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THE ROLE OF CHI3L1 IN BREAST CANCER

“A DRIVER OF IMMUNOSUPPRESSION ON THE ROAD OF TUMOR PROGRESSION”

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Abstract

The human epidermal growth factor receptor-2 (HER2) is one of the most valuable targets in cancer. HER2 is responsible for the pathogenesis of 20% of breast carcinomas (BC) and is associated with an aggressive phenotype. Despite the remarkable improvement in survival obtained by the anti-HER2 monoclonal antibodies, Trastuzumab and Pertuzumab, half of the patients still progress in less than two years. Because Antibody-dependent cytotoxicity (ADCC) mediated by NK cells is a major determinant of these antibodies' efficacy, we wanted to assess whether the development of resistance is associated with a decreased NK cell activity and to identify molecular mediators of resistance. We analyzed the sera from a cohort of HER2+ BC patients undergoing Trastuzumab treatment and showed that NK cells treated with non-responders' sera have impaired ADCC. We identified Chitinase 3 like-1 protein (CHI3L1/YKL-40) as a potential mediator of resistance as its levels were elevated in sera of resistant patients compared to complete remission patients and healthy controls. We found that CHI3L1 inhibits healthy NK cell and CD8 T cell cytotoxicity by interfering with the polarization of the microtubule-organizing center (MTOC) and lytic granules to the immune synapse. CHI3L1 mediates its effect by modulating the receptor of advanced glycation end products (RAGE) and its downstream JNK signaling leading to paralysis of the NK lytic machinery. In vivo, injecting mice with CHI3L1 drastically increased the growth of RMA-S (NK sensitive) tumors. Similarly, CHI3L1 overexpression enhanced the growth of HER2+ BC Xenografts and abrogated the control of tumor growth by ADCC in Trastuzumab treated mice. Finally, a CHI3L1 neutralizing antibody in combination with Trastuzumab was able to cure mice with HER2 xenografts by restoring NK cell activity. Our work identifies CHI3L1 as an important mediator of resistance to HER2 targeted therapy and a potential soluble immune checkpoint of NK cells and CD8 T cells beyond Trastuzumab and Breast cancer.

To the memory of my beloved brother

Imad

"A man's measure is his will"

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List of abbreviations

ADC: adoptive cell therapy
ADCC: antibody-dependent-cell-cytotoxicity
APC: antigen-presenting cell
BC: Breast cancer
CAFs: cancer-associated fibroblasts
CD: cluster of differentiation
CHI3L1: Chitinase 3 Like-1
CLPs: Chitinase like proteins
CTL: Cytotoxic T lymphocytes
CTLA4: cytotoxic T-lymphocyte–associated antigen 4
DAMPs: Danger-associated molecular patterns
DNAM-1: DNAX accessory molecule-1
dNK: NK cells in maternal deciduas
EGFR: epidermal growth factor receptor
GM-CSF: granulocyte-macrophage colony-stimulating factor
GVHD: graft versus host disease
HA: hemagglutinin antigens
HCMV: human cytomegalovirus
HLA: human leukocyte antigen
ICAM-1: intracellular adhesion molecule-1
IFN: interferon
Ig: immunoglobulin
IL: Interleukin
ITAM: immunoreceptor tyrosine-based activating motif
ITIM: immunoreceptor tyrosine-based inhibitory motif
JNK: c-Jun N-terminal kinase
KIRs: Killer Immunoglobulin-like receptors
KLRB1: Killer cell lectin-like receptor subfamily B member 1
LAMP-1: lysosomal-associated-membrane protein-1
LFA-1: lymphocyte function associated-antigen 1
LN: lymph node
mAb: monoclonal antibody
MFI: mean fluorescence intensity
MHC: Major histocompatibility complex
NCR: natural cytotoxicity receptors
NK: Natural Killer
o/n: overnight
PB: peripheral blood
PBMC: peripheral blood mononuclear cells

PMA: phorbol 12-myristate 13-acetate
RAGE: Receptor of advanced glycation end products
RT: room temperature
TAMs: tumor-associated macrophages
TANs: tumor-associated neutrophils
TGF-β: transforming growth factor-beta
TIL: tumor-infiltrating lymphocytes
TNF-α: tumor necrosis factor-alpha
TRAIL: TNF-related apoptosis-inducing ligand
Treg: regulatory T cells
VEGF: vascular endothelial growth factor

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Chapter 1: Introduction

1.1 Background

Breast cancer is a heterogeneous disease, with different molecular subtypes having distinct biological and clinical characteristics. Fourmajor subtypes can be distinguished based on the immunohistochemical (IHC) expression of the estrogen receptor (ER), the progesterone receptor (PgR), and human epidermal growth factor 2 (ERBB2; formerly HER2): Luminal A hormone receptor-positive/ERBB2 negative (30-45% of patients), Luminal B hormone receptor-positive/ERBB2 negative or positive (10-20%), ERBB2 positive (15%-20%), and triple-negative (tumors lacking the 3 standard molecular markers; 15%) (Harbeck *et al.*, 2019). The prognosis of patients with early-stage disease has improved in the past three decades thanks to the advancement of targeted therapies for HER2+ BC, hormone therapy for HR+ BC, and more recently immunotherapy for TNBC. Nonetheless, the management of advanced metastatic disease remains limited with a 22% 5-year survival rate (Waks and Winer, 2019). In the last few years, Immunotherapy, using immune checkpoint blockers or adoptive cell therapy, offered a new hope with unprecedented durable responses to a subset of patients with advanced hematological and solid malignancies, but has yet to make an impact in BC (Kruger *et al.*, 2019). Nonetheless, the revolution of immune checkpoints was long preceded by the introduction of other forms of immunotherapy, namely monoclonal antibodies to tumor-associated antigens (TAAs). Among these, Cetuximab, Trastuzumab and Pertuzumab target surface receptors of the epidermal growth factor receptor (EGFR) family and are used in the treatment of colorectal cancer and breast cancer, respectively. We have demonstrated that besides blocking receptor-ligand interaction, Cetuximab, also acts via the activation of the immune system (Pozzi *et al.*, 2016). Trastuzumab and Pertuzumab target the human epidermal growth factor receptor-2 (HER2) which is overexpressed in approximately 20% of breast cancers and is associated with poor clinical prognosis and patient survival. Despite the good improvement in progression-free survival (PFS) obtained by combining these antibodies, half the patients still progress in less than two years (Swain *et al.*, 2014). This highlights the need to understand how resistance is occurring despite the efficient blockade of HER2 signaling by the two antibodies. Data from preclinical models and patients, support that antibody-dependent cytotoxicity (ADCC) mediated by NK cells is a major determinant of Trastuzumab and Pertuzumab efficacy in Vivo. It has been shown that objective responses to

trastuzumab are dependent on FcγR2A and 3A polymorphisms (Musolino *et al.*, 2008; Tamura *et al.*, 2011) and that certain combined polymorphisms of Killer-cell immunoglobulin-like receptors (KIRs)/human leukocyte antigen (HLA) predict better response of patients to anti-EGFR therapy (Morales-Estevez, De la Haba-Rodriguez, Manzanares-Martin, Porras-Quintela, Rodriguez-Ariza, Moreno-Vega, Maria J Ortiz-Morales, *et al.*, 2016). Further, the amelioration of treatment efficacy by the combination therapy seems to be related to an increased ADCC in the presence of the two antibodies in vitro (Toth *et al.*, 2016). In agreement, trastuzumab F(ab')2 fragments (lacking Fc domain) showed little antitumor activity in vivo despite retaining their anti-proliferative and pro-apoptotic effects in vitro (Spiridon, Guinn and Vitetta, 2004). More precisely, depletion of NK cells abolished anti-HER2 mAb therapeutic activity in preclinical mouse models of HER2+ breast cancer (Park *et al.*, 2010; Jaime-Ramirez *et al.*, 2011; Stagg *et al.*, 2011). In the same context, considering that breast cancer cells isolated from patients resistant to trastuzumab are still susceptible to NK cell ADCC (Kute *et al.*, 2009), the major defect in trastuzumab resistance may be associated with an impaired NK cell activity. Although NK cell dysfunction has been reported in various cancers including breast cancer (Caras *et al.*, 2004), soluble molecules acting as negative regulators of their activity are not well characterized.

1.2 Aims

Our **first aim** was to identify molecules that could predict and explain innate and acquired resistance to Trastuzumab. Based on large evidence supporting ADCC as a major determinant of Trastuzumab efficacy, we focused our approach on this immune mechanism mediated by NK cells. We hypothesized that NK cells of non-responders might be dysfunctional which compromises ADCC and leads to treatment failure.

Our **second aim** was to study the effects of candidate molecule(s) on the cytotoxicity and biology of NK cells and cytotoxic T cells and to elucidate the molecular mechanism of its effects.

Our **third aim** was to develop a therapeutic tool that can be used to overcome resistance to Trastuzumab in HER2 BC or in combination with other immunotherapeutic modalities to restore and boost NK cell activity.

1.3 Approach

We collected sera from HER2+ metastatic breast cancer patients at basal (before Trastuzumab therapy) and subsequently until patients reached progressive disease (PD) or complete remission (CR) state (12 months). NK cells isolated from healthy donors were incubated with the patients' sera and tested for their ADCC activity in vitro. We then carried out a comparative proteomic analysis of sera from PD versus CR patients to identify differentially abundant molecules that could explain the effect of each group of sera on NK cell activity.

1.4 Main findings

We show that Sera from non-responders reduced healthy NK cells activity, while sera from responders did not affect NK cells activity. We found high levels of CHI3L1 (a.k.a YKL-40) protein in sera of trastuzumab-resistant patients but not in sera of responding patients where levels were comparable to healthy controls. We confirmed, in vitro and in mice models, that CHI3L1 is responsible for impairing NK cell ADCC by negatively modulating RAGE signaling, namely the JNK pathway, and consequently the correct polarization of the degranulation machinery. This suggested that CHI3L1 is a potential target to overcome or prevent trastuzumab resistance in HER2+ BC. To prove this, the combination of a CHI3L1 neutralizing antibody with Trastuzumab restored NK cell activity and lead to complete regression of HER2+ xenografts. Importantly, we also found that CHI3L1 inhibits NKG2D dependent NK cell cytotoxicity and CD8 T cell cytotoxicity. In this context, neutralizing CHI3L1 enhanced CAR-T cell cytotoxicity in vitro and in tumor models.

1.5 Conclusion

Our work identified a new mechanism of resistance to Trastuzumab and potentially other ADCC dependent antibodies. This offers a new therapeutic possibility for resistant patients. We also identified a new role of CHI3L1 protein as a general immunosuppressive molecule, in addition to its well-established role in fostering tumor growth and metastasis. Thus, targeting CHI3L1 could be a valuable tool to enhance the efficacy of other immunotherapeutics and could benefit patients beyond trastuzumab and breast cancer.

Chapter 2: Literature review

2.1 Introduction

The purpose of this chapter is to introduce the literature that has informed the foundations and inspired the progress of my PhD work. The following subjects will be covered:

1. Breast cancer and the immune involvement in this disease, with a focus on HER2 BC.
2. An overview of NK cell biology with a focus on the immunological synapse during NK cells cytotoxicity.
3. An overview of the evolutionary conserved and intriguing family of Chitinase-like proteins, with a focus on CHI3L1/YKL-40 and its role in cancer.

2.2 Breast cancer

Each year, 2.1 million women, are diagnosed with breast cancer worldwide making it the most frequent cancer in women. Although overall mortality for BC has declined over the years, thanks to the advancement of therapeutics and patient care, it remains the second most common cause of cancer death in women. In 2018, an estimated 627,000 women died from BC, approximately 15% of all cancer deaths among women (World Health Organisation, 2018). While BC is more frequent among women in more developed countries, rates are increasing in nearly every region globally. This highlights the importance of global research efforts to improve BC treatment and diagnosis.

The pathophysiology of BC is multifactorial and not well understood, with advancing age and female sex being the most common risk factors (Watkins, 2019). Genetic mutations, specifically BRCA 1 and 2, account for about 10% of breast cancers (Jonsson *et al.*, 2019). Other known risk factors include a history of ductal carcinoma in situ, high body mass index (BMI), family history of breast or ovarian cancer, late menopause, and postmenopausal hormone therapy use (Watkins, 2019).

In routine clinical practice, BC can be classified into prognostic and predictive subtypes based on the immunohistochemical (IHC) expression of the estrogen receptor (ER), the progesterone receptor (PgR), the human epidermal growth factor receptor type 2 (HER2), with in situ hybridization of equivocal cases (scored as 2+ at IHC), histological grade and the Ki-67 proliferation index. Accordingly, Luminal A-like, luminal B-like, HER2 enriched (non-

luminal) and triple-negative breast cancer (TNBC) are defined as clinicopathological surrogate groups having each different clinical characteristics and prognosis (Harbeck *et al.*, 2019) (**Figure 1**).

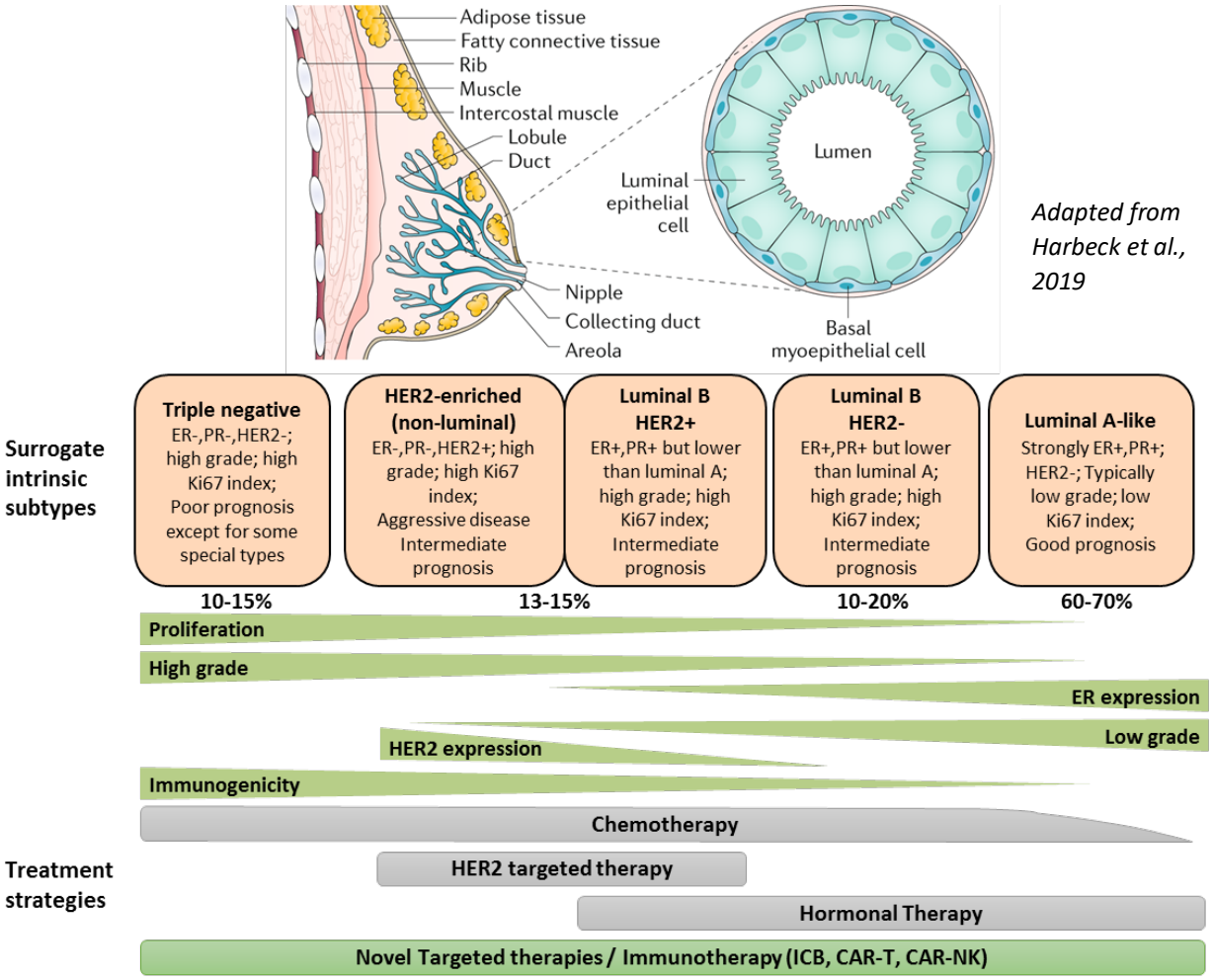


Figure 1: Breast cancer subtypes, characteristics, and treatments.
BC can arise in the milk ducts (ductal carcinomas) or the lobular units (lobular carcinomas). According to IHC score of expression of the estrogen receptor (ER), the progesterone receptor (PgR), human epidermal growth factor receptor type 2 (HER2), and the Ki-67 proliferation index, BC can be classified into: Luminal A-like, luminal B-like, HER2 enriched (non-luminal) and triple negative (TNBC). The relative placement of the boxes align with the characteristics (e.g proliferation and grade) and with the systemic treatment strategies (Grey boxes: currently adopted treatments, Green box: treatments in development). GES: gene expression signature, NST: no special type, ICB: Immune checkpoint blockers, CAR-T/NK: Chimeric antigen receptor T cells or NK cells.

2.3 Treatment of Breast cancer

A detailed overview of the management of BC is provided by (Harbeck *et al.*, 2019). Below is a summary of therapeutic strategies, guided by stage and subtype.

2.3.1.1 Early BC

This includes patients with In situ or non-invasive disease or locally invasive but operable tumors (up to stage III). Regardless of subtype, the main curative intervention for these patients is surgery to remove the tumor and excise the affected axillary lymph nodes that can be followed by postoperative radiation therapy. Surgery can be preceded with systemic therapy (neoadjuvant setting) in patients with larger tumors to decrease tumor burden before surgery. Systemic therapy after surgery (adjuvant therapy) is given to prevent recurrence in all subtypes of BC and is especially relevant in subtypes with high recurrence rates such as TNBC and HER2+.

2.3.1.2 Advanced BC

It includes patients with inoperable locally invasive tumors (not yet spread to distant organs), or metastatic (stage IV) tumors that have spread to distant organs (bone, lungs, and liver are most common sites). Advanced BC is still incurable, but can be managed with a wide arsenal of systemic therapies including chemotherapy and targeted therapies to prolong patient survival.

2.3.1.3 Systemic therapies for BC

As mentioned above, systemic therapy can be given in neoadjuvant setting, before surgery or if surgery is not applicable (Ex. metastatic disease) and in the adjuvant setting for eliminating residual disease and preventing recurrence. The choice of chemotherapeutics and/or targeted therapies depends on BC subtype (**Figure 1**):

- Hormone receptor-positive (HR+): endocrine therapy (Tamoxifen, Anastrozole, Fulvestrant…) with chemotherapy mostly for luminal B. Targeted therapy with kinase inhibitors (Ex. Palbociclib, ribociclib) was recently introduced for use in combination with hormone therapy for metastatic HR+ disease (Finn *et al.*, 2016) and Alpelisib for tumors with mutated PIK3CA (André *et al.*, 2019).
- Triple-negative (TNBC): Known as the hardest to treat due to the lack of classical molecular targets. Chemotherapy is the standard of care with the addition of PARP inhibitors for BRCA mutated tumors (Earl *et al.*, 2017) and Atezolizumab for PD-L1+ tumors (Heimes and Schmidt, 2019).

- HER2 enriched: In addition to chemotherapy, multiple class of targeted drugs are available for the treatment of HER2 BC:

  -Anti-HER2 monoclonal antibodies: Trastuzumab and Pertuzumab.

  -Antibody-drug conjugates: Trastuzumab emtansine (TDM-1) and Trastuzumab-deruxtecan

  -Small molecule kinase inhibitors: lapatinib and Tucatinib (HER2 specific), Neratinib (HER1, HER2 and HER4

HER2+ BC will be discussed in more depth in the next section.

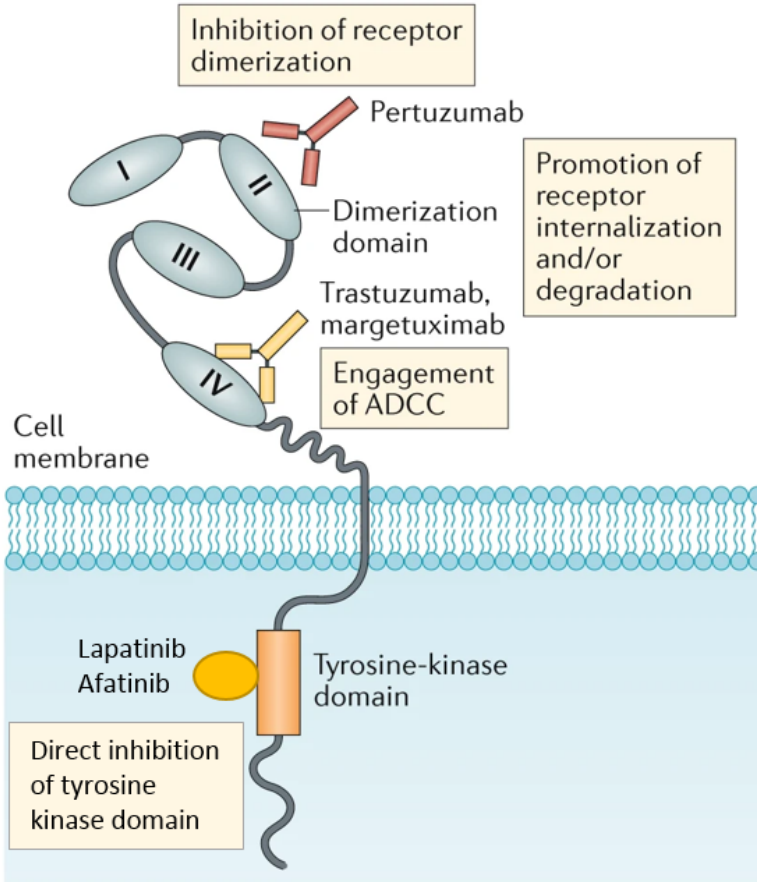
2.3.2 History and current status of HER2 BC

"*These data indicate that this gene may play a role in the biologic behavior and/or pathogenesis of human breast cancer*" Dennis J slamon and colleagues concluded in the 1987 paper marking the discovery of the role of HER2 oncogene in breast cancer (Slamon *et al.*, 1987). A few years earlier, Genentech scientists had successfully cloned members of the growth factor family of receptors (Ullrich *et al.*, 1984). Using DNA probes specific for HER2/neu in southern blot analysis, they found that almost a quarter of breast tumors overexpressed this oncogene and correlated it with an aggressive phenotype (Slamon *et al.*, 1987). Today, HER2 is a well-established tumor-associated antigen (TAA) and a therapeutic target of interest not only in BC but also in other tumor types such as Gastric, biliary tract, colorectal and bladder cancers (Oh and Bang, 2019).

HER2, also known as Receptor tyrosine-protein kinase erbB-2, is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases. Overexpression of this receptor leads to homodimerization and heterodimerization with other members of the EGFR family. This results in a cascade of cellular signaling responsible for uncontrolled proliferation and tumorigenesis (Yarden and Sliwkowski, 2001).

To harness the therapeutic potential of targeting this receptor, Trastuzumab was developed by Genentech (San Francisco, CA) in 1990 as the first anti-HER2 monoclonal antibody. Trastuzumab is a humanized IgG1 kappa antibody produced by recombinant DNA technology in Chinese hamster ovary (CHO) cells (Fendly *et al.*, 1990). In 2001, the addition of Trastuzumab to chemotherapy improved the overall survival (OS) in women with HER2-positive metastatic breast cancer (Slamon *et al.*, 2001), and this was followed by its approval in the adjuvant treatment of women with early-stage disease in 2005 (Piccart-Gebhart *et al.*,

2005; Romond *et al.*, 2005). Pertuzumab is another humanized anti-HER2 IgG1 monoclonal antibody, targeting a different epitope of HER2 (Domain II) from that of trastuzumab (Domain IV) (**Figure 2**).



Adapted from (Oh and Bang, 2019)

Figure 2: Mode of action of HER2 targeted agents.
The antitumor effects of monoclonal antibodies, such as Trastuzumab, Margetuximab and Pertuzumab are mediated through multiple mechanisms: inhibition of downstream signalling pathways, engagement of antibody-dependent cellular cytotoxicity, or, in the case of pertuzumab, inhibition of receptor dimerization. Alternatively, small-molecule inhibitors, such as lapatinib and afatinib are used to directly inhibit activation of the PI3K pathway by inhibiting tyrosine kinase activity.

Pertuzumab was found to synergize with trastuzumab in vitro and in vivo (Scheuer *et al.*, 2009). In the Phase III clinical trial CLEOPATRA, the combination of both antibodies increased O.S by 15.7 months compared to Trastuzumab alone (Swain *et al.*, 2014). For more than a decade, these antibodies have become the golden standard for the treatment of HER2 BC and have improved the lives of many women with this disease. Unfortunately, many others do not fully benefit and develop resistance to these antibodies, with half the metastatic patients still progressing in less than two years (Pohlmann, Mayer and Mernaugh, 2009; Swain *et al.*, 2014). Several mechanisms of action have been proposed for anti-HER2 monoclonal antibodies: inhibition of dimerization and the PI3K–AKT signaling pathway, receptor

internalization and/or degradation, and antibody-dependent cellular cytotoxicity (ADCC) (**Figure 2&3**). The latter being a major determinant of these antibodies' efficacy, led to the development of Margetuximab, a monoclonal antibody binding the same epitope of Trastuzumab but with an Fc engineered for enhanced ADCC ability (Y.J. *et al.*, 2017; Liu *et al.*, 2019).

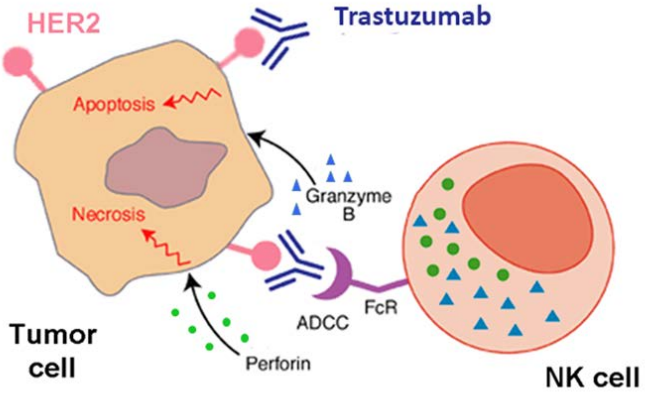


Figure 3: Antibody-dependent cellular cytotoxicity (ADCC).
NK cells, via their Fc receptors (FcR, CD16, FcγRIIIA) can recognize the Fc portion of antibodies (e.g. Trastuzumab) bound on their target antigens on tumor cells (e.g HER2). This leads to the release of cytotoxic molecules and tumor cell lysis.

Another HER2-targeted approved drug is Trastuzumab emtansine (T-DM1), designed to deliver a cytotoxic tubulin-binding agent (Mertansine or DM1) to HER2 tumor cells (Verma *et al.*, 2012). T-DM1 is currently used in second line to treat patients with advanced disease that progressed to adjuvant Trastuzumab and Pertuzumab plus taxane chemotherapy.

The approval of T-DM1 and Pertuzumab raised the question of whether a combination of the two might result in an additional benefit. However, the MARIANNE clinical trial did not show increased PFS of T-DM1 in combination with pertuzumab compared to standard treatment (trastuzumab plus chemotherapy) in first-line treatment of HER2 advanced BC patients (Perez *et al.*, 2017). On the other hand and in the same setting, a single agent Margetuximab plus chemotherapy was better than Trastuzumab plus chemotherapy in the phase III SOPHIA trial (O.S 26.1 months vs. 19.8 months respectively) and is now seeking FDA approval (Rugo *et al.*, 2019)

The reason remains unclear as to why, Margetuximab, an ADCC enhanced version of Trastuzumab, shows a survival benefit but the combo of two agents, TDM-1 and Pertuzumab doesn't. One can speculate (as we did in this work) that the major mechanism by which patients progress to HER2 targeted therapy is independent of the ability to bind and block

HER2 signaling per se. In other words, the real issue might depend on the failure of the immune component to support the complete action of HER2 monoclonal antibodies. The Immune component of BC will be reviewed next.

2.3.3 Immune involvement: Immunotherapy and Immune evasion

Cancer immunotherapy has witnessed a new renaissance with the advent of immune checkpoint inhibitors, which reactivate immune cells and foster endogenous anti-tumor responses. The excellent results in immunogenic tumors like melanoma, renal cancer, and lung cancer, inspired the oncology community to work on extending the benefits to all cancer types including BC. This led to the first approval of a checkpoint inhibitor in BC, the anti-PD-L1 atezolizumab for the treatment of TNBC (Heimes and Schmidt, 2019). This is not coincidental, as TNBC is the most immunogenic subtype of BC with fertile ground for immunotherapeutics, followed by HER2 BC. Luminal A and B subtypes are the least immunogenic (**Figure 1**) (Solinas *et al.*, 2017; Nagarajan and McArdle, 2018).

As in most cancers, the intensity of the host immune response in the tumor bed, reflected by the amount of tumor-infiltrating lymphocytes, positively correlates with BC patient's prognosis (Savas *et al.*, 2016). Neoantigen (tumor antigens) presentation by antigen-presenting cells (APCs) initiates the anti-tumor immune response by activating helper CD4+ T cells and cytotoxic CD8+ T cells. These latter are the main effector cells of the adaptive anti-tumor immune response. In parallel, innate immunity, comprising NK cells and NK T cells is capable of exerting direct tumor cell killing or indirectly through ADCC (especially relevant for Trastuzumab in HER2 BC). CD8+ T cells and NK cells kill tumor cells by secreting cytotoxic granules, perforin, and Granzymes. They also upregulate the expression of Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) on their membrane, which induces apoptotic pathways to kill tumor cells. To mimic these endogenous tumor responses, adoptive cell immunotherapies are being investigated in BC, namely, Tumor-infiltrating lymphocytes (TILs), CAR-T cells, and CAR-NK cells (Wu *et al.*, 2012; Wang and Zhou, 2017; Hu *et al.*, 2019).

To survive the host immunity, tumor cells are embedded in an immune hostile microenvironment, especially during the more advanced stages of the disease. Cancer-associated fibroblasts are the most abundant in BC stroma. Other important players in the tumor microenvironment (TME) include Regulatory T cells, M2 polarized macrophages, and

Neutrophils. Altogether these cell types have been widely associated with an immunosuppressive phenotype, resistance to therapy, and worst prognosis in BC (Buchsbaum and Oh, 2016; Williams, Yeh and Soloff, 2016; Soto-Perez-de-Celis *et al.*, 2017). They can act directly by engaging inhibitory receptors on the surface of T cells and NK cells or by secreting an array of cytokines and factors that can feed tumor growth or inhibit T cell and NK cell cytotoxicity. Consequently, a combinatorial approach, aiming to deliver cytotoxic cells while relieving immunosuppression is becoming more relevant for treatment success. The interplay between the effector immune response and tumor-associated cells in the TME of BC is depicted in **Figure 4**.

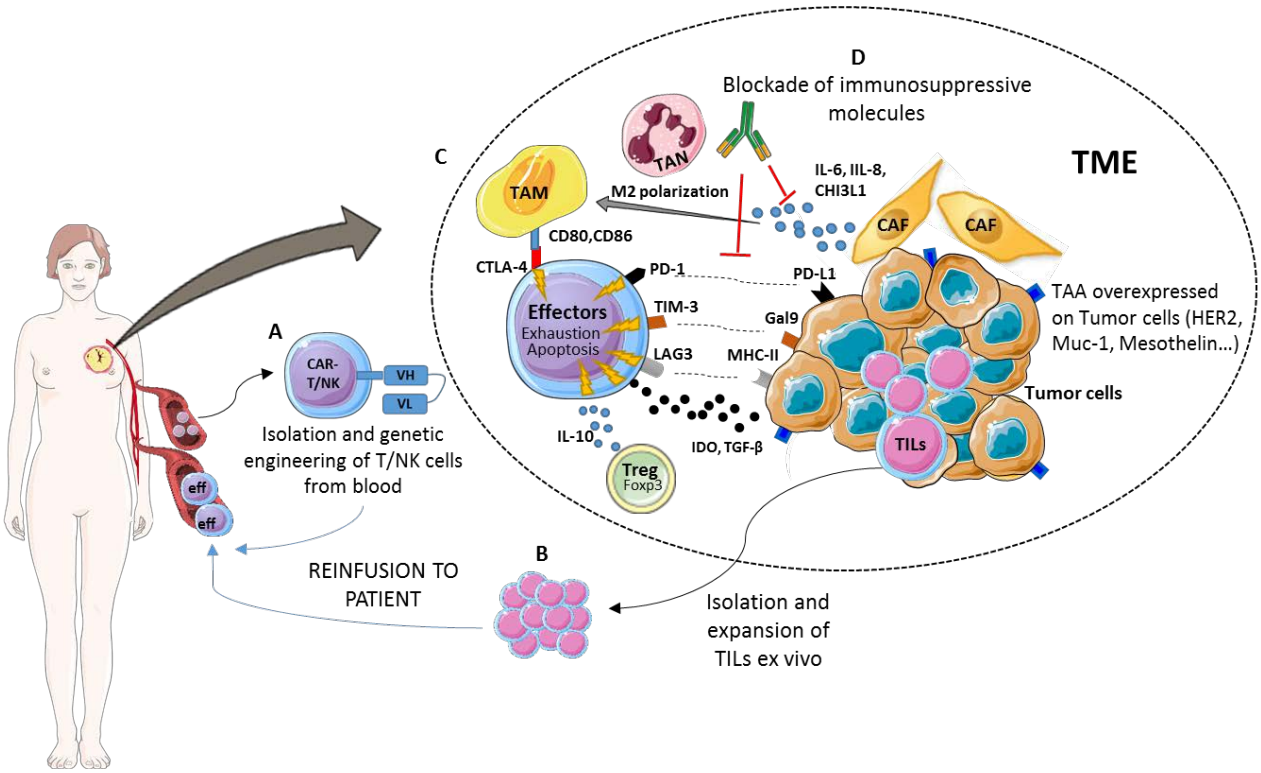


Figure 4: Inhibition of the effector immune response in the TME of BC by molecules of the tumor and tumor-associated cells.

(A) T cells or NK cells are harvested and transduced to express a tumor associated antigen (TAA) specific receptor (CAR-T/NK). **(B)** TILs are isolated from a tumor mass, expanded and selected for tumor recognition and killing. In both cases, tumor-specific effector cells are re-infused to the patient. **(C)** Once in the TME, endogenous or transferred effector cells interact with tumor-associated, immunosuppressive cells, including tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), regulatory T cells (Tregs), and cancer-associated fibroblasts (CAFs). These cells as well as tumor cells themselves express inhibitory molecules, including CD80/CD86, Gal9 and PDL-1, which bind to the inhibitory receptors CTLA-4, TIM-3 and PD-1, respectively. They also secrete soluble factors that either suppress immune cells directly or indirectly by M2 polarization of macrophages. All these factors serve to promote an "exhausted" phenotype in effector immune cells, characterized by upregulation of inhibitory signals, loss of cytotoxicity, and apoptosis. Combining adoptive cell therapy or the endogenous host immune response with specific blockade of these factors **(D)** could alleviate this inhibition and improve patients' response.

2.4 NK cells and how they killer "kiss" their targets.

2.4.1 Introduction

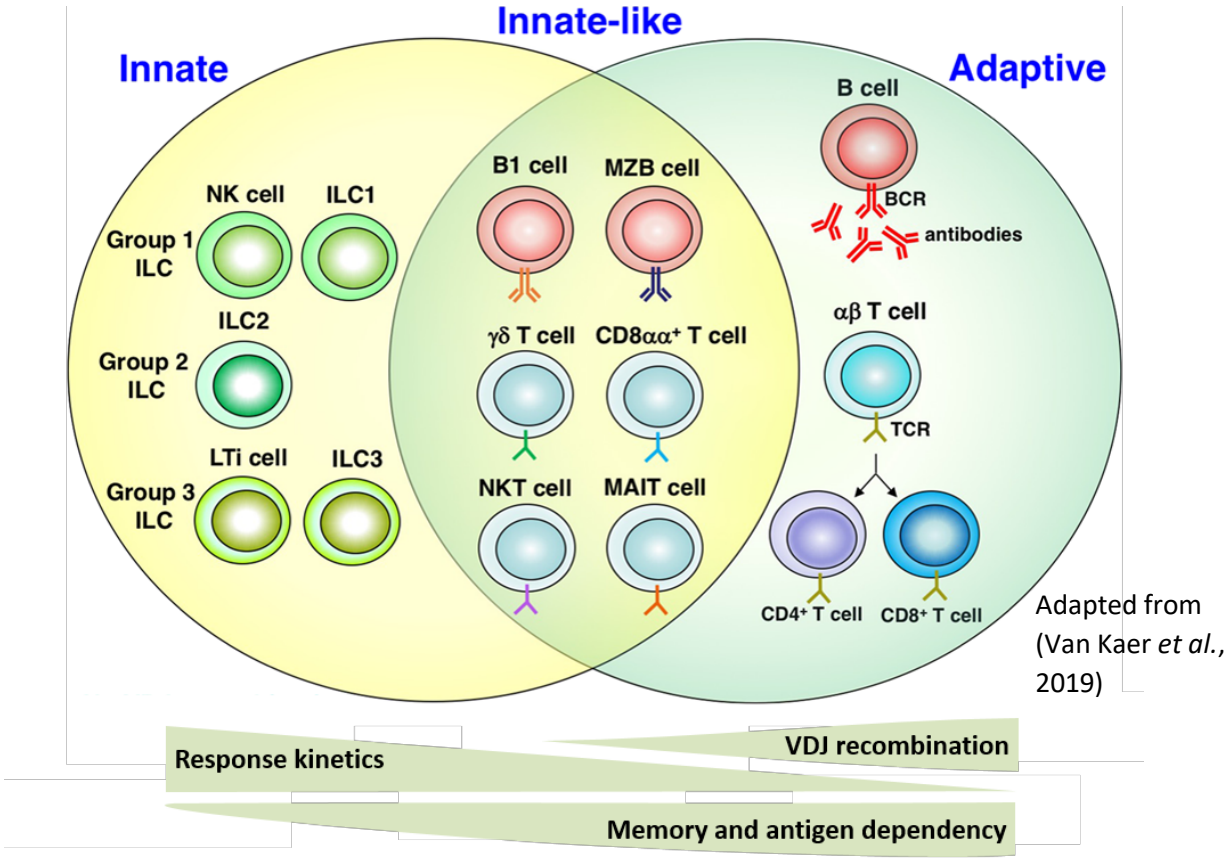
In this chapter, the biology and regulation of human NK cells will be discussed. The immune synapse they form with target cells will be described as well.

2.4.2 Overview of biology

The immune system of higher vertebrates have evolved to cope with an immense diversity of foreign bodies and to ensure cellular homeostasis. Approximately 500 million years ago, the adaptive immune response, mediated by B and T lymphocytes, emerged to complement its innate counterpart (Flajnik and Kasahara, 2010). Until the 1970s, these two cell types were thought to compose the entirety of the lymphocytic population. However, the discovery that antibody-coated target cells could be killed by naïve (unimmunized) lymphocytes, pointed to the existence of an additional player (Greenberg, 1973). Research began to identify the effector cells termed 'null' lymphocytes that are neither B nor T cells and that can spontaneously kill target cells without prior immunization or antibody coating. In 1975, Keissling et al. showed the "natural" cytotoxicity of these effectors against multiple tumor types and named them Natural Killer (NK) cells (Kiessling, 1975).

NK cells are an essential part of the innate immune system, predisposed to respond early and swiftly to kill transformed cells, virus-infected cells, and to regulate the immune response (Mandal & Viswanathan, 2015). More specifically, they are part of a family of innate lymphoid cells (ILCs) together with the more recently discovered members ILC1, ILC2, ILC3, and LTi cells (Spits *et al.*, 2013)(**Figure 5**).

NK cells constitute ~10% of circulating peripheral blood mononuclear cells (PBMCs) and are phenotypically defined as CD56+CD3- in humans. Blood NK cells are also known as "conventional" c-NK cells and are the most studied relative to tissue-resident NK cells (Vitale, Caligiuri and Sivori, 2020). There are two subsets of c-NKs according to CD56 and CD16 expression: CD56dim $CD16^{+}$ (90%) CD56bright $CD16^{-}$ (10%).



Adapted from (Van Kaer *et al.*, 2019)

Figure 5: The main subsets of lymphocytes, and their key properties.
BCR B-cell receptor, ILC innate lymphoid cell, LTi lymphoid tissue inducer, MAIT mucosal-associated invariant T, MZB marginal zone B, NK natural killer, NKT natural killer T, TCR T-cell receptor, VDJ variable diversity joining

The CD56dim cells are cytotoxic effectors containing a pool of cytotoxic granules and can exert natural and ADCC mediated cytotoxicity and secret INF-g upon activation (De Maria, 2011; Moretta, 2010). The CD56bright ones are minimally cytotoxic but play an immunomodulatory role via cytokine and chemokine secretion including IFN-γ, TNF-α, GM-CSF, CCL4, and HMGB1 (Poli *et al.*, 2009). An inverse scenario is found in tissues and secondary lymphoid organs with the predominance of CD56bright NK cells (Poli *et al.*, 2009; Vitale, Caligiuri, and Sivori, 2020). An interesting example of tissue-resident CD56bright NK cells is the uterine decidual NK (dNK) cells which constitute ~70% of resident lymphocytes and play an essential regulatory role in implantation and maintaining pregnancy (Hanna *et al.*, 2006). Strong evidence points that CD56bright cells are precursors of CD56dim cells during NK cell maturation (Sivori *et al.*, 2003; Freud and Caligiuri, 2006; Chan *et al.*, 2007; Romagnani *et al.*, 2007). Of note, a special feature of CD56dim human NK cells is the expression of perforin at basal state, which is uncommon to other cytotoxic cells like human CD8+ T cells (only 20% of healthy donors) or their murine counterparts (Pipkin, 2010; personal observation).

Altogether, perforin, CD56, CD57, CD16, and KIR/NCR expression (discussed next) can be considered markers of NK cell maturation and differentiation in humans (**Figure 6**) (Björkström *et al.*, 2010; Moretta, 2010).

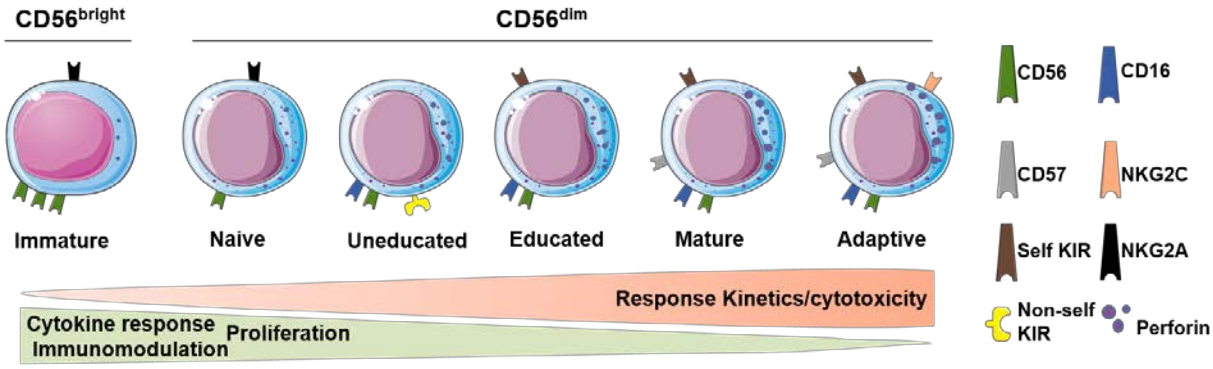


Figure 6: Overview of the distinct stages of human NK cell differentiation characterized by changes in their phenotypic and functional properties

At the end of the maturation spectrum, there is an adaptive or "memory-like" subset of NK cells. Although immunological memory and rearrangement of receptors is a hallmark of T and B cells (**Figure 5**), accumulating evidences have shown that NK cells display more efficient responses against some previously encountered antigens (Cerwenka and Lanier, 2016). For example, adaptive NK cells can be found in ~40% of cytomegalovirus (CMV) seropositive individuals (Q. and C., 2017). Of note, the improved function and persistence of this NK cell subset makes it valuable for adoptive cell therapy (Fehniger and Cooper, 2016).

Another divergence from T and B lymphocytes is that NK cells do not undergo positive or negative selection. So, it was initially assumed that they would express a minimum of one inhibitory receptor (a receptor that recognizes the self) to maintain tolerance to self (Raulet *et al.*, 1997). The discovery of NKG2A-KIR- NK cells (Two types of inhibitory receptors recognizing self MHC-I that are discussed later) in mice and humans disproved this (Cooley *et al.*, 2006). However, this population of NK cells was found to circulate in a hypo-responsive, nonfunctional state, to ensure self-tolerance. Indeed it is well established now, that during their maturation, NK cells are functionally tuned via inhibitory interactions between self-MHC and KIR or NKG2A in a process known as NK cell "education".

2.4.3 Killing mechanisms

NK cells largely exert their cytotoxic effect by the polarized release of cytotoxic granules into the target cells. These granules are secretory lysosomes and are formed through the fusion

of different vesicular structures (De Saint Basile & Fischer, 2010). Perforin and granzyme B are the two main components of cytotoxic vesicles, although CD56dim NK cells can also produce granzyme A and M, with CD56bright NK cells producing granzyme K (De Saint Basile, Ménasché and Fischer, 2010). Perforin is produced in the endoplasmic reticulum (ER) and then sorted into granules through the Golgi complex. It requires cathepsin L to be activated, while granzyme's activation requires cathepsin C (Nanut & Kos, 2014). Perforin is also kept inactive in acidic PH and by binding calreticulin (Fraser & Hudig, 2000). These regulations ensure that NK cells are protected from self lysis. Once activated and released, perforin attacks the target cell's membrane and oligomerizes to form pores allowing granzyme's entry. This induces apoptosis, both in a caspase-dependent and independent manner, leading to the production of reactive oxygen species as well as DNA and mitochondrial damage (Voskoboinik & Trapani, 2006).

Additionally, NK cells can employ the death receptor (DR) pathway by expressing Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) to induce apoptosis of target cells (Screpanti & Ljunggren, 2005).

Either way, a common step will be the formation of an NK cell- target cell conjugate which is referred to as the lytic immunological synapse that will be described later.

Another cytotoxic property of NK cells is their ability to engage and kill multiple targets in a relatively short time, known as "serial killing". For example, a resting or IL-2 activated NK cell can kill up to 4 and 6 targets, respectively (~20 min per target) (Prager *et al.*, 2019). This can be further enhanced by the use of ADCC inducing therapeutic antibodies (Bhat and Watzl, 2007; Kellner and Peipp, 2014). Of note, NK cells start relying more on the death receptor pathway during serial killing as a result of cytotoxic granules' depletion (Prager *et al.*, 2019). Interestingly, as little as 1% of the lytic granules content of an NK cell is sufficient to kill a target cell (Gwalani and Orange, 2018). Fortunately, the potent cytotoxicity of NK cells is tightly regulated and requires the integration of signaling from activating and inhibitory receptors before pulling the trigger (**Figure 7**). This will be discussed next.

2.4.4 Regulation, Receptors, and Ligands.

Since their discovery, interest in NK cells increased over the years with researchers focusing on deciphering how NK cells are regulated to better understand their function in health and

disease and to harness them for therapeutic application (Mandal & Viswanathan, 2015; Shimasaki, 2020). A major step on this path was taken by discovering their ability to recognize and kill major histocompatibility class I (MHC-I) deficient cells according to the seminar "missing-self" hypothesis (Ljunggren and Kärre, 1990) (**Figure 7**). However, the mechanisms regulating this ability were only understood with the subsequent discovery of NK cell receptors as the molecular switches controlling the outcome of NK cell-Target interaction (**Figure 8**) (Moretta *et al.*, 1997; Vitale *et al.*, 2015; Biassoni and Malnati, 2018). In this context, other than being a tumor cell or a virus-infected cell, a target can be an ally, where NK cells were shown to interact with dendritic cells to orchestrate the adaptive immune

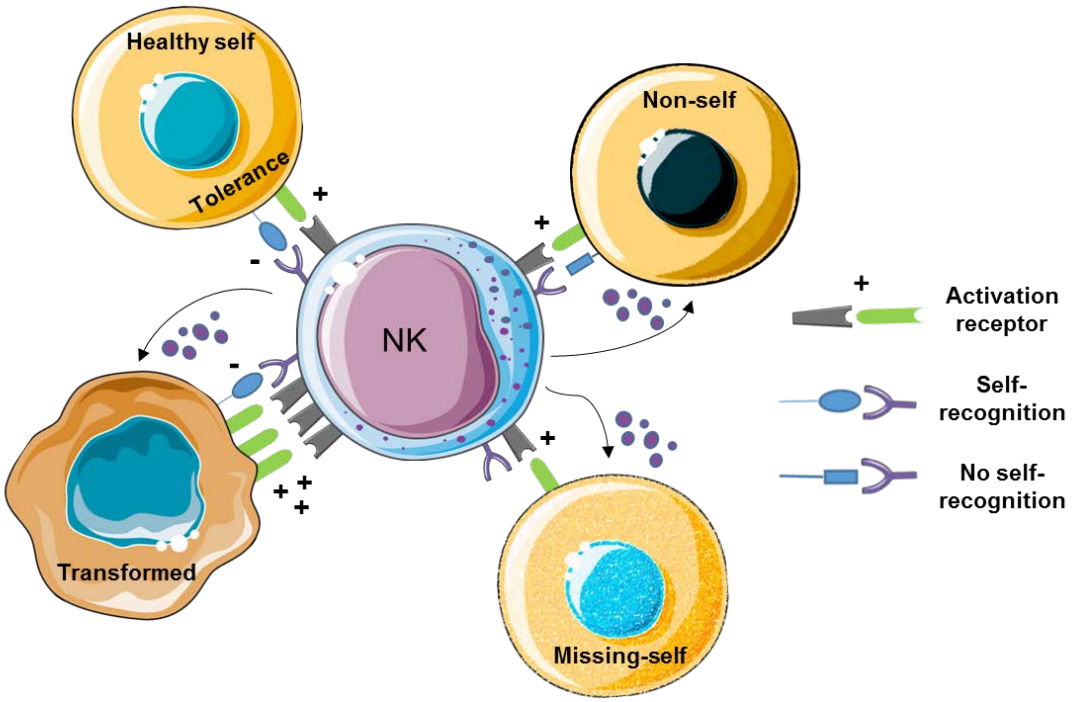


Figure 7: Overview of the different scenarios of NK-Target interaction.
The encouter bewteen NK cells and potential target cells can result in protection or lysis of targets based on the balance of signals received through activation and inhibitory receptors. Input from activating receptors prevails when target cells express low levels of MHC-I molecules or express non-self MHC-I. The overexpression by some tumor cells of activation ligands can overried inibition by MHC-I recognition.

response (Piccioli & Valiante, 2002; Terme & Zitvogel, 2008).

A sophisticated array of activation and inhibitory receptors that can bind MHC-I molecules or non-MHC ligands have been identified. The main families of receptors include Killer Ig-like receptors (KIRs), natural cytotoxicity receptors (NCRs), Natural-Killer group 2 (NKG2),

Nectin/Nectin-like, and the SLAM family of receptors (Moretta *et al.*, 1997; Vitale *et al.*, 2015; Biassoni and Malnati, 2018) (**Figure 8**).

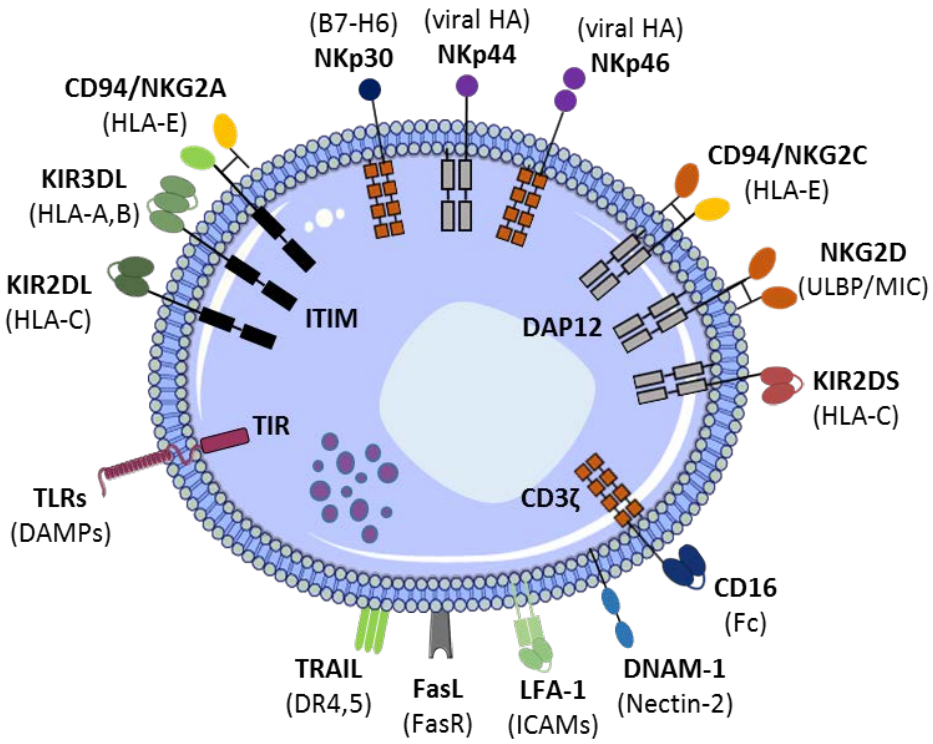


Figure 8: NK cell receptors and their ligands.
Visualization of the main cytotoxicity receptors on human NK cells (in bold), their ligands (between parethesis) and their intracellular signaling component. NCRs, NKG2D, CD16, DNAM-1, and TLRs are all examples of activation recptors. KIRs are mainly inhibitory receptors endowed with ITIM inhibitory signaling motifs. The discovery of these receptors led to a better understanding of how NK cells are regulated.

2.4.4.1 Killer Immunoglobulin-like receptor family (KIRs)

As mentioned above, MHC-I recognition is a hallmark of NK cell regulation. KIRs are transmembrane type I receptors of the Immunoglobulin-like receptor family and constitute the main group of inhibitory (with few activating) receptors allowing NK cells to recognize specific human leukocyte antigen (HLA) molecules (Moretta and Moretta, 2004). They are named based on the length of the cytoplasmic tail, short (S) or long (L), and the number of extracellular Ig-like domains (2 or 3). While the long cytoplasmic tail receptors contain immunoreceptor tyrosine-based inhibitory motifs (ITIM), the short tails contain immunoreceptor tyrosine-based activation motifs (ITAM) able to associate with the adaptor molecule DAP12 (Moretta and Moretta, 2004). Depending on the number of extracellular Ig-like domains, they can specifically recognize HLA-C, HLA-A, or HLA-B molecules. KIRs with two

Ig-like domains are specific for HLA-C alleles and were the first to be described, followed by those with three Ig-like domains that are specific for HLA-B and certain HLA-A alleles. The KIR locus is polygenic (15 distinct gene loci) and polymorphic and is one of the most variable regions in the human genome. Further, stochastic expression of KIR genes by epigenetic regulation, plus gene copy number variation, add more layers of diversity (Jiang et al., 2012; Li & Anderson, 2008). For simplicity, two main haplotypes (A and B) are used to group KIR genotypes within individuals (Middleton and Gonzelez, 2010). Haplotype A contains a restricted number of inhibitory receptors and one activating receptor, KIR2DS4. The less common haplotype B includes a larger repertoire of both inhibitory and activating receptors. The inhibitory receptors KIR2DL1, KIR2DL3 (HLA-C binders) and KIR3DL1 (HLA-B, A binder) are the most commonly studied examples. KIR2DS1 and KIR3DS1 which binds to HLA-C2 and HLA-F respectively are notable examples of activating receptors relevant among others for predicting good response to anti-EGFR therapy (Cognet *et al.*, 2010; Morales-Estevez, De la Haba-Rodriguez, Manzanares-Martin, Porras-Quintela, Rodriguez-Ariza, Moreno-Vega, Maria J. Ortiz-Morales, *et al.*, 2016).

2.4.4.2 Natural cytotoxicity receptors (NCRs)

Members of this family, NKp46, NKp30, and NKp44 are activating receptors critical for non-MHC-restricted cytotoxicity. NCR surface density on NK cells correlates with the magnitude of cytotoxicity against NK-susceptible target cells (Moretta *et al.*, 2001). NKp30 and NKp46 are expressed on resting and activated NK cells and signal via FceRIg and CD3z modules. Conversely, NKp44 is expressed only upon NK cell activation or stimulation by IL-2 and uses DAP12 adaptor for signaling (Kruse & Vivier, 2014). The ligand for NKp30 is B7-H6 and is expressed on tumor cell lines as well as on neutrophils and monocytes after toll-like receptor and pro-inflammatory cytokine stimulation (Chen & He, 2018). The ligands for NKp44 and NKp46 have been suggested to be viral hemagglutinins (Kruse *et al.*, 2014). In addition to its role in immune surveillance, NKp30 was found to be important in NK-DC interactions in tissues. Whereas NK cells can recognize and kill immature DCs through Nkp30, while mature DCs can modulate NK cell proliferation and activation via this receptor (Terme and Zitvogel, 2008). Of note, the expression of NCRs is downregulated by TGFβ1, thus affecting both anti-tumor killing and DC-NK cell communication (Han *et al.*, 2018).

2.4.4.3 NKG2 receptors

NKG2s come in different molecular flavors, from NKG2A to H. These receptors belong to the C-type lectin-like receptor superfamily, i.e. receptors containing a carbohydrate-binding protein domain, known as a lectin, and requiring calcium for binding (C-type). According to the presence of immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic domains and their binding with adaptor molecules, they are classified as inhibitory or activating receptors (Biassoni and Malnati, 2018). The molecular variants: A, B, C, E, and H have been shown to bind to the transmembrane-anchored glycoprotein CD94 which is required for NKG2 translocation to the surface. Of these, NKG2A and NKG2C share a common ligand, HLA-E, despite NKG2A being an inhibitory receptor containing ITIMs and NKG2C being an activating receptor associating with DAP12 for signaling (Borrego & Coligan, 2006). However, CD94/NKG2A is more affine than CD94/NKG2C in binding HLA-E, perhaps to preserve self-tolerance (Wada & Yamamoto, 2004). Other members, namely NKG2D and NKG2F, are the only ones within the NKG2s family to form homodimers instead of associating with CD94. NKG2D is an activating receptor and signals via a DAP10 adaptor. Its ligands ULBP1-4 and MICA/B are upregulated on target cells experiencing cellular stress making NKG2D one of the most important receptors of NK cells cytotoxicity and surveillance (Raulet & Jung, 2013). More recently, the role of NKG2D in NK cells education and self-tolerance has been emerging and challenges the narrow view on NKG2D as a cytotoxicity receptor only (Wensveen, & Polić, 2018). NKG2E and NKG2F are intracellular and have not been attributed to a clear function but their cytoplasmic association with DAP12 suggests that they might act as a regulator of this adaptor (Orbelyan *et al.*, 2014). NKG2B and NKG2H, meanwhile, are splice variants of NKG2A and NKG2E, respectively (Biassoni and Malnati, 2018).

2.4.4.4 Other activating receptors

Along with NCRs, NKG2C, and NKG2D, several other activating receptors exist which are equally important for the cytotoxic capability of NK cells, notably, CD16 and DNAX accessory molecule-1 (DNAM-1).

- **CD16**

As previously introduced, is the ADCC mediating receptor capable of binding the Fc portion of some IgG subtypes (e.g IgG1 in humans, IgG2a/b in mice). It is a type I transmembrane receptor and contains two extracellular immunoglobulin-like (Ig-like) domains (**Figure 8**).

Upon engagement of CD16, signaling is transduced by FcRg and the CD3z chain containing activation motifs (ITAMs) (Vivier *et al.*, 1992). A unique ability of this receptor is that it is sufficient alone to induce the activation of antibody-dependent redirected lysis in resting NK cells, while all other receptors tested (NKp46, NKG2D, 2B4, DNAM-1, or CD2) require the engagement of another receptor for NK cell activation (Bryceson & Long, 2006).

- **DNAM-1**

Also known as CD226, DNAM-1 functions both as a coactivating receptor for NK cells and as an adhesion molecule binding to the poliovirus receptor (PVR, CD155) and Nectin-2 (CD112), a tumor ligand. DNAM-1 expression correlates with education (discussed later), as well as adaptive-like NK cells in mice (De Andrade & Martinet, 2014). In humans, DNAM-1 expression is coordinated with lymphocyte function-associated antigen 1 (LFA-1) which undergoes conformational changes, as they co-localize at the immune synapse (Shibuya *et al.*, 1999).

2.4.4.5 Innate immune receptors

This class of receptors is responsible for sensing the presence of microorganisms and other potentially harmful molecules for the organism through the recognition of danger-associated molecular patterns or "DAMPs". This is the first crucial step in initiating a cascade of immune events up to the induction of adaptive immunity.

- **TLRs**

Toll-like receptors (TLRs) are among the most important and most studied group of innate immunity receptors or pattern recognition receptors (PRRs), responsible for a wide range of immune effects (O'Neill & Bowie, 2013). Structurally, they are composed of an extracellular domain with leucine-rich repeats (LRRs) that are responsible for binding and discriminating different DAMPs present in the cellular microenvironment. The intracellular Toll/interleukin (IL)-1 receptor (TIR) domain signals via MyD88/TRIF and is highly conserved among each subfamily of TLRs (O'Neill, Golenbock and Bowie, 2013). There are 13 TLRs described in mammals with 10 (TLR1-10) expressed in human tissues (surface or intracellular). All except TLR10 are expressed by NK cells at the protein level (Maldonado-Bernal and Sánchez-Herrera, 2020). TLRs are essential in modulating activation and induction of NK cytotoxic response and cytokine secretion to amplify the immune response (Maldonado-Bernal & Sánchez-Herrera, 2020; Noh & Jung, 2020). Because of this, the use of agonists of some TLRs is of interest for NK cell-based immunotherapy (Noh & Jung, 2020).

- **RAGE**

A less studied innate receptor in NK cells, but highly relevant for this work, is the receptor of advanced glycation end-products (RAGE).

RAGE belongs to the immunoglobulin superfamily and is composed of one V type domain, two C type domains (C1 and C2), a transmembrane domain, and a short cytoplasmic tail that associates with Diaphonus-1 (Dia-1) for signaling (**Figure 10**). While it's physiological role is less understood, vast literature implicates it in the pathogenesis of various inflammatory diseases including diabetic complications, neurodegenerative disorders, and cancer (Lü & Zhang, 2013). A salient property of this receptor is its ability to bind multiple families of structurally conserved ligands including S100/calgranulins, amyloid fibrils, amphoterins (e.g HMGB1), and advanced glycation end-products (AGEs) (Fritz, 2011). The latter after which this receptor is named. This multitude of ligands has diverse cellular implications mediated by multiple signaling pathways downstream of RAGE, including MAP kinases (p44/42, p38, and JNK), Jak/STAT pathways, rho, and Rac GTPases. Besides the membrane-bound receptor, a soluble form of RAGE exists (sRAGE) composed by V, C1, and C2. This soluble form is essential for regulating RAGE signaling by scavenging its ligands. Indeed, oppositely to membrane RAGE overexpression, sRAGE correlates with better outcomes in some inflammatory complications, especially in cancer, where its circulating levels are often decreased in advanced stages (Yan & Schmidt, 2010).

There are two reports on RAGE function in NK cells to date, both highlighting an activating function of this receptor (Narumi *et al.*, 2015; Parodi *et al.*, 2015). Precisely, RAGE was shown to induce NK cell cytotoxicity and chemotaxis by binding S100A8/A9 and HMGB1, respectively. In line with these studies, our work might add another important role of this receptor in regulating the cytotoxicity of NK cells by controlling the biology of the immune synapse.

The immune synapse between NK cells and their targets will be described next.

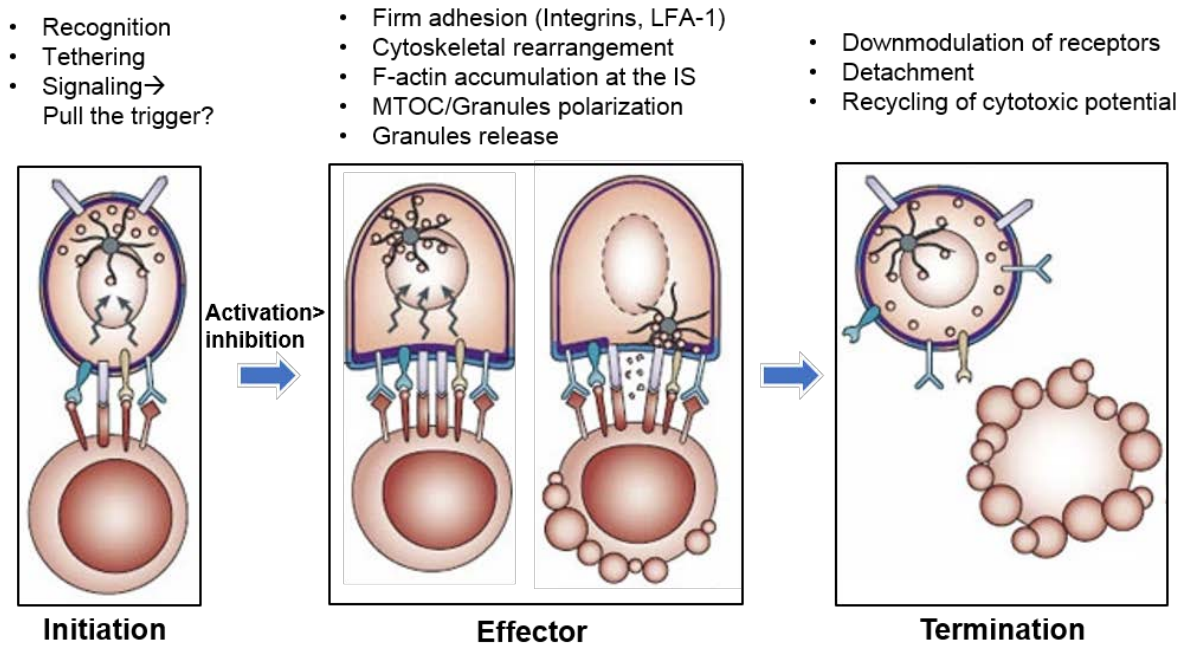
2.4.5 The Immune Synapse

A major way cells in our body communicate is by engaging in cell-cell contact events. This is especially relevant for immune cells where maturation, antigen recognition, and regulation happen through the formation of immune synapses (Dustin, 2014). An immune synapse can occur between an antigen-presenting cell (APC) and a T cell for example or between a

cytotoxic cell and a target cell. For clarity, the events described next are largely specific for the NK lytic synapse despite a high degree of overlap with that of T cells or other immune synapses.

The NK lytic synapse occurs in a complex and stepwise manner that would ensure the efficient elimination of target cells while preserving the effector cell and minimize bystandard killing of healthy cells.

It can be divided into three major stages: initiation, effector, and termination (**Figure 9**) (Orange, 2008a).



Adapted from Orange et al., 2008a

Figure 9: Formation of the NK lytic synapse

Following recognition of a potential target cell, engagement of activation/inhibition receptors to their respective ligands will dictate the nature of the IS (inhibitory synapse vs. lytic synapse). If activation signals overcome inhibitory ones NK cells are licensed for cytotoxicity. In the effector stage, key steps include actin reorganization, receptor clustering, polarization of the microtubule-organizing centre (MTOC) and lytic granules, and lytic-granule fusion with the plasma membrane. The main steps of the termination stage include a period of inactivity followed by detachment and potential recycling of cytotoxicity (for serial killing). The time required to progress through the various stages varies depending on the activation status of NK cells, recpetors engaged (ADCC, natural cytotoxicity) and the nature of the target cell.

2.4.5.1 Initiation

An NK cell can passively or actively encounter its potential target cell. In the latter, chemotactic signals can guide NK cell migration to where their effector functions are needed (tumor or infection site). Once there, an initial weak NK cell-target cell association occurs in

which CD2 on NK cells can bind sialyl Lewis X (CD15) on target cells (Warren & Parish, 1996). Low-affinity binding members of the Selectin family can be involved in this first contact as well (Orange *et al.*, 2003). Consequently, CD2 accumulation at the immune synapse is considered as a marker of early synapse initiation. As the Adhesion of NK cells becomes stronger, higher affinity receptors become involved. Namely, the integrin family of adhesion molecules is important in firm adhesion. Indeed, LFA-1 integrin, composed by CD11a and CD18 subunits, clusters rapidly at the synapse and is involved, not only in adhesion but also in signaling for synapse maturation (Zheng & Tian, 2009). Nonetheless, LFA-1 accumulation at the synapse does not necessarily indicate cytolytic commitment. At this point, an NK cell is at a crossroads between detaching from its target (inhibitory synapse through KIR engagement) and proceeding with cytolytic commitment to the effector phase. This is dictated by the balance and intensity of the activation and inhibition signals as previously discussed (**Figure 7**). Of note, at the CTL immunological synapse, a TCR can associate with up to six CD3 molecules containing a total of ten ITAMs, whereas NK-cell activating receptors couple with fewer ITAM-containing molecules (Orange, 2008a). This might explain why most NK cell activating receptors require co-stimulation with the exception of CD16 where its ligation alone is sufficient to induce activation and cytokine secretion by resting NK cells (Bryceson *et al.*, 2006).

2.4.5.2 Effector stage

After synapse initiation has occurred, and in the absence of overriding inhibitory signals, NK cells proceed with cytoskeletal rearrangement of their lytic machinery. This will allow the assembly and recruitment of lytic granules dispersed in the cell to the synapse and the fusion of the lytic-granule membrane with the plasma membrane for the release of the lytic-granule contents into the target cells.

A hallmark of early NK cell lytic commitment is the accumulation of F-actin (from monomeric G-actin) to form a mesh at the immune synapse that is responsible for the characteristic change in the shape of engaged NK cell. F-actin reorganization depends on VAV1 activity downstream of activation receptors and on Wiskott–Aldrich syndrome protein (WASP) (Orange et al., 2002; Riteau & Long, 2003). This latter is part of the protein machinery that promotes F-actin branching. Other events occur concomitantly with F-actin rearrangement,

such as receptor clustering, further activation signaling, lipid-raft aggregation, and lytic-granule redistribution (Orange, 2008a). The repertoire of receptors that cluster at the synapse might differ according to the type of target cells and the ligands they express. Also, the organization structure of these receptors has not been shown to parallel that of T cells which follows a "bull's-eye model" with the formation of central, peripheral, and distal supramolecular activation clusters (c-SMAC, p-SMAC, and d-SMAC). An exception to this is the clustering of KIRs in an inhibitory synapse (the supramolecular inhibitory cluster) (Treanor *et al.*, 2006).

Concomitantly with the accumulation of LFA-1, F-actin, and activation receptors at the synapse, the next key step is the polarization of the microtubule-organizing center (MTOC) with the granules to the synapse. This begins with the movement of the lytic granules along microtubules to the MTOC (minus-end movement). Next, The MTOC together with the granules will travel to the immune synapse (towards positive-end anchored at F-actin). The exact motor responsible for these movements is not known but evidence suggests that dynein is involved (Combs *et al.*, 2006). Essential events controlling MTOC polarization in NK cells include the phosphorylation of ERK (extracellular-signal-regulated kinase), JNK (c-Jun N-terminal kinases), and the activation of VAV1, PYK2 (protein tyrosine kinase 2), Diaphanous-1 (DIA1) (Butler & Cooper, 2009; X. Chen& Strominger, 2007; C. Li et al., 2008; Orange, 2008). Additionally, the integrity and reorganization of the F-actin network is required for MTOC and lytic-granule polarization to the synapse, suggesting linearity in the occurrence of steps during synapse formation (First F-actin then MTOC polarization).

Once the granules (~500nm each) reach the proximity of the plasma membrane, they pass through a pre-formed channel (1-4µm) within a small central region of the F-actin network. Importantly, microtubules destabilizing agents do not interfere with the disassembly of F-actin to form this channel suggesting that its formation precedes MTOC polarization during synapse maturation (Orange, 2008a). Next, the transit of the MTOC together with the granules, through these clearances in the F-actin network requires additional plus-end motor functions for further approximation to the membrane. In particular, Myosin II is required for this function as its downregulation does not interfere with MTOC polarization but prevents degranulation (Andzelm & Strominger, 2007).

The last steps of the effector phase are the docking of lytic granules to the synapse, and their priming for membrane fusion and subsequent release into the cleft between an NK cell and its target. The RAB family of small GTPases and the SNARE (soluble N-ethylmaleimide-sensitive-factor accessory-protein receptor) family are responsible for granules priming for fusion and the fusion to the plasma membrane, respectively (Orange, 2008a). Once degranulation occurs, lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) becomes detectable on the surface of NK cells and is widely used as a marker for degranulation (Alter & Altfeld, 2004).

2.4.5.3 Termination

This stage comes after the granules have been released. It includes downmodulation of the accumulated activating receptors such as NKG2D, NKp46, and CD16 shedding (Srpan *et al.*, 2018). This is followed by a period of inactivity (~10-20min) that aims to preserve the cleft and concentrate the amount of lytic granules at the synapse (increased efficacy while avoiding bystander killing) (Orange, 2008a). Finally, an NK cell will detach and can immediately engage new targets (see serial killing above). Otherwise, it can start to recycle its killing ability by synthesizing new granules and de-novo upregulating its activation receptors in the presence of favorable cytokines such as IL-2, IL-15, and IL-12 (Bhat and Watzl, 2007).

2.5 CHI3L1/YKL-40 an ancient molecule with new roles.

In this section, the biology, regulation, and role in health and disease of CHI3L1 will be reviewed. Its immune functions will be highlighted as well.

2.5.1 From Archea to humans

Second to cellulose, chitin is the most abundant polysaccharide in nature where it is a major component of fungal cell walls, the exoskeleton of insects, and the shells of crustaceans. The glycoside hydrolase 18 (GH18) family of chitinases is an ancient gene family expressed in archaea, prokaryotes, and eukaryotes (Funkhouser and Aronson, 2007; Adrangi and Faramarzi, 2013). These enzymes hydrolyze chitin and are essential for the remodeling of chitin-containing structures during the growth and development of insects for instance. Besides, certain bacteria use these enzymes to metabolize chitin as a nutrient source while they are considered part of the innate immune defenses against chitinous pathogens in some plants (Langner and Göhre, 2016). Intriguingly, humans and other mammals are devoided of these primitive functions (we neither synthesize chitin nor use it as a nutrient source), yet our genome still encodes several protein members of the GH18 family, some of which circulate our blood in abundant levels (Kzhyshkowska & Goerdt, 2007). This highlights an important evolutionary function of these proteins beyond their enzymatic activity. Indeed, only two of these proteins, chitotriosidase, and AMCase, remain true chitinases in humans (Adrangi and Faramarzi, 2013). The other members have lost their enzymatic function while retaining their ability to bind their substrate (chitin). They are therefore named chitinase-like proteins (CLPs) or Chi-lectins. Three CLPs have been identified in humans: chitinase 3-like 1 (CHI3L1; also known as YKL-40), chitinase 3-like 2 (CHI3L2; also known as YKL-39), and oviductin (OVGP1 or MUC9) (Adrangi and Faramarzi, 2013). Among them, Chitinase 3 like-1 is the most studied due to its prominent role in inflammation and its involvement in multiple human pathologies.

2.5.2 Overview

CHI3L1 is a secreted heparin and chitin-binding glycoprotein, first discovered in 1988 as a 39KD whey protein from bovine mammary secretions during the nonlactating period (Rejman and Hurley, 1988). Thus, it was initially named breast regression protein-39 (BRP-39). Subsequently, The human homolog was identified as one of the most abundantly secreted proteins from MG-63 osteosarcoma cells (Johansen & Price, 1992), synovial cells, and articular

chondrocytes (Hakala & Recklies, 1993). It was named YKL-40 for its N-terminal amino acid sequence (tyrosine, lysine, and leucine) and its apparent molecular weight of around 40KD. One year after, the murine CHI3L1 (also called BRP-39) was identified as a novel 39KD protein secreted from breast cancer cells derived from the HER2/neu initiated tumors (Morrison and Leder, 1994). More studies came out reporting the expression of this protein by vascular smooth muscle cells and as a marker for late-stage monocyte to macrophage differentiation (Krause et al., 1996; Shackelton & Millis, 1995). The *chi3l1* gene encoding YKL-40 was then cloned in 1997. This gene is located on chromosome 1 and consists of 10 exons encoding the full mature protein (Rehli & Andreesen, 1997). Structure wise, a hallmark of YKL-40 and other chitinase like-proteins is the presence of a conserved chitin-binding domain (CBD). Despite this, they lack chitinase activity due to mutations in their catalytic site (Fusetti & Dijkstra, 2003). Of note, a unique structural feature of the human YKL-40 is the presence of a glycosylation on Asn (residue 60) that is absent in chitinases and mutated to proline in other mammalian chitinase-like proteins (Fusetti *et al.*, 2003). This highlights an important role of this protein in mammals, which might be highly conserved but with potential inter-species variations. Intriguingly, the exact physiological function of YKL-40 is not fully understood but is known to be associated with tissue remodeling and repair according to its expression pattern. Conversely, there is a consortium on its role as an inflammation-related protein, where its aberrant expression is associated with the pathogenesis of an array of human diseases including asthma, neurodegenerative diseases, joint inflammation, metabolic diseases, and many cancer types where it correlates with bad prognosis and shorter patient survival (Eurich, 2009; Kazakova & Sarafian, 2013; Kzhyshkowska et al., 2007; Libreros, 2013; Mizoguchi, 2006). The role of CHI3L1/YKL-40 in health and disease, especially in cancer, will be discussed next.

2.5.3 Physiological role

CHI3L1 circulates our blood at ~30ng/ml, with no significant differences according to sample type (serum/plasma), sex, circadian cycle, and mild exercise (Johansen et al., 2008, personal observation). Although not affected over short periods (2-3 years), CHI3L1 levels correlate with age and are significantly increased to the upper normal levels in the elderly population (>70 years old) (Johansen et al., 2008, personal observation). This is not surprising, as aging is associated with chronic low-grade inflammation and increased basal levels of inflammatory

cytokines (e.g TNF-α, IL-6) and acute phase proteins, some of which are involved in YKL-40 induction (regulation discussed later). Although different non-malignant cell types of different tissue origin express YKL-40, its expression in cells and tissues with high proliferation, high metabolic activity, and turnover is most prominent (M. H. Kazakova & Sarafian, 2009; Ringsholt & Christensen, 2007). In this context, YKL-40 is expressed by and stimulates the proliferation of human connective tissue cells (fibroblasts, chondrocytes, synovial cells) in a dose-dependent manner (Recklies & Ling, 2002). This concurs with the sharp increase of its expression during mammary gland involution (remodeling), while it is expressed at low levels before and during pregnancy and lactation (Arcaro *et al.*, 2012). In line, it is expressed at higher levels in the uterus during proliferation, in secretory cells from various endocrine and exocrine glands, and all germ layers during the early development of the human musculoskeletal system where it seems to be associated with cell proliferation, differentiation and tissue morphogenesis (Johansen *et al.*, 2007; Ringsholt *et al.*, 2007). In addition to proliferation, YKL-40 regulates apoptosis and cell survival and consequently contributes to wound healing and fibroproliferative responses in barrier tissues like the skin, lungs, and intestines (Lee *et al.*, 2011; Zhou *et al.*, 2014). Moreover, YKL-40 is an angiogenic molecule expressed by vascular smooth muscle cells and contributes to the development of highly vascularized organs such as the placenta and the developing mouse heart (Han and Kim, 2017). In this latter, CHI3L1 expression coincides with morphological changes involving cell migration, altered cell adhesion, and remodeling which is consistent with its ability to stimulate the migration and adhesion of different cell types including vascular endothelial cells (Nishikawa and Millis, 2003). Despite all these diverse functions, CHI3L1/BRP-39 knockout mice develop normally (at least by broad examination of their physiology). Nonetheless, these mice have been extensively examined in their responses during pathological conditions and show protection from diverse inflammatory diseases, especially those characterized by TH2 inflammation (Lee and Elias, 2010; Stephania Libreros and Iragavarapu-Charyulu, 2015).

Overall, many nucleated cell types express CHI3L1 in their cytoplasm, some secrete it and contribute to its blood levels with the intensity of its expression correlating with cellular activity. Importantly, it is the same physiological properties (growth, survival, remodeling of ECM, neovascularization, and migration) that make this protein a foe during pathological

conditions characterized by inflammation and exaggerated expression of CHI3L1. This is discussed next.

2.5.4 Role in inflammation and cancer

As previously introduced, CHI3L1 circulating levels are significantly increased in various pathologies characterized by inflammation. These include infections, lung diseases, cardiovascular diseases, metabolic diseases, inflammatory bowel disease, neurodegenerative diseases, and multiple malignancies (Prakash *et al.*, 2013). In many of these, CHI3L1 levels correlate with disease activity, therapeutic response, and prognosis and have been proposed as a marker and or/therapeutic target. For example, patients with streptococcus pneumonia bacteremia had elevated serum levels of YKL-40 compared with age-matched healthy subjects. Antibiotics treatment of these patients decreased serum YKL-40 levels to normal within a few days, before the serum C-reactive protein (CRP) reaches its normal levels (M. et al, 2016). In particular, CHI3L1 is a master regulator of Th2 type of inflammation (Ahangari et al., 2015; Kwak et al., 2019; Lee & Elias, 2010) where one of its earliest involvement in diseases was shown to be in asthma and allergen-induced airway inflammation (Sohn et al., 2009). For this purpose, the group of Jack Elias in brown university generated the first CHI3L1 knockout mice and showed that these mice are defective in mounting Th2 immunity in response to allergens (Sohn et al., 2009). This is nicely illustrated in previous studies from the same group showing that elevated levels of this protein in sera of patients with asthma correlate with its levels in lung tissue and disease severity (Ober *et al.*, 2008). Apart from airway inflammation, CHI3L1 KO mice were protected from obesity, diabetic complication, atherosclerosis, and exaggerated inflammation in response to some pathogens. In humans, multiple studies highlighted that polymorphisms in chi3l1 gene correlated with the levels of circulating YKL-40, and consequently, the presence of asthma, lung function, and susceptibility to infections, rheumatoid arthritis, coronary artery disease, schizophrenia, solid tumors, and death in the elderly among others (Abd El-Fattah & Mohamed Kamal, 2018).

2.5.4.1 In cancer

As Inflammation is a hallmark of cancer (Colotta & Mantovani, 2009), it might not be surprising to see increased levels of CHI3L1 in many cancer types. Nonetheless, in the last two decades, an increasingly large number of studies, not only correlated but functionally linked

CHI3L1 overexpression to worst prognosis in cancer (**Figure 10**) (Eurich et al., 2009; Kzhyshkowska & Mitrofanova, 2016; S. Libreros & Iragavarapu-Charyulu, 2015; Stephania Libreros et al., 2013; Prakash et al., 2013).

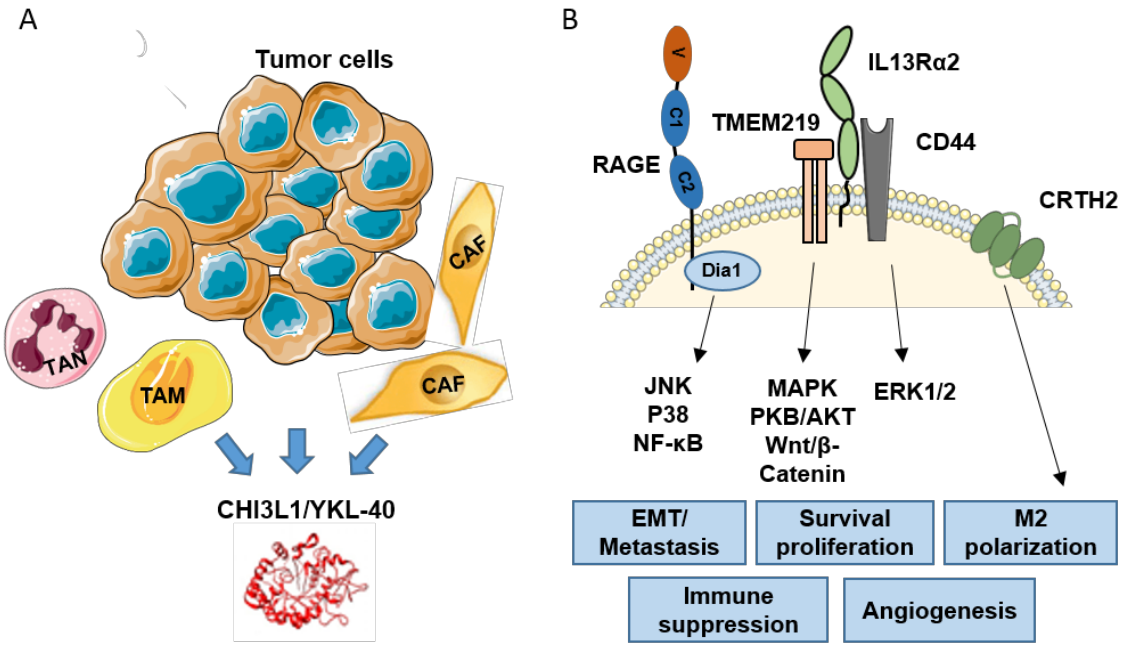


Figure 10: Overview of CHI3L1 in cancer

(**A**) CHI3L1 is secreted at exagerated levels by tumor cells and/or tumor associated cells such as TANs, TAMs, and CAFs (especially relevant in BC). Activated monocytes and Neutrophils in the blood can aslo contribute to circulating CHI3L1 levels. (**B**) CHI3L1 acts on target cells via several receptors inculing RAGE, CRTH2, and IL13Rα2. This latter has a short cytoplasmic tail and thus associates with other receptors/signaling modules to mediate cellular effects such as the transmembrane receptors TMEM219 and CD44, , essential for for IL13Rα2-CHI3L1 mediated effects.
This scheme focuses on the extrinsic role of secreted CHI3L1 protein. The intracellular protein as well seems to play an important role in cancer and immune-regulation that has begun to be elucidated recently.

Early reports showed that CHI3L1 induces angiogenesis and radioresistance (Pelloski et al., 2005; Francescone et al., 2011). In line, a YKL-40 neutralizing antibody inhibited angiogenesis and decreased the growth of Glioblastoma xenografts synergistically with radiotherapy in vivo (Faibish & Shao, 2011). CHI3L1 is particularly relevant in Glioblastoma as it is highly expressed in an aggressive subtype of this disease characterized by the worst prognosis (Qin *et al.*, 2017). Of note, it acts independently of other angiogenic factors as an anti-VEGF neutralizing antibody did not interfere with CHI3l1-induced migration (Francescone *et al.*, 2011). Consistent with its physiological growth properties, exaggerated levels of CHI3L1 in cancer promotes excessive proliferation and resistance to apoptosis in response to chemotherapy in mice models and patients with melanoma, prostate, ovarian, breast, colorectal and small cell

lung cancer among others (Boisen et al., 2016; Hamilton & Burghuber, 2015; Huang et al., 2020; Darr et al., 2018; Xu & Hao, 2014). In parallel, the ability of CHI3L1 to stimulate migration and regulate adhesion underlines its strong association with metastatic disease where its levels were found to correlate with disease stage and presence of metastasis (S. Libreros and Iragavarapu-Charyulu, 2015). In line, overexpressing CHI3L1 resulted in increased tumor growth and enhanced metastasis formation in different syngeneic (e.g B16, 4T1, and DA-3) and xenograft (e.g MDA-MB-231, HCT116, U87-MG, and OVCAR3) tumor models. The other way around, downmodulating CHI3L1 expression using shRNA, siRNA or neutralizing the secreted protein busing antibodies or chitin results in decreased tumor growth and a strong inhibition of metastasis (Jeet & Nelson, 2014; Stephania Libreros et al., 2012; Lin et al., 2019; Qin et al., 2017; Michael Faibish & Shao, 2011; Kim et al., 2018, Qiu et al., 2018). Besides, CHI3L1 was shown to mediate other pro-tumorigenic processes such as inducing stemness properties and epithelial to mesenchymal (EMT) transition (Hao et al., 2017; Jefri & Chen, 2015; Lin et al., 2019).

Interestingly, a recent study demonstrated that CHI3L1 can directly bind to P53 and inhibit its stability to promote lung tumors (Park *et al.*, 2020).

A nice illustration of the function of CHI3l1 on the borderline of inflammation and cancer comes from a study showing that DSS treated CHI3L1 KO mice develop severe colitis, but are protected from colitis-associated carcinogenesis (Low *et al.*, 2015).

Overall, the serum level of CHI3L1/YKL-40 was found to be an independent marker of serum cancer antigen (CA-125) in ovarian cancer, serum carcinoembryonic antigen (CEA) in colorectal cancer, serum human epidermal growth factor receptor 2 (HER-2) in metastatic BC, serum prostate-specific antigen (PSA) in metastatic prostate cancer and serum lactate dehydrogenase (LDH) in small cell lung cancer (Abd El-Fattah *et al.*, 2018). Despite this, CHI3L1 would not be suitable as a biomarker in cancer as its expression could be confounded with many other inflammatory conditions. Nonetheless, CHI3L1 expression has a strong prognostic value as it could predict the evolution of the disease and the response to therapy within the same individual.

2.5.5 Immune interaction and regulation

Apart from directly acting on its target cells and tissues, CHI3L1 mediates many of its effects by modulating the immune response. This is becoming increasingly relevant in cancer as CHI3L1 has been recently shown to promote an immunosuppressive microenvironment concomitantly with its direct effects on tumor cells (Cohen *et al.*, 2017; Kim *et al.*, 2018). Before that, the ability of this protein to modulate immune responses came to light as a master regulator of Th2 responses. In particular, CHI3L1 was shown to induce M2 macrophage polarization, and the accumulation of dendritic cells, eosinophils, and Th2 cells in asthma and allergic responses (Lee *et al.*, 2009). These effects as well as IL-13 tissue responses and fibrosis were largely abrogated in CHI3l1 KO mice (Lee *et al.*, 2009). CHI3L1 is not expressed by resting monocytes and is induced only in differentiated macrophages. A process that requires the binding of the SP1 transcription factor to the chi3l1 gene promoter (Rehli *et al.*, 2003). It is hence regarded as a marker of macrophage differentiation. In fact, the major producers of CHI3L1 in cancer are tumor-associated macrophages (TAMs), neutrophils, and CAFs (especially in BC) (S. Libreros and Iragavarapu-Charyulu, 2015). Recently, other immune cells, including T cells and NK cells were shown to express CHI3l1, although at relatively low levels that are increased after activation (Kim et al., 2018, personal observation). Consistent with the above, CHI3L1 knockout T cells exhibit strong TH1 cytotoxic responses and efficient control of tumor growth and metastasis (Kim *et al.*, 2018). Despite favoring TH2 immunity, CHI3L1 is induced by various TH2 and in-TH2 proinflammatory cytokines including IL-6, IL-13, IFN-γ, TNF-α, and IL-1β (Johansen, 2006). Some hormones such as vasopressin and parathyroid hormone-related protein can induce CHI3L1 expression as well (Johansen, 2006). In turn, CHI3L1 stimulates the secretion of pro-inflammatory and chemotactic molecules CXCL2/IL-8, CCL2/MCP-1, and MMP-9 by macrophages and TGF-β by tumor cells, all together promoting immunosuppression and tumor growth (Lazennec and Richmond, 2010; Qiu *et al.*, 2018).

As previously mentioned, CHI3L1 is also upregulated during diverse infections of mucosal tissues consistent with its expression in Th17 cells (Capone *et al.*, 2016). For example, it is highly expressed in inflammatory conditions of the gut such as colitis where it might function in both promoting and dowmodulating inflammation by regulating host-microbial interaction. In particular, Its expression in the inflamed colonic mucosa promotes Escherichia coli, and

Salmonella typhimurium infection by enhancing the adhesion of these bacteria to intestinal epithelial cells (IECs) (Mizoguchi, 2006; Tran *et al.*, 2014). Precisely through interacting with chitin-binding protein (CBP) expressed by these bacteria (Kawada *et al.*, 2008). Additionally, LPS released from gram-negative bacteria is known to strongly induce CHL31 by activating the NF-kB pathway (Lei and Guo-Ping, 2009). Conversely, in the lungs, CHI3L1 was shown to protect against S. pneumonia infection, by enhancing the ability of macrophages to kill this bacteria and increasing host tolerance to immune-induced lung damage (Cruz *et al.*, 2012). Similarly, it was also shown to be important for controlling Candida albicans corneal infection (Gao and Yu, 2015). Besides bacteria and fungi, CHI3L1 was found to be a critical mediator of airway Inflammation in patients infected with respiratory Syncytial Virus (RSV) and neutralizing it was able to protect infected mice from pulmonary complications (Kim *et al.*, 2019). Overall, these reports highlight the importance of host-microbiota interaction in regulating CHI3L1 levels, reminiscent of its evolutionary function. In turn, they also highlight its functional involvement in the resolution or exacerbation of infections.

Another involvement of CHI3L1 in the immune context is in autoimmune diseases where high levels of this protein were shown to correlate with disease activity in rheumatoid arthritis patients, and are decreased after treatment with anti-rheumatic drugs (Jafari-Nakhjavani et al., 2019; Väänänen et al., 2017). In this case, high levels of this protein are the result of articular inflammation and joint destruction but have not been functionally linked to the pathogenesis of RA. An exception is found in a rare subset of patients with autoantibodies to CHI3L1 or its closely related family member CHI3L2 (Sekine *et al.*, 2001).

In summary, CHI3L1 has a prominent immune identity and is induced in response to different inflammatory cues. In turn, it functions to limit tissue damage by controlling the immune response and favoring Th2 immunity while signaling for tissue repair and ECM remodeling. In case of sustained injury or inflammation, this turns into a pathogenic scenario, where exaggerated levels of CHI3L1 exacerbate a variety of diseases such as lung complications, fibrosis, and cancer.

2.5.6 Receptors and signaling

As highlighted in the previous sections, CHI3L1 exhibits many cellular and tissue functions. This suggests the existence of multiple receptors or binding partners of this protein,

responsible for modulating multiple signaling pathways. Indeed the first receptor was identified in 2013, where YKL-40 was shown to bind interleukin-13 receptor α2- (IL-13Rα2) with a high affinity (Kd=12pM) (C. H. He *et al.*, 2013). This receptor was initially thought not to signal and to act as a decoy receptor for IL-13 because of its short cytoplasmic tail. However, it proved to play a central role in regulating many of CHI3L1/YKL-40 tissue responses including oxidant injury, apoptosis, pyroptosis, inflammasome activation, antibacterial responses, melanoma metastasis, and TGF-β1 production through the activation of MAPK, AKT, and Wnt/β-catenin signaling pathways (C. H. He *et al.*, 2013). More recently, other receptors of CHI3L1 have been identified although shown to be involved in specific settings. Of note, most of these have been identified by the same group in brown university led by Jack Elias who is one of the main contributors to the field of chitinase-like proteins. Among these receptors, the Prostaglandin DP2 receptor (CRTH2) was shown to be important for CHI3l1 mediated M2 macrophage differentiation and to play a critical role in pulmonary fibrosis (Zhou *et al.*, 2015, 2019). In this context, Galectin-3 (Gal-3) was shown to interact with CHI3L1 and regulate its binding to IL-13Rα2 while favoring its binding to CRTH2 (Zhou *et al.*, 2018). CD44V3 was also shown to interact with CHI3L1 within an IL-13Rα2-CD44-CHI3l1 complex. This interaction promotes metastasis and epithelial-to-mesenchymal transition through β-catenin/Erk/Akt signaling in gastric cancer (Geng *et al.*, 2018).

Another receptor of CHI3L1 with high relevance to this work is RAGE (see RAGE in NK cells). By binding this receptor, it induces the survival and proliferation of intestinal epithelial cells during chronic inflammation and promotes colitis-associated cancer. Specifically, by competing with S100A9 on RAGE binding and activating STAT3, β-catenin and NF-κB signaling pathways.

Overall, CHI3L1 has evolved to bind multiple mammalian receptors including both innate (RAGE) and adaptive immune receptors (IL-13Rα2). In their totality, these receptors could be expressed in almost any tissue and mediate multiple signaling pathways. This might help to clarify how CHI3L1 can mediate many of its effects. Nonetheless more studies to identify other important receptors are needed to see the full picture.

Chapter 3: Material and Methods

Patients

Women with histological diagnosis of locally advanced invasive or metastatic BC were considered eligible for the study if classified as HER-2 positive, i.e. IHC score 3+ or IHC score 2+ and FISH amplified. We excluded enrollment of HER-2 positive patients with ER or PgR receptor-positive disease to avoid interference of hormonal therapy in our study.

Response to therapy was evaluated based on clinical, pathological, and radiologic examination of disease burden before and after treatment. The revised RECIST criteria (version 1.1) was used to evaluate the treatment response which was classified as complete response (CR), stable disease (SD), or progressive disease (PD).

Table 1:Time of sample collection in the discovery and validation cohorts

	Patient ID	Sample Time (Days)	
Cohort 1 (Discovery)	PD1	0	241
	PD2	0	214
	PD3	0	161
	PD4	0	105
	PD5	0	273
	PD6	0	455
	PD7	0	277
	PD8	0	371
	PD9	0	322
	CR1	0	357
	CR2	0	343
	CR3	0	362
	CR4	0	346
	CR5	0	330
	CR6	0	368
	CR7	0	364
	CR8	0	154
	SD1	0	364
	SD3	0	349
	SD4	0	364

	Patient ID	Sample Time (Days)	
Cohort 2 (Validation)	PD1	0	361
	PD2	One time point at PD	
	PD3	0	88
	PD4	0	130
	PD5	0	404
	CR1	0	438
	CR2	0	105
	CR3	0	318
	SD1	0	440

-In the **discovery cohort (2011-2013)**: Twenty patients were enrolled and underwent a first-line chemotherapy in combination with trastuzumab. Blood samples were collected at basal (before Trastuzumab therapy) and subsequently until patients reached PD, SD (~12 months from enrollment), or CR state (**Table 1**). This study was approved by the Ethics Committee of the European Institute of oncology hospital, Milan, Italy.

-In the **validation cohort (2018-2020)**: Nine patients were enrolled in this study and received trastuzumab plus docetaxel and pertuzumab as first-line treatment based on the conclusion and recommendations of the CLEOPATRA study. Blood was collected during response (at the

time of proven control of disease/ no evidence of disease) and considered as reference (basal time point), and at the time of change of clinical outcome (to PD or CR) or if the patient stayed in remission without achieving CR by the end of the study (18 months from enrollment) (**Table 1**). This study was approved by the Ethics Committee of Humanitas clinical institute, Milan, Italy.

Written informed consent was obtained from all patients.

Serum proteomic analysis by Luminex technology

We used the Luminex 100 system (Luminex Corp., Lagitre s.r.l.) to evaluate the sera from 11 HER2 breast cancer patients from our discovery cohort (6 PD and 5 CR/SD) and 5 healthy subjects. Levels of 12 proteins (GDF15, VitD-BP, RBP4, BDNF, CHI3L1, ICAM-1, IGFBP-3, Angiogenin, sCD14, CRP, Cystatin, and DPPIV) that were among the most significant hits from a larger screen using the proteome Profiler Human XL Cytokine Array Kit (ARY022, R&DSystems) were assessed. We used the R&DSystems Kit (LXSAHM, R&DSystems) according to manufacturer's protocol. Briefly, 25 µl of diluent and 25 µl of serum were added to each well followed by addition of mixed micro-beads (25 µl). The plate was incubated and agitated for 1 h, washed and re-incubated with 25 µl of detection antibody for 30 min. The plate was washed again and incubated with 25 µl of Streptavidin-Phycoerythrin for 30 min. The plate was then washed twice and the beads were re-suspended in the plate with 100 µl sheath fluid and analyzed using the Luminex 100 system. The readout for the concentration of each cytokine was detected as mean fluorescence intensity (MFI) by the instrument. These values were subsequently converted to pg/ml of cytokine based upon the MFI values from a set of standards that were run simultaneously in the assay.

Cell lines

The different cell lines used in this work are shown in **Table 2**.

Table 2: Cell lines, culture method, and use.

Cell line	origin	Type	Culture medium	Use
SKBR3	human	HER2+ BC	RPMI 1640 *1	ADCC assays
HCC156			RPMI 1640 *1	Tumor xenograft
JIMT-1			DEMEM *1	Tumor xenograft/ ex-vivo ADCC
MEC-1		B-cell leukemia	DEMEM *1	CTL killing assays
K562		Erythroleukemia	RPMI 1640 *1	NK cell killing assays
NK-92		NK cell lymphoma	α-mem w/o nucleosides *2	In vitro characterisation CHI3L1-RAGE axis
RMA-S	Murine	Leukemia	RPMI 1640 *1	Tumor model/ex-vivo NK cytotoxicity
B16F10		Melanoma	RPMI 1640 *1	Metastasis tumor model
4T1-luc		TNBC	RPMI 1640 *1	Tumor + metastasis model

*1: 10% heat-inactivated fetal bovine serum ,20 mM Glutamine.
*2: Glutamine, 1 mM sodium pyruvate, non-essential amino acids, 12.5% horse serum + 12.5% FBS + 100UI/ml IL-2

4T1-luc mammary tumor cell line expressing firefly luciferase was kindly provided by Dr. Matteo Massara (Humanitas University, Milan.). RMA-S lymphoma cell line was kindly provided by Dr. Sebastian kobold (LMU, Munich). All other cells were purchased from ATCC. JIMT-1 cells were transfected with PcDNA3.4 vector encoding murine CH3L1 or with mock vector (GeneArt, Life Technologies) and were selected with 400ug/ml G418 for stable expression. Expi-CHO cell line was purchased from Thermo Fisher Scientific Ltd and handled according to the manufacturer's instructions. These cells were used for in house production of recombinant proteins CHI3L1 and Fc-chimerized CHI3L1 receptors: sRAGE-Fc, and sIL13Rα2-Fc.

Primary cells

Human PBMCs were isolated from buffy coats of healthy donors by ficoll density gradient separation using ficoll-paque (GE Healthcare). Primary NK cells or CD8 T cells were purified by magnetic separation using the NK cell and CD8 T cell purification kits (Miltenyi Biotec). Cells used in different assays had >93% purity. NK cells and CD8 T cells were then cultured with RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum, 20 mM Glutamine, 1 mM sodium pyruvate, 1:100 non-essential amino acids (Life Technologies). Before cytotoxicity assays, NK cells and CD8 T cells were cultured overnight with 100UI/ml rhIL-2 (Peprotech) and then treated or not with different concentrations of rhCHI3L1 (RnD systems or produced in house). For murine NK cells, single-cell suspensions of murine spleens were prepared and lysed with ammonium chloride lysis buffer. NK cells were isolated using murine NK cell isolation kit (MiltenyiBiotec) and cultured in 10 ng/ml IL-15 and 100uI/ml Il-2 (Peprotech). On day 3, NK cells were treated or not with rmCHI3L1 (Sino biological or produced in house) for 1h before incubation with SKBR3 targets in ADCC.

For the therapeutic experiment in NSG mice requiring NK cell adoptive transfer in combination with Trastuzumab, isolated primary human NK cells were activated and expanded for two weeks to reach sufficient numbers using the Miltenyi Biotec expansion and activation kit. Briefly, NK cells were cultured in NK MACS medium (Miltenyi Biotec) supplemented with 500Ui/ml IL-12 and 5ul/10^6 NK cells of anti-CD2/Anti-NKp46 agonistic antibodies.

CAR-T cell generation

HER2 CAR gene segments were fused by overlap-extension PCR (Heckman, Nature Protocols 2007). The human aHER2-CD28-CD3z chimeric receptor construct (HER2CAR) is made up of an anti HER2 (Uniprot entry P04626) single-chain variable fragment, human CD28 (Uniprot Entry P10747 AA 153-220) and human CD3z (Uniprot Entry P20963 AA 52-164). For virus production retroviral pMP71 (kindly provided by C. Baum, Hannover) vectors carrying the sequence of the HER2 CAR were stably introduced in packaging cell lines. Single-cell clones were subsequently generated and indirectly screened for the highest level of virus production by determining the transduction efficiency of primary T cells. This method was used to generate the producer cell lines 293Vec-RD114-CAR-HER2. Human T cells have been differentiated from healthy donor PBMC after a MACS CD3 positive selection (Miltenyi Biotec) and 48 h stimulation with human TCM and 8.25 µl per 10^6 cells of human anti-CD3-anti-CD28 dynabeads (eBioscience). Cells could then be transduced using previously described protocols (Rapp et al. Oncoimmunology 2016) or directly taken into culture with human TCM in concentrations of 10^6 T cells per ml medium. Primary human T cells were cultured in RPMI 1640 containing 2.5 % human serum, 1 % PS, 1 % L-glutamine, 1 % NEAA, 1 % sodium pyruvate (human TCM). 50 µM β-mercaptoethanol, 10 IU/ml IL-2, and 100 µg/ml IL-15 were added to human TCM when culturing the T cells. Transduced or untransduced T cells were cultured with 10ug/ml IL13Rα2-Fc or IgG-Fc for 2-3 days before and replenished during cytotoxicity assays.

CAR-T cell cytotoxicity was measured using the XCELLigence® Real-Time Cell Analysis system according to the manufacturer's protocol (ACEA Biosciences). This method is based on the ability of electrodes in the bottom of wells to conduct electric current. Adhesion of cells to these electrodes impedes electric current, providing highly sensitive measurements of the number of cells present and their adherence to the plate surface. Consequently, xCELLigence is able to monitor CAR-T cell cytotoxicity by measuring changes in conductivity which correlates with the number of lysed target cells.

Animal experimentation

Mice were purchased from Charles River Laboratories and were cared for and used under specific pathogen-free conditions according to the guidelines established in the Principles of Laboratory Animal Care (directive 86 /609 /EEC). **Table 3** summarizes the use of mice and cell

lines in tumor experiments. The detailed experimental procedure can be found in the results section as a scheme before each experiment.

Table 3: Mice and cells used in tumor experiments.

Tumor model	Mice	Sex and age	Amount of cells and Injection route
RMA-S	C57BL/6 J	Female 7-8 weeks	5×10^5 s.c right flank
B16F10			2.5×10^5 i.v
4T1-luc	BALB/c	Female 8-10 weeks	2×10^5 s.c 4th mammary area
JIMT-1	Nude (CD1 and BALB/c)	Female 4-6 weeks	$3\text{-}4\times10^6$ (as indicated) s.c right flank
HCC1569	NSG	Female 6-8 weeks	2×10^6 s.c right flank

s.c: subcutanous
i.v: Intravenous

Tumor cells at 70% confluence were detached, washed twice, and resuspended in cold PBS and kept on ice during the injection procedure. Tumor measurements and endpoints were registered by an observer blinded to the treatment groups except for the 4T1 experiment where group assignment and tumor measurement were based on bioluminescence readout. Tumor size (>1.5cm) and necrosis, more than 15% weight loss or a decrease in general health condition (judged by decreased mobility, general weakness, hunched posture, or ungroomed hair) are defined as humane surrogate endpoints for survival and are later referred to as survival of mice.

Cytotoxicity assay

NK cell and CD8 T cell-mediated cytotoxicity in vitro was determined using the DELFIA® EuTDA Cytotoxicity Reagents (PerkinElmer Life Sciences, Waltham, MA, USA), according to manufacturer's instructions. Briefly, target cells were labeled with BATDA (a fluorescence enhancing ligand) in culture medium for 30 min at 37°C and then washed. Next, labeled target cells were transferred into a V-bottom sterile plate. In ADCC assays, SKBR3 cells were treated with 10ug/ml Trastuzumab (Roche) for 20 min at 37°C. In CD8 T cells cytotoxicity assays, MEC1 targets were loaded with 2ug/ml SEB sAg (SIGMA) for 30 min at 37°C. Effector cells were treated as described in each experiment and then co-cultured with target cells in a v-bottom plate for 1h30min in case of ADCC and 2 hours in case of natural NK cytotoxicity or CD8 T cells cytotoxicity (effector/target ratio of 5:1). After incubation, 20 µl of supernatant from each well was transferred to 180 µl of europium (Eu) solution and the fluorescence was measured by time-resolved fluorometry (Victor3, PerkinElmer). The percent of specific release (=specific

lysis) was calculated using the formula (experimental release – spontaneous release)/(maximum release – spontaneous release) X 100(%).

In neutralization experiments, anti-CHI3L1 (clone MaY) (EMD Millipore) or mIgG1 Isotype were pre-incubated with the rhCHI3L1 for 1h at 37°C. The mixture was then added to NK cells and incubated for 1h before co-culture with target cells. In RAGE blocking experiments, NK cells were treated with anti-RAGE antibody, Isotype control, or rage antagonistic peptide (RAP) overnight before cytotoxicity experiments.

Confocal microscopy

For imaging NK cells alone, cells were treated as described and then cultured on poly-L-lysine coated slides (Thermo fisher scientific) for 2 hours at 37°C in a humidified chamber. For imaging the immune synapse, NK cells, and target cells were co-incubated at E:T ratio of 2:1 for 15 min in a v-bottom plate to allow conjugate formation. Conjugates were then gently resuspended and deposited on poly-L-lysine coated slides and incubated in a humidified chamber for 30 min at 37°C. Cells were then fixed and permeabilized for 10 min at RT in cytofix/cytoperm solution (BD bioscience), washed, and then blocked for 10 min with PBS, 10% FBS, in perm buffer. The indicated primary antibodies were added to the slide in blocking buffer for 30 min at RT or 18 h at 4°C. Slides were mounted with Vectashield H1000 mounting medium with DAPI (Vectorlabs) and imaged on Leica TCS SP8 system (Leica-microsystems) using 60×/1.40 NA oil objective. Image analysis and quantifications were carried out using National Institutes of Health ImageJ software. The accumulation of F-actin and LFA-1 at the synapse were used to ensure correct synapse formation while localization of the MTOC staining at the synapse was used to determine the polarization of NK cells. For each replicate of each experiment, a minimum of 50 NK:target conjugates were counted.

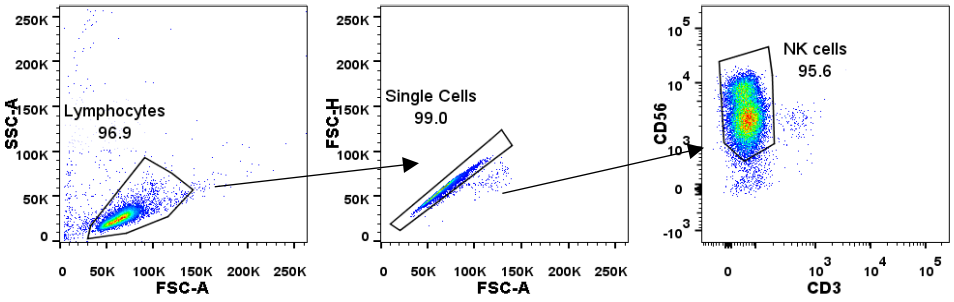
Live cell imaging

$5x10^4$ SKBR3 cells were seeded in a µ-Slide 8 Well Glass Bottom overnight (ibidi). 10^5 NK cells were washed once with phenol red free RPMI-1640 medium, supplemented 20mM HEPES (Sigma Aldrich), 2mM L-glutamine, and non-essential amino acids (Life Technologies) and stained with SiR-tubulin and verapamil (Spirochrome) (500 nM and 10 µM, respectively) for 1 h at 37 °C. LysoTracker Red DND-99 (Life technologies) was then added to NK cells (10 µM) for another 30 min at 37 °C. Following incubation, NK cells were washed three times and treated or not with 100ng/ml CHI3L1 for 1h. In parallel, SKBR3 targets were treated with 10

ug/ml Trastuzumab for 20 min. After mounting the slide with the SKBR3 target cells, the medium containing Trastuzumab was removed before addition of NK cells. Eight fields per condition were recorded for two hours with 180 seconds intervals. Live imaging was performed at 37 °C using environmental chambers on a Leica SD AF Spinning Disc Confocal (Leica-microsystems).

Flow cytometry and phosphoflow experiments

Single-cell suspensions were incubated with Fc receptor-blocking antibody before staining with primary antibodies (except for CD16 staining). Cell doublets were excluded by comparison of side-scatter width to forward-scatter area. Dead cells were excluded by live/dead staining using fixable live/dead stain dyes (Thermofisher). NK cells were gated as singlets, FSC-.SSC appropriate for lymphocytes, CD3neg, CD56pos cells (as shown below). Intracellular staining was performed using a fixation and permeabilization buffer (BD Bioscience).



For phosphoflow experiments, NK cells were fixed with 4% PFA for 12 min at 37°C immediately after treatment ends. Cells were permeabilized with Ice cold PermIII buffer (BDbioscience) for 30 min on ice. Stained cells were analyzed on FACSCanto II cytometer (BD Biosciences). Flow cytometry data were analyzed with FlowJo v10 software (Tree Star).

For FACS based apoptosis assays, the Annexin-PI staining kit (BDbiosciences) was used according to manufacturer's instruction.

ELISA and binding studies

Human and murine CHI3L1 levels in different sample types (patient and mice sera) were measured using ELISA kits from R&Dsystems. Blood was always processed in less than two hours after collection and was kept at 4°C to avoid non-specific release of CHI3L1 from Neutrophils.

Apparent dissociation constants (KD) of CHI3L1 binding to RAGE alone or in competition with RAGE ligands was measured by in house ELISA as previously described (Syedbasha *et al.*,

2016). Briefly, plates were coated with 50ul sRAGE-Fc (10ug/ml) and incubated at 4°C overnight. The next day, 50ul of increasing molar concentration of rhCHI3l1-his tagged were added and incubated for 2h at RT. In competition assays, 4ug/ml of CHI3l1 were pre-incubated with an increased concentration of S100A8/A9, HMGB1, or RAP. Plates were washed with 200ul PBS-Tween 01% three times before the addition of HRP-conjugated His-tag antibody (Genescript) for 1h at RT. After background subtraction and normalization, data points were fitted to a one-site specific binding model.

Absorbance was measured at 450nm using Clariostar™ Plate reader (BMG Labtech).

Western blot

The endogenous human CHI3L1 secreted from HCC1569 cells or the murine CHI3L1 secreted by transfected JIMT-1 cells were detected by western blot analysis of supernatant from 70% confluent culture (20ul) using goat anti-human CHI3l1 polyclonal antibody and rat anti-mouse antibody, respectively.

Cell proliferation

The Cyquant assay (Thermofisher Scientific) was used to determine the effect of CHI3L1 treatment or overexpression on the proliferation of tumor cells in vitro before conducting tumor experiments with these cells. Cells were plated at 1×10^4 cells/well in 96 well flat-bottom plates. Some plates were harvested the same day, a time when very little proliferation had occurred and considered as T0. Parallel culture plates, either control or treated, were then harvested after 48h or day 72h. The assay was conducted according to manufacturer's instruction and acquired with a matrix-based scanning acquisition of each well on Clariostar™ reader (BMG Labtech).

Cohorts analysis (public databases)

Gene expression data were obtained from the R2 Genomics Analysis and Visualization Platform (http://r2.amc.nl) or TCGA database and CHI3L1 expression data were median centered per array and plotted with PRISM (GraphPad Software) or using the GEPIA plotter (http://gepia.cancer-pku.cn) (for TCGA data vs. normal breast). The univariate survival analysis of different patient cohorts were performed using the R2 Kaplan-Meier analysis module or Kmplotter (http://kmplot.com) which included comprehensive information on the relevant clinical and prognostic factors selected for analysis. Probesets in each database with the highest average signals were selected for analysis.

Statistics

The statistical significance between the two groups was determined with unpaired Student's t-test, whereas the comparison of multiple groups was carried out by one-way or two-way ANOVA, followed by Bonferroni's post-test for correction of multiple comparisons using GraphPad Prism software (San Diego, CA). The univariate survival analysis of different breast cancer and gastric cancer cohorts were performed using the R2 Kaplan-Meier analysis module or Kmplotter (http://kmplot.com) both based on the log-rank test. The cutoff was defined to yield the most significant (likelihood ratio test) split separation with a minimum group size of 30. $P \leq 0.05$ was considered to denote significance.

Table 4: List of antibodies

Target	*Fluorophore*	*Isotype*	*Reactivity*	*Application*	*Use at*	*Provider*	*Code*	*Clone*
CD56	PerCP-Cy5.5/ FITC	Mouse IgG1	Human	Phenotyping FC	1:75	BD	560842	B195
NKp46	PerCP-Cy5.5/ FITC	Rat IgG2a, κ	Mouse		1:100	Biolegend	137601	29A1.4
CD3	V500	Mouse IgG1	Human		1:100	BD	561416	UCHT1
	V500/FITC	Rat IgG2b, κ	Mouse		1:100	Biolegend	100201	17A2
CD4	AF488	Rat IgG2b, κ	Mouse		1:100	Biolegend	100405	GK1.5
CD8	PECy7	Mouse IgG1	Human		1:100	BD	557746	RPA-T8
	APCCy7	Rat IgG2a, κ	Mouse			Biolegend	100707	53-6.7
CD16	PECY7	Mouse IgG1	Human		1:100	Biolegend	302016	3G8
NKG2D	PE	Mouse IgG1	Human		1:100	BD	561815	1D11
	BV421/FITC	Rat IgG1, κ	Mouse		1:100	Biolegend	101301/2	CX5
NKG2A	BV421	Mouse IgG2a	Human		1:100	BD	747923	131411
PD-1 (CD279)	PE-Cy7	Mouse IgG1	Human		1:100	Biolegend	329918	EH12.2H7
DNAM-1	FITC	Mouse IgG1	Human		1:100	Biolegend	337104	TX25
CD44	PE	Rat IgG2b, κ	Mouse		1:100	Biolegend	103024	IM7
CD62L	AF488	Rat IgG2a, κ	Mouse		1:100	Biolegend	104412	MEL-14
CD69	APCCy7	Mouse IgG1	Human		1:100	BD	560912	FN50
CD11a	A647	Mouse IgG1	Human	FC/IF	1:100/1:50	Biolegend	301218	HI111
CD18	FITC	Mouse IgG1	Human	FC/IF	1:100/1:50	Biolegend	302105	TS1/18
LFA1	A488	Mouse IgG1	Human	FC/IF	1:100/1:50	Biolegend	363403	m24
CD107a	BV421/FITC	Mouse IgG1	Human	Degranulation/ cytotoxicity (FC/IF)	1:75	BD	562623	H4A3
Perforin (active)	A488/A647/PE	Mouse IgG2b	Human	Active perforin (FC/IF)	1:50	BD	563764	δG9
Perforin (mouse)	A488/A647/PE	Rat IgG2a	Mouse	FC	1:75	Novus	NBP1-97512AF488	CB5.4
Granzyme A	PE/A594	Mouse IgG1	Human	Granzyme A stain (FC/IF)	1:50	Biolegend	507206	CB9
Granzyme B	A647	Mouse IgG1	Hu/Mu	Granzyme B stain (FC/IF)	1:50	BD	560212	GB11
INFg	APC	Rat IgG1	Mouse	INF-g stain tumor model	1:100	Biolegend	505810	XMG1.2
RAGE (AGER)	-	Rabbit IgG	Hu/Mu/Rat	RAGE expression analysis IF-FC	2ug/ml	abcam	ab37647	Polyclonal
	A488	Rabbit IgG	Hu/Mu/Rat	RAGE expression analysis IF-FC	1:100	Bioss	bs-4999R-A488	Polyclonal
	-	Mouse IgG2b	Human	RAGE Blocking assay	4-12ug/ml	abcam	ab89911	MM0520-8D11
	-	Poly Goat IgG	Hu/Mu/Rat	RAGE Blocking assay	1ug	RnD	AF1145	Polyclonal
IL13ra2	FITC/APC	Human IgG1	Human	FC IL13ra2 expression	1:50	Miltenyi	130104670	REA308
CHI3L1	-	Mouse IgG1	Human	Neutralizing in vitro and In vivo	-	Millipore	MABC196	mAY
	-	Rat IgG2b	Mouse	WB,IHC	1:500 WB	RnD	MAB2649	321924
		Goat IgG	Human	CHI3L1 expression FC,WB,IHC			AF2599	Polyclonal
Stathmin	AF488	Rabbit IgG	Hu/Mu/Rat	ICC/IF, FC	1:100	abcam	ab199735	EP1573Y
ERK1/2 (pT202/pY204	A488	Mouse IgG1	Human	Phosphoflow	1:50	BD	612592	20A
ZAP70 (pY319)/Syk (Y35	PE-Cy7	Mouse IgG1	Human	Phosphoflow	1:50	BD	561458	17A/P-ZAP70
Akt (pS473)	PE	Mouse IgG1	Human	Phosphoflow	1:50	BD	560378	M89-61
Stat3 (pY705)	A647	Mouse IgG1	Human	Phosphoflow	1:50	BD	557815	4/P-STAT3
JNK (pT183/pY185)	A647	Mouse IgG1	Human	Phosphoflow	1:50	BD	562481	Clone N9-66
Trastuzumab		Human IgG1	Human	Cytotoxicity assay/In vivo	10ug/ml	Roche		

Chapter 4: Results

4.1 Introduction

Results are divided into two parts: the first follows the main project of my thesis on NK cells and Trastuzumab resistance in HER2 BC. The second part is an annex of early results that were inspired during the progress of the main project.

PART 1

CHI3L1 promotes resistance to HER2 targeted therapy by impairing NK cell ADCC

Modulation of NK cell activity with sera from HER2+ BC patients

Previous work in our group investigated whether soluble factors in patients' blood could be impairing NK cell ADCC and thus promoting resistance to Trastuzumab. For this aim, sera from Trastuzumab treated patients were used to modulate the ADCC activity of healthy NK cells against trastuzumab coated SKBR3 cells. The modulation of NK cell activity by sera from each patient was determined by measuring the ADCC with a reference serum sample (at basal before starting trastuzumab therapy) and a serum sample at time of clinical outcome (remission or progression). We found that sera from non-responders reduced NK cell activity while those from responders did not affect (SD) or improved (CR) NK cell activity (**Figure 1a, b**).

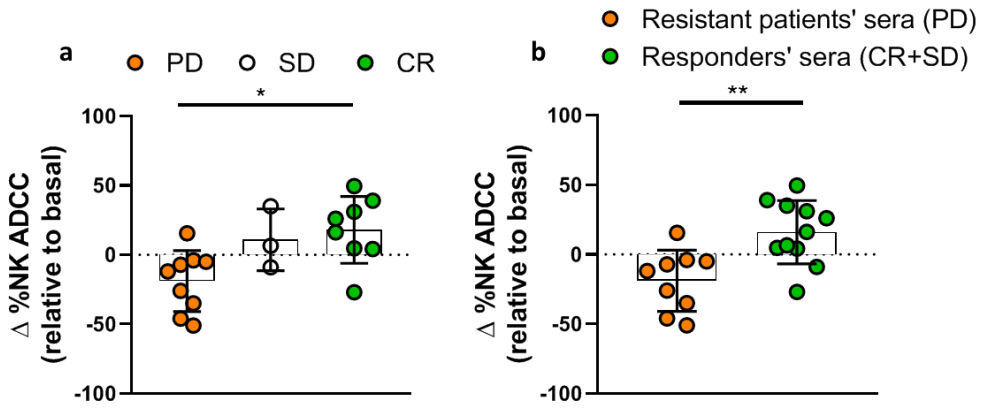


Figure 1: Sera of Trastuzumab resistant patients negatively modulate NK cell activity.
Blood samples were collected at basal (before Trastuzumab therapy) and when patients reached progressive disease (PD) N=9, complete remission (CR) N=8, or stable disease (SD) N=3 within 12 months follow-up. Plotted is delta NK activity as determined by measuring ADCC of healthy NKs treated with a reference serum sample (at basal before starting trastuzumab therapy) and the endpoint serum sample when the patient reached a clinical outcome. (**a**) shows the three groups of patients and (**b**) is a pool of SD and CR patients as responders. Values represent the means ± SD (95% CI, *$p < 0.05$, **$p < 0.01$).

We then focused on identifying differentially abundant molecules in PD patient's sera that could explain their inhibitory effect on NK cells. We initially performed a large scale proteomic analysis using the XL human proteome array (R&Dsystems) and selected the most significant molecular hits for the construction of a Luminex panel to assay 12 proteins: GDF15, VitD-BP, RBP4, BDNF, CHI3L1, ICAM-1, IGFBP-3, Angiogenin, sCD14, CRP, Cystatin, and DPPIV. We confirmed that some of these candidates were differentially expressed between healthy donors and patients (whether CR or PD) (**Figure 2a**). These included previously identified BC associated molecules with prognostic value such as sCD14 and GDF15 (Breit *et al.*, 2011; W. He *et al.*, 2013). Cystatin C levels were also increased which might indicate a worsening of renal function is these heavily treated patients (He *et al.*, 2019). Among these, only Chitinase 3-like 1 protein (CHI3L1) levels could distinguish between PD and CR/SD sera. The levels of this protein remained unchanged during treatment in sera of responders and were comparable to healthy controls. Whereas its levels were higher at basal in sera of some PD patients and increased further at time of disease progression (**Figure 2b,c**).

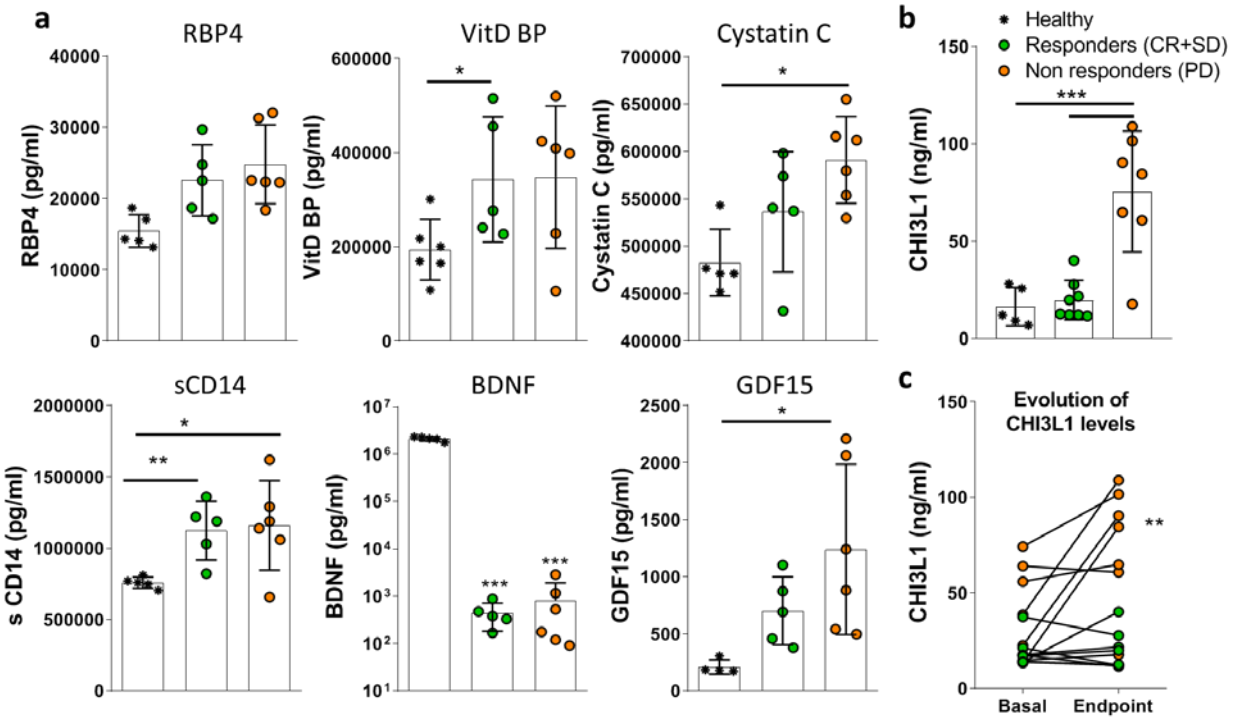


Figure 2: CHI3L1 levels correlate with decreased NK cells ADCC and disease progression in Trastuzumab resistant patients.

Results from Luminex multiplex measurements of **(a)** differentially expressed molecules in sera of HER2 BC patients compared to healthy donors Responders (N=5), Non responders (N=6), and healthy controls (N=5). ELISA measurements of CHI3L1 levels at clinical endpoint (**b**) and at basal vs endpoint (**c**), Responders (N=8), Non responders (N=7), and healthy controls (N=5). Values represent the means ± SD (95% CI, *p < 0.05, **p < 0.01, ***p < 0.001).

To validate our findings from this discovery cohort which was recruited before the introduction of Pertuzumab for the treatment of HER2+ BC, we decided to recruit a second cohort of patients receiving the current standard therapy (Trastuzumab + Pertuzumab + chemotherapy). This time, the first blood sample was taken during therapy when patients had no evidence of disease, and once they had a clinical outcome (PD, CR, or SD after 18 months). In accordance with the previous cohort, PD patients sera had an inhibitory effect on healthy NK cells ADCC (**Figure 3a**) and contained higher levels of CHI3L1 compared to sera of CR patients and healthy subjects (**Figure 3 b,c**). Interestingly, one responder patient was diagnosed with advanced-stage Essential Thrombocythemia, a rare form of blood cancer, and had high CHI3L1 levels in accordance with a previous study (Andersen *et al.*, 2014).

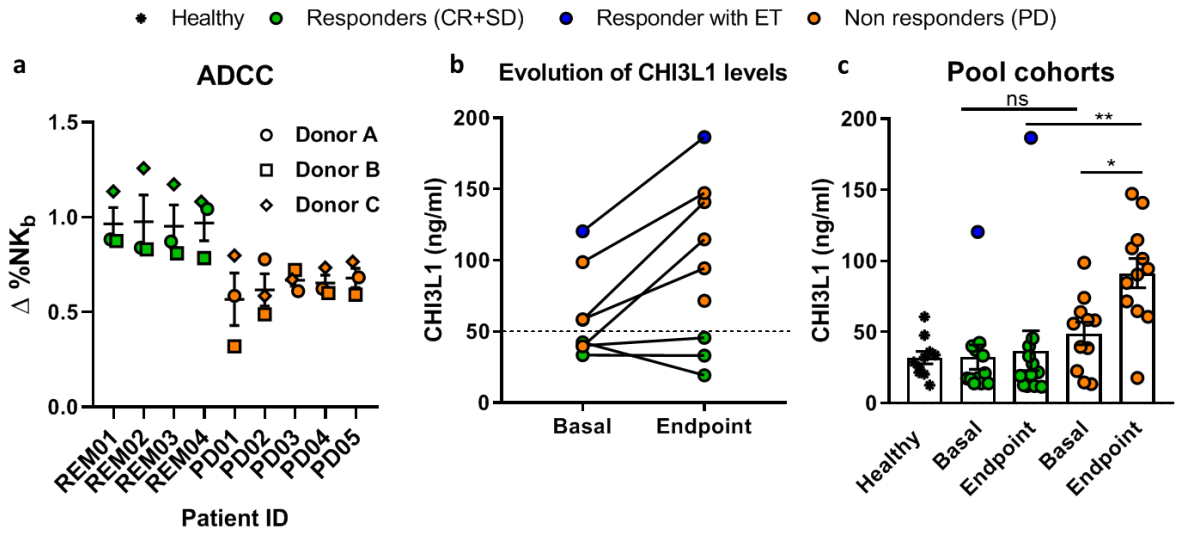


Figure 3: Validation of the correlation between the inhibitory effect of progressor's sera and elevated CHI3L1 levels

(a) Effect of sera on ADCC (compared to basal) of NK cells from three healthy donors and (b) evolution of CHI3L1 levels in the sera of patients from the validation cohort (N=4 responders/ N=5 PD), one responder with Essential Thrombocythemia (ET) is highlighted in Blue (c) Pool of CHI3L1 levels at basal or at time of clinical endpoint from the two cohorts (N=10 healthy, N=12 Responders and N= 12 PD). Values represent the means ± SEM (95% CI, *p < 0.05, **p < 0.01,, NS, not significant).

To extend the relevance of our results we assessed CHI3L1 involvement in BC by analyzing publically available gene expression databases. We found that CHI3L1 expression was most elevated in HER2+ and TNBC subtypes which parallels the findings from our cohorts (**Figure 4**). Furthermore, higher expression of this protein in primary tumors correlated with worse overall survival (OS), progression-free survival (PFS), and recurrence in different BC cohorts (**Figure 5a-d**) and HER2 gastric cancer cohorts where Trastuzumab is also a therapeutic option

(**Figure 5e,f**).

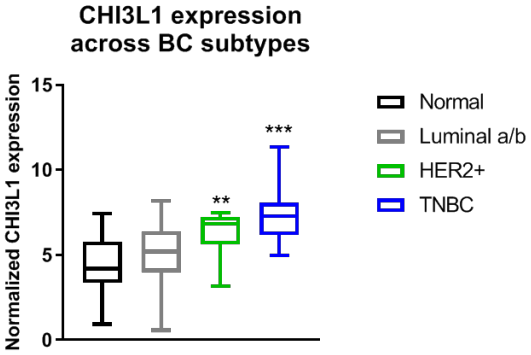


Figure 4: CHI3L1 expression is elevated HER2+ BC and TNBC

Normalized expression values of *chi3l1* in normal breast and primary BC tumors from 159 patients from all subtypes were obtained from **GSE1456** using R2 Genomics Platform (http://r2.amc.nl). Values represent the means ± SD (95% CI, *p < 0.05, **p < 0.01, ***p < 0.001)

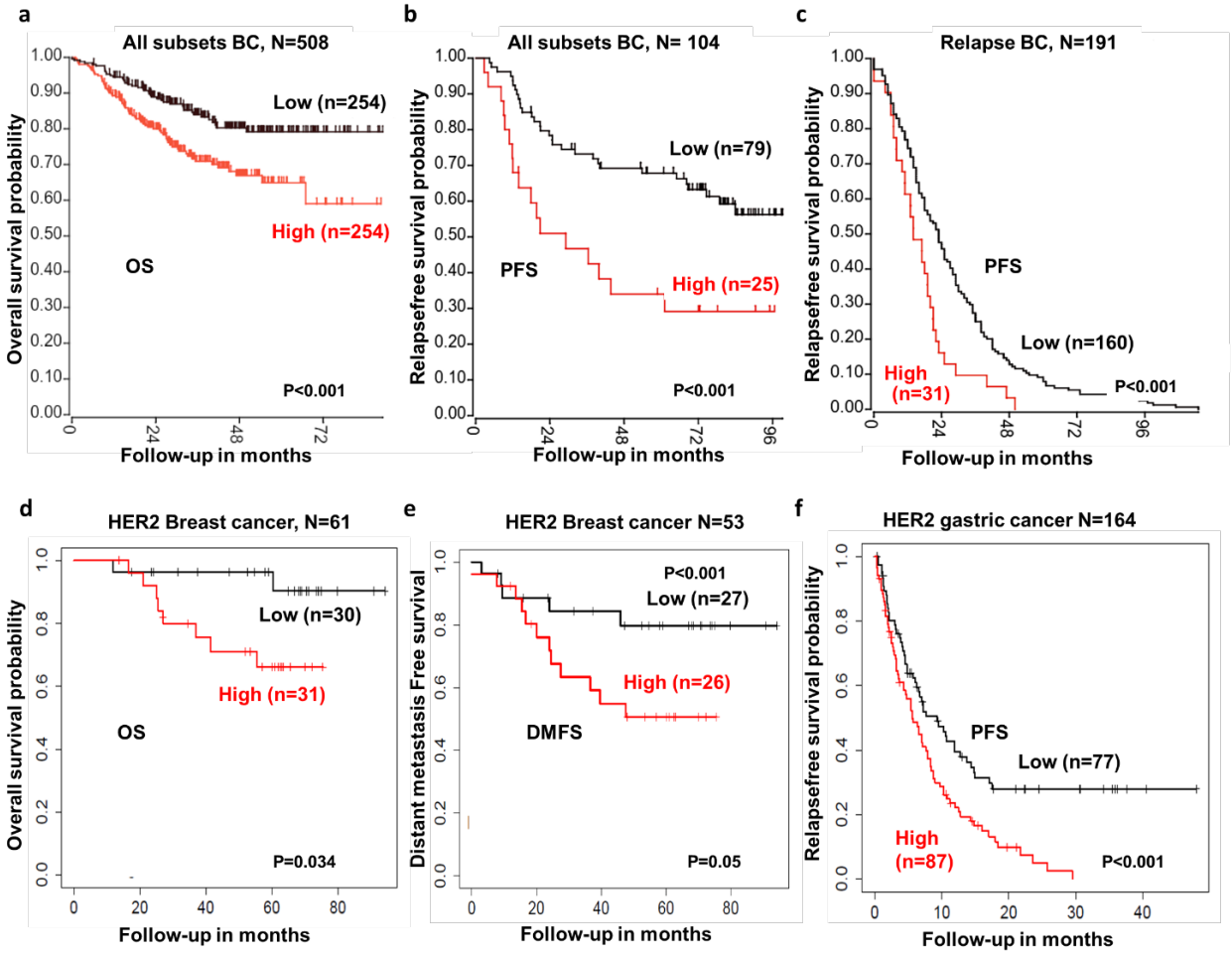


Figure 5: High CHI3L1 expression correlates with worst prognosis in BC

Kaplan Meier analysis of plublically available databases according to CHI3L1 gene expression in primary tumors (**a**) overall survival (OS) of breast cancer patients from all subtypes (n=508, Cutoff=Median), source**: E-GEOD-25066**. (**b**) and (**c**) progression free survival (PFS) of BC patients (Cutoff=most significant), source: **E-GEOD-42568** and **GSE29271** respectively. (**d**) and (**e**) OS and distant metastasis free survival (DMFS) of HER2+ BC patients (Cutoff=Median), source= **Kmplotter** (**GSE45255**). (**f**) PFS of HER2+ Gastric cancer patients, source= **Kmplotter** (multiple cohorts).

CHI3L1 inhibits NK cells cytotoxicity in vitro.

Based on the previous results we decided to focus on CHI3L1 in our analysis and to test if it is directly responsible for the decreased NK cytotoxic activity. We found that treatment with recombinant CHI3L1 impairs antibody-dependent and natural cytotoxicity of human and murine NK cells in a dose-dependent manner (**Figure 6, a-d**). We confirmed the involvement of CHI3L1 in the observed phenomenon by co-incubating with a CHI3L1 neutralizing antibody which abrogated its inhibitory effect (**Figure 6e**). Intriguingly, CHI3L1 did not affect CD16 or NKG2D expression levels or the amount of degranulation as measured by CD107a staining (**Figure 6 f-h**).

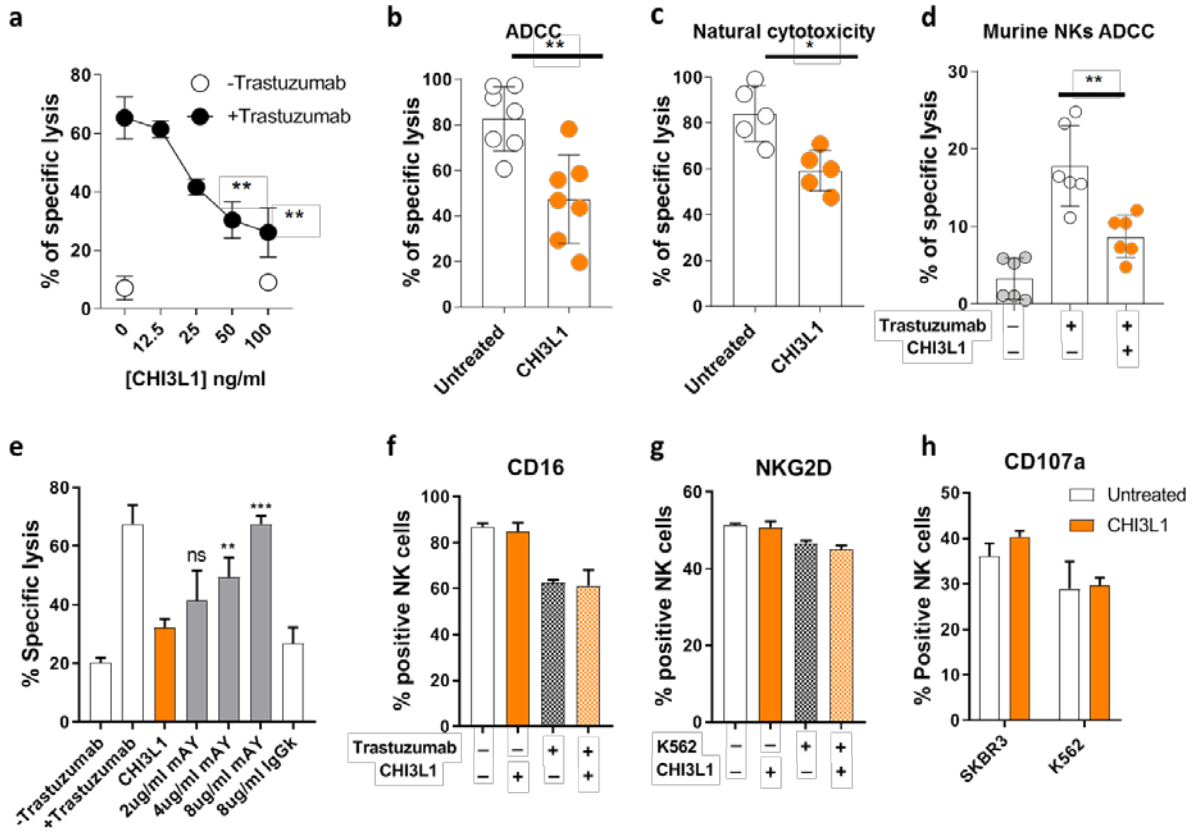


Figure 6: CHI3L1 inhibits ADCC dependent and Natural cytotoxicity of NK cells.
(**a**) ADCC Specific lysis of SKBR3 tumor cells by NK cells treated with increased dose of rhCHI3L1 (**b**) Pooled data of NK cells ADCC treated with recombinant human rhCHI3L1 (100ng/ml) for 1h before assay (n=7 healthy donors). (**c**) NK cells natural cytotoxicity against K562 targets after treatment with rhCHI3L1 (100ng/ml) (n=5 healthy donors). (**d**) ADCC specific lysis of SKBR3 cells by murine NK cells treated with recombinant mouse rmCHI3L1 (100ng/ml)(n=6 BALB/c mice). (**e**) ADCC Specific lysis of SKBR3 targets in the presence of anti-CHI3L1 Neutralizing antibody (mAY) or Isotype control (IgGk). (**f**), (**g**) and (**h**) Flow cytomtery analysis of the frequencies of CD16 , NKG2D and CD107a positive NK cells alone (basal) or following incubation with SKBR3 (ADCC) or K562 (natural cytotoxicity) targets with or without rhCHI3L1 (100ng/ml), respectively. All plots are representative of at least two independent evaluations. Values in all graphs represent the means ± SD (95% CI, *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant).

Similarly, other receptors involved in modulating NK cell cytotoxicity such as NKG2A, LFA-1, DNAM-1, and early activation (CD69) were unaffected by CHI3L1 treatment (**Figure 7**).

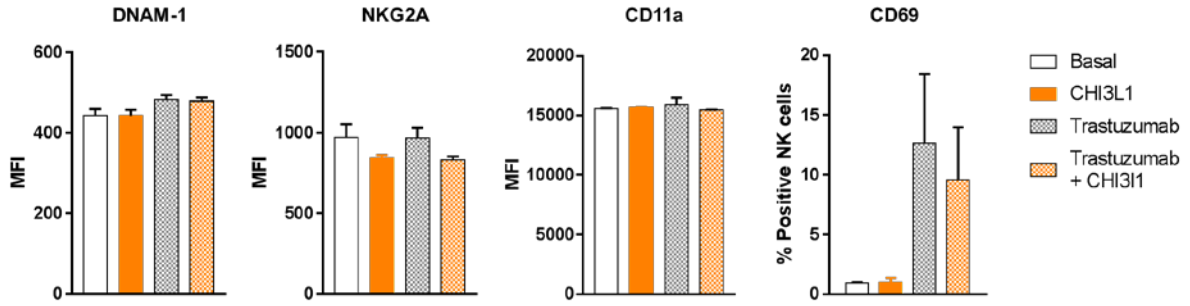


Figure 7: CHI3L1 effects are independent of the expression of cytotoxicity receptors on NK cells. Flow cytometry analysis of the expression of different cytotoxicity receptors on NK cells after treatment with 100ng/ml rhCHIL1 in ADCC or at basal. All plots are representative of at least two independent evaluations. Values in all graphs represent the means ± SD (95% CI, *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant).

Altogether, these results indicate that CHI3L1, which is overexpressed in patients that progressed to trastuzumab, is capable of inhibiting the cytotoxic activity of NK cells (both classical and ADCC). This inhibition does not seem to occur via the modulation of cytotoxicity receptors.

Defective polarization of the lytic machinery and loss of lytic granules in CHI3L1 treated NK cells.

The finding that CHI3L1 exerted inhibitory effects on both NK cells and CD8 T cells (see in Annex II) independently of modulating surface receptors or the amount of degranulation, suggested that CHI3L1 might be affecting the lytic granules machinery as a common mechanism of killing by these cells. In NK cells, the immune synapse (IS) occurs in a stepwise controlled manner: they first form conjugates with target cells, followed by cytoskeletal rearrangement and the formation of a dense F-actin mesh at the immune synapse, the granules then accumulate at the microtubule organizing center (MTOC) and together polarize towards the target cell surface (Orange et al., 2008). We used confocal microscopy to study these major steps and found that CHI3L1 treatment impaired Perforin polarization to the IS despite correct accumulation of F-actin and LFA-1, two early requirements of correctly formed lytic conjugates (**Figure 8a**).

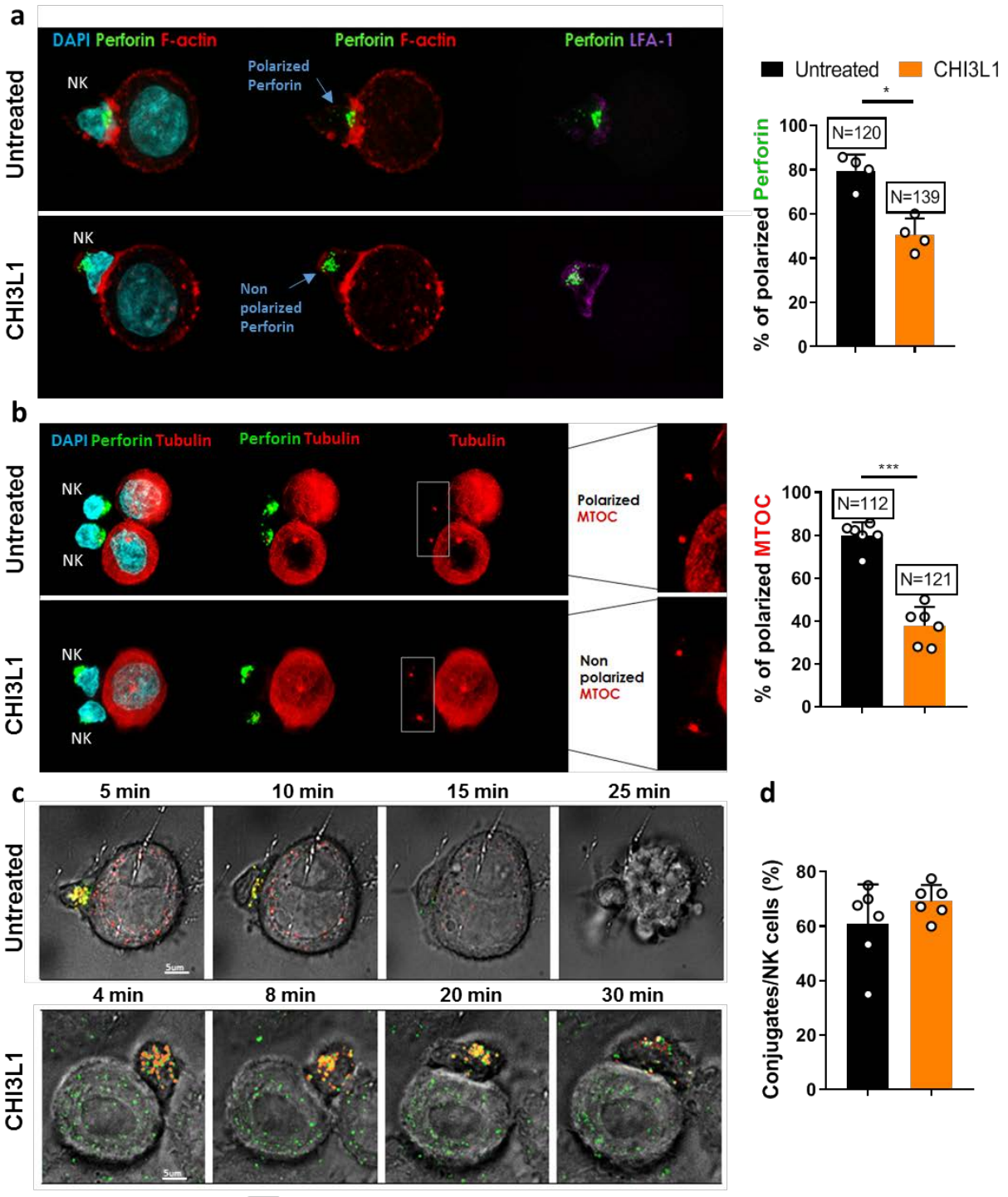


Figure 8: The decreased cytotoxicity of NK cells is related to defective MTOC and granules polarization in CHI3L1 treated NK cells.

Confocal microscopy Images of the immune synapse between NK cells treated or not with CHI3L1 (100ng/ml) and SKBR3 cells treated with Trastuzumab. (**a**) Staining of perforin and early polarization markers (F-actin and LFA-1). (**b**) Staining for perforin and the microtubule organizing center (MTOC). (**c**) Live cell imaging of NK cells in ADCC with SKBR3 targets at 2:1 E:T ratio and followed for two hours (Red: Lysotracker for granules, Green: Sir-tubulin for MTOC) (**d**) Frequency of NK:Target Conjugates (N= number of conjugates counted). Confocal Imaging Data representative of 5 independent experiments and 6 different healthy donors. Live cell imaging representative of 3 healthy donors. Values in all graphs represent the means ± SD (95% CI, *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant).

We next analyzed MTOC polarization and we found that Perforin was accumulating at the MTOC, but at this point, NK cells failed to polarize their MTOC to the IS (**Figure 8b**). We also confirmed these findings by live-cell imaging where we observed that NK cells were correctly engaging their targets followed by the accumulation of the granules at the MTOC but will end up detaching without productive release towards the target cells (**Figure 8c,d**). To further elucidate the effect on the lytic machinery we treated NK cells and evaluated the content of their lytic granules. We found that CHI3L1 treatment decreased the levels of Perforin, Granzyme A, and Granzyme B in a time and dose-dependent manner (**Figure 9a-c**).

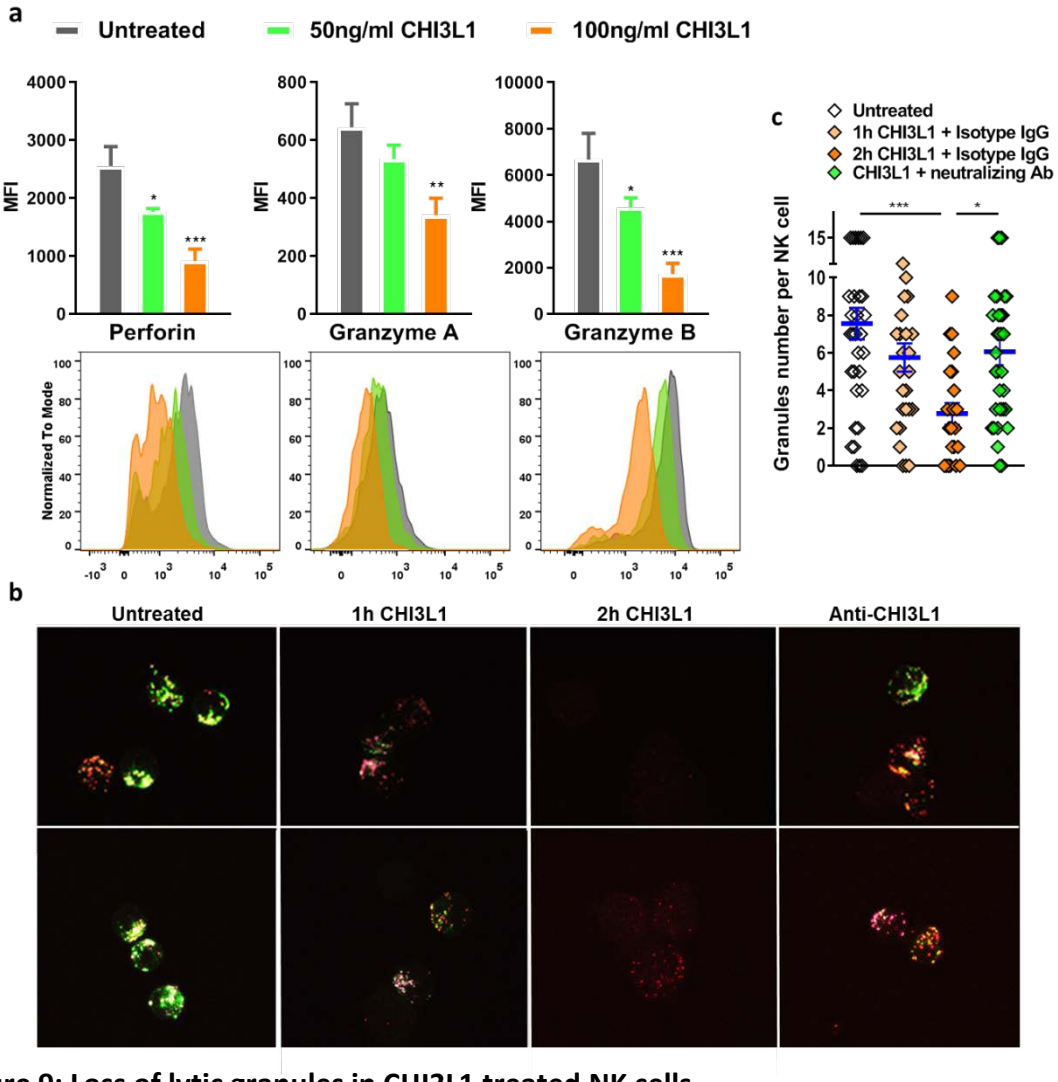


Figure 9: Loss of lytic granules in CHI3L1 treated NK cells

(**a**) Flow cytometry analysis of lytic granules expression after treatment with CHI3L1 for 2 hours. (**b**) Confocal images showing the effect of treatment with CHI3L1 on lytic granules content with time. (**c**) Quantification of the average number of granules per NK cell from >40 NK cells per condition (Sum of **perforin**, **granzyme A**, **granzyme B**/3). Flow cytometry data representative of 3 independent experiments. Confocal data representative of 4 independent experiments for the time points and two for the neutralization assay. Values in (a) represent the mean ± SD and (c) the mean ± SEM (95% CI, *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant).

CHI3L1 exerts its effects by modulating RAGE signaling in NK cells.

To elucidate the molecular mechanism by which CHI3L1 is acting on NK cells. We first analyzed the expression of known receptors of this protein. Several receptors have been shown to bind CHI3L1, including Interleukin 13 receptor subunit alpha 2 (IL13Rα2), receptor of advanced glycation end products (RAGE), and Prostaglandin DP2 receptor (CRTH2)(C. H. He *et al.*, 2013; Low *et al.*, 2015; Zhou *et al.*, 2015). CRTH2 is expressed on a small subset of NK cells (Chen, Perussia, and Campbell, 2014) while flow cytometry and confocal analysis showed no expression of IL13Rα2 on NK cell surface but in the cytoplasm (**Figure 10a,b**). Of note, IL13Rα2 was not mobilized to the surface upon treatment with IL-2, IL-15, CHI3L1, or IFN-g (not shown). Thus we excluded the involvement of this receptor in CHI3L1 mediated effects in our setting.

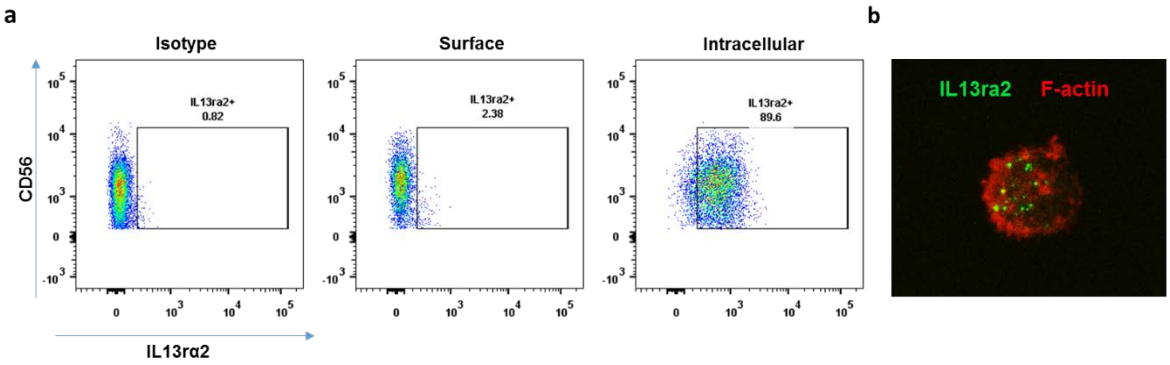


Figure 10: IL13Rα2 is expressed intracellularly in NK cells.
Expression of IL13Rα2 by (**a**) Flow cytometry analysis on the surface (non permeabilized) and intracellular (permeabilized) NK cells and (**b**) Confocal imaging showing clusters of recpetor inside NK NK cells (cytoplasm). Flow cytometry data representative of NK cells from 4 healthy donors. Confocal data representative of a single donor.

Conversely, consistent with a previously described activator function of RAGE in NK cells (Narumi *et al.*, 2015; Parodi *et al.*, 2015) we found this receptor to be expressed at higher levels on the CD56dim population (**Figure 11a**). Further, the expression of RAGE was downmodulated after incubation with CHI3L1 suggesting it is internalized upon CHI3L1 binding (**Figure 11b**). Importantly, we found that RAGE accumulates at the immune synapse (IS) during ADCC highlighting a functional involvement of this receptor in the IS biology (**Figure 11c**).

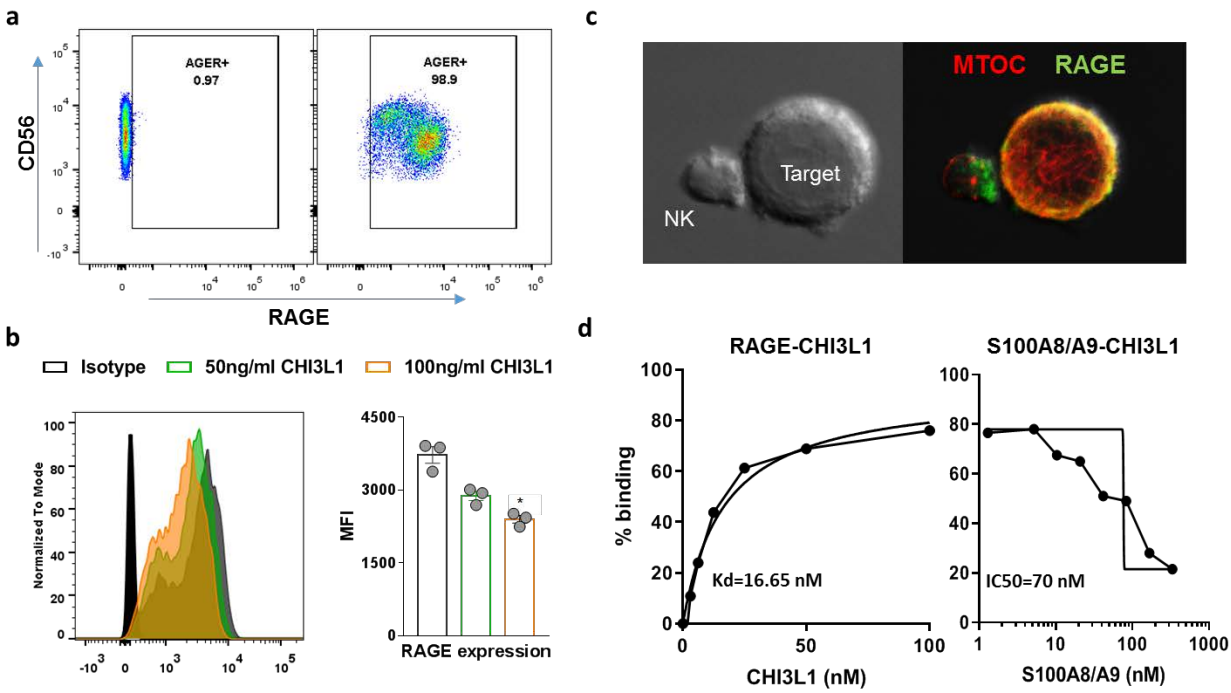


Figure 11: RAGE is expressed on NK cells and interacts with CHI3L1

Expression of RAGE by (**a**) Flow cytometry on the surface of NK cells, in (**b**) after treatment with CHI3L1 and (**c**) by confocal imaging of the lytic synapse during ADCC showing the clustering of RAGE at the synaptic front concomitantly with MTOC polarization. (**d**) Saturation curve of CHI3L1 binding to RAGE (left). Competitive binding of 20nm CHI3L1 (physiological blood levels) with increased concentration of S100A8/A9 (Right). Binding was measured by ELISA. Expression data representative of NK cells from 4 (a), 3 (b) and 2 (c) healthy donors. Elisa measurements representative of two repetitions. KD and IC50 were fitted with linear regression assuming one binding site using Graphpad prism.

We next tried to validate the role of RAGE in NK cell cytotoxicity by using RAGE blocking antibody or RAGE antagonistic peptide (RAP). We found that blocking RAGE, similarly to treatment with CHI3L1, inhibited NK cell cytotoxicity against K562 and SKBR3 targets, MTOC polarization to the immune synapse (IS) and induced a decrease of lytic granules (**Figure 12 a-c**).

This suggested to us that CHI3L1 played an inhibitory function through RAGE binding. To address this issue, we evaluated the effect of CHI3L1 ligation on the signaling pathway downstream of RAGE. Specific signaling modules such as ERK1/2, p38 MAPK, SAPK/JNK, and NF-κB are triggered by different ligands/RAGE interaction in different cell types (Xie & Aguilar-Hernández, 2013), but their involvement in NK cells was unknown.

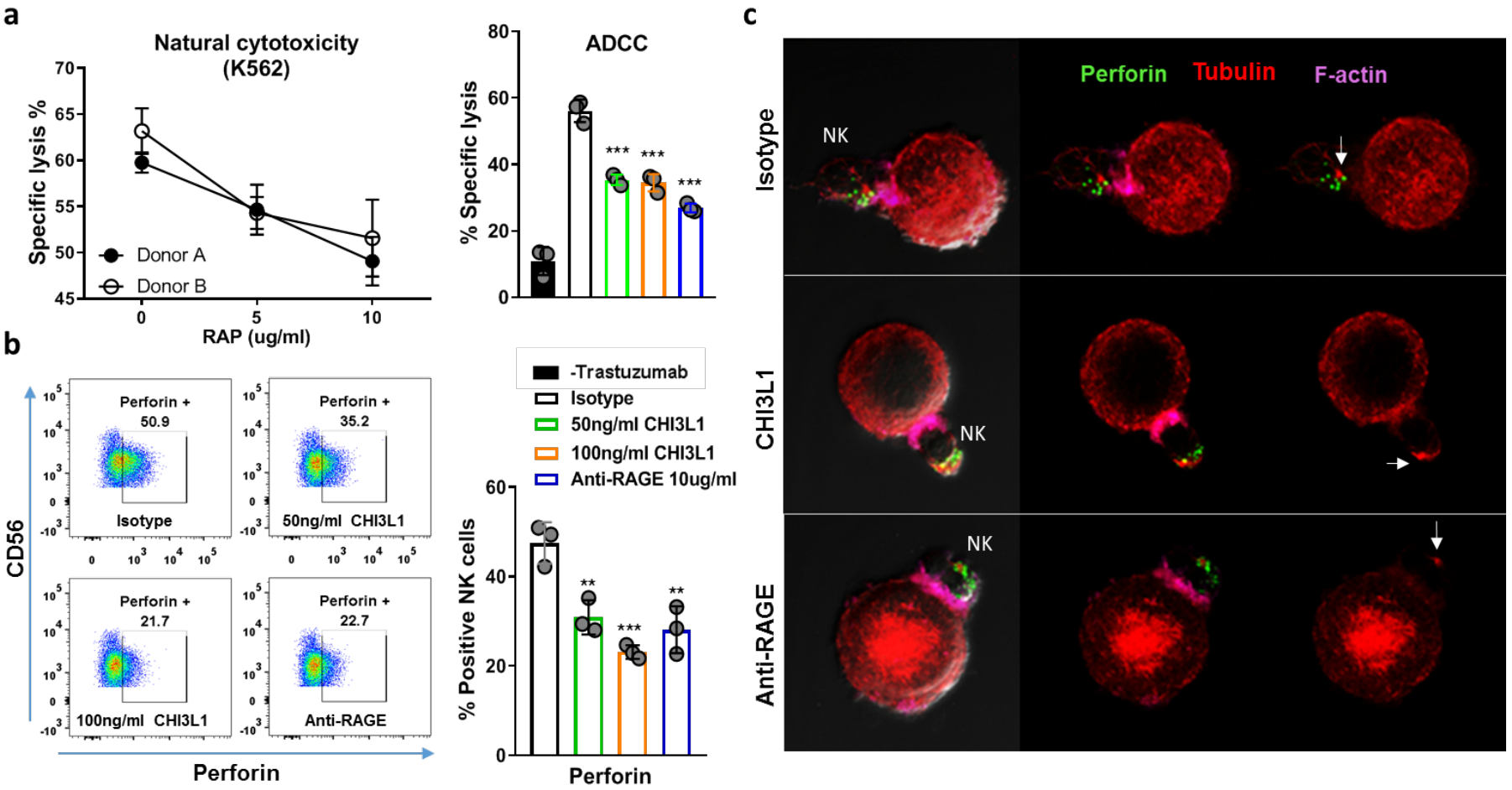


Figure 12: RAGE blockade inhibits NK cell cytotoxicity by interfering with the NK lytic machinery.

(**a**) Natural cytotoxicity (left panel) and ADCC (right panel) specific lysis of K562 and SKBR3 tumor cells respectively by NK cells treated with RAGE antagonistic peptide (RAP), rhCHI3L1 or anti-RAGE Blocking antibody (10ug/ml) for 1h before the assay. (**b**) Flow cytomtery analysis of the frequencies of perforin+ NK cells treated with rhCHI3L1 or anti-RAGE Blocking antibody (10ug/ml) for 2h. (**c**) Confocal images of the immune synapse between NK cells treated with rhCHI3L1 or anti-RAGE Blocking antibody (10ug/ml) for 1h and SKBR3 tumor cells, Stained for perforin, F-actin and Tubulin (MTOC indicated with an arrow). All plots are representative of at least two independent evaluations. Values represent the means ± SD, 95% CI, *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant).

We focused on pathways that have been shown to be important for NK cell cytotoxicity, including granules biology, and MTOC polarization, namely ERK1/2, AKT, STAT3 and JNK (X. Chen et al., 2007; Kabanova & Baldari, 2018; C. Li et al., 2008). While CHI3L1 treatment did not impact ERK1/2, AKT, or STAT3 phosphorylation levels (**Figure 13a**), basal P-JNK levels were decreased in a dose-dependent manner in CHI3L1 treated NK cells (**Figure 13b**).

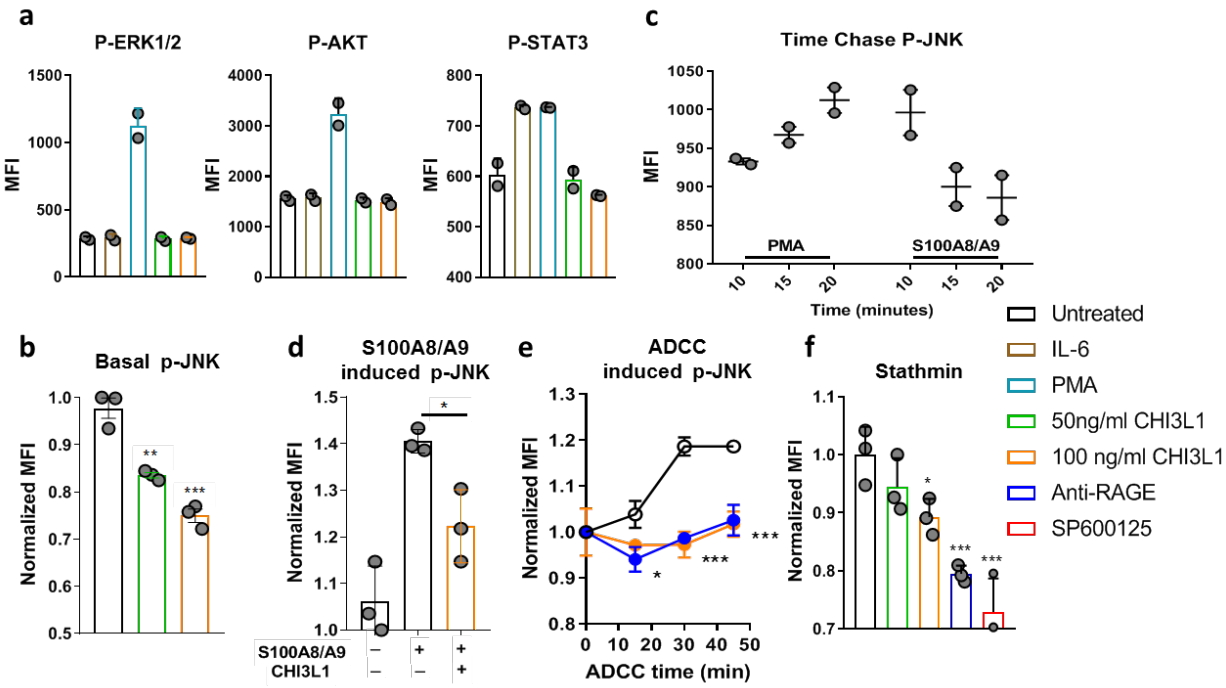


Figure 13: CHI3L1 exerts its effects by inhibiting JNK signaling downstream of RAGE.
Phosphoflow cytometry analysis of (**a**) the effect of CHI3L1 on key signaling mediators of NK cell cytotoxicity and microtubule function, (**b**) Basal p-JNK levels in NK cells treated with CHI3L1 for 1 hour (**c**) p-JNK induction kinetics by S100A8/A9 or PMA as positive control (**d**) p-JNK levels in NK cells after 10 min of stimulation by RAGE ligand S100A8/A9 (10ug/ml) alone or in competition with rhCHI3L1 (100ng/ml), and (**e**) P-JNK levels during ADCC of NK cells pretreated with rhCHI3L1 or anti-RAGE (10ug/ml) for 1h and incubated with SKBR3 targets at (5:1) E:T ratio. (**f**) MFI of Stathmin expression in NK cells treated with rhCHI3L1, anti-RAGE Blocking antibody (10ug/ml) or JNK inhibitor SP600125 (10uM) for 2 hours. All plots are representative of at least two independent evaluations. Values represent the means ± SD, 95% CI, *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant).

Consequently, we decided to evaluate the effect of CHI3L1 on ligand-induced JNK activation. In particular, we used the RAGE agonist S100A8/A9 (Calprotectin), which has been described to activate murine NK cells through RAGE (Narumi *et al.*, 2015), and to compete with CHI3L1 on RAGE binding (Low *et al.*, 2015). In line, S100A8/A9 rapidly induced JNK phosphorylation whereas pre-Incubation of S100A8/A9 with CHI3L1 prior to treating NK cells, opposed this activation (**Figure 13c,d**). In this context, we determined that CHI3L1 bound RAGE with a good affinity (Kd~16nm) (**Figure 12d**), higher than that of most S100 family members which is in

the µM range (Leclerc et al., 2009). This was apparent from competition ELISA as ~4 folds of S100A8/A9 was needed to decrease by 50% CHI3L1 binding (**Figure 12d**). Besides affecting basal JNK levels and ligand-induced JNK activation, we wanted to assess whether CHI3L1 affects JNK signaling also during NK cell cytotoxicity. As expected JNK was induced in a time-dependent manner during ADCC (**Figure 13e**). Importantly, pretreatment of NK cells with CHI3L1 or anti-RAGE significantly reduced this induction suggesting suboptimal JNK activation during ADCC in CHI3L1 and anti-RAGE treated NK cells (**Figure 13e**). In line, Treating NK cells with the JNK inhibitor SP600125 strongly impaired their ADCC (**Figure 14a**) and MTOC polarization (**Figure 14b**) consistent with a previous study reporting that JNK is required for MTOC polarization in NK cells (C. Li *et al.*, 2008).

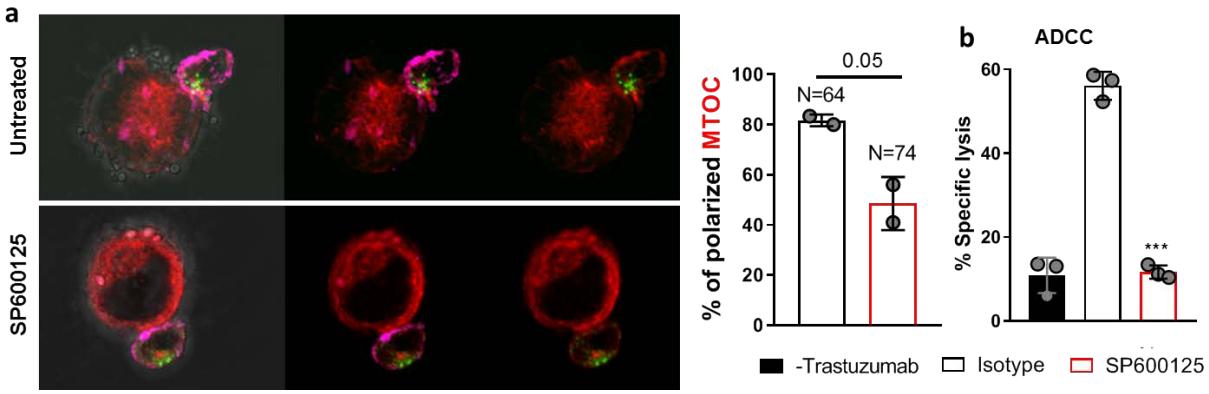


Figure 14: JNK inhibition impairs MTOC polarization and ADCC.
(**a**) Confocal images of the immune synapse between NK cells treated or not with SP600125 JNK inhibitor for 1h and SKBR3 tumor cells, Stained for **perforin**, **F-actin** and **Tubulin**. N=number of conjugates counted from two different donors. (**b**) ADCC specific lysis of SKBR3 targets by NK cells treated or not with SP600125 JNK inhibitor for 1h. Data representative of NK cells from two healthy donors. Values represent the means ± SD, 95% CI, *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant).

To further elucidate the mechanism by which impairment of JNK signaling by CHI3L1 is acting on MTOC stability, we analyzed the microtubule regulatory protein Stathmin as a potential target protein of JNK. Stathmin is regulated by a series of serine phosphorylations mainly by STAT3 and JNK (Dominic et al., 2010; Dominic et al., 2006). As STAT3 was not affected by CHI3L1 treatment (**Figure 13a**), we speculated that JNK inhibition was leading to the deregulation of Stathmin levels. Of note, Stathmin is essential for MTOC polarization in T cells and its loss impairs this process (Filbert et al., 2012). CHI3L1 treatment, consistent with RAGE blockade and JNK inhibition, led to decreased levels of Stathmin in NK cells as shown by flow cytometry analysis of Stathmin expression (**Figure 13f**).

Altogether, these results support a model in which CH3L1 acts on NK cells by downmodulating JNK signaling through RAGE binding. In turn, the inhibition of the JNK pathway affects microtubule stability, by decreasing the levels of the microtubule regulatory protein Stathmin (**Figure 15**).

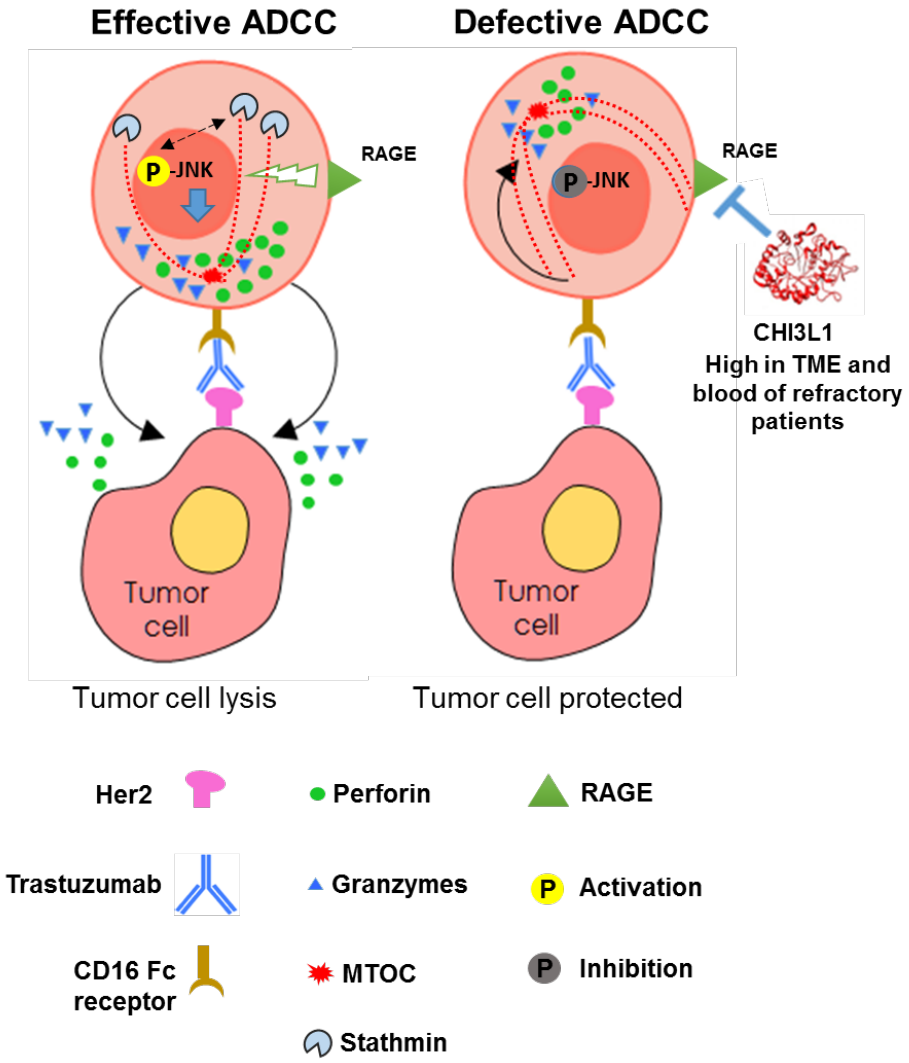


Figure 15: Control of NK cell ADCC by CHI3L1 in HER2 BC.
NK cells recognize Trastuzumab bound on HER2 tumor cells through their Fc receptors. This induces a strong signaling cascade including JNK phosphorylation, an essential step for the polarized release of Lytic granules into the IS and target cell lysis. Precisely, active JNK controls the levels of Stathmin, a microtubule regulatory protein. CHI3L1 treated NK cells recognize and engage target cells correctly. Next, lytic granules are polarized to the microtubule organizing center (MTOC), but NKs fail at this point to polarize their MTOC to the IS due to CHI3L1 binding to RAGE and its impairment of the downstream JNK- Stathmin pathway. This microtubule destabilizing event ultimately leads to a defective ADCC.

CHI3L1 overexpression enhances the growth of HER2+ xenografts and abrogates tumor control by trastuzumab.

To validate our findings from patients and from in vitro experiments, we used a HER2+ BC xenograft model that would allow us to evaluate whether CHI3L1 overexpression can lead to Trastuzumab resistance. In particular, we used JIMT-1 cells which are intrinsically resistant to Trastuzumab (Tanner *et al.*, 2004). However, tumor growth from these cells can still be partially controlled in vivo thanks to NK cell ADCC if Trastuzumab treatment is started when the tumor size is around 50-100mm^3 (Barok *et al.*, 2007). In other words, a loss of Trastuzumab efficacy in this model directly reflects on the ability of NK cells to mediate efficient ADCC, without other confounding factors such as inhibition of HER2 signaling. To study the effect of CHI3L1 in this system, we transfected JIMT-1 cells to stably overexpress the murine CHI3L1 protein (**Figure 16a**). We chose to overexpress the murine protein to avoid an autocrine enhancement of tumor growth as mCHI3L1 does not act on human cells. Indeed overexpression of CHI3L1 did not affect the proliferation of JIMT-1 cells in vitro (**Figure 16b**).

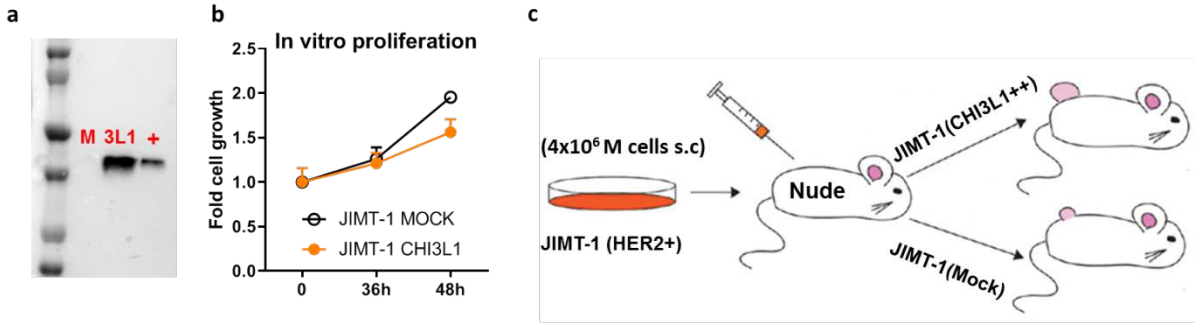


Figure 16: Experimental Setup for the JIMT-1 HER2 tumor experiment
(**a**) Western blotting to detect CHI3L1 from supernatant of JIMT-1 Mock (M), JIMT-1 CHI3L1 (3L1), or recombinant protein as positive control (+) Band size~42KD. (**b**) Cyquant measurement of in vitro proliferation of JIMT-1Mock vs. JIMT-1CHI3L1 cells. (**c**) JIMT-1 cells overexpressing CHI3L1 (JIMT-1CHI3l1) or transfected with empty plasmid (JIMT-1 Mock) were injected subcutaneously in the flank of BALB/c (inbred) or CD1 (outbred) nude mice.

In a first experiment, we injected nude mice with JIMT-1Mock and JIMT-1CHI3L1 cells to establish tumor growth (**Figure 16c**). We used inbred (BALB/c) and outbred (CD1) nude mice. These latter are less used in tumor studies because of their genetic diversity and hostility to xenograft growth but were interesting to evaluate CHI3L1 interaction with tumor growth in different genetic backgrounds.

Tumor growth was significantly increased in mice injected with JIMT-1CHI3L1 cells regardless of the genetic background, although CD1 mice were more resistant to tumor development (**Figure 17a,b**). This was paralleled with higher CHI3L1 levels in sera of mice with CHI3L1 overexpressing tumors (**Figure 17c**). Interestingly, In CD1 mice some mock tumors completely regressed while all CHI3L1 overexpressing ones continued to grow. To investigate this difference, we analyzed NK cells (as the major cytotoxic effectors in nude mice) and found that, although their frequency was unaltered, in mice with CHI3L1 tumors, NK cells were less activated than in mice with mock tumors as indicated by NKG2D and CD69 expression (**Figure 17d**).

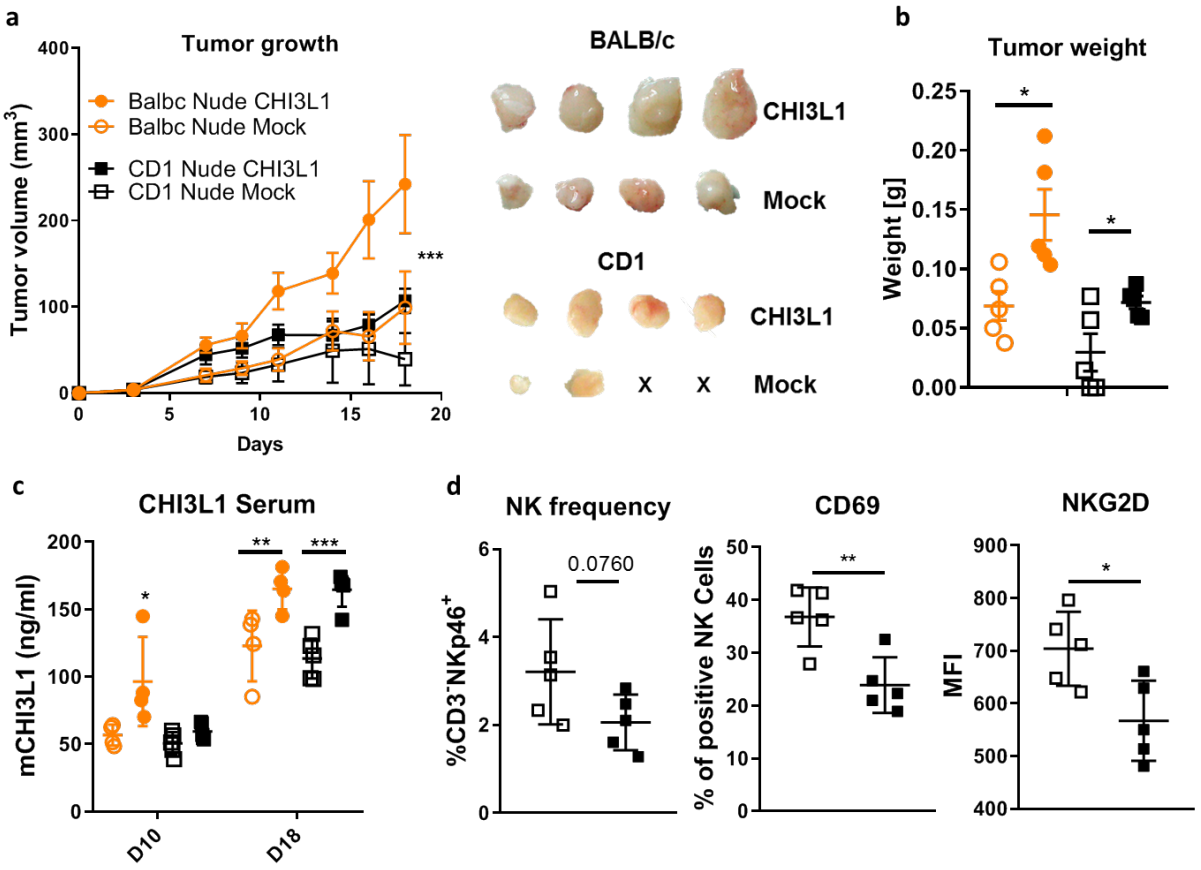


Figure 17: CHI3L1 overexpression enhances the growth of JIMT-1 HER2 BC xenografts.
Tumor growth (**a**) and weight (**b**) of JIMT-1Mock vs JIMT-1CHI3L1 cells in BALB/c and CD1 nude mice and representative images of tumors from each group, (x) signify tumors that regressed (3/5 Mock tumors in CD1 mice). (**c**) CHI3L1 levels in the sera of mice at D10 and at D18 (sacrifice). (**d**) FACS analysis of NK cell frequency and activation in the blood of CD1 mice to explain the regression of Mock tumors. All plots are representative of at least two independent evaluations. Values represent the means ± SEM in (**a**), (**b**), (**c**) and the means ± SD in (**d**) , 95% CI, *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant).

We next proceeded to our main aim from this experiment which was to assess the effect of CHI3L1 on the efficacy of Trastuzumab (ADCC) in controlling tumor growth. We decided to inject less JIMT-1CHI3L1 cells to adjust for the differential tumor growth kinetics we observed in the previous experiment and to minimize the time gap of starting the treatment (**Figure 18a**). As expected, Trastuzumab partially but significantly inhibited the growth of JIMT-1 mock tumors. However, this effect was abrogated with CHI3L1 overexpression where tumors were rendered completely insensitive to Trastuzumab injections (**Figure 18b-d**). This was paralleled with higher CHI3L1 sera levels in these mice (**Figure 18e)**.

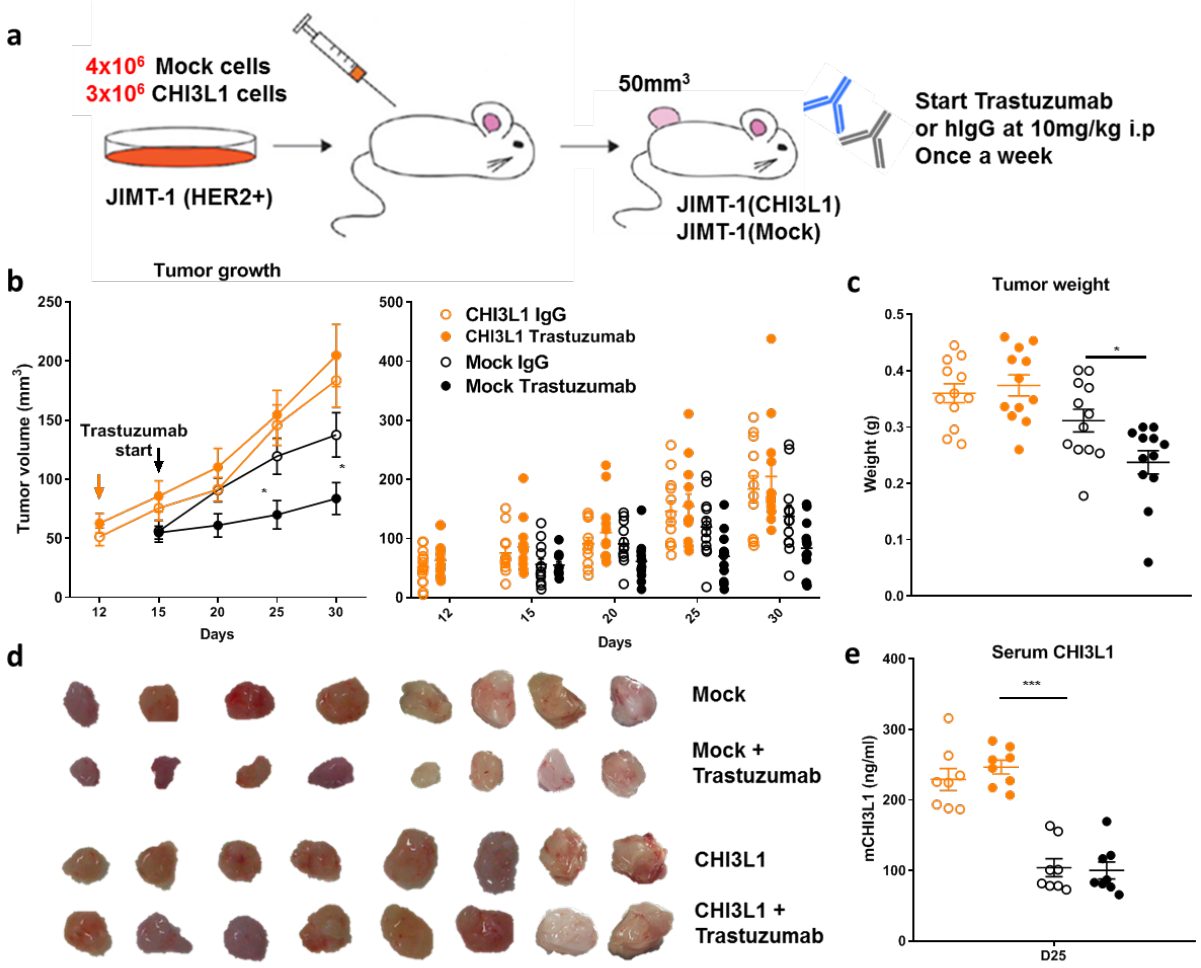


Figure 18: CHI3L1 overexpression abrogates the ability of Trastuzumab ADCC to control JIMT-1 tumors

(**a**) Experimental scheme. Tumor growth (**b**) and weight (**c**) in Trastuzumab or hIgG treated mice. (**d**) Representative images of tumors from different groups. (**e**) ELISA measurement of CHI3L1 sera levels. Data is pooled from two independent experiments (N=6 mice per group for each). Values represent the means ± SEM, 95% CI, *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant).

In line with the differences in Trastuzumab efficacy, mice with CHI3L1 tumors had decreased NK cell activation and cytotoxic parameters in the Blood (**Figure 19a**) and Spleen (**Figure 19b**).

In accordance, ex vivo ADCC assay using splenocytes against JIMT-1 targets showed a decreased ADCC of splenocytes isolated from mice with CHI3L1 overexpressing tumors but without differences in degranulation (**Figure 19c,d**).

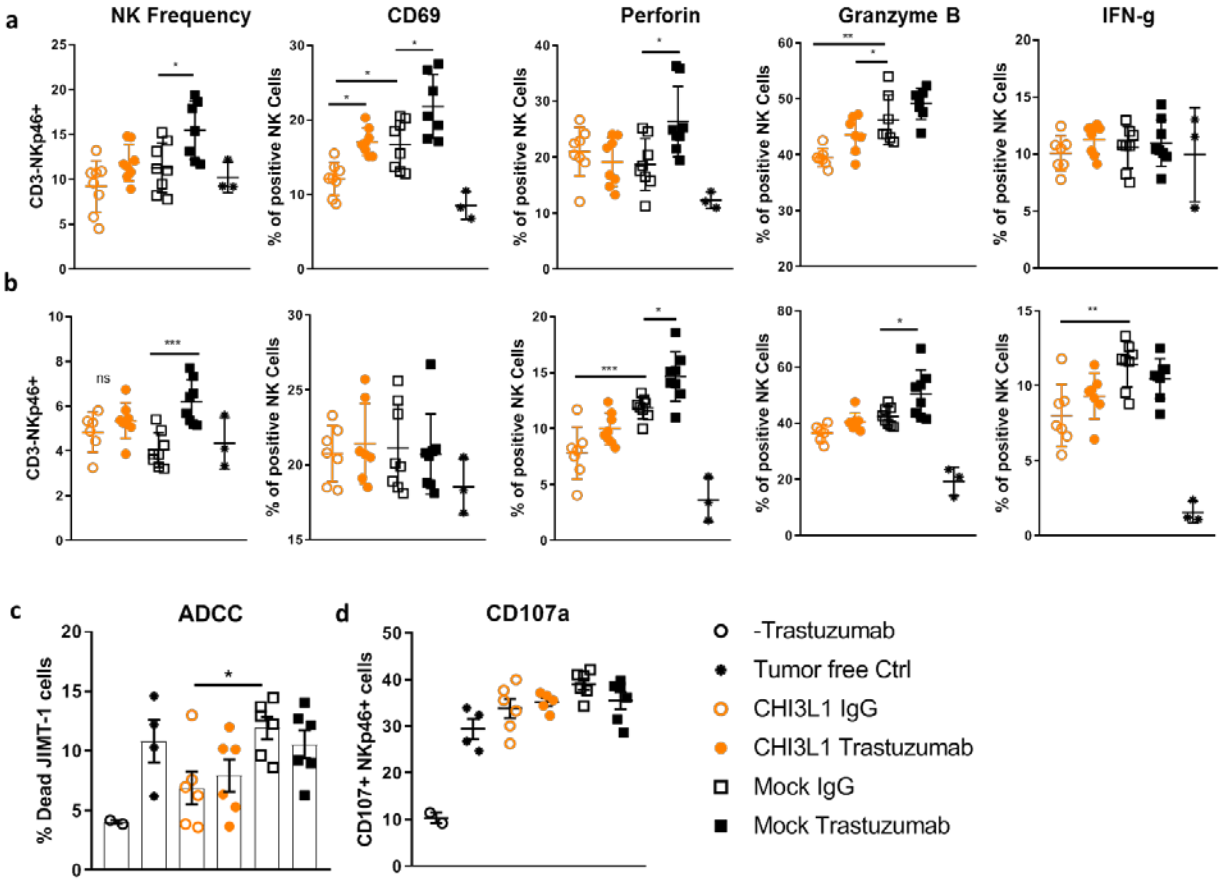


Figure 19: CHI3L1 overexpression dampens Trastuzumab induced NK cell activation and cytotoxicity ex-vivo

FACS anaylsis of NK cell frequency and cytotoxic parameteres in the blood (**a**) and spleens (**b**). (**c**) Ex-vivo ADCC assay with freshly isolated splenocytes against JIMT-1 cells (E:T 40:1). (**d**) Measurment of degranulation by CD107a staining from the ex-vivo cytotoxicity assay. Data representative of two independent experiments. Ex-vivo cytotoxicity of one experiment. Values represent the means ± SD, 95% CI, *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant).

CHI3L1 neutralizing antibody and Trastuzumab synergistically cure mice with HER2+ xenografts.

Our results so far established the inhibitory role of CHI3L1 on NK cell ADCC and as a direct consequence, its negative impact on Trastuzumab efficacy in patients and mice. This suggested that inhibiting this protein could potentially enhance Trastuzumab efficacy by restoring NK cells activity. To test this hypothesis we used the HCC1569 HER2+ BC tumor cells that endogenously express high levels of CHI3L1 (**Figure 20a**).

To reconstruct a relevant humanized therapeutic setting, we injected these cells in NSG mice and transferred human NK cells followed by treatment with Trastuzumab alone or in combination with anti-human CHI3L1 neutralizing antibody, or its isotype control (**Figure 20b**). We observed a partial tumor control by Trastuzumab alone, whereas NK cell transfer did not have any additional benefit (**Figure 20c,d**). However, when mice were treated with CHI3L1 neutralizing antibody, NK cell transfer in combination with Trastuzumab led to a complete regression of tumors (**Figure 20c,d**). Of note, NK cells persistence was low by the end of the experiment and was not affected by treatment (**Figure 20d**).

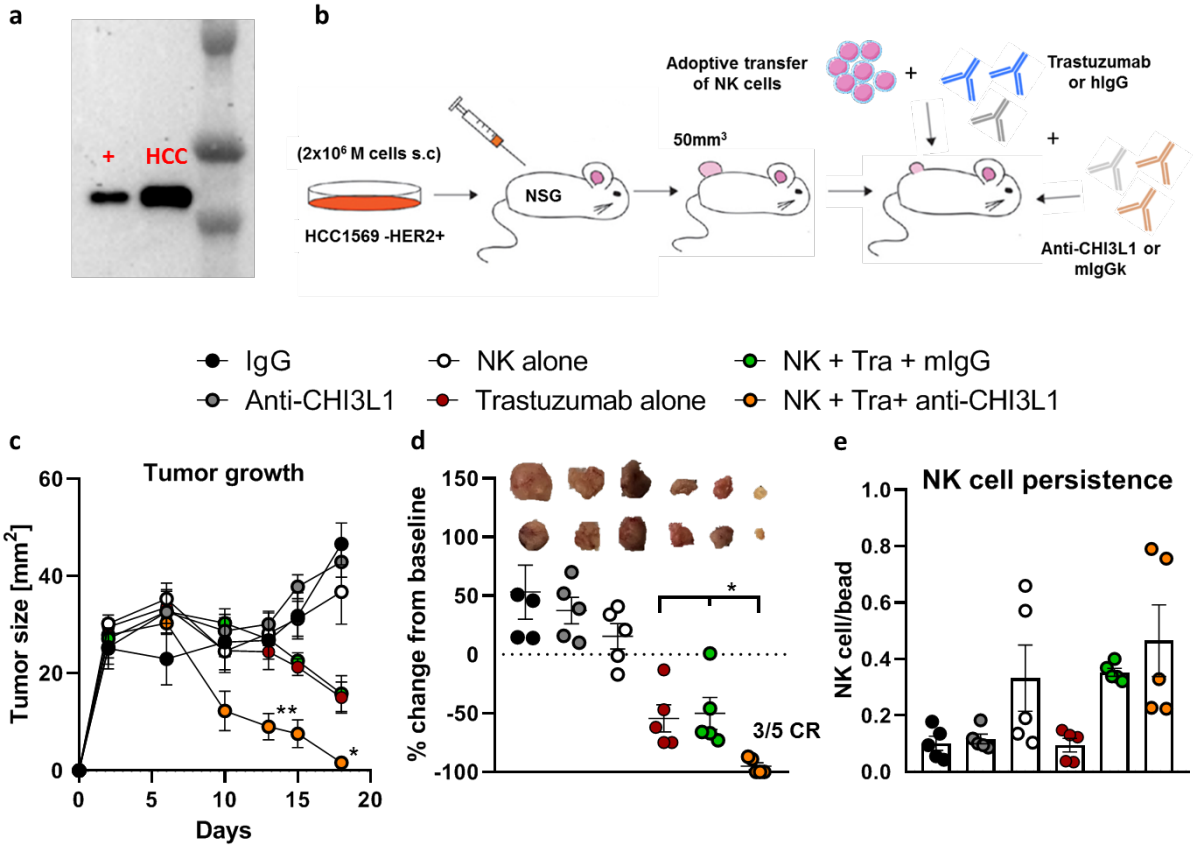


Figure 20: Neutralizing CHI3L1 boosts the efficacy of Trastuzumab by unleaching NK cells.
(**a**) Western blotting of CHI3L1 from supernatant of HCC1569 cells (+:rhCHI3L1). (**b**) HCC1569 cells were injected in the flank of NSG mice. Once tumors reached 50mm^3 mice were assigned into groups of 5 and 8x10^6 expanded human NK cells were transferred via the tail vein to some groups (D0). Anti-CHI3L1 treatment started on D-1 (200ug/mouse every two days) and Trastuzumab on D1 (200ug/mouse once a week) of NK cell transfer. Tumor growth (**c**) and change from baseline (**d**) of different treatment groups. In (**d**) two representative tumor images are shown above tumor size variation of each group (**e**) FACS analysis of spleens to check for CD56+ human NK cells. Values represent the means ± SEM, 95% CI, *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant).

These results demonstrate the therapeutic potential of targeting CHI3L1 to restore NK cell activity and boost Trastuzumab (ADCC) Efficacy in a robust preclinical model closely mimicking the human disease.

ANNEX I

Beyond Trastuzumab and HER2 breast cancer

CHI3L1 administration drastically increased the growth of NK cell-sensitive tumors

Besides ADCC, we also observed that CHI3L1 inhibited NK cell natural cytotoxicity in vitro. Hence we thought to investigate the effect of CHI3L1 on NK-mediated tumor surveillance in vivo. We used the RMA-S lymphoma model where tumor growth is largely controlled by NK cell activity. RMA-S cells are TAP2 deficient and thus are unable to express MHC-I molecules on their surface, making them susceptible to NK cell lysis in an NKG2D/perforin dependent mechanism (Ljunggren & Kärre, 1991; van den Broek, Kägi & Hengartner, 1995). We injected a limiting amount of cells that would allow us to reveal the effects of CHI3L1 on tumor growth (**Figure 21a**). Of note, RMA-S do not express CHI3L1 and their proliferation was not affected by treatment with the recombinant protein in vitro (**Figure 21b,c**).

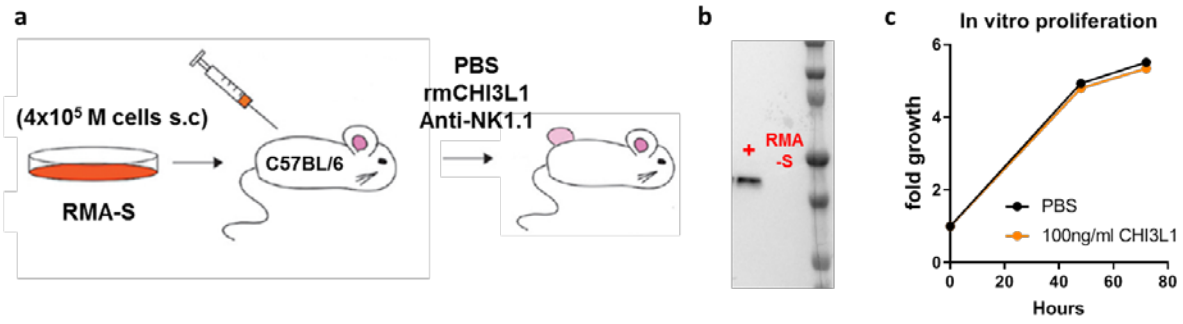


Figure 21: Experimental setup for the RMA-S NK-sensitive model.
(**a**) RMA-S lymphoma cells were injected s.c in the flank at a low number of cells ($4x10^5$) at D0. Mice were randomized in three groups to receive PBS, rmCHI3L1 (0.5ug/mouse i.p every other day starting D0), or anti-NK1.1 (150ug/mouse on D0, D2, D4). (**b**) Western blot for CHI3l1 detection in the supernatant of RMA-S cells (+:rmCHI3L1). (**c**) Cyquant measurement of in vitro proliferation of RMA-S cells treated or not with rmCHI3L1.

Conversely, Tumor take and tumor growth were markedly enhanced in CHI3L1 treated mice, to the extent of mice treated with anti-NK1.1 antibody for NK cell depletion (**Figure 22a-c**).

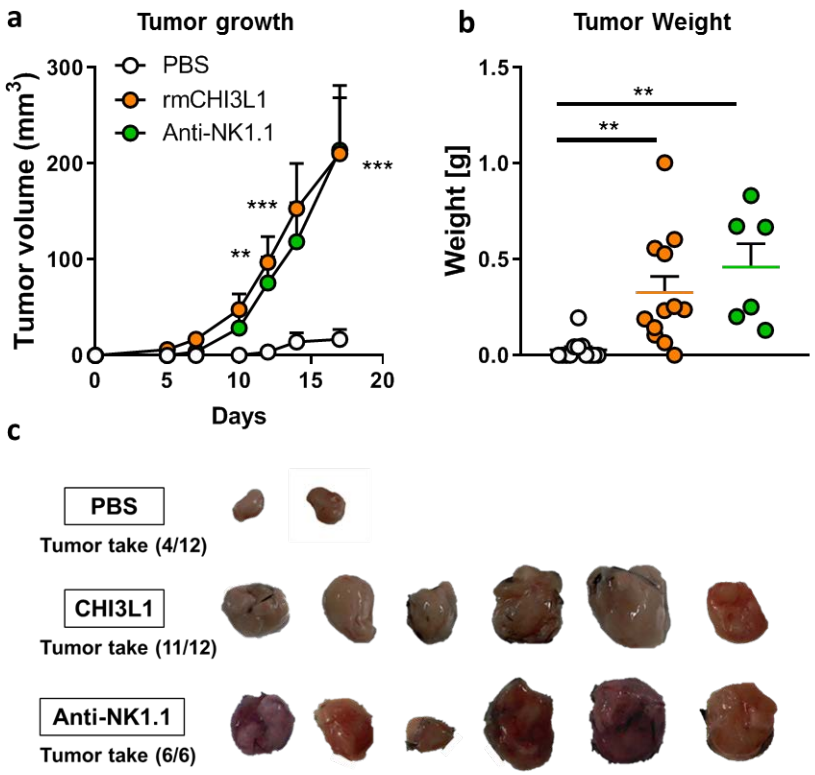


Figure 22: CHI3L1 injections increase tumor take and enhance the growth of RMA-S tumors. Measurements of Tumor growth (**a**) and weight (**b**). (**C**) Representative images of tumors from one of experiment. Data is pooled from two independent experiments for PBS and CHI3L1 groups and one experiment for NK1.1 treated group. Values represent the means ± SEM, 95% CI, *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant).

In line with the observed effect on tumor growth, splenocytes from CHI3L1 or NK cell depleted mice were less cytotoxic ex vivo against RMA-S cells (**Figure 23a**). However, we did not observe changes in NK cell activation or degranulation except for a decrease in DX5 expression (**Figure 23b,c**). This parallels our observation on human NK cells where CHI3L1 affects the killing machinery instead of activation parameters. This could be explained as well by the method of CHI3L1 administration (injection every other day) which allows for short-lived effects conversely to endogenous or genetic overexpression which maintains high CHI3L1 levels. Indeed, we found similar levels of CHI3L1 in the sera of PBS, CHI3L1, and NK1.1 treated mice (**Figure 23d**).

Overall, data from this model indicate that CHI3L1 is responsible for enhancing tumor growth at least in part by shutting NK-mediated tumor surveillance, especially where NK cell activity is highly required.

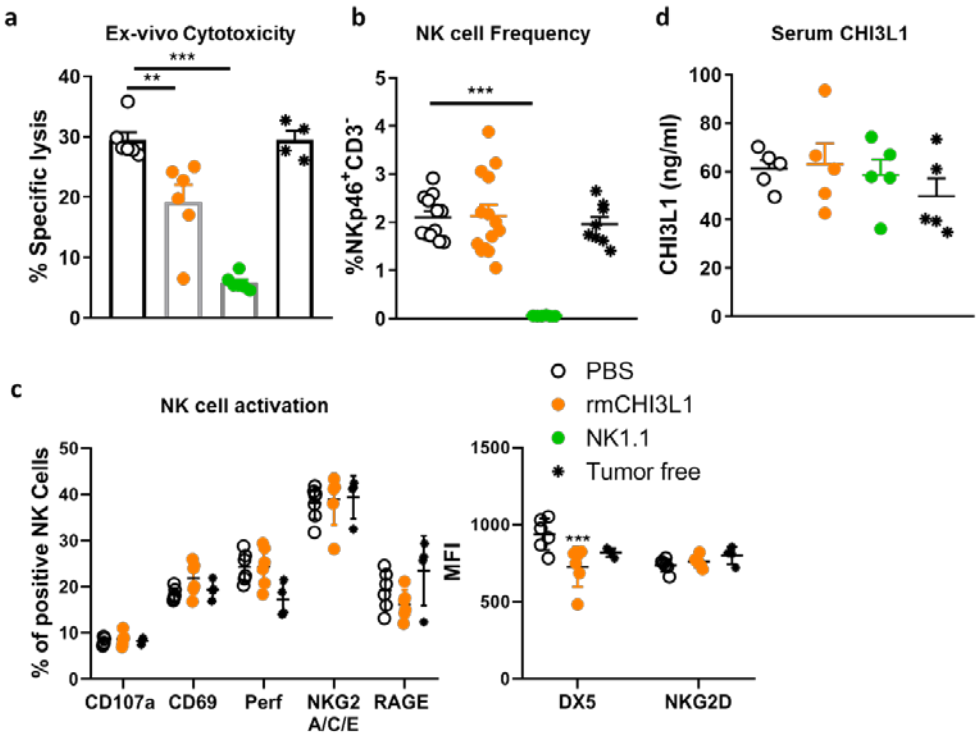


Figure 23: NK cell cytotoxicity but not activtion is inhibited by CHI3L1 injections.
(**a**) Ex-vivo cytotoxicity assay against RMA-S cells using splenocytes cultured O.N with 10ng/ml IL-15 and 100UI/ml IL-2 (E:T 40:1). FACS analysis of NK cell frequency (**b**) and NK cell cytotoxic parameters (**c**) from Spleens of mice from different treatment groups or tumor free controls. (d) ELISA measurement of CHI3l1 sera lebels.Values represent the means ± SEM, 95% CI, *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant).

Treatment with soluble RAGE enhances anti-tumor immunity and decreases tumor growth and metastasis in TNBC and Melanoma tumor models.

In our initial analysis of patient cohorts we found that CHI3L1 was expressed at the highest levels in TNBC among BC subtypes (**Figure 4**). This is in line with another report in the literature showing that CHI3L1 is among the top 10 upregulated genes in TNBC (Mansi Desai et al., 2019) Because CHI3L1 mediated inhibition of NK cells and CD8 T cells (see annex II) could be a general mechanism of immune escape in cancers with high CHI3L1 expression, we thought to extend our findings in HER2+ BC to TNBC. CHI3L1 has been shown to be involved in tumor growth and metastasis in the 4T1 TNBC model where it is endogenously expressed at high levels consistent with the human disease (Libreros *et al.*, 2012).

We wanted to assess if in line with our in vitro data, neutralizing CHI3L1 could enhance anti-tumor immune function. Unfortunately, a neutralizing antibody to the mouse CHI3L1 is not available. We used instead an Fc-silenced chimerized soluble form of RAGE (sRAGE-Fc) as a mean to neutralize CHI3L1 and to assess the involvement of RAGE in CHI3L1 mediated effects.

We followed primary tumor growth and lung metastasis of 4T1-Luc cells by bioluminescence in vivo imaging (**Figure 24a**).

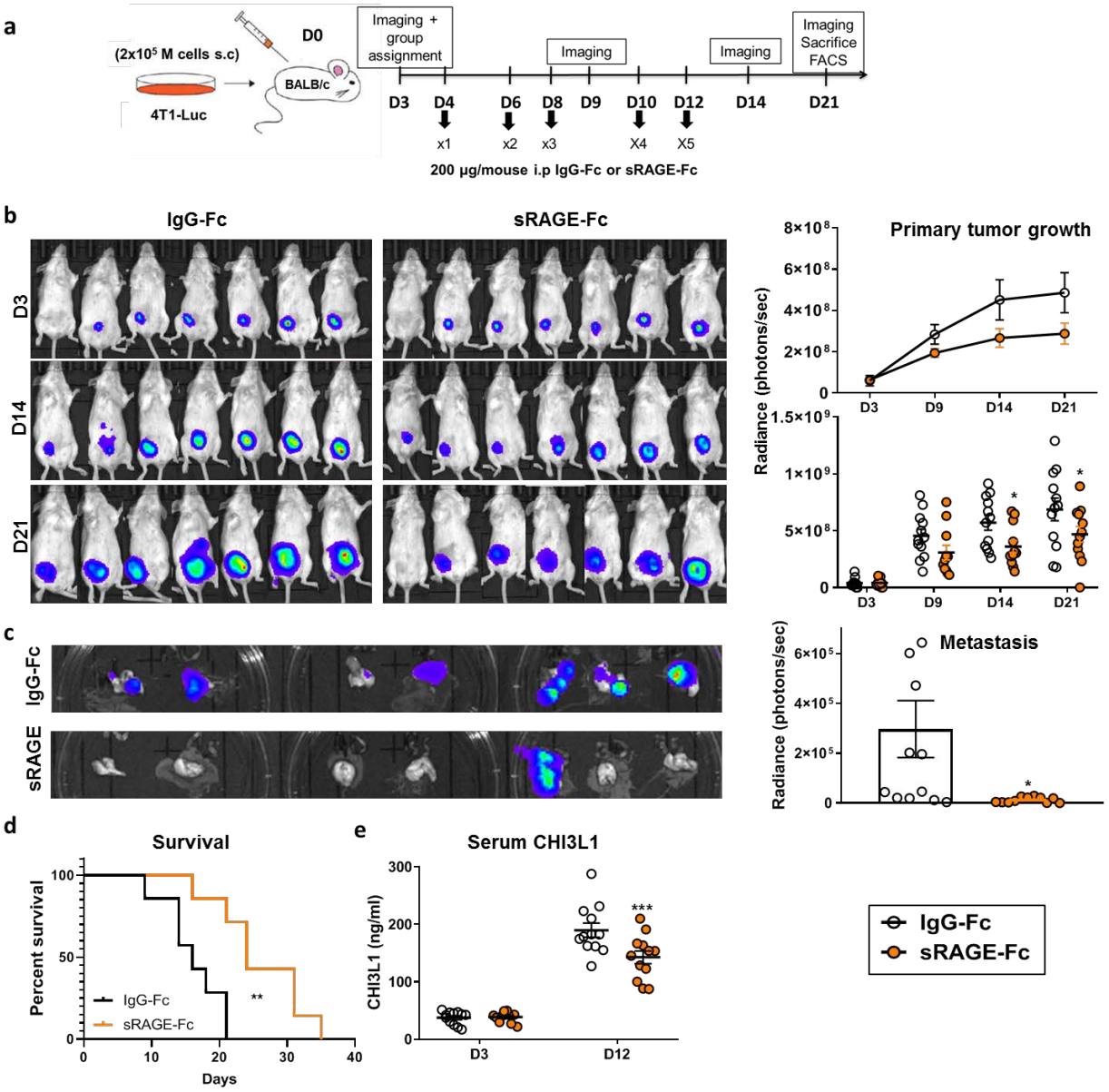


Figure 24: Neutralizing CHI3L1 with sRAGE injections enhances the immune response and decreases tumor growth and metastasis.

(**a**) Mice were injected with 4T1-luciferase tagged cells in the 4th mammary area and assigned into equal groups according to bioluminescence signal. Tumor bearing mice were treated with sRAGE-Fc or IgG-Fc (200ug/mouse x5 i.p injections) and analyzed for tumor growth and metastasis by in vivo imaging system. (**b**) Ventral image of IgG-Fc isotype or sRAGE-Fc treated tumor-bearing mice showing mice from one of two independent expriments and (Right panel) corresponding quantification of tumor-specific bioluminescence at D3, D14 and D21 after tumor inoculation (pool of two independent experiments, N=12 mice per group). (**C**) Ex vivo images of lungs from 4T1 tumor bearers, showing mice from one of two independent experiments and Quantification of bioluminescence (photons/sec) in lungs (pool of two independent experiments) (**d**) Survival of mice (N=7 mice per group) followed in a seperate experiment and scored as described in materials and methods. (**e**) ELISA measurement of CHI3L1 levels in the sera of mice at D3 (Before) and D14 (after) treatment initiation. Values in panels (**b**), (**c**) and (**e**) represent the means ± SEM (95% CI, *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant).

Injection of sRAGE decreased primary tumor growth and strikingly inhibited lung metastasis formation (**Figure 24b,c**). This correlated with longer survival and decreased CHI3L1 levels in sRAGE treated mice (**Figure 24d,e**).

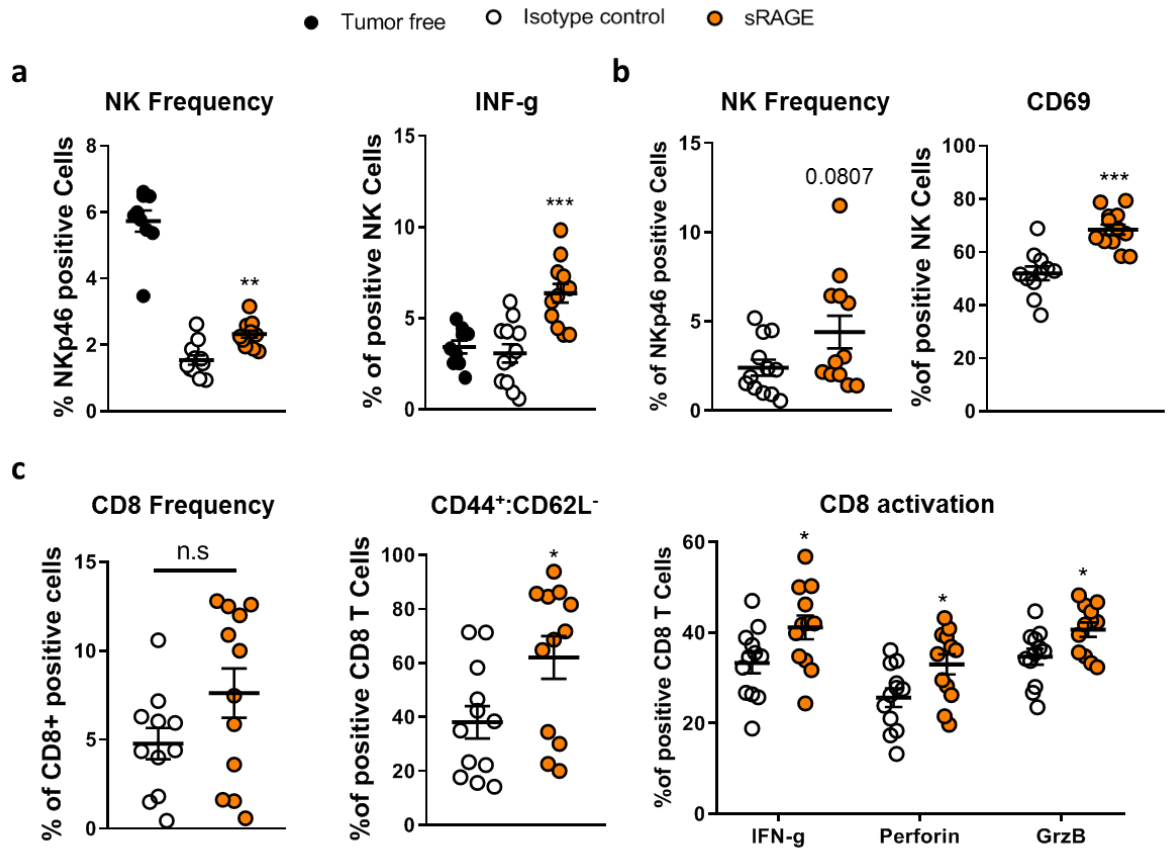


Figure 25: Improved NK cell and CD8 T cell function in sRAGE 4T1 tumor-bearing mice.
(**b**) and (**c**) FACS analysis of NK cells from spleens and tumors, respectively. (**d**) FACS analysis of CD8 T cells from tumors (shown are pooled results from two different experiments N=6 mice per group each). Values in all graphs represent the means ± SEM, (95% CI, *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant).

Flow cytometry analysis showed higher frequencies of NK cells that produced higher levels of INFγ in the spleens of sRAGE treated mice (**Figure 25a**). Although we did not observe a significant increase of tumor infiltration by NK cells and CD8 T cells, these cells were more activated as suggested by increased expression of CD69 on NK cells and marked increase of CD44+CD62L- T cells (**Figure 25b,c**). In line with this, tumor NK cells produced higher levels of INFg and CD8 T cells had higher levels of INFg, Perforin, and Granzyme B (**Figure 25c**). Altogether, these results show that CHI3L1 inhibition in vivo correlates with the activation of NK and CD8 T cells both systemically and intratumorally which translates in an impairment of tumor growth and metastasis.

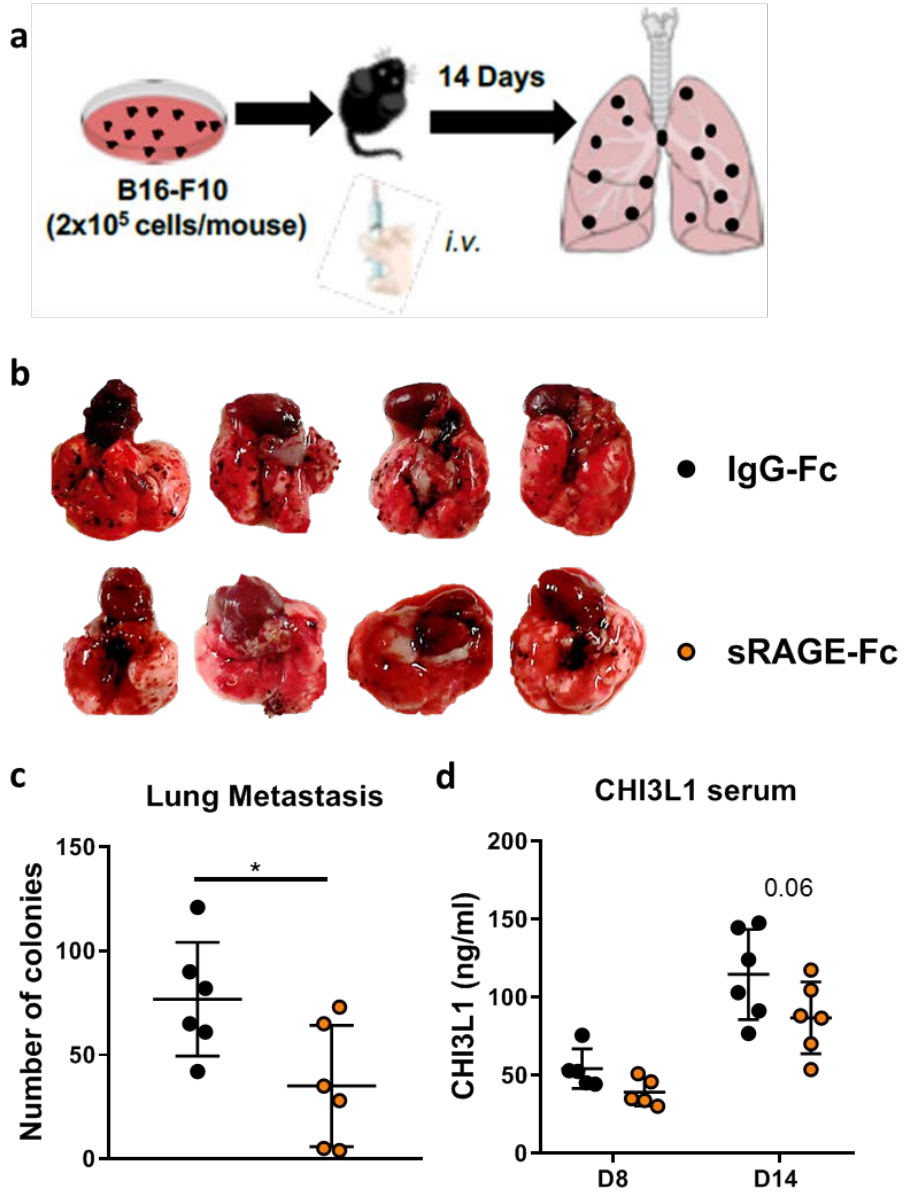


Figure 26: sRAGE-Fc injections decrease B16-F10 melanoma lung metastasis.
(**a**) Mice received B16-F10 cells i.v via the tail vein and were randomized at D0. Mice started receiving i.p injections of sRAGE or IgG-Fc (200ug/mouse every other day). Lungs were taken out at D14 and counted for metastatic colonies (black dots). (**b**) Representative images of lungs and (**c**) metastatic score. (**d**) ELISA measurement of CHI3L1 sera levels. Values represent the means ± SD, (95% CI, *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant).

Since we observed a pronounced reduction of lung metastasis in the 4T1 model by sRAGE injections, we thought to investigate if we would observe a similar effect in the B16F10 lung metastasis model. We chose this latter because it has been used to underline the key role of CHI3L1 in metastasis formation. Indeed, CHI3L1 or its receptor IL13Rα2 knockout mice are highly resistant to metastasis formation in the B16 model (C. H. He *et al.*, 2013). Interestingly, we observed that sRAGE competes with IL13Rα2 on CHI3L1 binding in competitive ELISA. So

it was plausible that this effect could be occurring in vivo as well, in addition to sRAGE mediated downmodulation of membrane-bound receptor signaling. We injected mice with B16F10 cells via the tail vein and started treatment with sRAGE-Fc or IgG-Fc every other day (**Figure 26a**). sRAGE treated mice had significantly less metastatic colonies in the lung (**Figure 26b,c**) and a trend towards lower CHI3L1 sera levels (**Figure 26d**). Similarly to the 4T1 model, NK cells and T cells were more activated in the spleen and lungs of sRAGE treated mice (**Figure 27**).

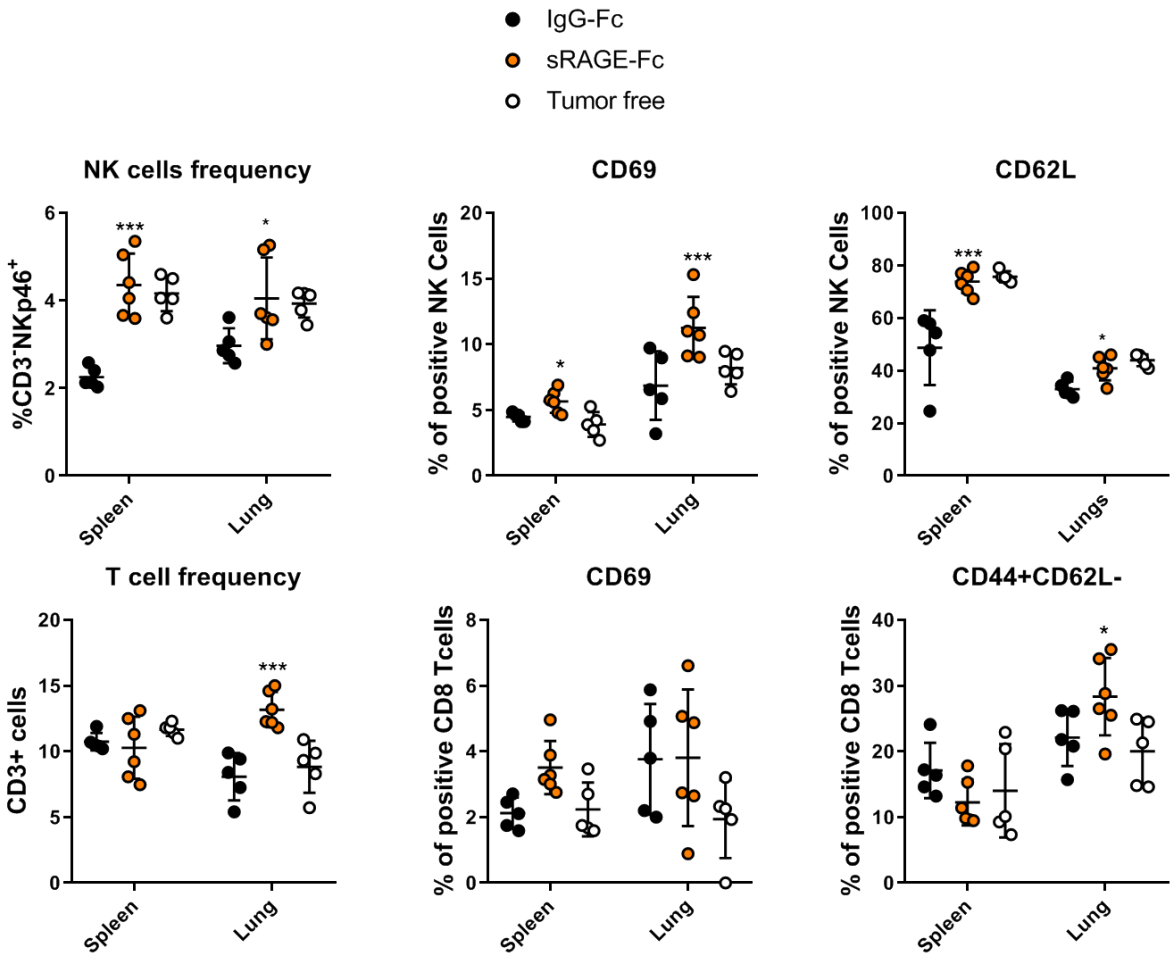


Figure 27: Increased activation and lung infiltration of effector cells in sRAGE treated mice. FACS analysis of NK cells (upper panels) and T cells (lower panels) from spleens and lungs of treatment groups or Tumor free controls. Values represent the means ± SD, (95% CI, *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant).

ANNEX II

Beyond NK cells: CHI3L1 modulation of T cell activity.

Early in our investigations of CHI3L1 effects, we found that recombinant CHI3L1 inhibited T cell-mediated cytotoxicity against MEC-1 cells loaded with super-antigen (MEC-1 is a B cell leukemia cell line used for antigen presentation) (**Figure 28a**). Similarly to NK cells, this was happening independently of the amount of degranulation (**Figure 28b,c**). We also found a prominent T cell enhancement in the 4T1 and B16 syngeneic models after inhibition of CHI3L1 with sRAGE-Fc.

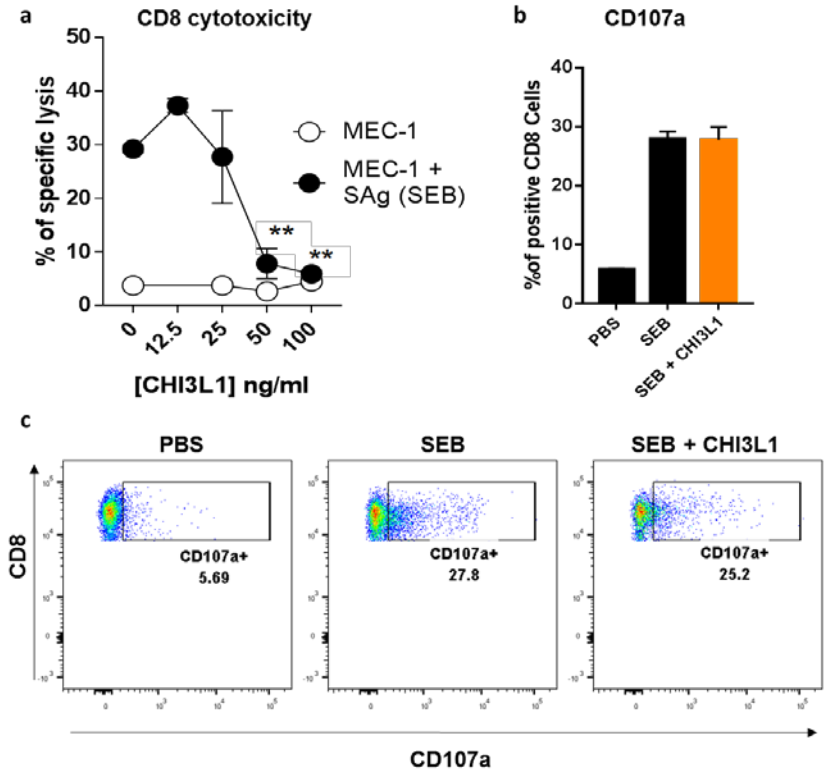


Figure 28: CHI3L1 inhibits the cytotoxicity of CTLs
(**a**) Specific lysis of MEC-1 cells loaded or not with Staphylococcus enterotoxin B (SEB) (2ug/ml) by healthy CD8 T cells treated with increased dose of rhCHI3L1 for 1h. (**b**) and (**c**) FACS analysis of degranulation after co-incubation of T cells treated or not with CHI3L1 and target cells. Plots are representative of two independent experiments using T cells from 4 healthy donors. Values represent the means ± SD, (95% CI, *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant).

More recently, Kim et al. found that murine T cells from CHI3L1 KO mice elicit strong Th1 cytotoxic responses to inhibit lung metastasis in the B16 model (Kim *et al.*, 2018). This was an eye-opening finding that highlights the role of intracellular CHI3L1 in regulating immune function, especially that we had previously found that also human NK cells and T cells expressed CHI3L1 (**Figure 29**).

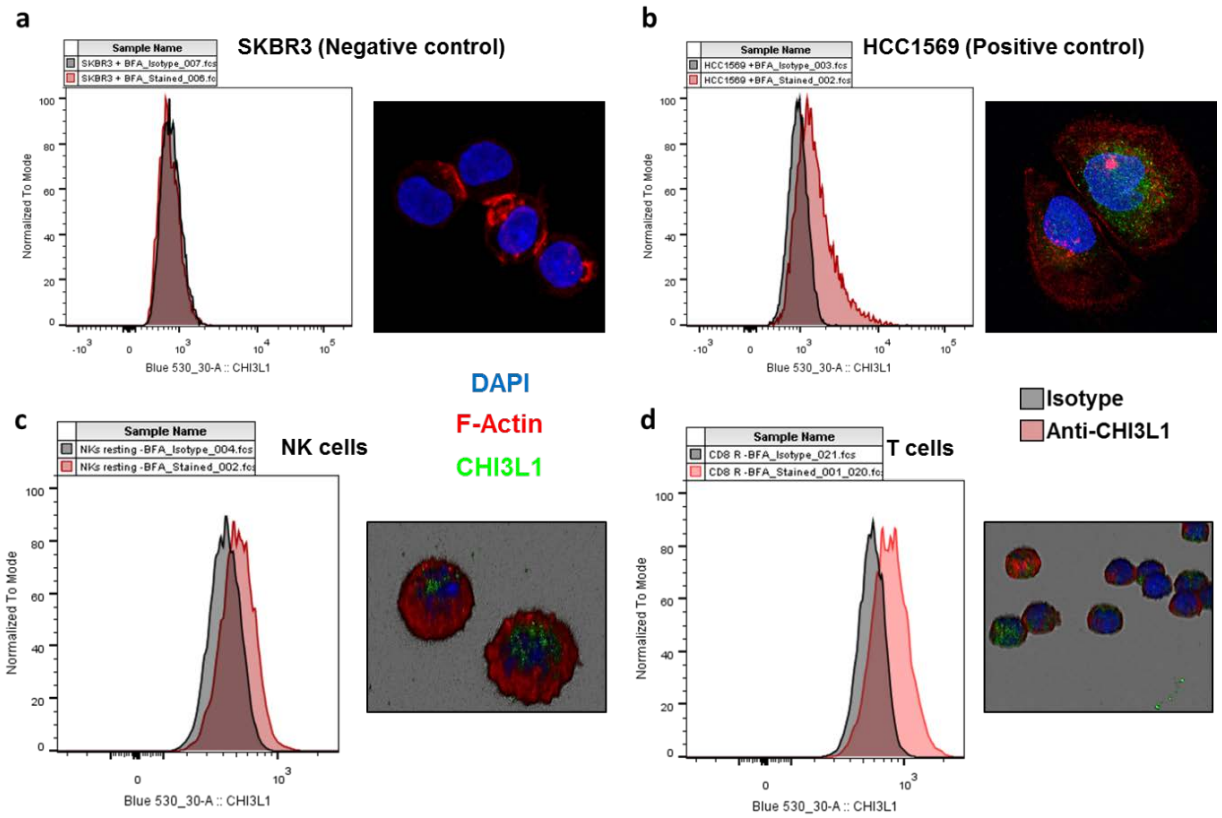


Figure 29: Human NK cells and CD8 T cells express CHI3L1 protein at low levels.
Flow cytometry and confocal analysis of CHI3L1 expression in SKBR3 cells (no expression), HCC1569 (high expression) and resting primary human NK cells and CD8 T cells (low expression).

As a breakthrough application of T cell-based immunotherapies, CAR-T cells showed great promise in blood cancers but their success has been limited in treating solid tumors. We thought to take advantage of modulating the CHI3L1 axis and to investigate whether CHI3L1 inhibition could boost CAR-T cell activity and potentially their efficacy to treat solid malignancies. We advised two different approaches to do this: (1) a genetic approach by CRISPR-Cas9 knockout of *chi3l1* and (2) a pharmacological approach by culturing CAR-T cells with a CHI3L1 neutralizing ligand. The optimization of CRISPR condition and selection of the best guide RNA is underway. In parallel, we produced in CHO cells the recombinant human IL13Rα2 (Extracellular domain-Fc chimerized) as a mean of neutralizing CHI3L1. The high affinity of this receptor (Kd=12pM) to CHI3L1 would ensure prolonged and efficient neutralization of CHI3L1. We began this study by transducing human T cells isolated from healthy donors with a HER2 specific CAR. We chose a HER2 CAR because this would allow us to employ the experimental methods we already developed for the Trastuzumab study. We first found that CHI3L1 expression increased upon transduction of T cells and culturing them with anti-CD3 and anti-CD28 activating antibodies (**Figure 30a**). Similarly, early activation

markers such as CD69, PD-1, and TIM-3 were induced upon transduction (**Figure 30b**). Interestingly, CAR-T cells cultured with IL13Rα2 showed a higher increase of CD69 and PD-1 expression (**Figure 30b**). Next, we wanted to see if this reflects into a better functionality of these cells so we measured their cytotoxicity against the HCC1569 HER2+ positive cells using the Xcelligence system. We observed a marked increase in the killing kinetics of CAR-T cells cultured with IL13Rα2-Fc compared to those cultured with IgG-Fc (**Figure 30c**).

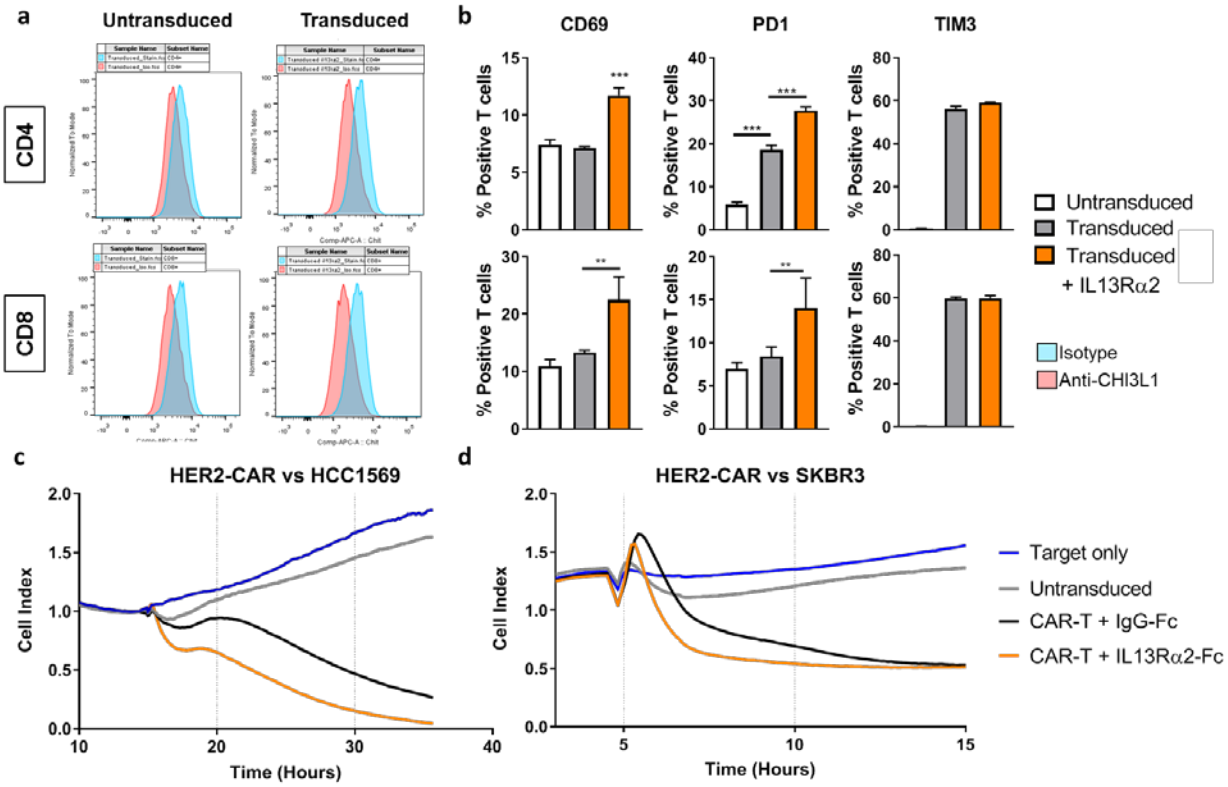


Figure 30: Modulation of CAR-T cells with IL13Rα2 boosts their cytotoxicity in vitro.
FACS analysis of the expression of CHI3L1 (**a**) and early activation markers (**b**) in CD4 and CD8 CAR-T cells cultured with IgG-Fc or IL13Rα2. Xcelligence assay to measure the killing kinetics of HCC1569 cells (**c**) and SKBR3 cells (**d**) by HER2-CAR T cells treated with IgG-Fc or IL13Rα2. Values represent the means ± SD, (95% CI, *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant).

To exclude the effect of exogenous CHI3L1 secreted by HCC1569 cells on the observed phenotype and to limit what we see to the neutralization of CHI3L1 secreted by T cells themselves, we used the SKBR3 cells as targets that do not express CHI3L1. We observed a similar enhancement in the killing kinetics with IL13Rα2-Fc in this setting as well, albeit less pronounced than with HCC1569 targets (**Figure 30d**). This highlights the cooperative role of exogenous (tumor or microenvironment secreted) and endogenous (T cell-secreted) CHI3L1 in regulating T cell activity.

We were next interested to see if these findings could be translated into a therapeutic mouse model, namely the HCC1569 HER2-BC model we used for the Trastuzumab study. We treated NSG tumor-bearing mice with a single dose of HER2 specific CAR-T cells alone or in combination with IL13Rα2-Fc or IgG-Fc (**Figure 31a**).

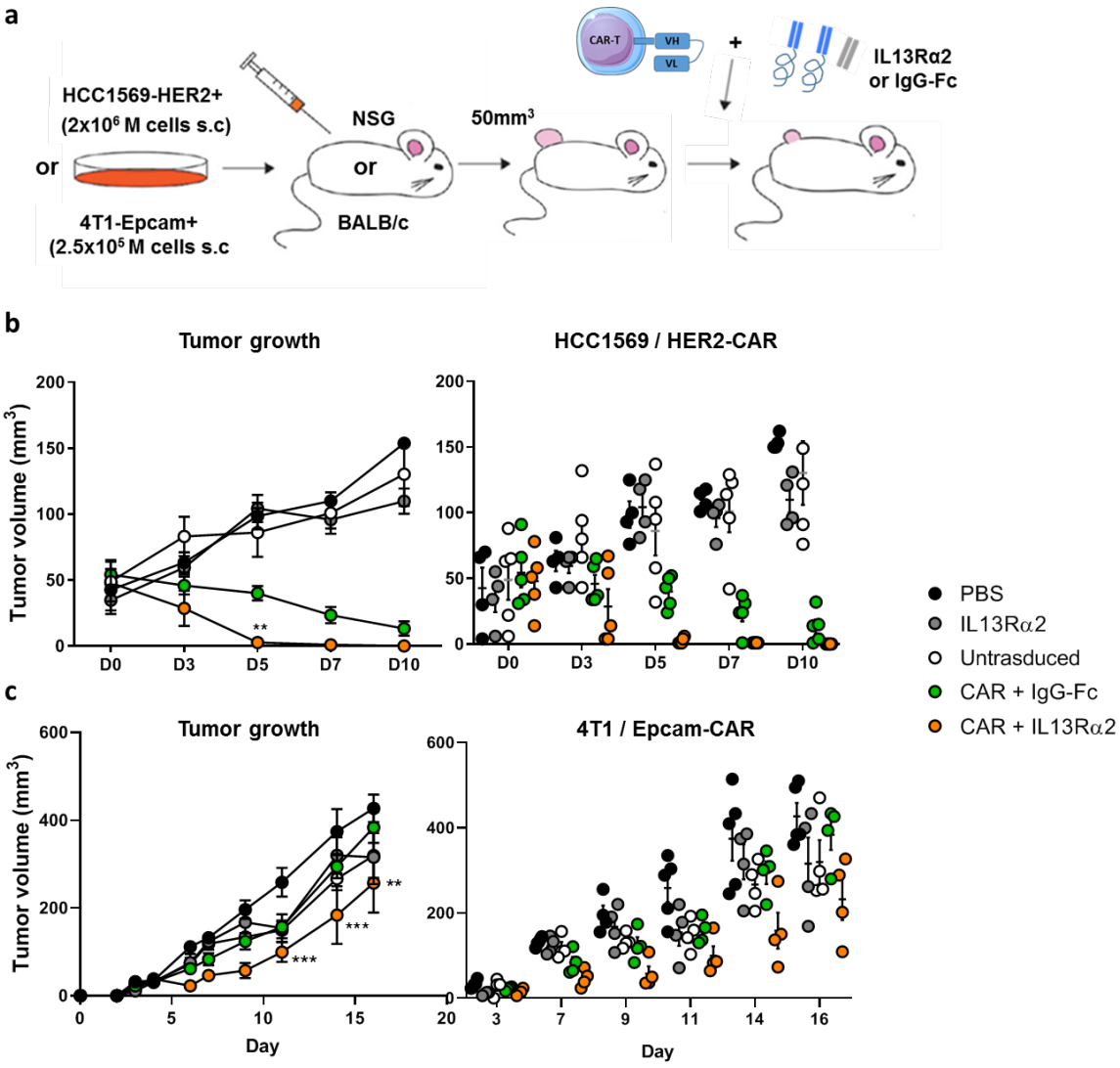


Figure 31: IL13Rα2 enhances the efficacy of CAR-T cells in controlling solid tumors.
(**a**) HCC1569 or 4T1-Epcam tumor bearing mice were treated with a single infusion of HER2 ($8x10^6$) and Epcam ($5x10^6$) specific CAR-T cells, respectively, alone or in combination with IL13Rα2. Growth measurements of HCC1569 tumors (D0=start of treatment) (**b**) and 4T1-Epcam tumors (**c**). Experiments were done once with 5 mice per group. Values represent the means ± SEM, (95% CI, *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant).

CAR-T cells with IgG-Fc were effective in controlling tumor growth and exhibited a steady decrease in tumor size (**Figure 31b**). Meanwhile, we observed a striking effect of CAR-T cell infusion in mice treated with IL13Rα2-Fc, where tumors completely regressed at day 5 after CAR-T cell transfer (**Figure 31b**). Importantly, IL13Rα2-Fc alone did not affect tumor growth

suggesting that the observed effect is dependent on the enhancement of CAR-T cell activity. As all this work was done with one type of CAR-T cells (HER2-CAR) we wanted to verify that this effect can be extended to other CAR types in other cancers, namely syngeneic ones. So we chose to use the 4T1 model we previously established as a highly immunosuppressive and challenging model for CAR-T cell efficacy. This time we used 4T1-Epcam overexpressing cells and Epcam specific murine CAR-T cells to target them. IL13Rα2-Fc alone and CAR-T cells alone had limited efficacy in this model (**Figure 31c**). However, the combination of both therapeutics significantly ameliorated tumor control especially at earlier time points after CAR-T cells infusion (**Figure 31c**).

Altogether these results show that CHI3L1 inhibition could be a promising approach for enhancing CAR-T cell efficacy for the treatment of solid tumors.

This is currently being investigated.

ANNEX III

CHI3L1 in NK-92 cells: at the interface between immune and tumor identity

While trying to study the mechanism of action by which CHI3L1 was inhibiting NK cells, we decided to use the NK-92 cell line as a more homogenous and accessible tool than primary NK cells. This cell line is derived from the PBMCs of a patient with non-Hodgkin's lymphoma and is dependent on IL-2 in culture. Apart from being a model cell line for NK cell studies, the NK-92 cell line is a valuable tool for off the shelf NK cell-based immunotherapies such as CAR-NK cells (Zhang *et al.*, 2017). Indeed, different variants have been generated that are IL-2 sufficient or equipped with CD16 for mediating ADCC.

We began by characterizing the CHI3L1-RAGE axis in this cell line. Unexpectedly, we found that NK-92 cells expressed high levels of CHI3L1 and were not inhibited by treatment with the recombinant protein or with a CHI3L1 neutralizing antibody (**Figure 32a,b**). On the other hand, RAGE blockade markedly decreased their cytotoxicity against K562 targets (**Figure 32b**). As CHI3L1 was shown to regulate the survival and proliferation of other "tumor" cells through RAGE (Low *et al.*, 2015), we hypothesized that it might play a similar role in NK-92 cells. To verify this, we cultured them for 48 hours (versus overnight for cytotoxicity treatment) with recombinant CHI3L1, its neutralizing antibody, or anti-RAGE blocking antibody. Surprisingly, we found that the addition of CHI3L1 induced a massive proliferation of NK-92 cells while neutralizing it had a slight but non-significant effect on survival (**Figure 32c-e**). On the other hand, anti-RAGE antibody blocked the survival and induced apoptosis of these cells (**Figure 32c,e**). These intriguing findings led us to study the signaling pathways induced by CHI3L1 in these cells. Paradoxically, we found that both, CHI3L1 and anti-RAGE induced a strong activation of JNK phosphorylation while anti-CHI3L1 induced Akt activation (**Figure 31f**). ERK1/2 signaling was not involved in the CHI3L1-RAGE axis.

Whether these results are specific for the NK-92 cell line because of its malignant identity or would be applied to highly expanded primary NK cells remains to be answered. Nonetheless, these results add another layer of complexity to the CHI3L1-immune interaction by regulating survival and proliferation. At the same time, they offer new insights for harnessing the biology of this protein in NK cell-based immunotherapies.

This, as well as the implication and limitation of the results of my thesis work, will be addressed next in the discussion section.

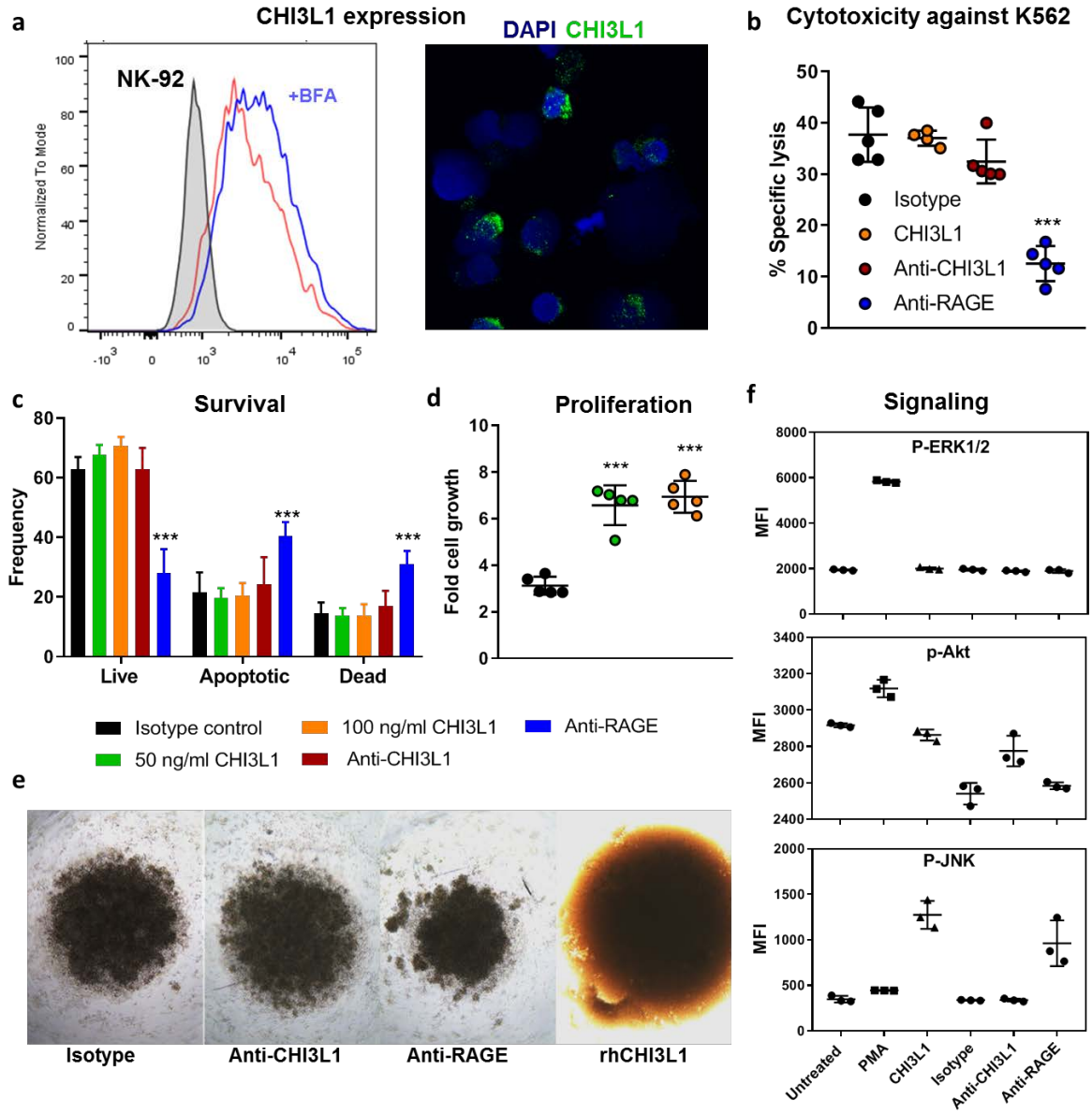


Figure 31: CHI3L1 regulates the survival and proliferation of NK-92 cells through RAGE.
(a) CHI3l1 expression by FACS and confocal imaging in NK-92 cells treated or not with BrefeldinA (BFA) to inhibit protein secretion. (b) Specific lysis of K562 targets by NK-92 cells treated O.N with CHI3L1, anti-CHI3L1, or anti-RAGE (5:1 E:T). (c) Annexin PI stainig to study the effect of different treatments (for 48h) on the survival of NK-92 cells. (d) Cyquant measurment of the effect of CHI3L1 on proliferation. (e) Representative images of NK-92 culture in 96well plate showing the effect of different treatments. (f) Phosphoflow cytomtery analysis of some signaling modules dowstream of RAGE. Each plot is representative of at least two different repetitions. Values represent the means ± SD, (95% CI, *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant).

Chapter 5: Discussion and future perspectives

This project aimed to identify mechanisms of tumor escape from Trastuzumab mediated ADCC in HER2 BC. For this purpose, we looked for molecules that could explain why NK cells, main effectors of ADCC, are inactivated by sera of Trastuzumab resistant patients. That is how we identified CHI3L1, as its levels were increased in the sera of non-responders from two different patient cohorts. This protein belongs to the evolutionary conserved family of Chitinases which are essential components of the ecosystem in lower organisms. Through evolution, most human Chitinases have lost their enzymatic function while retaining their ability to bind chitin and thus are called Chitinase-like proteins (CLPs). This highlights an important function of these proteins that has only begun to be elucidated recently. Major Interest in the biology of CLPs was brought to light by the infamous CHI3L1 protein as dysregulation of its expression has been associated with an array of human pathologies including cancer. Studies using different tumor models have shown that CHI3L1 overexpression leads to increased tumor growth and metastasis by inducing angiogenesis, proliferation, and resistance to apoptosis (Libreros *et al*., 2015). In parallel, CHI3L1 has been shown to promote a type 2 immune response which is known to contribute to tumor development. Indeed, studies focusing on lung diseases showed that CHI3L1 is highly increased in Th2 inflammatory conditions and is induced by OVA/Alum, house dust mite (HDM), and in IL-13 transgenic mice (Lee *et al.*, 2009). In line, CHI3L1 KO mice show reduced Th2 inflammation, and overexpression of YKL-40 reverses this phenotype. In particular, IL-13 production by T cells in the lungs is significantly reduced in Chi3L1 KO mice (Lee *et al.*, 2009). However, IFNγ, Perforin, and Granzyme B levels are significantly increased in T cells by CHI3L1 deficiency. In this context, a specific ablation of CHI3L1 from murine T cells was shown to elicit a strong Th1 response able to control lung metastasis (Kim *et al.*, 2018). Although these studies identified CHI3L1 as a significant immune modulator, a direct effect of this protein on cytotoxic cells such as NK cells in cancer patients has not been characterized. Moreover, mechanistic insights on how it might interact with these cells are still lacking.

Here we show by a series of in vitro and mice experiments the direct involvement of CHI3L1 in inhibiting NK cell ADCC. We also identified the paralysis of the NK lytic machinery as the mechanism by which CHI3L1 impairs ADCC. IL13Rα2 is a central receptor for CHI3L1 and mediates a broad range of its effects, however, we observed only an intracellular expression

of this receptor in human NK and T cells. This excluded a possible involvement of this receptor in the activity of exogenous CHI3L1 and suggested a potential role by binding the intracellular protein which we also found to be produced by both cell types. On the other hand, we observed that RAGE, another receptor of CHI3L1 is expressed at good levels on NK cells. There are two reports on RAGE function in NK cells to date, both highlighting an activating function of this receptor (Narumi *et al.*, 2015; Parodi *et al.*, 2015). Precisely, RAGE was shown to induce NK cell cytotoxicity and chemotaxis by binding S100A8/A9 and HMGB1, respectively. Here we show that CHI3L1, behaves like a blocking agent for RAGE, and inhibits its downstream JNK signaling which is a key event for MTOC polarization. Interestingly, Parodi et al. showed that several cytoskeletal or cytoskeleton-associated proteins were upregulated following RAGE ligation by HMGB1 in NK cells (Parodi *et al.*, 2015). In line, we found that RAGE accumulated at the immune synapse which is characterized by major cytoskeletal rearrangement needed for the correct delivery of lytic granules into target cells. Altogether, these results highlight a new function of RAGE receptor in controlling, the cytoskeleton and granules biology in NK cells and consequently their migration and cytotoxicity. This is relevant considering that Diaphonous-1 (Dia-1), the signaling adaptor of RAGE, directly interacts with, and regulates F-actin and tubulin structures (Bershadsky *et al.*, 2006). Importantly, the loss of DIA-1 in NK cells impairs MTOC polarization but without affecting synapse formation and F-actin accumulation (Butler and Cooper, 2009). This closely parallels our observation on CHI3L1 mediated effects through RAGE. We conducted preliminary experiments in which we observed DIA-1 accumulation at the synapse during NK cells cytotoxicity, a process that is decreased by CHI3L1 treatment. Of note, some donors seem to be refractory to the activity of CHI3L1, but its inhibitory activity is restored if NK cells are cultured with IL-2/IL-15. This observation could be explained by the upregulation of RAGE receptor and JNK signaling in preactivated NK cells thus decreasing the threshold of sensitivity to CHI3L1 inhibitory effects.

To support our findings in vitro, we conducted two xenograft studies of HER2 BC. In the first model, CHI3L1 overexpression rendered JIMT-1 tumors insensitive to Trastuzumab treatment. This indicated a dysfunction of NK cells in mice bearing CHI3L1-overexpressing tumors as Trastuzumab efficacy is fully dependent on NK cell ADCC in this model (Barok *et al.*, 2007). This was confirmed by ex-vivo ADCC assay. Next, we established a HER2 therapeutic model that would allow us to assess the potential of combining CHI3L1 blockade with Trastuzumab. In particular, we used the HCC1569 cell line endogenously expressing high

levels of CHI3L1. Intriguingly, these cells were derived from a resistant patient although they still respond partially to Trastuzumab mediated HER2 inhibition in vitro. This argues for the real reason as to why this patient was resistant to Trastuzumab (Partial responsiveness to HER2 inhibition or CHI3L1 overexpression?). To reconstruct a relevant humanized therapeutic setting, we injected these cells in NSG mice followed by the transfer of human NK cells. The presence of NK cells did not have any additional benefit to Trastuzumab alone suggesting that NK cells are inhibited in these mice with a CHI3L1 rich environment. Only when mice were treated with a CHI3L1 neutralizing antibody, NK cell transfer in combination with Trastuzumab led to a complete regression of tumors. These results demonstrate that inhibiting CHI3L1 can restore NK cell activity and boost Trastuzumab (ADCC) efficacy in a robust preclinical model closely mimicking the human disease. Very recently, the first two studies associating HER2 BC with CHI3L1 were published. One group showed that CHI3L1 facilitates migration and invasion of HER2 tumor cells by stimulating EMT (Morera *et al.*, 2019). The other showed that CHI3L1 is overexpressed in cerebrospinal fluid of patients experiencing breast to brain metastasis (most frequent site in HER2 BC) and inhibiting it impairs this process (Ansari *et al.*, 2020). These and our findings come many years after discovering the murine CHI3L1/BRP-39 protein as one of the most overexpressed proteins in tumor cells derived from HER2/neu oncogene activation (Morrison and Leder, 1994). In the same context, our effort is one of many that have identified potential mechanisms of resistance to HER2 targeted therapy. Whereas most of these were focused on tumor cells (e.g mutations in HER2 signaling) our approach focused on ADCC which has been extensively shown to be a major determinant of this antibody's efficacy. Indeed, the promising results obtained by Margetuximab (ADCC-enhanced version of Trastuzumab) (Rugo *et al.*, 2019), and by Cetuximab in combination with the anti-NKG2A Monalizumab (Cohen *et al.*, 2019), argue in favor of boosting NK cell activity to increase patients' response. Interestingly, in some patients higher CHI3L1 levels preceded the clinical diagnosis of progression although there was no statistically significant difference between basal levels of responders and non-responders owing to the small sample size. This does not mean that CHI3L1 would be a good biomarker for HER2 BC because of its association with other cancer types and many other inflammatory diseases. Indeed, one patient in our cohorts that was on remission from HER2 BC presented with elevated levels of CHI3L1 because of an underlying comorbidity, Essential Thrombocythemia, a rare type of blood cancer previously associated with elevated CHI3L1 levels (Andersen *et al.*, 2014). Nonetheless, the evolution of

CHI3L1 levels could be used to monitor clinical response within the same patient and this warrants investigation in larger patient cohorts.

Beyond ADCC, we also found that CHI3L1 could inhibit the natural cytotoxicity of NK and T cells both of which are dependent on the lytic granules machinery. This suggested a broader activity of this protein on the effector cytotoxic response, which was apparent by the drastic increase in tumor take and tumor size in the RMA-S NK sensitive model as a result of CHI3L1 injections. The similarity of this result to that obtained by NK cell depletion, and the absence of effect of CHI3L1 on RMA-S proliferation in vitro, would argue that the phenotype we observed is due to NK cell inhibition. Yet, we cannot completely exclude other direct effects of CHI3L1 on tumor cells in this model. Similarly, in the previously discussed JIMT-1 model, CHI3L1 overexpression per se led to a marked increase in tumor size in inbred and outbred nude mice despite not affecting cell proliferation in vitro. These findings are reminiscent of the well documented pro-tumorigenic properties of CHI3L1 (angiogenesis for example (Francescone *et al.*, 2011)) to which we would add the cooperation of its immunosuppressive effects. Indeed in outbred mice (more hostile to xenografts), we could appreciate the immune phenotype as mock tumors regressed but the CHI3L1 overexpressing ones continued to grow concomitantly with a decreased NK cell activation. Thus, based on data from these models, it is safe to say that CHI3L1 mediates its effects at least in part by shutting down NK-mediated immune surveillance, especially where NK activity is highly required.

Concerning T cells, we faced two additional layers of complexity, receptor expression, and intracellular derived CHI3L1. First, CHI3L1 inhibited CD8 T cell cytotoxicity via RAGE independent mechanism as we couldn't detect its expression on resting or briefly activated CD8 T cells in accordance with previous studies (Durning *et al.*, 2016). We cannot exclude that in the tumor microenvironment T cells may upregulate RAGE, as we did observe its expression on cytokine induce killer (CIK) T cells. Thus, the involvement of other receptors in mediating the effects of exogenous (tumor-derived) CHI3L1 on CD8 cytotoxicity remains to be investigated. As previously mentioned, IL13Rα2 is also not expressed on the surface of T cells but in the cytoplasm. Accordingly, it might be contributing to the effects of Intracellular CHI3L1 which we found to be expressed by NK and T cells. In this context, Kim et al. showed that murine T cells from CHI3L1 KO mice elicit strong Th1 cytotoxic responses to inhibit lung metastasis in the B16 model (Kim *et al.*, 2018). We tried to build upon this finding and our findings, and use CHI3L1 inhibition in combination with CAR-T cell therapy for solid tumors,

where their efficacy remains limited. For this purpose, we produced the soluble IL13Rα2-Fc that would bind the CHI3L1 produced by T cells with high affinity and neutralize it. We found that combining CAR-T cells, either the human HER2 specific or the murine Epcam specific, with IL13Rα2 led to improved killing kinetics in vitro which was translated in better tumor control, up to complete remission of HER2 xenografts. Altogether these encouraging results lay the foundation for exploring the potential of targeting CHI3L1 to boost the efficacy of CAR-T cells in different types of solid tumors. Of note, such application would also be valid in settings without high levels of tumor-derived CHI3L1 as neutralizing the protein produced by T cells is sufficient for boosting their activity.

To sum up the previous findings on NK cells and T cells and to evaluate the benefit of targeting CHI3L1 in cancers other than HER2 BC, we set up two syngeneic models well known for their immunosuppressive effects and CHI3L1 involvement. In particular, we used the 4T1 (TNBC) and B16 (Melanoma) models and treated mice with soluble RAGE-Fc injections as a mean of neutralizing CHI3L1 activity by competing with CHI3L1 binding to the membrane bound receptor. Importantly, in both models, tumor cells are able to form lung metastasis which allowed us to assess NK cell activity as sentinels against metastasis formation. Interestingly, sRAGE injections decreased tumor growth and markedly reduced lung metastasis, concomitantly with enhanced NK, and T cell activation in both models. These findings point towards a strong protumorigenic and metastatic role of CHI3L1 via controlling immune cell cytotoxicity. Arguably, the effectiveness of RAGE treatment in controlling lung metastasis is potentially due to its broad binding and neutralizing avidity for other pro-metastatic molecules (Nasser *et al.*, 2015).

The previous point features the main limitation of our work whether in these models or the CAR-T cell models, as we relied on soluble receptors to neutralize CHI3L1. The receptors we produced and used, bind at least one other molecule (IL-13 for IL13Rα2) or multiple other ligands (in the case of RAGE). Nonetheless the high affinity of RAGE and an even higher affinity of IL13Rα2, concomitant with elevated CHI3L1 levels in mice, would favor the preferential binding of this protein. Within this limitation, our data still reveal the importance of regulating the signaling of membrane-bound RAGE by its soluble form as an innate control mechanism in cancer. This might explain why the decreased levels of sRAGE in multiple cancers, including BC, correlates with worse prognosis (Yan *et al.,* 2010).

To overcome the problem of specificity in the CAR-T cell setting we are currently optimizing a genetic approach of CRISPR-Cas9 Knockout of this protein out of T cells.

The final part of our ongoing work was focused on the NK-92 cell line. We initially aimed to use these cells as a tool to better understand the CHI3L1-RAGE axis in NK cells. However, we were surprised by the high expression of CHI3L1 in these cells which is reminiscent of their tumor identity. In this case, the addition of the recombinant protein did not affect the cytotoxicity of these cells but rather induced a strong increase in their proliferation. On the other hand, blocking RAGE impaired their cytotoxicity by limiting their survival and inducing apoptosis. Interestingly, CHI3L1 binding to RAGE was shown to be important for the pathogenesis of colitis-associated cancer by preventing apoptosis (Low *et al.*, 2015).

These findings can be exploited by using the recombinant protein to stimulate the expansion of NK-92 cells in culture as these cells constitute a valuable tool for off the shelf CAR-NK immunotherapy. Importantly, our data raises some concerns regarding the non-immune tumor identity of these cells as they secrete high CHI3L1 amounts and potentially other molecules that might help feed the tumors they are intended to treat. Thus, blocking CHI3L1 in combination with NK-92 adoptive therapy might be a way of increasing their efficacy in vivo.

In conclusion, our data demonstrate a new function of CHI3L1 as an immune checkpoint molecule that differently from the others, acts directly on the cytotoxic machinery which is fundamental to achieve tumor cell killing. The premise of targeting CHI3L1 will be to simultaneously hit an important molecule for tumor cells and to restore immune activation. Further, given its pronounced effect on metastasis, inhibiting this protein may also be proposed as adjuvant treatment in patients with minimal residual disease to avoid tumor cell spreading. Finally, a combination of CHI3L1 inhibition with Trastuzumab could offer new hope for resistant patients and increase the number of responders.

Future perspectives.

Continuous efforts to treat cancer have been translated into several breakthroughs that culminated in the use of monoclonal antibodies to checkpoint molecules or cellular therapies. Yet, metastatic cancer remains mostly incurable because of immune-escape mechanisms. Thus, a combinatorial approach would be crucial for durable responses. We believe based on our data that CHI3L1 is a valuable target and inhibiting it would constitute an important step forward in overcoming or preventing resistance to different therapies. Thanks to our findings and those of others, we devised that optimal targeting of this molecule should consider two important properties: the inhibition of its binding to different receptors, including IL13Rα2 and RAGE (1) and its extracellular and intracellular functions (2). Importantly, any approach targeting the intracellular protein shouldn't neglect its potential effect on lymphocyte survival. Based on this, we already performed in silico characterization of the optimal target epitope within CHI3L1/YKL-40 (**Figure 32**). We have since started an immunization campaign using an engineered mouse platform that would allow us to generate a fully human antibody fit for clinical development. The best candidate antibody will be used to uncover the full potential of targeting CHI3L1 in different applications (ADCC, ICB, CAR-T cells, vaccination, or NK-92-CARs). Such antibody could also be used in diseases beyond cancer where this protein plays a proven pathogenic role (e.g severe asthma, lung inflammation, and fibrosis). Following the opposite logic, the administration of recombinant CHI3L1 per se could be explored in settings requiring the stimulation of immune tolerance such as autoimmunity or GVHD.

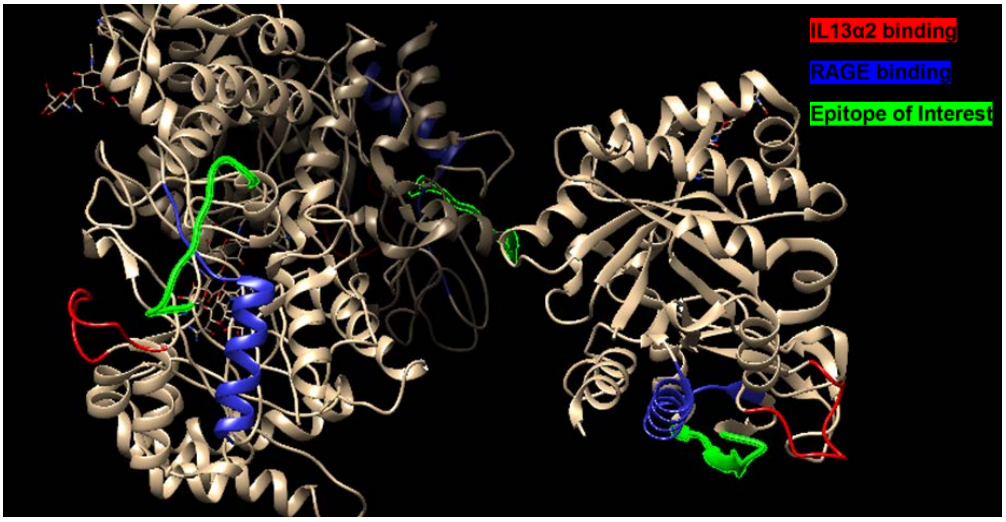


Figure 32: Epitopes of interest within YKL-40.
3D modelling was generated with chimera (UCSF) using PDB sequences generated by Phyre2 fold recognition server (Kelley LA et al. (2015))

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