumour suppressor genes BCRA1 and BCRA2, and stimulated by cofactors such as BMI, physical activity, or reproductivity and menstrual history (Mavaddat et al., 2010; Nagini, 2017). Histologically, breast cancer can be divided into different subtypes: hormone receptor-positive (positive for progesterone receptor, oestrogen receptor-positive (or both)), human epidermal growth factor receptor 2 (HER2) positive, or triple-negative (negative for progesterone receptor, oestrogen receptor, or HER2) (Harbeck & Gnant, 2017; Nagini, 2017). Patients diagnosed with triple-negative breast cancer (TNBC) have poorer overall survival compared to non-TNBC patients. Important for the overall survival of breast cancer is the moment of diagnosis. Since metastasis of the tumour cells is the major driver for mortality, early diagnosis and early treatment, have a tremendous impact on the outcome of the disease (Chan et al., 2020; Jafari et al., 2018).

Currently, the role of the immune system during development, progress, and the effectiveness of therapy against breast cancer is an upcoming topic of interest. Especially T cells are extensively studied for efficiently targeting the disease. However, the use of T cells for immunotherapy is limited because of difficulties in regeneration of sufficient neoantigen-reactive T cells, and the presence of exhausted T cells due to irradiation (Hall et al., 2016; Terranova-Barberio et al., 2020; Turcotte et al., 2013). Further research and development of new immunotherapeutic targets and or biomarkers are therefore highly important. The innate immune response elicited by NK cells should be considered because of its immediate response and potential anti-tumour effect in early stages of tumour progression (Cózar et al., 2021; Wu et al., 2020). This review focuses on the role of NK cells in breast cancer, and whether these cells can be used as a therapeutic target against breast cancer.

2. Biology of NK cells

Before analysing the role of NK cells in breast cancer, it is important to understand the biology of NK cells and their function in the innate and adaptive immune response. NK cells are immune cells of lymphoid origin and represent 10-15% of all the lymphocytes in the periphery (Navarro et al., 2015). They are formed in the bone marrow followed by release in the bloodstream of the periphery (Moretta et al., 2008). Maturation occurs in various distinct tissues, such as the bone marrow itself, thymus, lymph nodes, spleen, and liver (Simoni et al., 2017). NK cells are heterogeneous, even in the same organ or tissue, and can be divided, according to their location, into the conventional NK and tissue-resident NK cells. Conventional NK cells are located in the periphery able to migrate towards the tissue, and tissue-resident cells can be found in tissues (Simoni et al., 2017; Wu et al., 2020). Within these groups, different types of NK cells can be identified based on surface markers and function, i.e. cytotoxic NK cells, antigen-presenting NK cells (AP-NK), helper NK cells (hNK) and regulatory NK cells (NKreg) (Wu et al., 2020). In addition, studies showed that prestimulation of NK cells with interleukin (IL)12, IL15, and IL18 leads to increased Interferon γ (IFN-γ) upon reintroduction of these cytokines or stimulation with leukaemic cells. Stimulation of NK cells with IL12, IL15, and IL18 combined with priming of primary NK cells can also enhance a tumour-specific attack (Pal et al., 2017; Romee et al., 2012). These data suggest that NK cells remember previous stimulants and can differentiate towards a memory NK (mNK) phenotype.

Generally, NK cells can be characterized by the expression of CD56 and CD16 and lack expression of CD3 and CD4. NK cells can be divided into the subpopulations $CD56^{dim}$ $CD16^{+}$ and $CD56^{bright}$ $CD16^{-}$ (Chiossone et al., 2018; Cózar et al., 2021; Navarro et al., 2015). $CD56^{dim}$ $CD16^{+}$ NK cells are mainly found in the peripheral blood, whereby CD16 is important for inducing antibody-dependent cell cytotoxicity (ADCC), and $CD56^{bright}$ $CD16^{-}$ NK cells show similarities towards helper cells with lower cytotoxicity (Wu et al., 2020).

2.1 Migration

Upon infections or presence of tumour cells, NK cells first migrate towards the inflamed tissue in response to chemotactic factors and adhesion molecules (Moretta et al., 2008). Migration of $CD56^{dim}$ $CD16^{+}$ is influenced by integrin β1/2 and E and P-selectins, and express CXCR1 and CX3CR1 for migration towards inflamed tissue. $CD56^{bright}$ $CD16^{-}$, on the other hand, expresses CCR7 for interaction with c-c motif chemokine ligand (CCL) 21 and CCL19 in the lymph node, and L-selectins for interaction with lymph node high endothelial venules (Liu et al., 2021; Moretta et al., 2008). For the transendothelial migration process, adhesion molecule DNAX accessory molecule 1 (DNAM-1) seems to be important (Moretta et al., 2008; Reymond et al., 2004).

2.2 Activation

When arriving at the side of infection or damaged tissue, NK cells become activated upon interaction with pathogen-derived substances, cytokines, or ligand-receptor interaction. Important, the outcome of NK cell function depends on the balance between signals released from inhibitory and activating receptors (figure 1) (Moretta et al., 2008). The inhibitory receptors, receptors CD94/natural killer group 2 member A (NKG2A) heterodimer and receptors belonging to the killer cell immunoglobulin-like family, play an important role in recognition of major histocompatibility complex 1 (MHC-1) expressed by other cells. Activation of these receptors by MHC-1 stimulates the inhibitory signal leading to the inactivation of NK cells(Cózar et al., 2021). However, upon neoplastic transformation and viral infections, cells lose the expression of MHC-1 molecules leading to activation of NK cells (Sivori et al., 1996). In addition, low levels of MHC-1 molecules can be found on tumour cells as a result of an escape mechanism against the anti-tumour response by $CD8^{+}$ T cells (Wu et al., 2020). CD94/NKG2A is expressed by both subsets, whereby the killer cell immunoglobulin-like receptors are mainly expressed by the $CD56^{dim}$ $CD16^{+}$ NK subpopulation (Gaynor & Colucci, 2017).

Besides the interaction with inhibitory receptors, stimulation of activation receptors is also necessary for maintaining NK cell activation and response. Activating receptors expressed by NK cells are killing receptors (natural killer protein (NKp)30, NKp40, NKp44, NKp46, and CD16), non-self-

antigen receptors (LY49D, LY46H), and other receptors, such as NKp80, CD19, CD2, and TLR3/9 (Cózar et al., 2021; Paul & Lal, 2017). Among these receptors, NKps identify a wide range of ligands and molecules from, bacterial, parasites, and tumour cells (Kruse et al., 2014).

In addition to signalling through inhibitory and activating receptors, NK cells also rely on stimulation by complex molecules, such as IL12, IL15, and IL18, released by stromal cells (Wu et al., 2020).

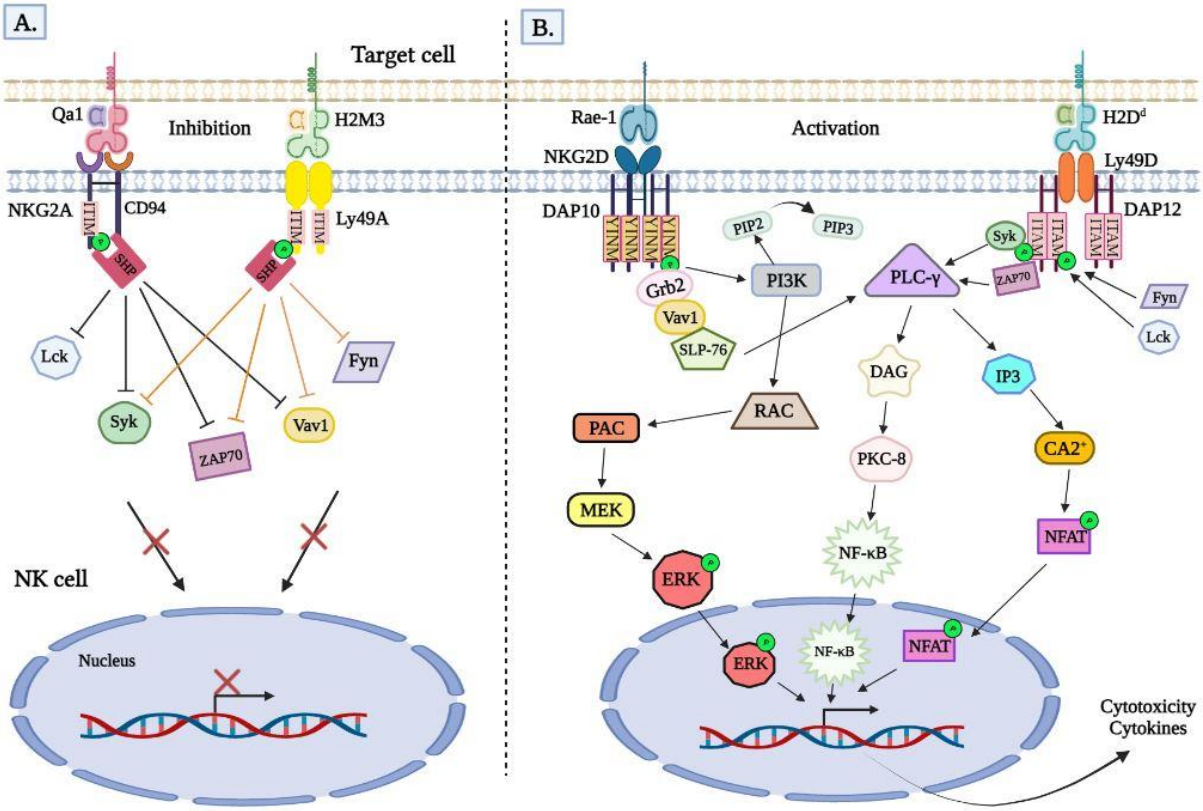


Figure 1 Inhibition and activation receptors NK cells.
Schematic representation of inhibition and activation signalling in NK cells. (A) Interaction between NK cell receptors NKG2A/CD49 and Ly49a with MCH1 molecules Qa1 and H2M3, leads to phosphorylation of ITIM, followed by recruitment of SHIP stimulating inhibition of activation signalling through Lck, Syk, ZAP70, Vav1, and Fyn. (B) Interaction between NKG2D and LY49 with Rae-1 and H2D^d induces phosphorylation of YINM and ITAM, enhancing PI3K, Syk/ZAP70, and GrB2/Vav1/SLP-76 recruitment. Activation of the GrB2/Vav1/SLP-76 complex results in P13K activation inducing downstream activation of the MEK/ERK pathway. Together with Syk/ZAP70, PLC-γ is activated followed by activation of IP3 and DAG, leading to downstream activation of NK-κB and NFAT. Translocation of ERK, NF-κB and NFAT into the nucleus results in cytotoxicity and release of cytokine/chemokine by NK cells. DAG, Diacylglycerol; Grb2, growth factor receptor-bound protein 2; MEK, mitogen-activated protein kinase kinase; NKG2A, natural-killer group 2, member A; NKG2D, natural-killer group 2, member D; IP3, Inositol triphosphatase; ITAM, immunoreceptor tyrosine-based activation motifs; ITIM, immunoreceptor tyrosine-based inhibitor motif; Lck, lymphocyte-specific protein tyrosine kinase; MEK, mitogen-activated protein kinase kinase; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PIP, plasma membrane intrinsic protein; PKC-8, protein kinase C8; PLC-γ, phospholipase C gamma; SHP, Scr homology domain-containing tyrosine phosphatase; SLP-76, SH2 domain-containing leukocyte protein; SyK, spleen tyrosine kinase; YINM. tyrosine-based signalling motif; Zap70, zeta-chain-associated protein kinase 70. Figure according to findings by Paul et al. 2017 and created by Biorender (Paul & Lal, 2017).

2.3 Cytotoxic immune response

Activated NK cells can lyse target cells (Moretta et al., 2008; Wu et al., 2020). The $CD56^{dim}$ $CD16^{+}$ NK population is mainly responsible for this cytotoxic response. Hereby, CD16 plays an important role during the degranulation, seen in ADCC (Yeap et al., 2016). The cytotoxic response can be triggered upon MHC-1 mismatch, detection of antibody-coated target cells or interaction between NKGD2 and its ligands (Abdel-Latif & Youness, 2020). Generally, there are four major steps for inducing cell lysis. First, immunological synapses are formed between NK cells and target cells, accompanied by rearrangement of the actin cytoskeleton. Second, polarization of the microtubule-organizing center occurs together with migration of the secretory lysosome towards the lytic synapse. Third, the lysosome docks and fuses with the plasma membrane, followed by release of cytotoxic particle granzyme and perforin (Paul & Lal, 2017). When released, perforin creates pores in the cell membrane of target cells, assisting the entrance of granzyme into the cell. Granzyme then induces apoptosis by activating, caspases, such as caspase 3 (An Pardo et al., 2002; Moretta et al., 2008; Topham & Hewitt, 2009). Immunoreceptor tyrosine-based activation motif receptors, such as NKp46, NKp44, NKP30 might be sufficient for inducing this degranulation (Bryceson et al., 2005; Freud et al., 2013). *In vivo* results showed that the perforin-dependent cytotoxic response is essential for tumour control in, among others, prostate and mammary carcinoma's, and lymphoid tumours (Street et al., 2001; van den Broek et al., 1995). Besides cytotoxicity by perforin and granzymes, NK cells are also able to induce cell lysis via death receptors, such as DR4/5 and Fas/CD95, and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) (Al Absi et al., 2018). In addition, DNAM-1 seems to play a role in stimulation cell lysis upon detection of CD155 on tumour cells (Bottino et al., 2003).

2.4 Effector immune response

Besides induction of cytotoxic response, as described above, NK cells also interact with cells of the innate immune system, such as dendritic cells (DC), mast cells, eosinophils and neutrophils (Moretta et al., 2008; Wu et al., 2020) as well as with T-cells of the adaptive immune response (Chiossone et al., 2018).

Cytokine release

Upon activation, NK cells release a wide range of cytokines, such as tumour necrosis factor (TNF)α, IFN-γ, IL5, IL10, IL13, granulocyte-macrophage colony-stimulating factor (GM-CSF), and chemokines, including IL8, macrophage inflammatory protein 1α/β, and CCL5, to interact with other cells (Paul & Lal, 2017). Release of IFN-γ stimulates, together with TNFα, Th1 polarization as well as destruction of activated T cells, preventing expansion of the population (Chiossone et al., 2018; Guido Ferlazzo & Morandi, 2014; Wu et al., 2020). Also, IFNγ can modulate the expression of FasL, TRAIL and caspases stimulating the anti-tumour response (Paul & Lal, 2017). Furthermore, release of XC-chemokine ligands 1 and 2, and CCL5, stimulate migration of DCs, whereby secretion of NKp30 stimulates IFN-γ enhancing further activation of DCs (Chiossone et al., 2018).

Crosstalk between NK cells and DCs

Crosstalk between NK cells and DCs also stimulated maturation of DCs upon antigen uptake and induces release of cytokines influencing NK cell behaviour (figure 2) (Moretta et al., 2008). For example, a study by Erick et al. showed that expression of IL15 by DCs stimulates activation of NK cells (Lucas et al., 2007). Next, release of IL12 induces NK cell cytotoxicity as well as secretion of IFN-γ (Guido Ferlazzo & Morandi, 2014; Moretta et al., 2008). Hereby, IFN-γ might further stimulate IL1, IL18 and TNF-α release as well IL15 on DCs (Morandi et al., 2009; Ortaldo et al., 2006). Contrary to IL12, release of transforming growth factor (TGF) inhibits secretion of TNF-α, TNF-γ and GM-CSF (Bellone et al., 1995; Esebanmen & Langridge, 2017). Moreover, exposure of NK cells to IL12 and IL18 contributes to Th1 polarization (Guido Ferlazzo & Morandi, 2014). Th1 cells are the main controllers of type one immunity and are important for elimination of tumours and intracellular pathogens, whereby IL-2 and IFN-α are major key factors (Xu et al., 2019).

Besides the cytokine-induced interaction between NK cells and DCs, NK cells also control the DCs population (Moretta et al., 2008). Upon infection, and antigen uptake, DCs maturate and express increased levels of MHC1. However, immature or not fully maturated DCs express lower levels of MHC1 and are therefore sensitive for cell lysis (G Ferlazzo et al., 2003). Receptors involved in detection of immature DCs are, among others, NKp30 and DNAM-1 (G Ferlazzo et al., 2002; Pende et al., 2006). Previous studies showed that absence of NK cells leads to strongly biased $CD4^+$ T cells towards a Th2 phenotype (Byrne et al., 2004; J. D. Coudert et al., 2002), suggesting that NK cells use their ability to lyse DCs to control T cell priming (Moretta et al., 2008).

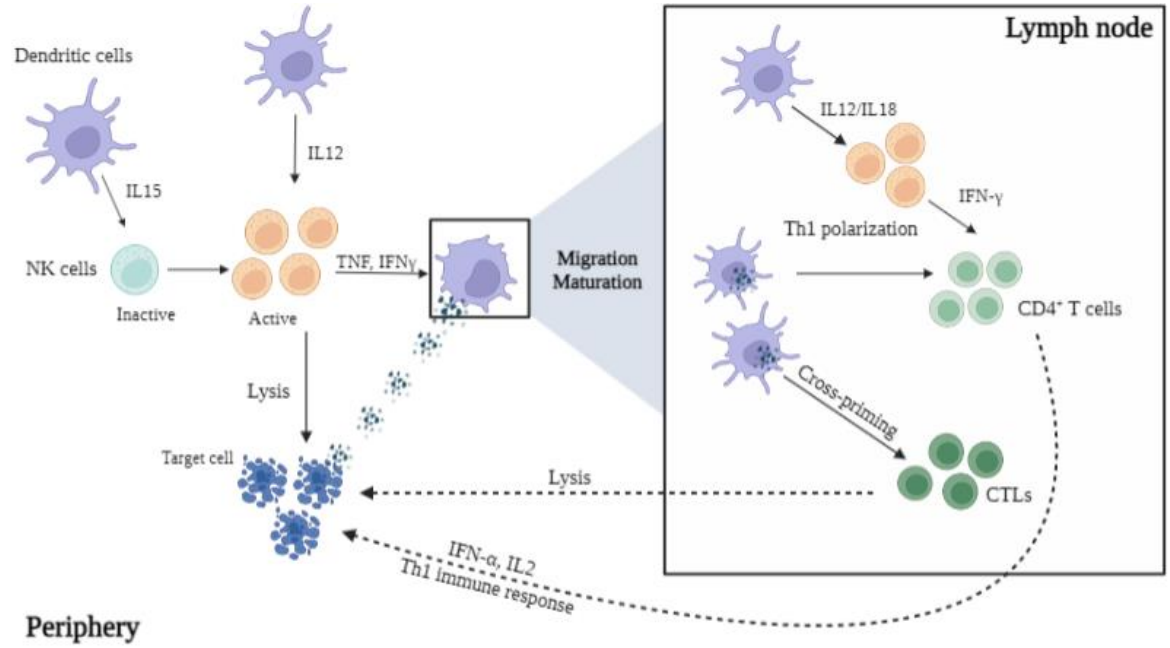


Figure 2. Crosstalk between NK cells and DCs
Schematic representation of crosstalk between NK cells and DCs, and T cell priming. NK cell activation can be promoted by IL15, released by DCs. When activated, lysis of target cells is induced upon stimulation with IL12 as well as release of TNF and IFNγ. TNF and IFNγ support maturation of immature DCs together with migration towards the lymph nodes. Here, target cell antigens are presented inducing cross-priming of CTLs. Th1 polarization is induced upon antigen presentation by DCs and IL12/IL18 induced IFN-γ released by NK cells. DCs, Dendritic cells; TNF, tumour necrosis factor; IFNγ, interferon γ; CTLs, cytotoxic T cells. Created by Biorender.

3. NK cells in Breast cancer

Apart from the effect of NK cells on target cells, target cells can also modulate NK cells for their benefit. Especially in cancer, the immunomodulation of immune cells, by tumour cells, may contribute to the disease progress, resistance against therapies and limited survival (Cózar et al., 2021).

3.1 Tumorgenesis and NK cell activation

In breast cancer, it is seen that tumour cells decrease their MHC1 expression to evade the anti-tumour response by $CD8^+$ T cells (Garrido et al., 2018). As mentioned before, this decrease stimulates detection and activation of NK cells (Cózar et al., 2021; Wu et al., 2020). Tumour cells can also be detected upon interaction between B7-H7, expressed by tumour cells, and NKp20 located on NK cells (Textor et al., 2016). Stimulation by IL12, IL15, and IL18, released by stromal cells, further induces the detection as well as accumulation of NK cells in tumour tissue, promotion anti-tumour response *in vivo* and *in vitro* and formation of mNK cells (Ni et al., 2012; Wu et al., 2020). Activation of NK cells is enhanced by polypeptide-related sequence (MIC) A and MICB present on tumour cells membranes (Bauer et al., 1999). The anti-tumour response can also be stimulated by MICA released by monocytes, together with antigen-presenting by DCs (Campbell et al., 2017; Wu et al., 2020). In NK cells, IL12 induces elevated expression of NKG2A, and IL2 stimulates increases in NKG2D, which ligand is highly expressed by tumour cells, resulting in increased detection of tumour cells (Sáez-Borderías et al., 2009). In addition, IL 2 also stimulates development, survival, and activation of NK cells as well as increased cytotoxicity towards breast cancer tumour cells (Hu et al., 2019).

3.2 Immune control and escape

Upon interaction with tumour cells, NK cells induce an immune response to target these cells as described in previous chapter, whereby ADCC is induced by $CD56^{dim}CD16^+$ NK cells and $CD56^{bright}$ NK cells release cytokines such as IFN-γ, GM-CSF and TNF-β. In addition, mNK cells release perforin and granzyme to stimulate cell lysis of tumour cells (Petricevic et al., 2013; Wu et al., 2020).

The lethality of breast cancer caused by metastasis is mainly due to immune escape as a result of immune suppression by, among others, immune suppressor cells and release of anti-tumour molecules. Several studies demonstrated strongly decreased expression of activating receptors in breast cancer biopsies, while inhibitory receptors were highly expressed, correlating with impaired NK cell cytotoxicity(Mamessier et al., 2011; Nieto-Velázquez et al., 2016). Downregulation of, among others, NKG2D can be mediated by factors such as cellular activity and physicochemical features of the TME. Within the TME, the expression of activin-A, podocalyxin-like protein 1, TGF-β, prostaglandin E2 (PGE_2) and indoleamine-pyrrole 2,3-dioxygenase (IDO) stimulate the downregulation of NKG2D (Duan et al., 2019). Hereby, IDO inhibits IL2 induced expression of NKG2D via the c-JUN N-terminal kinase pathway (Fan et al., 2018), and PGE_2 inhibits upregulation of NKG2D by IL15 and therefore blocking the transcription via the cAMP/ adenylate cyclase /protein kinase pathway by binding to EP2/4 (Holt et al., 2012; Martinet et al., 2010). Also, *in vivo* studies showed that repeated exposure of NK cells to tumour cells leads to downregulation of NKG2D, resulting in NK dysfunction, whereby the effector response cannot be stimulated (J. Coudert et al., 2008; Deng et al., 2015). Interestingly, in breast cancer, cells release soluble NKG2D to avoid immune detection, whereby soluble NKG2D is associated with poor prognosis (Dhar & Wu, 2018).

When interacting with tumour cells, an increase in CD25 is seen (Shenouda et al., 2017). Stimulation of this marker induces an anti-tumour response by enhanced glycolysis and cell survival accompanied by activation mTORC1/cMYC pathway in early stages (Loftus et al., 2018). However, in later stages, this stimulation becomes harmful because of insufficient nutrients available in the TME, due to the high activation status of metabolic pathways in tumour cells (Steggerda et al., 2017). A study by Loftus et al. showed that mTOR signalling can be suppressed under low glutamine or arginine conditions, which indirectly affects IL-2 associated stimulation processed via cMYC (Loftus et al., 2018). Since cellular energy levels regulate mTOR activation, low energy levels due to this competition may therefore decrease the functionality of NK cells (Hare & Harvey, 2017).

Tumour cells can also stimulate their progression, migration and proliferation by increased expression of CD155, which can be detected by DNAM-1 on NK cells inducing cell lysis. However, CD155 has a higher affinity for T cells immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibition motif domain stimulating inhibition of the anti-tumour response (Lupo & Matosevic, 2020). A study by Yu-Chen et al. showed that overexpression of CD155, in breast cancer, correlates with elevated tumour cell proliferation, dysfunction of immune cells in the TME and reduced overall survival (Yoshikawa et al., 2021; Yu-Chen et al., 2020).

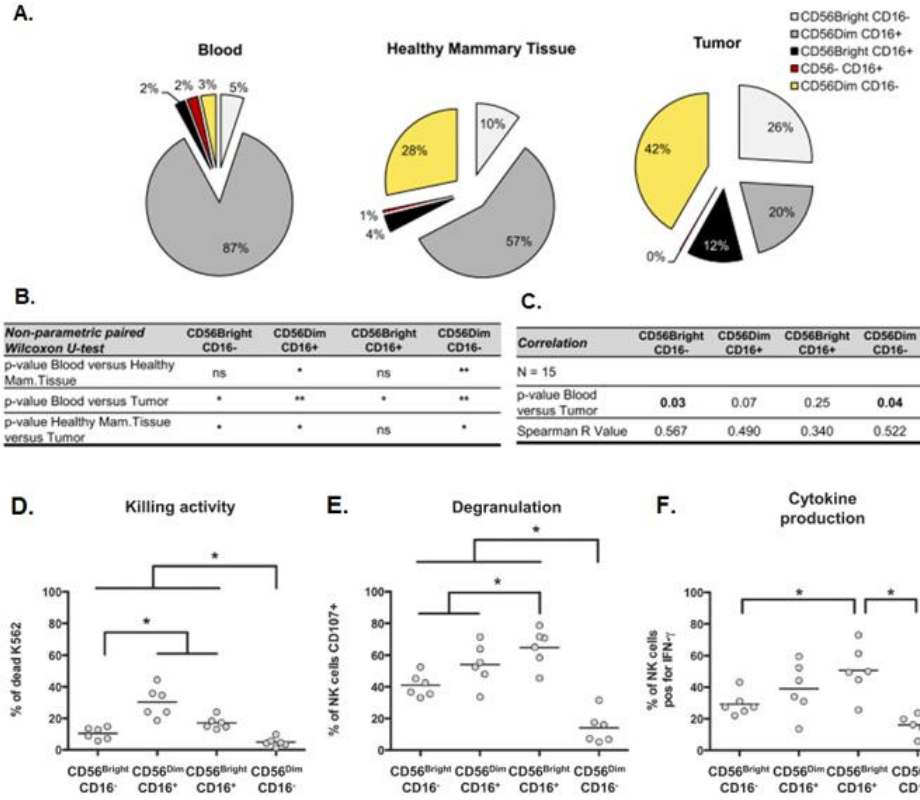
Interestingly, tumour cells can also induce formation of the $CD73^+$ phenotype, expressing increased levels of checkpoint inhibitors, such as PD-1, PD-L1, and LAG3. Activation of these receptors, for example, PD-L1 inhibits NK cell activation. In breast cancer patients, $CD73^+$ NK cells correlate with increased tumour size (Neo et al., 2020). Besides the $CD73^+$ phenotype, tumour cells are also able to impair mNK cells (Neo et al., 2020). It is suggested that TFG-β plays a role in NK cell dysfunction and therefore contributes to metastasis. Especially in aggressive breast tumours, an increase in TGFβ producing cells was found which correlated with elevated NK cell impairments (Mamessier et al., 2011). In addition, a study by Slattery et al. showed impaired IFN-γ production, decreased TRAIL and K562 tumour cell cytotoxicity of NK cells in metastatic breast cancer, together with increased expression of TGFβ receptor glycoprotein-A repetitions predominant (GARP) (Slattery et al., 2021). Hereby, the impairment was associated with several metabolic deficiencies, among others diminished oxidative phosphorylation response upon stimulation with cytokines and mitochondria alterations such as enhanced fragmentation (Slattery et al., 2021). When neutralizing TGFβ, these metabolic and functional impairments were improved supporting the role of TGFβ in NK cell dysfunction (Slattery et al., 2021).

3.3 NK cells and tumour prognosis

Previously described information showed the effect of NK immunomodulation on the progression of breast cancer. It is important to analyse if there is a correlation between the number of NK cells, and subtypes, and cancer progression, or if the overall effect may be induced by other factors. A study by Mamessier et al. showed similar numbers of NK cells in mammary tumours compared to healthy tissue (Mamessier et al., 2011). However, different phenotypes were found between healthy and malignant tissue, whereby $CD56^+$ NK cells were more frequent in tumour tissue compared to healthy control (Mamessier et al., 2011). Similarly, Levi et al. showed significant enriched $CD56^+$ cells in tumour breast tissue, compared to peritumoral breast tissue. Also, the majority of the NK cells infiltrated in breast carcinoma are $CD56^+CD16^-$ (Levi et al., 2015). These studies may suggest that especially $CD56^+$ NK cells play a role in breast cancer, whereby Taouk and coworkers found high heterogeneity intra- and intertumoral of this marker (Taouk et al., 2019).

Several studies have been done analysing the correlation between $CD56^+$ NK cells and cancer progression. Muntasell and coworkers showed that $CD56^+$ NK cells were associated with positive survival(Muntasell et al., 2019). Mamessier et al. showed that in malignant breast cancer tissue $CD56^{bright}$ NK cells express higher levels of NKG2A, CD94, NKG2D, and NKp46, compared to NK^{dim} subsets, supporting the beneficial aspects of NK cells in the immune response towards tumour cells (Mamessier et al., 2013). However, contrary to the study of Muntasell, Rathhore et al. showed negative overall survival upon the presence of $CD56^{bright}$ NK cells (Rathore et al., 2014).

NK cells and subsets were analysed in different stages of the disease by Mamessier et al. Regarding the NK cell subsets, the subsets $CD56^{dim}CD16^+$, $CD56^{bright}CD16^-$, $CD56^{dim}CD16^-$, $CD56^{bright}CD16^+$, and $CD56^-CD16^+$ were found. Furthermore, malignant tumour tissue showed a decrease in $CD56^{dim}CD16^+$ and increases in $CD56^{bright}CD16^-$, $CD56^{bright}CD16^+$, and $CD56^{dim}CD16^-$ compared to healthy mammary tissue and peripheral blood. (figure 3A, B). When analysing the killing activity, degranulation, and cytokine production of these subsets *in vitro*, the results showed that $CD56^{bright/dim}CD16^-$ subsets had lower efficient cytotoxicity towards malignant cells (figure 3D-F). When comparing different stages of the disease, they found increased $CD56^{bright}CD16^-$, and $CD56^{bright}CD16^+$ NK cells in locally advanced cancer and metastatic breast cancer compared to benign breast cancer tumours and invasive localized cancer. Also, these subsets were increased in tumour tissue compared to peripheral blood, whereby the $CD56^{bright}CD16^-$ was also increased compared to healthy mammary tissue (Mamessier et al., 2013).



Non-parametric paired Wilcoxon U-test	CD56Bright CD16-	CD56Dim CD16+	CD56Bright CD16+	CD56Dim CD16-
p-value Blood versus Healthy Mam.Tissue	ns	*	ns	**
p-value Blood versus Tumor	*	**	*	**
p-value Healthy Mam.Tissue versus Tumor	*	*	ns	*

Correlation	CD56Bright CD16-	CD56Dim CD16+	CD56Bright CD16+	CD56Dim CD16-
N = 15				
p-value Blood versus Tumor	**0.03**	0.07	0.25	**0.04**
Spearman R Value	0.567	0.490	0.340	0.522

Figure 3. Distribution of NK cell subsets and their capacity (Mamessier et al., 2013)
Percentages NK cells subsets analysed in peripheral blood, healthy breast tissue, and malignant tissue of breast cancer patients (A). Statistical analysis was performed using the non-parametric paired Wilcoxon U test (B) together with the correlation of the subsets between peripheral blood and breast cancer patient tumours (C). The functionality of subsets was analysed upon interaction with leukemic HLA-I12 K562 cell, focusing on killing activity (D), degranulation (CD107a/c) (E), and cytokine production (IFN-g)(F). E/T ratio = 1:1. Statistical analysed using nonparametric paired Wilcoxon U test (*p, 0.05, **p, 0.005)

4. NK cells and Immunotherapy

In breast cancer modulation of NK cells can stimulate progression of the disease, therefore studies focused on the use of NK cells for immunotherapies in breast cancer (Cózar et al., 2021; Wu et al., 2020).

As mentioned before, NK cells become impaired upon interaction with tumour cells. Avelumab an IgG1 anti-PD-Li checkpoint inhibitor is analysed for restoring activation of NK cells. A clinical study already showed that Avelumab induced increased tumour detection in TNBC patients with PD-L1 positive compared to PD-L negative tumour-associated immune cells (Dirix et al., 2018). In addition, Pfizer (NCT02554812) is currently analyzing combination treatments with Avelumab for novel immunotherapies. A study by Juliá et al. showed that Avelumanb induces NK cell cytotoxicity (ADCC) towards TNBC cells, with increased sensitivity towards cells with elevated PD-L1 levels *in vitro* (Juliá et al., 2018). In addition, cytokine production upon Avelumab was increased by stimulation with IL2 and IL15 (Juliá et al., 2018). These data suggest that the use of Avelumab may be a promising therapy for targeting tumour cells, whereby combination with IL2 and/or IL15 modulators could be used to increase the effect of Avelumab, especially in TNBC.

Furthermore, the use of chimere antigen receptor (CAR) NK cells are currently analysed in clinical trials. Hereby, CAR NK cells contain a similar structure as CAR T cells, consisting of an intracellular domain, transmembrane domain, and hinge domains, whereby the extracellular domain binds to a specific receptor on tumour cells (Hu et al., 2019). Clinical trials are analysing the use of CD19, CD7, NKG2D, MUC1 and HER (NCT02742727, NCT02892695, NCT02839954, NCT03383978, NCT03415100) in leukaemia, lymphoma's, HER2 positive glioblastoma, and solid tumours. In addition, mesothelin, Her2, and EGFRvIII are used as targets for treatment of solid cancers, including colorectal cancer, ovarian cancer, breast cancer, and glioblastoma *in vivo*, and are associated with improved anti-tumour activity (Li et al., 2018; Schönfeld et al., 2015).

Next, cytokines interacting with NK cells are being analysed, such as the use of agonists. One of these agonists is ALT-803(N-803). ALT-803 consist of IgG1Fc fused to an IL15 receptor α-subunit able to bind IL15 mutein. When interacting with NK cells, ALT-803 is shown to support cytotoxicity in ovarian cancer *in vivo* and *in vitro* (Felices et al., 2017). A study by Wolfsen et al. showed that stimulation of NK cells with N-803 is insufficient to *in vitro* suppress tumour growth in TNBC. However, when combined with Fulvestrant, an oestrogen receptor blocker, tumour cells become sensitive for N-803 resulting in suppression of tumour growth (Wolfson et al., 2021). In addition, when combining N-803 with PD-L1 t-haNK, an engineered NK-92 cell line which expressed increased affinity CD16, IL2, and PD-L1 chimeric antigen receptor, cytotoxicity towards tumour cells increased upon stimulation with Fulvestrant in TNBC. Both N-803 and PD-L1 engineered t-haNK cells entered clinical trials for further investigation (NCT04927884) (Wolfson et al., 2021).

Finally, the safety and efficacy of autologous NK cell administration, after Trastuzumab treatment, in HER2 positive breast cancer and gastric cancer patients are being/have been analysed (NCT02030561). Trastuzumab is an already approved treatment against Her2 overexpression in breast cancer patients. A previous performed clinical study showed that patients that did respond to Trastuzumab treatment had increased NK cells activity and ADCC compared to healthy controls. However, this activity and ADCC response were lower in patients that did not respond to the treatment (Beano et al., 2008).

5. Conclusion/Discussion

NK cells play an important role in the first-line defence using their cytotoxic capacity against target cells, including tumour cells (Abdel-Latif & Youness, 2020; Cózar et al., 2021; Moretta et al., 2008; Paul & Lal, 2017). Besides their role in the innate immune response, NK cells also perform an effector role supporting cross-priming of CTLs, and Th1 priming of $CD4^+$ T cells upon interaction with DCs. In breast cancer, NK cells target tumour cells inducing elimination of these cells as part of the protective mechanism of the human body. However, tumour cells can modulate NK cells impairing their cytotoxic capacity as well as their effector function, promoting progress of the disease resulting in metastasis (Mamessier et al., 2011).

Increased levels of $CD56^{bright}$ NK cells were found in breast cancer tissue, suggesting that an effector response is induced within the tumour tissue, stimulating cross-priming of CTLs and Th1 polarization of $CD4^+$ cells, which would then support tumour cell targeting. However, the presence of $CD56^{bright}$ NK cells showed contradictory outcomes in overall survival (Mamessier et al., 2013; Rathore et al., 2014). $CD56^{bright}$ NK cells contain CD16 in early stages of the disease, which disappears in later stages of breast cancer, resulting in lower cytotoxic capacity. This suggests that loss of CD16 may contribute to disease progression (Mamessier et al., 2013).

For development of new therapies, NK cells should be considered due to their contributing role in the progression of cancer. Hereby, it is important to restore the impairment, thus increasing detection and elimination of tumour cells. Inhibitors could be used to dampen the inhibitory signal induced by NKG2A, as well as inhibiting CD25 and GARP for preventing metabolic overactivation and impairment of NK cells and to dampen the effect of TGFβ (Hare & Harvey, 2017; Slattery et al., 2021). Also, agonists for NKp30, NKp46, NKG2D, DNAM1 and CD16 may be beneficial for stimulation of tumour detection, NK cell activation, and the cytotoxic response. Moreover, released cytokines, such as TGFβ, podocalyxin-like protein 1, TGFβ, PGE_2 and IDO, as well as soluble NKG2D could be neutralized preventing modulation of NK cells (Duan et al., 2019). However, when inhibiting, stimulating, or neutralizing receptors and compounds it is important to also analyse their function in healthy cells.

However, for targeting NK cells, it is important to know the distribution of the different NK cell types and subpopulations. Besides the $CD56^{dim}CD16^+$ and $CD56^{bright}CD16^-$ subsets, corresponding for cytotoxic and effector/helper NK cells, there are more phenotypes, such as mNK cells, AP-NK cells, NKreg cells, each with different markers and functions (Leong et al., 2014; Pal et al., 2017; Romee et al., 2012). Therefore, it is interesting that many studies characterize NK cells only based on the markers which distinguish them from other immune cells. However, characterizing might be challenging since NK cells undergo phenotypic changes during different stages of breast cancer.

Treatments should be personalized due to this change as well as the NK cell heterogeneity found between tumours and among breast cancer patients (Dorner et al., 2004; Sun et al., 2009). CAR NK-cells would therefore be ideal. When using CAR NK cells, patients NK cells are used and enriched with a specific receptor improving, detection of tumour cells. Also, CAR NK cells could be enriched by multiple markers, which are decreased in breast cancer, stimulating cytotoxicity and crosstalk with the adaptive immune system (Mamessier et al., 2011; Petricevic et al., 2013). Moreover, one of the advantages of CAR NK cells is the use of patients own NK cells, which may prevent toxic effects as seen in some clinical studies using transplantation of autologous NK cells (NCT02030561).

Furthermore, it is important to analyze whether the effect of tumour cells on NK cells is locally or if it also can be induced in NK cells distant from the tumour tissue. For example, downregulation of NKG2D is induced upon repeated exposure to tumour cells or by interaction with the TME (J. Coudert et al., 2008; Deng et al., 2015; Duan et al., 2019). If tumour cells would also impair NK cells distant from the tumour cells, then the recruited NK cells would already be impaired contributing to the progress of the disease and metastasis. Similar to tumour secreted cytokines; are they TME bound, or can they migrate to distant locations in the periphery, interacting and impairing other NK cells? Therefore, it is important to further analyse the interaction between NK cells and tumour cells in breast cancer.

Based on the role of NK cells, it can be concluded that they are important in breast cancer tumour progression and that these cells should be considered for developing cancer treatments. For this development, it is important to also focus on different subsets instead of the general NK cell population. Since breast cancer is a complex disease whereby other immune cells, such as DCs and T cells, also play a role, it is suggested that targeting NK cells only would not cure the disease but may contribute to survival.

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