Role of NK cells in the efficacy of anti-HER therapeutic antibodies in breast cancer:

from biomarkers to functional checkpoints enhancing ADCC responses

Mariona Cabo

DOCTORAL THESIS UPF – 2019

SUPERVISOR

Dr. Aura Muntasell Castellví

Institut Hospital del Mar
d'Investigacions Mèdiques

Departament de Ciències Experimentals
i de la Salut, Universitat Pompeu Fabra

TUTOR:
Prof. Miguel López-Botet Arbona

“Un esforç total és una victòria completa”
– Mahatma Gandhi

És veritat el que diuen de que un doctorat és molt més que una etapa formativa, i que et transforma no només a nivell científic sinó també a nivell personal. Després d'aquests quatre anys, voldria donar les gràcies a totes aquelles persones que d'una forma o d'una altra han contribuït a que avui estigui escrivint aquestes línies.

En primer lloc voldria donar les gràcies a l'Aura per la seva formació i el seu suport científic, per fer-me de guia i per ser "la cabeza pensante" d'aquest projecte. També m'agradaria donar les gràcies al Miguel per haver-me obert les portes del seu laboratori i haver ajudat a que aquest projecte i el meu doctorat hagin estat possibles.

A toda la gente que ha pasado por MLB lab durante mi estancia, gracias por vuestra calidad humana y por crear un ambiente de trabajo tan bueno. Las muchas horas que hemos pasado en el laboratorio se hubieran hecho mucho más duras con otros compañeros. En especial a Michelle, Mireia, Sara, Marcel, Gemma, Andrea, Anna, Èlia, Eli y Laura, muchas gracias por el apoyo y las comidas que cargan pilas! Un agraïment especial pel Marcel, per la teva paciència i amabilitat, va ser un plaer ser la teva Padawan en els inicis; y a Sara, por el soporte técnico y emocional, por tu locura e intensidad que ayudan a superar cualquier obstaculo.

Voldria donar també les gràcies a tots els donants de sang i pacients per la seva contribució en aquest estudi, així com a les infermeres de l'IMIM, al Servei de Citometria i al Servei de Microarrays pel seu suport tècnic.

I ja fora del PRBB m'agradaria donar les gràcies a tots els amics i família que m'han recolzat en els moments difícils, que han aguantat rotllos de ciència tot i que els sonessin a xino, i que m'han ajudat a veure les coses amb una mica més de perspectiva. Gràcies a les de sempre: Natàlia, Gemma, Glòria i Elena, per ser-hi sempre; als immunitos: Paula, Gemma, Helena, Meritxell, Arnau, Laia, Sergio i Alexandra, per les converses existencials sobre futur i per entendre millor que ningú els maldecaps de la ciència; a la família Nadeu-Prat per preocupar-vos sempre per mi; a tota la meva família i especialment als meus pares: muchas gracias por vuestro apoyo y dedicación, y por creer tanto en mi; i finalment al Ferran per la teva calma i paciència, pel teu gran optimisme i suport en tots els sentits possibles. Moltes, moltes gràcies, no trobaria a ningú altre amb qui fer un equip millor.

ABSTRACT

NK cells contribute to the efficacy of tumor antigen-specific monoclonal antibodies (TAA mAbs) and are involved in the orchestration of tumor-specific adaptive immunity in breast cancer murine models. On the other hand, NK cell dysfunction owing to immunosuppressive factors (i.e. TGF-β1) has been related to disease progression in breast cancer patients with metastatic disease. Hence, strengthening NK cell function and persistence is envisaged as a relevant option for enhancing the clinical efficacy of TAA mAbs.

In the present study, immunophenotypic analysis of circulating NK cells in patients with HER2+ primary breast cancer evidenced an inverse correlation between baseline CD57+ NK cell numbers and response to anti-HER2 antibody-based treatment, independently of age, conventional clinicopathological factors and CD16A 158F/V genotype. Circulating CD57+ NK cells displayed lower CXCR3 expression and CD16-induced IL-2-dependent proliferation as compared to the CD57- population, yet showing comparable trastuzumab-induced *in vitro* degranulation against HER2+ breast cancer cells. Remarkably, CD57+ NK cells were reduced in breast tumor-associated infiltrates as compared to paired peripheral blood samples, suggesting that deficient homing, proliferation and/or survival in the tumor niche could underlie CD57+ NK cell association with resistance to anti-HER2 antibodies.

In the second part of the study, we demonstrated the ability of CD137 (4-1BB) agonistic antibody urelumab for overcoming TGF-β1-mediated inhibition of human NK cell proliferation and sustained antitumor function. Transcriptomic, immunophenotypic and functional analysis evidenced that CD137 costimulation modified the transcriptional program induced by TGF-β1 on activated NK cells by limiting Smad4-dependent inhibition of proliferative (CD25, Myc), activating (NKG2D, CD16) and

effector (granzyme B, IFN-γ) molecules while maintaining Smad4-independent acquisition of tumor-retention features (CXCR3, CD103). Activated NK cells cultured in the presence of TGF-β1 and CD137 agonist showed preserved antibody-dependent cytotoxicity against breast cancer cells, secretion of T cell-helper cytokines (i.e. CCL5, IFN-γ) upon restimulation *in vitro* and gained the capacity for producing IL-9. Treatment of multicellular breast carcinoma cultures with anti-HER2 antibodies and urelumab recapitulated CD137 induction and fostered tumor-infiltrating CD16+ NK cells. Overall, our data point to NK cell aging as a limiting factor for breast tumor infiltration and clinical benefit to anti-HER2 antibodies and provide the rationale for combinatorial strategies including CD137 agonists for enhancing anti-HER2 antibody efficacy in breast cancer.

RESUM

Les cèl·lules NK contribueixen a l'eficàcia dels anticossos específics d'antígen tumoral, i regulen la immunitat adaptativa antitumor en models murins. D'altra banda, la seva disfunció degut a factors immunosupressors com el TGF-β1 s'ha relacionat amb la progressió de la malaltia en pacients amb càncer de mama metastàtic. Així doncs, estratègies que potenciïn la funció NK i la persistència d'aquestes cèl·lules en el tumor podrien ser útils per tal d'incrementar l'eficàcia clínica dels anticossos específics de tumor. En aquest estudi els anàlisis immunofenotípics de les cèl·lules NK circulants en pacients amb càncer de mama primari $HER2^{+}$ van evidenciar una correlació inversa entre el nombre de cèl·lules NK CD57+ i la resposta al tractament amb anticossos anti-HER2, independent de l'edat, d'altres factors clinicopatològics i del genotip de CD16A (158 F/V). Les cèl·lules NK CD57+ circulants presentaven menor expressió de CXCR3 i proliferació depenent de IL-2 postactivació via CD16, encara que mostren una degranulació comparable contra cèl·lules de càncer de mama HER2+ en presència de trastuzumab. A més, la proporció de cèl·lules NK CD57+ en el tumor era notablement menor que en mostres aparellades de sang perifèrica, suggerint que deficiències en la seva capacitat de migració, proliferació i/o resistència en l'entorn tumoral, podrien explicar la seva associació amb la resistència al tractament amb anticossos anti-HER2.

En la segona part de l'estudi, hem demostrat la capacitat de l'anticòs agonista contra CD137 (4-1BB) urelumab de contrarestar la inhibició per TGF-β1 sobre la proliferació NK, mantenint la subseqüent funció antitumor d'aquestes cèl·lules. Anàlisis de transcriptòmica, immunofenotíp i funcionals han evidenciat que la co-estimulació per CD137 modifica el programa transcripcional induït per TGF-β1 en les cèl·lules NK activades, limitant la inhibició Smad4-depenent de

molècules proliferatives (CD25, Myc), activadores (NKG2D, CD16) i efectores (granzima B, IFN-γ), però preservant l'adquisició Smad4-independent de característiques associades a la retenció dels limfòcits en el tumor (CXCR3, CD103). Les cèl·lules NK activades i cultivades en presència de TGF-β1 i de l'anticòs agonista contra CD137 conserven la citotoxicitat mediada per anticòs contra cèl·lules de carcinoma de mama, i la secreció de citocines que regulen la funció dels limfòcits T com la CCL5 o l'IFN-γ, després de ser re-estimulades *in vitro*, mentre que adquireixen la capacitat de secretar IL-9. El tractament amb trastuzumab i urelumab de cultius multicel·lulars derivats de tumors de mama reprodueix la inducció de CD137 i preserva la presència de cèl·lules NK infiltrants de tumor CD16+. En conjunt, les nostres dades apunten cap a l'envelliment del compartiment NK com a factor limitant de la infiltració en tumor i el benefici clínic dels anticossos contra HER2, i aporten les bases per l'ús d'agonistes de CD137 en teràpies combinades amb l'objectiu d'augmentar l'eficàcia dels anticossos anti-HER2 en el context de càncer de mama.

PREFACE

Breast cancer is a major health care problem worldwide, with an estimated 1.67 million women diagnosed annually. HER2 overexpression accounts for approximately 15-20% of breast tumors, and mainstay therapy for these patients includes HER2-specific antibodies in combination with chemotherapy. Although the efficacy of anti-HER2 antibodies has significantly improved disease control, resistance to treatment eventually develop, accounting for dismal outcomes and evidencing the need for biomarkers and therapeutic strategies improving clinical management and patient survival. Natural killer cells (NK) are cytotoxic innate lymphocytes specialized in the defense against virus infected and transformed cells. Recognition of antibody-coated targets triggers the activation of NK cell effector functions leading to target cell death and the release of soluble factors regulating the development of subsequent antitumor immunity. Several observations support the contribution of NK cells to the efficacy of anti-HER2 therapeutic antibodies in breast cancer and NK cell dysfunction, owing to immunosuppressive factors present in the tumor, has been related to tumor progression in metastatic patients. In this context, strengthening NK cell responses is envisaged as a relevant option for enhancing the therapeutic benefit of anti-HER2 mAbs. In this work we provide novel insights on the role of NK cells in the efficacy of anti-HER2 antibodies in breast cancer by describing NK cell aging as a factor potentially limiting the efficacy of anti-HER2 antibodies, and disclose the potential of CD137 costimulation for enhancing NK cell effector function despite immunosuppression. Understanding the mechanisms underlying the heterogeneity of NK cell anti-tumor responses, including actionable checkpoints enhancing NK cell tumor-homing and persistence will pave the way for biomarker development and personalized therapeutic strategies.

TABLE OF CONTENTS

INTRODUCTION

1. NK cell biology

1.1. NK cell characterization and distribution

Natural killer (NK) cells are innate cytotoxic lymphocytes specialized in the early response against virus-infected and transformed cells (1–3). In humans, NK cells are phenotypically defined based on the lack of CD3 and expression of the neural cell adhesion molecule 1 (NCAM-1 or CD56) and comprise 5-25% of human peripheral blood lymphocytes. Circulating NK cells have been traditionally subdivided into the $CD56^{bright}$ subset, expressing high levels of CD56 and low or no expression of the activating receptor CD16A (FcγRIIIA, hereafter CD16), and the $CD56^{dim}$ subset, expressing low levels of CD56 and high levels of CD16. $CD56^{bright}$ NK cells are preferentially found in secondary lymphoid organs, present low cytotoxic capacity and produce pro-inflammatory cytokines in response to soluble factors such as IL-12 and IL-18 (4,5). The $CD56^{dim}$ subpopulation accounts for 90% of peripheral blood NK cells and display higher cytotoxic potential in response to target cell-mediated activation and can recognize antibody-opsonized target cells (6,7). $CD56^{bright}$ and $CD56^{dim}$ NK cells do also differ in their chemokine receptor profile. $CD56^{bright}$ NK cells are characterized by high expression of CCR7, CXCR3 and CD62L, while $CD56^{dim}$ NK cells express CXCR1, CXCR2 and CX3CR1 in resting conditions, yet expression of chemokine receptors is modulated upon NK cell activation (8,9).

In the recent years, the landscape of innate lymphocytes has broaden by the description of tissue-resident non-cytotoxic subsets globally referred to as innate lymphoid cells (ILCs) (10,11). ILCs were initially characterized in mice and later on identified in human, yet additional studies are needed to further understand ILCs biology and function. Both human and mice ILCs have been broadly classified into three main groups

based on their cytokine profile and transcription factor expression: ILC1, which includes conventional NK cells and ILC1 cells, express T-bet and secrete IFN-γ; ILC2, which produce IL-5, IL-9 and IL-13, and require GATA3, as well as ROR-α in mice, for their development and function; and ILC3, which express ROR-γ and produce IL-17A and IL-22 (11–14). As compared to ILC1, conventional NK cells are also dependent on the transcription factor Eomes for their development and function, and emerge as a cytotoxic subset mirroring CD8+ T cells, whereas ILC1s are considered a helper subset producer of IFN-γ with diminished cytotoxic capacity, resembling CD4+ Th1 helper cells. Nonetheless, certain functional plasticity has been described for ILCs depending on the microenvironment (15,16). For instance, both human ILC2 and ILC3 have been shown to convert into ILC1-like cells producing IFN-γ in the presence of IL-12 and IL-1β, or IL-2 and IL-15, respectively (17–20). In mice, TGF-β has also been shown to induce the conversion of NK cells towards an ILC1-like phenotype with diminished cytotoxic capacity (21,22).

1.2. Regulation of NK cell function

NK cells express on their cell surface an array of germ-line encoded activating and inhibitory receptors that modulate NK cell activation upon encounter with target cells expressing their cognate ligands (**Figure 1**). NK cell tolerance against healthy cells depends on inhibitory receptors specific for HLA class I molecules (HLA-I), which prevent NK cell activation against cells expressing normal HLA-I levels (3,23). On the contrary, HLA-I levels are down-regulated in some viral-infected and transformed cells allowing activation of NK cells in the presence of ligands for activating NK cell receptors (NKR) (24).

NK cell activation results in the polarized release of preformed cytotoxic granules containing perforin and granzymes, the secretion of pro-inflammatory cytokines (i.e. TNF-α, IFN-γ) and chemokines [i.e. MIP1α, MIP1β, CCL5 (RANTES)] and the expression of ligands for death receptors of the TNF receptor superfamily (TNFRSF) (i.e. TNF-α, TRAIL, Fas ligand). The coordinated action of perforin and granzymes, and signaling through TNF receptor superfamily (TNFRSF) death receptors causes target cell death (25). Moreover, secretion of pro-inflammatory cytokines and chemokines contributes to the development of subsequent adaptive immune responses (26–29).

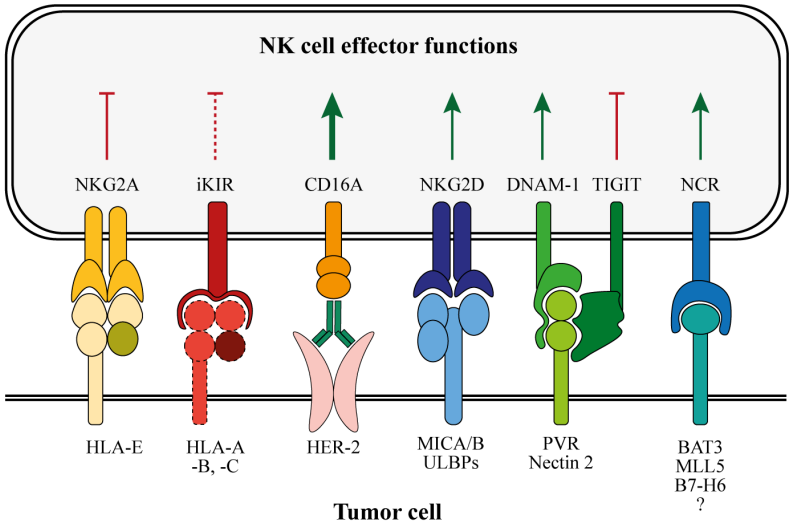

Figure 1. Main NK cell receptors involved in tumor cell recognition. iKIR: inhibitory KIR, NCR: NKp30, NKp44, NKp46. *Adapted from Muntasell A, Front Immunol, 2017* (283).

1.2.1 Inhibitory NK cell receptors

Main inhibitory NKR with constitutive expression on NK cells recognize HLA-I molecules and include some members of the killer cell Ig-like receptor (KIR) superfamily (i.e. KIR2DL1/L2/L3/L5; KIR3DL1/L2), specific for epitopes shared by groups of alleles of classical HLA-I molecules (i.e. HLA-A, -B, -C) (30,31); the CD94/NKG2A receptor, specific for the non-classical class Ib molecule HLA-E (32); and LILRB1, recognizing a broad range of HLA-I molecules including HLA-G (33,34).

KIR molecules are a polygenic and highly polymorphic family. KIR2DL2/L3 recognize HLA-C1 epitopes, containing Asn in position 80, while KIR2DL1 preferentially interacts with HLA-C2 epitopes, containing Lys in position 80. KIR3DL1 binds to the Bw4 epitope, carried by subsets of HLA-A and HLA-B alleles. KIR3DL2 interacts with HLA-A3/11 molecules (31,35).

KIR and NKG2 families do also include activating members (i.e. KIR2DS1/S2/S3/S4/S5, KIR3DS1, CD94/NKG2C) which in some cases (i.e. KIR2DS1 and CD94/NKG2C) bind to the same ligand as their inhibitory counterparts (i.e. KIR2DL1 and CD94/NKG2A), yet with lower affinity. Identification of ligands for some activating KIR receptors remains elusive (36).

Other inhibitory receptors expressed by NK cells and which are relevant for the recognition of transformed cells are TIGIT (T cell immunoglobulin and ITIM domain) and CD96 (TACTILE), which bind to nectin and nectin-like adhesion molecules highly expressed in many types of tumors (37,38). In human NK cells, both TIGIT and CD96 are expressed in resting conditions and the former is further up-regulated after activation (37,39,40). TIGIT binds to poliovirus receptor (CD155, PVR) and nectin-2 (CD112, PVRL2), yet presents higher affinity for PVR (41). The main

ligand for CD96 is likewise PVR, but association to nectin-1 (CD111) has also been described (42). Both TIGIT and CD96 compete with the activating receptor DNAM-1 for the binding of PVR and nectin-2 (43).

All inhibitory NKR signal through immunoreceptor tyrosine-based inhibitory motifs (ITIMs) present in their cytoplasmatic tail, which enable the recruitment and activation of SHP-1 and -2 phosphatases that subsequently switch off the activating signaling cascade initiated by activating NKR by dephosphorylating signaling molecules such as Syk, ZAP70 or Vav1, among others (44).

1.2.2. Activating NK cell receptors

NK cell activating receptors with constitutive expression comprise CD16, NKG2D, natural cytotoxic receptors (NCR) NKp30 and NKp46, CD94/NKG2C, members of the KIR family (i.e. KIR2DS1/S2/S3/S4/S5, KIR3DS1) and the coreceptor/adhesion molecule DNAM-1. NKp44, member of the NCR family, is not expressed in resting NK cells but induced upon activation (45). With the exception CD16, activation of resting NK cells relies on the cooperation between of signaling through different activating receptors (46). Due to the lack of signaling domains in their cytoplasmic tails, all activating NKR but DNAM1 signal through the association with adaptor molecules containing immunoreceptor tyrosine-based activation motifs (ITAMs) such as FcεRIγ (i.e. CD16, NKp46), CD3ζ (i.e. CD16, NKp30, NKp46) and DAP12 (i.e. NKp44, NKG2C, KIR2DS1-5, KIR3DS1), or with the adaptor protein DAP10 containing the Tyr-Ile-Asn-Met (YINM) motif (i.e. NKG2D) (47–49). Upon receptor-ligand binding the two tyrosines in the ITAM domains are phosphorylated by Src-kinase family members, inducing the recruitment of ZAP70 and Syk tyrosine kinases. These kinases phosphorylate transmembrane adaptors such as LAT and NTAL, resulting in the

clustering and activation of phospholipase C (PLC)γ-1/2, Vav2/3 and phosphoinositide 3-kinase (PI3K), ultimately triggering degranulation and secretion of effector molecules via Ca^{2+} flux and cytoskeletal reorganization. Conversely, NKG2D signaling through DAP10 does not require Syk or ZAP70, nor LAT or NTAL (50). Phosphorylation of DAP10 by Src-family kinases induces the recruitment of PI3K or the small adaptor Grb2, leading to activation of PLCγ-1/2 and Vav1 (50).

Ligands for NKG2D include self-molecules such as MICA/MICB and the ULBP family (ULBP1-6), which are poorly expressed by normal cells but up-regulated under stress conditions (51,52). NKp30 and NKp44 recognize self-molecules exposed in damaged cells (i.e. BAT3 and MLL5, respectively) or induced by inflammatory stimuli (i.e. B7-H6, ligand for NKp30), yet the specific nature of some ligands for NCRs remains unknown (53–55). As stated above, CD94/NKG2C and some activating KIRs bind to HLA-I molecules (e.g. CD94/NKG2C and HLA-E) yet with lower affinity than their inhibitory counterparts (56); while DNAM-1 competes with TIGIT and CD96 for the recognition of PVR and nectin-2 (57).

CD16 is the most potent activating NK cell receptor and the only one capable of inducing NK cell responses in resting NK cells by its own (46). Expression of CD16 is limited to $CD56^{dim}$ NK cells and certain T lymphocyte subsets (i.e. γδ T cells). Upon recognition of the constant fragment (Fc) of cell-bound immunoglobulin G (IgG), CD16 triggers antibody-dependent cell cytotoxicity (ADCC) by signaling through association with dimers of CD3ζ or FcεRIγ adaptors, containing three and one ITAMs, respectively (23). CD16 binds with higher affinity IgG1 and IgG3, as compared to IgG2 or IgG4 (58). An allelic dimorphism where a T>G substitution changes valine (V) to phenylalanine (F) on position 158 in the IgG binding domain of CD16 dictates receptor surface expression

levels and the affinity of the receptor for IgG. Cells bearing CD16 158V/V receptors show higher expression levels and higher affinity for IgG, as compared to cells carrying CD16 158V/F, and more notably CD16 158F/F receptors (59).

1.2.3. The diversity of the NK cell receptor repertoire

Beyond the conventional subdivision of NK cells into $CD56^{bright}$/$CD56^{dim}$, $CD56^{dim}$ subset includes different subpopulations at distinct differentiation status and harboring different NKR combinations by the influence of genetic (i.e. KIR and HLA-I) and environmental factors [i.e. infection by human cytomegalovirus (HCMV)], which results in a high degree of inter-individual heterogeneity in the NK cell receptor *repertoire* (**Figure 2**).

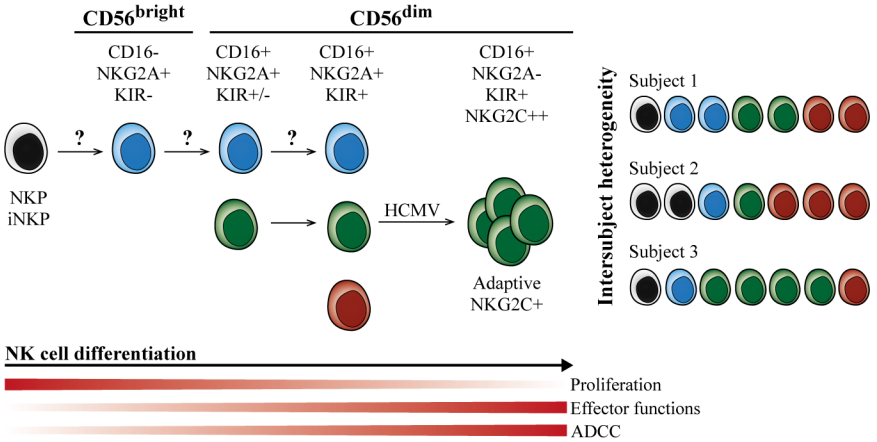

Figure 2. Diversity of the NK cell *repertoire*. NKP: NK cell progenitor; iNK: immature NK cell; HCMV: human cytomegalovirus; ADCC: antibody-dependent cell cytotoxicity. Different colors represent stochastic expression of KIRs in different NK cell clones. *Adapted from López-Botet M, Front Immunol, 2017* (61).

According to the currently accepted NK cell differentiation scheme, $CD56^{bright}$ NK cells, which originate from CD34+ hematopoietic stem cells (HSCs) and express high levels of the hematopoietic stem cell

marker CD117 (c-Kit) (60), differentiate to CD56^{dim} NK cells. Most CD56^{bright} NK cells express NKG2D, NCR and NKG2A, the first HLA-I-specific inhibitory receptor expressed along ontogeny, but lack KIR and CD16 expression (61). Clonal acquisition of KIR receptors occurs during the transition of CD56^{bright} NK cells towards CD56^{dim} CD16+ cells (62). The high variety of haplotypes in the KIR loci together with stochastic expression of KIR and NKG2 receptors during NK cell development contributes to the diversification of the NKR repertoire (63). Stochastic expression of KIR receptors, which has been reported to be epigenetically regulated (64), is followed by a positive selection of NK cells expressing KIRs specific for self HLA-I, which will subsequently acquire functional competence (65). Moreover, CD56^{dim} NK cells further differentiate by acquiring LILRB1 and CD57 expression (62,66). The CD57 epitope is a glycan carbohydrate modification of different surface glycoproteins, which is barely detected in newborn NK cells, but increases with age (67–70). NK cell differentiation occurs concomitant to progressive loss of their proliferative capacity in response to pro-proliferative cytokines, and the enhancement of their cytotoxic activity (6,62,71).

Infection by HCMV further shapes the NK cell repertoire by inducing, in some individuals, the persistent expansion of NK cells with adaptive features characterized by high expression of the activating NKR CD94/NKG2C in the absence of its inhibitory counterpart CD94/NKG2A (56). As compared to other NK cell subsets, adaptive NKG2C+ NK cells display an oligoclonal pattern of inhibitory KIRs specific for self HLA-C molecules, express reduced levels of NCR, and include high proportions of LILRB1+, CD57+ and FcεRIγ- cells (56,72–75). Besides, adaptive NKG2C+ NK cells show variable epigenetic silencing of adaptor/signaling molecules (i.e. FcεRIγ, Syk, Eat2) and transcription factors (i.e. PLZF, Helios and IKZF2) (76,77). Functionally, adaptive NK

cells display increased TNF-α and IFN-γ production and higher granzyme B levels, and are proficient in mediating ADCC responses likely due to FcεRIγ down-regulation and enhanced CD16 coupling to CD3ζ chain adaptor (78–80).

1.2.4. Cytokines

Different cytokines do further regulate NK cell activation, effector function, proliferation and survival. IL-2 and IL-15 increase NK cell proliferation and survival, as well as IFN-γ production and cytotoxic capacity (81). Binding to their shared β (IL-2/IL-15Rβ) and γ (γc) receptor units induces signaling through STAT5 (81). Specific α chains (IL-2/IL-15Rα) enhance affinity and stabilize ligand binding (81). IL-12 and IL-18 signaling through STAT4 enhances NK cell cytotoxicity and IFN-γ secretion (82,83). Both IL-12 and IL-18 receptors are heterodimeric complexes formed by two subunits (IL-12Rβ1/β2, IL-18Rα/β). Type I interferons (i.e. IFN-α/β) regulate NK cell effector functions through the up-regulation of perforin, Fas ligand and IFN-γ production. IFN-α/β signaling occurs through binding to heterodimeric IFN-α/β receptor (IFNAR) 1/2, and STAT1/2 and IFN regulatory factor (IRF)-9 complex formation (84). Conversely, TGF-β has been shown to antagonize with mTOR signaling pathway in NK cells, leading to reduced proliferation, expression of various activating receptors and cytotoxic activity (85).

1.2.5. Activation-inducible receptors

Another layer of regulatory ckeckpoints for NK cell function are activation-induced stimulatory and inhibitory receptors. However, in spite of being extensively characterized in cytotoxic CD8+ T cells, their expression and function has been considerably less investigated in NK cells. Most extensively studied inducible receptors are going to be revised in the following lines.

CD137 (4-1BB, *TNFRSF9*), OX40 (CD134, *TNFRSF4*) and GITR (glucocorticoid-induced tumor necrosis factor, *TNFRSF18*) are stimulatory receptors from the tumor necrosis factor receptor superfamily (TNFRSF) expressed by NK cells upon activation with IL-2 and IL-15 or upon CD16-dependent target cell recognition (86–89). CD137L, OX40L and GITRL are predominantly expressed on activated professional antigen presenting cells (APCs), including dendritic cells, macrophages and B cells. Circumstantially, expression has also been detected in other hematopoietic (i.e. activated NK, ILCs, mast and T-activated cells) and non-hematopoietic cells (i.e. epithelial and endothelial cells) (90–92). The role of CD137, OX40 and GITR in NK cell function remains unclear with reports showing increased or reduced NK cell cytotoxicity and IFN-γ production by ligation of specific TNFRSF members (86–89,93–99).

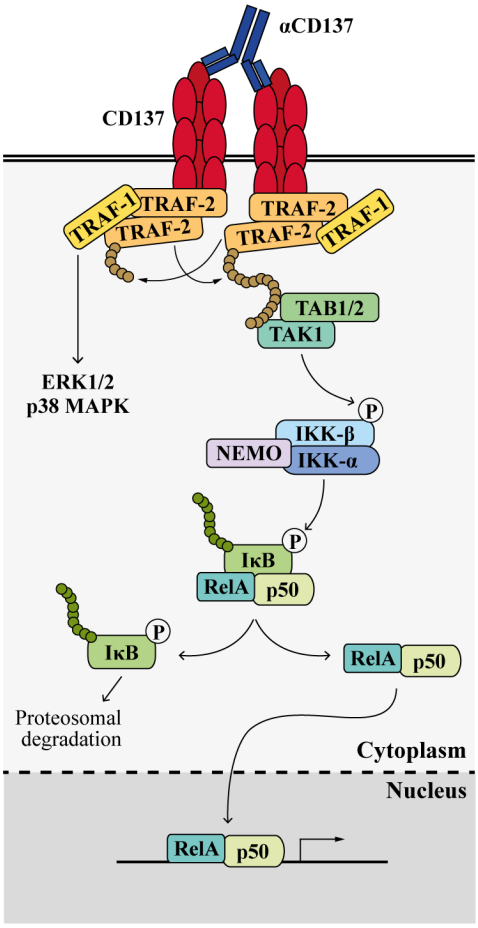

Figure 3. CD137 signaling pathway upon crosslinking with agonistic antibodies as described in CD8+ T cells.

Among TNFRSF members, signaling through CD137 has been extensively studied in CD8+ T cells. CD137 ligation by CD137L trimers or multivalent agonistic antibodies induces the recruitment of TRAF1 and TRAF2 into

CD137 cytoplasmatic tail. Transubiquitination of TRAF2 results in activation of TAK1-TAB1/2 (transforming growth factor beta-activated kinase 1-TAK-1 binding proteins 1 and 2) and phosphorylation of nuclear factor κ-B kinase (IKK)-β leading to canonical activation of NF-κB, as well as ERK1/2 and p38 MAPK signaling pathways (100–103) **(Figure 3)**.

Inducible costimulatory (ICOS), which belongs to the B7-CD28 superfamily, has been described to be up-regulated in NK cells by cytokine stimulation (i.e. IL-2, IL-12, IL-15, IL-18) (104–106). Binding of ICOS to its cognate ligand, ICOSL, mainly expressed by APCs, enhanced cytotoxicity and IFN-γ production by NK cells (105,107,108).

Regarding inhibitory receptors, programmed cell death-1 (PD-1) expression has been mainly reported under pathological conditions such as cancer or chronic infections in human NK cells, but also in mature $CD56^{dim}$ NK cells with a NKG2A- KIR+ CD57+ phenotype from healthy donors (109–112). PD-1+ NK cells showed reduced cytokine-induced proliferation, and impaired degranulation and cytokine production upon encounter with tumor targets *in vitro* (112). Blockade of the PD-1/PD-L1 axis enhanced NK cell-mediated antitumor responses both *in vitro* and *in vivo* in human and mice (110,113,114).

Finally, expression of cytotoxic T lymphocyte-associated protein 4 (CTLA-4) has been described in mouse NK cells upon IL-2 mediated activation, as well as in tumor-infiltrating NK cells in tumor-bearing mice (115,116). Engagement of CTLA-4 by CD80 in mouse NK cells has been shown to inhibit IFN-γ production in response to mature dendritic cells (115). Expression of CTLA-4 in human NK cells remains elusive.

2. NK cells and cancer

2.1. NK cell tumor immunosurveillance

NK cell immunosurveillance of primary tumor formation, tumor recurrence and metastasis has been described in multiple mouse models of spontaneous and induced carcinomas since the 1980s (117–123). NK cell cytotoxicity may be particularly important for the elimination of tumors with reduced or absent HLA-I expression that evade CD8+ T cell-mediated killing, and CD16, NKG2D, NCRs and the costimulatory molecule DNAM-1 are considered the main activating receptors involved in the recognition of transformed cells (123,124). In humans, high NK cell cytotoxic activity in *in vitro* assays was associated to lower risk of cancer in an 11-year follow up epidemiological study (125). Moreover, presence of tumor-associated NK cells in different tumor types has been related to good prognosis and/or low risk of metastasis in patients (27,126–132).

Despite the fact that infiltrating NK cells in solid tumors are usually scarce, they appear important for tumor control not only because of their cytotoxic capacity but also because of their immunoregulatory role (26–29). In melanoma and breast cancer mouse models, secretion of CCL5 (RANTES), XCL-1/2 and FLT3 ligand by infiltrating NK cells contributed to the recruitment and maintenance of conventional type 1 dendritic cells (cDC1) and CD8+ T cells, necessary for an effective antitumor response (26,27). In melanoma and head and neck squamous cell carcinoma patients, presence of NK cells within the tumor correlated with the frequency of cDC1 and response to anti-PD-1 immunotherapy (27). In this sense, a limited number of NK cells infiltrating the tumor might be enough to set in motion optimal anti-tumor responses.

2.2 TGF-β as a NK cell immunoevasion mechanism in solid tumors

Different mechanisms are used by tumor cells in order to limit NK cell antitumor function: (i) shedding of ligands for NK cell activating receptors (e.g. MICA/B, B7-H6) (133,134); (ii) up-regulation of ligands for NK inhibitory receptors (e.g. non-classical HLA-I) (135,136) and (iii) dysregulated expression of death receptors (e.g. Fas, TRAIL-R) (137,138) or apoptosis-related molecules (e.g. BH3 family members) (139,140). Moreover secretion of immunosuppressive soluble factors such as TGF-β, prostaglandin E2 or idoleamine 2,3-dioxygenase (IDO) by tumor cells themselves or other regulatory cells present in the tumor microenvironment further impair NK cell survival and function (26,141–143).

Transforming growth factor-β1 (TGF-β1) is a pleiotropic cytokine involved in many cellular processes in the developing embryo and adult organism including cell differentiation, cell homeostasis, tissue regeneration and immune regulation (144). TGF-β belongs to a superfamily of 32 members grouped into the TGF-β and the bone morphogenic protein (BMP) subfamilies. The TGF-β subfamily includes, among others, three TGF-β ligands (TGF-β1, -β2, and –β3), which are the most relevant members of the family in immune regulation. Specifically, TGF-β1 is the most abundant and ubiquitously expressed isoform in mammalian tissues. TGF-βs are synthesized in a latent form as pro-proteins, and are activated by the action of proteases and metalloproteases such as MMP-9 and MMP-2, thrombospondin-1, or integrins such as αVβ6 or αVβ8; in the presence of acidic conditions; or by the exposure to reactive oxygen species (ROS) (145). TGF-β family members signal through heterodimeric complexes of specific type I and type II receptors

containing serine/threonine kinase domains in their cytoplasmic tails. Upon ligand binding, TGF-β-RII, a constitutively active kinase, phosphorylates TGF-β-RI (ALK5), triggering its activation and downstream signaling. The coreceptor TGF-β-RIII (also known as betaglycan) has been shown to enhance the affinity of TGF-βs for TGF-β-RII (146). Signal transduction downstream of TGF-β-RI occurs via phosphorylation of receptor-regulated Smads (R-Smads), Smad2 and Smad3. Activated R-Smads form heteromeric complexes with common-partner Smad4 or, alternatively, TIF-1γ (also known as TRIM33 or Ectodermin), which facilitates their translocation to the nucleus (147–149). Once in the nucleus, Smads regulate transcriptional programs by cooperating with lineage-specific transcription factors, co-activators and corepressors including AP-1 (encoded by *JUN* and *FOS*), FOXO1-FOXO4, RUNX3, E2F4, ATF3 or ID1 (150). Inducible Smad7 acts as a negative regulator of the signaling pathway mainly by suppressing phosphorylation of Smad2/3 (151). TGF-β-RI and –RII can also activate non-canonical Smad-independent signaling such as MAPK (ERK, JNK and p38 MAPK) or PI3K/AKT (147) **(Figure 4)**.

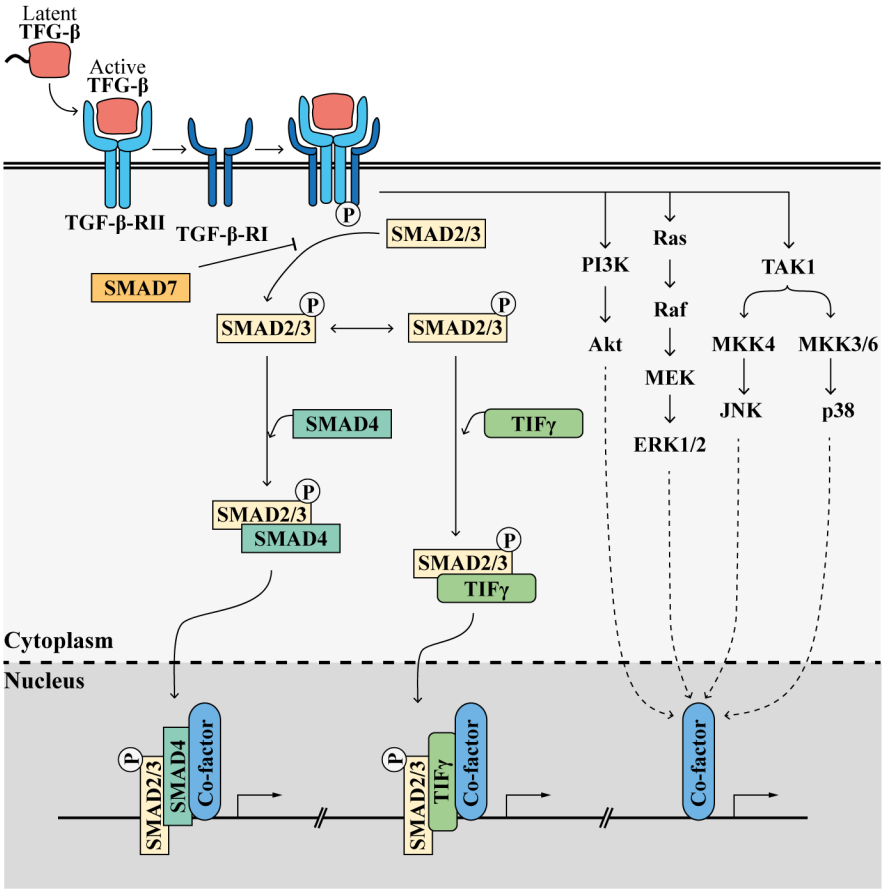

Figure 4. TGF-β signaling pathway. Canonical and non-canonical Smad4-dependent and –independent signaling branches are shown.

TGF-β can elicit a tumor suppressor or tumor promoting role depending on the cell context and tumor stage (152). In early tumor stages, TGF-β may act as a tumor suppressor, inducing apoptosis in pre-malignant cells and inhibiting proliferation of carcinoma cells. However, aberrant regulation of TGF-β signaling in tumor cells turns TGF-β into a pro-oncogenic factor contributing to angiogenesis, epithelial-mesenchymal transition (EMT), metastasis dissemination, drug resistance and immune suppression in evolved solid tumors (147). Being produced in high amounts by tumor cells, cancer-associated fibroblasts and regulatory

immune cells such as tumor-associated macrophages, TGF-β inhibits Th1 helper and cytotoxic T cell responses, induces a T regulatory phenotype, and promotes dendritic cell differentiation towards myeloid suppressor cells, among others (144). Elevated TGF-β serum levels have been found in poor prognosis patients with hematologic malignancies and solid tumors and associates to systemic inhibition of immune function, including impaired NK cell responses (153–157).

In human NK cells silencing of IFN-γ and T-bet through Smad-dependent TGF-β signaling has been described in resting and CD16-activated NK cells (158,159). Down-regulation of activating receptors (i.e. NKp30, NKG2D, DNAM-1) and effector molecules (i.e. granzymes and perforin) due to TGF-β has been reported in resting, IL-15- and CD16-activated NK cells, leading to impaired direct and antibody-mediated killing of target cells (159–161). TGF-β1 has been shown to antagonize with IL-15-dependent NK cell proliferation and mTOR activation, resulting in reduced NK cell metabolic activity (85,161). Global changes induced by TGF-β promote NK cell differentiation into poorly cytolytic CD16- CD9+ CD103+ subset, resembling decidual NK cells (162). In cancer mouse models, TGF-β signaling in the tumor microenvironment promoted NK cell differentiation towards an ILC1-like phenotype (i.e. increased CD49a, CD73 and TRAIL expression, decreased EOMES and granzyme B levels) with diminished cytotoxic activity facilitating for tumor immunoevasion (21,163).

3. Breast cancer

Breast cancer is the most frequent cancer type in women worldwide, and the second most common cause of cancer death after lung cancer (http://globocan.iarc.fr/old/FactSheets/cancers/breast-new.asp). Breast cancer is a highly heterogeneous disease, both histologically and molecularly (164–166). Breast carcinomas have been traditionally classified based on the expression of the receptors for steroid hormones estrogen (ER) and progesterone (PR), and the overexpression of the epidermal growth factor receptor tyrosine kinase HER2, yet 5 molecular subtypes (i.e. luminal A, luminal B, HER2-enriched, basal-like and claudin-low) have been defined in the recent years according to transcriptional profiles (167,168). However, to date, breast cancer classification on daily clinical practice does mostly rely on the expression of traditional tumor markers ER, PR and HER2 only. Accordingly, 3 major breast cancer subtypes can be distinguished: hormone receptor+/HER2- tumors; HER2+ breast tumors, that comprise 15-20% of total cases; and triple-negative breast cancer, lacking expression of ER, PR and HER-2. Patients with HER2+ breast cancer benefit from HER2-targeted therapy, including anti-HER2 monoclonal antibodies (i.e. trastuzumab, pertuzumab, T-DM1) and small-molecules tyrosine kinase inhibitors (i.e. lapatinib) (169,170). Specifically, HER2 dual targeting with trastuzumab and pertuzumab together with chemotherapy is the first line treatment for primary HER2+ tumors in the neoadjuvant setting and metastatic disease (171). Moreover, T-DM1 (i.e. trastuzumab-emtansine, an antibody-drug conjugate including the antimitotic agent DM1) has been approved for advanced HER2+ breast cancer patients with progressive disease following trastuzumab/pertuzumab plus chemotherapy regimens (172). Combination of lapatinib and trastuzumab/chemotherapy is also used for the treatment of refractory patients with advanced disease

(173,174). As compared to ER+/PR+ HER2- breast carcinomas, HER2+ breast tumors were associated with a more aggressive disease and decreased survival (175). Approval of HER2-targeted therapy brought a significant improvement to the clinical outcome of HER2+ breast cancer patients. Mechanisms of action of anti-HER2 mAb include prevention of HER2 dimerization with other members of the family, required for HER2 signaling, increased endocytosis and HER2 degradation and immune cell activation by antibody-dependent cell cytotoxicity (ADCC) (176). Nonetheless, disease progression eventually occurs due to *de novo* or acquired resistance to treatment in a proportion of patients (177).

3.1. NK cell-mediated ADCC in HER2+ breast cancer

All anti-HER2 mAb (i.e. trastuzumab, pertuzumab, T-DM1) approved for clinical use are IgG1 capable of inducing ADCC responses. Several studies point towards a role of NK cell-mediated ADCC in the clinical efficacy of tumor antigen-specific mAb, together with other immune cells such as tumor-specific T lymphocytes. In mouse models, deficiency of CD16, NK cells or CD8+ T cells, as well as treatment with F(ab')2 fragments, impaired the antitumor effect of HER-specific antibodies (178–181). In breast cancer patients, treatment with trastuzumab and T-DM1 induced increased tumor infiltration by lymphocytes, particularly NK cells (182–184). Indeed, presence of tumor infiltrating lymphocytes has been associated to response to neadjuvant treatment with anti-HER2 mAb (185–189). We have recently identified tumor infiltrating-NK cells in diagnostic core biopsies as an independent biomarker predictive of response to anti-HER2 mAb-based neoadjuvant treatment in primary HER2+ breast cancer patients (132). Presence of NK cell in HER2+ tumors correlated with activated dendritic cells and CD8+ T cells, as measured by gene expression signatures (132). In addition, impaired

ADCC responses have been described in patients at advanced or metastatic stages of the disease and the magnitude of NK cell-mediated ADCC against trastuzumab-coated tumor cell lines in *in vitro* assays correlated with response to treatment in metastatic patients treated with anti-HER2 mAb (182,190–194).

3.2. Immunotherapy in breast cancer

Immunotherapy in breast cancer is still far from other solid tumors, but an increased interest in the recent years has led to more than 200 clinical trials currently ongoing in the field (195). Among different breast cancer subtypes, presence of tumor-infiltrating lymphocytes is more pronounced in triple-negative and HER2+ tumors, which are considered the more immunogenic breast cancer subtypes (196). The majority of the trials currently ongoing are aimed at the treatment of triple-negative breast tumors, the subtype showing the worst prognosis to date (195). Blocking antibodies targeting the PD-1/PD-L1 axis showed great efficacy in preclinical models of breast cancer, and account for most of the immunotherapeutic agents being evaluated in clinical trials (181,184). Anti-PD-L1 antibody atezolizumab has been recently approved in combination with chemotherapy for the treatment of metastatic triple-negative breast cancer, yet clinical efficacy is rather low (197). Anti-CD137 agonistic antibody utomilumab is being tested with anti-HER2 mAb trastuzumab or T-DM1 alone (NCT03364348), or in combination with chemotherapy and anti-PD-L1 mAb avelumab (NCT03414658) in patients with metastatic HER2+ breast cancer in two different clinical trials. Other strategies being addressed include vaccines, oncolytic viruses and adoptive cell therapy. Adoptive transfer of tumor-infiltrating lymphocytes recognizing tumor antigens in combination with IL-2 and pembrolizumab (anti-PD-1 mAb) has been recently reported to lead to

complete durable regression of the tumor in a patient with metastatic ER+ HER2- breast cancer refractory to chemotherapy (198).

Altogether, there is still a long way to go in the field of immunotherapy in breast cancer. Good clinical responses have been reported in some patients, yet biomarkers of response as well as novel strategies are needed to broaden the number of patients benefiting from these therapeutics.

HYPOTHESIS AND AIMS

One of the mechanisms of action of anti-HER2 therapeutic antibodies is the triggering of NK cell-mediated antibody-dependent cytotoxicity (ADCC). Natural killer (NK) cells are cytotoxic innate lymphocytes that can contribute to ADCC and facilitate the development of tumor-specific adaptive immunity. Several evidences support the contribution of NK cell-mediated ADCC to the clinical efficacy of anti-HER2 therapeutic antibodies in breast cancer and NK cell dysfunction has been associated to disease progression in metastatic patients.

We hypothesized that the heterogeneity on the clinical benefit of anti-HER2 antibody-based treatments could be related to genetic and epigenetic differences dictating the expansion of relevant NK-cell subpopulations in distinct patients. On the other hand, molecular tools targeting immune checkpoints could be used for strengthening NK cell-mediated ADCC responses and enhancing the clinical efficacy of HER2-specific antibody-based therapies.

To asses this issue, the following aims have been addressed:

1. To analyze the influence of the NK cell repertoire on the clinical efficacy of anti-HER2 mAb in HER2+ breast cancer patients.

2. To study the suitability of CD137 (4-1BB) stimulatory co-receptor for enhancing NK cell-mediated ADCC responses against HER2+ breast cancer cells.

CHAPTER 1

High numbers of circulating CD57+ NK cells associate with resistance to HER2-specific therapeutic antibodies in HER2+ primary breast cancer

Aura Muntasell, Sònia Servitja, **Mariona Cabo**, Begoña Bermejo, Sandra Pérez-Buira, Federico Rojo, Marcel Costa-García, Oriol Arpí, Manuela Moraru, Laia Serrano, Ignasi Tusquets, María Teresa Martínez, Gemma Heredia, Andrea Vera, María Martínez-Garcia, Laura Soria, Laura Comerma, Sara Santana-Hernández, Pilar Eroles, Ana Rovira, Carlos Vilches, Ana Lluch, Joan Albanell and Miguel López-Botet

Muntasell A, Servitja S, Cabo M, Bermejo B, Pérez-Buira S, Rojo F, et al. High Numbers of Circulating CD57+ NK Cells Associate with Resistance to HER2-Specific Therapeutic Antibodies in HER2+ Primary Breast Cancer. Cancer Immunol Res. 2019 Aug;7(8):1280-1292. DOI: 10.1158/2326-6066.CIR-18-0896.

CHAPTER 2

CD137 (4-1BB) costimulation counteracts TGF-β1 impairment of antibody-dependent NK cell responses against breast cancer cells

METHODS

1. Human blood samples

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood samples of volunteer healthy adults by Ficoll-Hypaque gradient (Lymphoprep) and kept overnight in complete RPMI 1640 GlutaMAX medium (Invitrogen) [i.e. penicillin/streptomycin (100 U/ml and 100 µg/ml, respectively, Invitrogen), sodium pyruvate (1 mM, Gibco), 10% FBS (Gibco)] supplemented with recombinant IL-2 (200 U/ml, Proleukin). NK cell purification was performed by negative selection using NK isolation kit (Miltenyi) according to the manufacturer instructions. HCMV serology was assessed by standard clinical diagnostic tests.

A heparinized blood sample was obtained from a patient with juvenile polyposis syndrome (JPS). The patient had been diagnosed as harboring a c.403C>T (p.Arg135*) (rs377767326) *SMAD4* mutation in heterozygosis leading to SMAD4 haploinsufficiency (199). PBMC were obtained as stated for healthy volunteers.

2. Human breast carcinoma samples

Remnant breast carcinoma samples from diagnosis were selected by the pathologist from patients undergoing surgery. After clearance of fat pieces, the specimen was mechanically disrupted and digested for 40 minutes under agitation with collagenase type IV (1 mg/ml, Gibco) and DNAse ($50x10^3$u/ml, Sigma Aldrich). The digested tissue was filtered through 100 µm and 40 µm filters, and multicellular suspensions were recovered by centrifugation at 400xg for 7 minutes at 4 ºC, counted and cultured overnight in the presence of IL-2 (200 U/ml) and trastuzumab (210 ng/ml, Herceptin®, Roche) in flat-bottom 96-well plates. CD137, PD-1 and CD16 expression was checked after overnight culture by flow

cytometry on DAPI- CD45+ CD3- CD56+ cells. Alternatively, multicellular suspensions were culture in the presence of IL-2 (200 U/ml), trastuzumab (210 ng/ml) and urelumab (50 µg/ml, Bristol-Myers Squibb) for 6 days, and phenotyped by flow cytometry for the expression of CD16, CD137, NKG2D, CD103 and PD-1.

3. Ethics Statement

All healthy volunteers and patients gave written informed consent for the analysis of peripheral blood and tumor biopsies for research purposes. Study protocol was approved by the local ethics committee (Clinical Research Ethics Committee, Parc de Salut Mar nº 2015/6038/I for healthy volunteers; nº 2015/6038/I and 2019/8584/I for cancer patients).

4. Cell lines

The human HER2+ breast cancer cell line SKBR3 was grown in complete DMEM/F-12 medium (Sigma-Aldrich) supplemented with L-glutamine (0.5 mM, Gibco). The mouse mastocytoma cell line P815 and the HLA class I -negative human erythroleukemic cell line K562 were cultured in complete RPMI 1640 GlutaMAX medium [i.e. penicillin/streptomycin (100 U/ml and 100 µg/ml, respectively, Invitrogen), sodium pyruvate (1 mM, Gibco), 10% FBS (Gibco)].

5. NK cell expansion in CD16-coated plates

Purified NK cells were cultured at a density of $3x10^5$ cells/ml in flat-bottom plates previously coated overnight at 4 ºC with purified anti-CD16 mAb (0.5 or 5 µg/ml, clone KD1, kindly provided by Dr. A. Moretta). TGF-β1 (10 ng/ml, Peprotech), anti-CD137 agonist antibody urelumab (50 or 1 µg/ml) or control human IgG4 (50 µg/ml, Bristol-Myers Squibb) were added at 8 hours post activation (unless specifically stated). Cultures

were maintained up to 6 days and NK cells were subsequently used for functional, phenotypic or transcriptomic analysis. In some experiments the ALK receptor inhibitor SB-431542 (20 μM, Sigma-Aldrich) was added prior activation with anti-CD16 (5 μg/ml) and maintained along the 6 days culture.

6. NK cell immunophenotyping by multiparametric flow cytometry

Total PBMC, primary or expanded purified NK cells were pre-treated with aggregated human IgG (10 μg/ml) to block Fc receptors, incubated with specific unlabelled mAb (i.e. anti-TNF-RI) or hybridoma culture supernatants (i.e. KIRmix), washed and further incubated with secondary antibodies polyclonal goat anti-mouse IgG+IgM-PE (Jackson Immunoresearch) or polyclonal goat anti-mouse IgG-APC/Cy7 (Biolegend). Subsequently, samples were stained with combinations of directly labelled antibodies. In experiments in which intracellular staining was performed (i.e. for the detection of γ-chain, phospho-NF-κB p65, TNF-α, IL-9 and granzyme B), cells were fixed and permeabilized (fixation/permeabilization kit, BD Bioscience) prior to incubation with directly-labelled antibodies specific for intracellular antigens. Data was acquired on a FACS-Fortessa or FACS-LSRII (BD Bioscience) and analyzed with FlowJo vX software (FlowJo LLC). Antibodies used for flow cytometry are listed in **Table 1**.

Monoclonal antibody	Clone	Source
Active caspase 3-FITC	C92-605	BD Bioscience
CCR7-PE/Cy7	3D12	BD Bioscience
CD3-PerCP	SK7	BD Bioscience
CD16-PE/Cy7	3G8	BD Bioscience
CD16-APC-eFluor 780	CB16	eBioscience
CD25-PE	BC96	eBioscience
CD25-APC/Cy7	BC96	Biolegend

CD45-Alexa Fluor 700	2D1	eBioscience
CD56-APC	CMSSB	eBioscience
CD56-APC-eFluor 780	HCD56	eBioscience
CD56-HV510	NCAM16.2	BD Bioscience
CD57-FITC	HCD57	Biolegend
CD103-FITC	B-Ly7	eBioscience
CD103-PE/Dazzle 594	Ber-ACT8	Biolegend
CD107a-FITC	H4A3	BD Bioscience
CD117 (cKit)-PE	104D2	eBioscience
CD137-PE	4B4-1	eBioscience
CXCR3-eFluor 660	CEW33D	eBioscience
FCεRI γ subunit-FITC		Merck
Granzyme B-Pacific Blue	GB11	Biolegend
IL-9-PE	REA1038	Miltenyi
NF-κB p65 (pS539)-PE/Cy7	K10-895	BD Bioscience
NKG2A-CF-Blue	Z199	Dr. A Moretta, Immunostep (*)
NKG2C-PE	134591	R&D systems
NKG2D-APC	BAT221	Miltenyi Biotec
NKp46-BV605	9E2	Biolegend
OX40-APC	ACT45	eBioscience
PD-1-PE-eFluor 610	J105	eBioscience
TNF-α-CF Blue	Infliximab	Jannsen, Immunostep (*)
TNF-RI	16803	R&D systems
TNF-RII-PE	22235	R&D systems

(*) Anti-NKG2A (clone Z199, kindly provided by Dr. A. Moretta) and anti-TNF-α infliximab (Remicade®, Janssen) were labelled with CF-Blue by Immunostep.

Hybridoma culture supernatant	Clone	Source
KIRmix, mix of the following hybridoma supernatants:		
KIR2DL1, -2DS1/S3/S5	HPMA-4	Dr. M. López-Botet
KIR2DL2/L3, -2DS2	CHL	Dr. S. Ferrini
KIR2DL5	UP-R1	Dr. M. López-Botet
KIR2DL1/L2, -2DS2/S4/S5, -3DS1	5.133	Dr. M. Colonna
KIR3DL1	DX9	Dr. L. Lanier

Table 1. Antibodies used for flow cytometry analyses.

7. viSNE (t-SNE) analysis

viSNE (t-SNE) analysis were implemented for the analysis of activated NK cells at 6 days. Raw flow cytometry data was imported into R (v3.5.1) using flowCore and openCyto packages. A compensation matrix generated in FlowJo (v10.0.7, Tree Star) was exported an applied into R. NK cells were gated on forward and side scatter and data from 2,000-5,000 NK cells were concatenated. Barnes-Hut t-SNE was conducted using the Rtsne package. Graphics were produced using packages gplots, ggplot2, and RColorBrewer.

8. NK cell ADCC assays

Primary or CD16-expanded NK cells were cultured in the presence of target cells (i.e. SKBR3, P815 or K562) in complete RPMI 1640 GlutaMAX medium in U-bottom 96-well plates. Co-culture with target cells was done at an effector:target (E:T) ratio 4:1. Trastuzumab was added at 210 or 2.1 ng/ml in the indicated conditions. NK cell degranulation was checked by monitoring CD107a mobilization by flow cytometry after 4-hour-co-culture including the anti-CD107a-FITC (clone H4A3, BD Bioscience) and monensin (5 µg/ml, Sigma-Aldrich). When indicated TNF-α (infliximab-CF Blue) was also analyzed by intracellular staining at the end of the co-culture in the presence of brefeldin A (10 µg/ml, Sigma-Aldrich). Co-culture experiments assessing CD137/OX40 induced expression were generally analyzed at 24 hours, unless stated in the corresponding figure.

In indicated experiments, recombinant human TNF-α (10 ng/ml, Peprotech), anti-TNF-α neutralizing antibody infliximab (50 µg/ml, Remicade®, Janssen) or TNF-RI and -RII blocking antibodies (10 µg/ml,

#16805 and #22210 respectively, R&D Systems) were added in the co-culture and maintained along the assay.

For P815-redirected activation, NK cells were co-cultured in with P815 cells (E:T 1:1) previously incubated for 20 min with anti-CD56 (clone C218), -CD16 (clone KD1), -NKp30 (clone AZ20) or –NKp46 (clone Bab281) supernatants (all of them kindly provided by Dr. A. Moretta). NK cell degranulation and CD137 expression was analysed by flow cytometry in 4-hour- or 24-hour-co-cultures, respectively.

9. NK cell cytotoxicity assays

Activation of caspase 3 in SKBR3 cells was checked by intracellular flow cytometry after co-culture with purified NK cells in the presence of trastuzumab (210 ng/ml) at different effector target ratios as indicated in each figure. SKBR3 cells were gated based on forward and size scatter (FSC/SSC) and by CD45 exclusion. Levels of active caspase 3 levels in SKBR3 cells in the absence of NK cells were subtracted in each other condition.

10. Cytokine stimulation of primary NK cells

CD137 expression was analyzed by flow cytometry in NK cells treated with recombinant IL-2 200 U/ml or recombinant IL-15 10 ng/ml (Peprotech) for 24 and 48 hours.

11. Phospho-NF-κB p65 analysis by flow cytometry

Blocking activity of anti-TNF-Rs mAbs was confirmed pre-incubating NK cells with anti-TNFRI and –RII for 20 minutes prior to treatment with recombinant human TNF-α (10 ng/ml) for 10 minutes. Phosphorylation of NF-κB p65 was analysed by intracellular flow cytometry (clone K10-895, BD Bioscience). Alternatively, phosphorylation of NF-κB p65 in NK cells

was checked in 30-minute- to 24 hour-co-culture with SKBR3 cells (E:T 1:1) and trastuzumab (210 ng/ml), in the presence or absence of anti-TNF-α antibody infliximab (50 μg/ml).

12. IL-9 intracellular staining in CD16-expanded NK cells

6-day CD16-activated NK cells (5 μg/ml) in the presence of IL-2, TGF-β1 and/or urelumab (50 or 1 μg/ml) were re-stimulated or not with PMA/ionomycin (10 ng/ml and 1 μg/ml, respectively; Sigma-Aldrich) for 6 hours and treated with brefeldin A (10 μg/ml) for the last 4 hours. IL-9 was checked by intracellular flow cytometry (clone REA1038, Miltenyi).

13. Proliferation assays and estimation of NK cell numbers

PBMC were labeled with 0.5 μM CFSE (CellTrace CFSE Cell Proliferation Kit, Invitrogen) prior to NK cell purification. Briefly, PBMC were incubated with CFSE diluted in PBS (10^7 cells/ml) at room temperature in the dark for 8 minutes. Afterwards, an equivalent volume of FBS was added and left for 5 minutes prior to three washes in complete RPMI 1640 Glutamax medium. NK cells were purified and activated by plate-bound anti-CD16 following protocol 4. After 6 days CFSE fluorescence was checked by flow cytometry and results were expressed as percentage of NK cells undergoing ≥4 divisions, as indicated by CFSE dilution peaks.

Alive NK cell numbers were calculated by cell counts in Neubauer chamber upon trypan blue exclusion. Numbers of CD16+ NK cells were estimated based on the percentage obtained by flow cytometry in each condition.

14. Reverse transcription quantitative PCR (RT-qPCR)

Total RNA was isolated (RNeasy® Micro Kit, Qiagen) from purified NK cells or total PBMC after overnight activation with anti-CD16 (5 μg/ml) or anti-CD3 (1 μg/ml, clone SPV.T3b, kindly provided by Dr J. de Vries), respectively. cDNA was synthesized from RNA with Superscript III Reverse Transcriptase (Invitrogen). cDNA expression was analyzed by quantitative PCR using LightCycler 480 SYBR Green I Master (Roche). The expression of target genes was calculated and normalized to the expression of reference gene β2-microglobulin using the $2^{-\Delta\Delta CT}$ method. Primers pairs used (Isogen, except *TNFRSF9* which are from Invitrogen) for RT-qPCR are listed in **Table 2**.

Gene	Forward primer	Reverse primers
TNFRSF9	CACTCTGTTGCTGGTCCTCA	CACAGGTCCTTTGTCCACCT
TNFRSF4	CACTGTGTCGGGGACACCTA	ACCACGTCGTTGTAGAAGCC
CTLA4	CTACCTGGGCATAGGCAACG	CCCCGAACTAACTGCTGCAA
PDCD1	CAGTTCCAAACCCTGGTGGT	GGCTCCTATTGTCCCTCGTG
B2M	TTAGCTGTGCTCGCGCTACTCT	TGGTTCACACGGCAGGCATACT

Table 2. Primers used for RT-qPCR.

15. Microarray data processing and analysis

Microarray hybridization and analysis were performed by the microarray core facility at Hospital del Mar Medical Research Institute, Barcelona.

15.1. Sample processing and microarray hybridization

Total RNA was isolated (RNeasy® Micro Kit, Qiagen) from purified NK cells after 5 days activation with anti-CD16 (5 μg/ml), in the presence of IL-2, TGF-β1 and urelumab (50 μg/ml). RNA intregrity was assessed using Agilet 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) prior to sample processing. Amplification, labelling and hybridization was performed according to protocol GeneChip WT PLUS Reagent kit (P/N

703174 2017), and then hybridized to Human Clariom S Array (Thermo Fisher Scientific) in a GeneChip Hybridization Oven 640. Washing and scanning were performed using the Expression Wash, Stain and Scan Kit (Thermo Fisher Scientific) and the GeneChip System (GeneChip Fluidics Station 450 and GeneChip Scanner 3000 7G) according to the Expression Wash, Stain and Scan User Manual (P/N 702731 2017).

15.2. Microarray analysis

For the statistical analysis, R programming (Version 3.4.3) was used, together with different packages from Bioconductor (200) and the Comprehensive R Archive Network (CRAN 2017). After quality control of raw data, samples were background corrected, quantile-normalized and summarized to a gene-level using the robust multi-chip average (RMA) (201) obtaining a total of 20893 transcript clusters. Clustering methods were used and a possible batch effect was discarded. An empirical Bayes moderated t-statistics model (LIMMA) (202) adjusting for the variable "sample" as a random effect was built to detect differentially expressed genes between the studied conditions. Specifically, genes with a p-value<0.05 and a |log fold-change|>0.585 were considered to be significantly different between conditions. Genes differentially expressed between α-CD16+TGF-β1 and α-CD16+TGF-β1+urelumab were loaded into the ingenuity pathway analysis (IPA) software to analyse the biological functions, pathways and networks modulated by urelumab. Gene set enrichment analysis (GSEA) of differentially expressed genes were performed against the Molecular Signatures Database (MSigDB) collection "C7: immunologic gene sets" available at the Broad Institute website (software.broadinstitute.org/gsea/msigdb). Differentially expressed genes between murine ILC1s and NK cells isolated from intestine and liver were generated from the GEO dataset GSE37448 (203) and used for GSEA.

16. IFN-γ, IL-9 and CCL5 analysis by ELISA

Production of IFN-γ, CCL5 and IL-9 secretion was measured in 6-day cell-free culture supernatants (centrifuged at 1250xg, 10 minutes) with #88-7316-88, Invitrogen; DY278, R&D and DY209, R&D commercial ELISAS, respectively, following manufacturer instructions. Alternatively, IFN-γ production was measured in overnight cell-free culture supernatants from 6-day-expanded NK cells re-estimulated by anti-CD16 mAb (5 μg/ml) and recombinant IL-12 (10 ng/ml, Peprotech). Optical density was determined on Infinite 200 Pro plate reader (Tecan). Readings at 570 nm and blank were subtracted from readings at 450 nm, and protein concentration was calculated by an 8-point standard curve.

17. TGF-β-RII, Smad4/7 and β-actin expression by Western Blot

NK cells or PBMC dry pellets ($5x10^5$) cells were lysed in RIPA buffer (40μl/10^6 cells; 0.15M NaCl, 1% Nonidet P-40, 0.1% SDS, 50mM Tris (pH 7.5), supplemented with protease and phosphatase inhibitors: 1mM PMSF, 2mM Na_3VO_4, 5mM NaF, 10mM β-glycerol-phosphate, 5mM EDTA, and 1 mM protease inhibitor cocktail Sigma (P8340, Sigma-Aldrich). Protein extracts were denatured in Laemmli buffer, boiled 5 minutes at 95 ºC, separated by SDS-PAGE 10% under reducing conditions (170 V, 1-2 hours), and transferred (wet transfer at 4 ºC, 100 V, 1 hour 30 minutes) onto PVDF membranes (Immobilon, Millipore). Blots were blocked for 2 hours at room temperature or overnight at 4ºC in 5% bovine serum albumin or non-fat dry milk in TBS + 0.05% Tween 20. Specific antibodies were subsequently used following manufacture's recommendations: anti-Smad4 (#46535, Cell Signaling, 1:1000); monoclonal mouse anti-Smad7 (MAB2029, R&D Systems, 1:1000), anti-TGFβ-RII (sc-17791, Santa Cruz Biotechnology, 1:100) and anti-β-actin

(A5441, Sigma-Aldrich). Proteins were detected with HRP-labelled anti-rabbit or mouse antibodies (GE Healthcare, 1:3000 except for β-actin 1:10000) and ECL substrate (SuperSignal West Pico or Femto Chemiluminescent Substrate, Thermofisher). The intensity of Western Blot bands was quantified by ImageJ (National Institutes of Health).

18. Statistics

Statistical analyses were performed using Graphpad Prism 6.0 software. All data were analyzed with a paired or unpaired Mann-Whitney test. A *P* value of ≤0.05 was considered significant.

RESULTS

1. Regulation of CD137 expression in CD16-activated NK cells

1.1. Identification of CD137 (4-1BB) as a targetable receptor in activated NK cells

Several immunomodulatory monoclonal antibodies (mAbs) targeting activation-induced stimulatory (e.g. TNFRSF members) and inhibitory (e.g. PD-1, CTLA-4) receptors on immune effectors cells have been approved or are in clinical development with the aim of re-establishing T cell responses against tumors (204,205). Despite the fact that NK cells share diverse effector functions with CD8+ T cells, the hierarchy of activation-induced receptor expression and the effect of immunomodulatory mAbs on NK cell antitumor function needs further investigation. In order to identify actionable checkpoints for enhancing NK-cell mediated ADCC responses against tumor cells, we evaluated the expression profile of activation-induced stimulatory and inhibitory receptors in CD16-activated NK cells.

Microarray analysis previously performed in the laboratory using polyclonal primary NK cells activated through plate-bound anti-CD16 agonistic antibody showed the up-regulation of stimulatory (i.e. *TNFRSF9, TNFRSF18, TNFRSF4, TNFRSF1B* respectively encoding for CD137 or 4-1BB, GITR, OX40 and TNF-RII molecules) rather than inhibitory (i.e. *CTLA4* and *PDCD1* encoding for PD-1) receptor mRNA expression at 12 hours. Among TNF receptor superfamily (TNFRSF) members, CD137 was the one showing the highest induction (**Figure 1A**). Induction of CD137 in the absence of PD-1 mRNA in CD16-activated NK cells was confirmed by reverse transcription quantitative PCR (RT-qPCR) (**Figure 1B**). In contrast, CD3 crosslinking resulted in the up-regulation of CD137, OX40, CTLA-4 and PD-1 mRNA in PBMCs (**Figure 1C**).

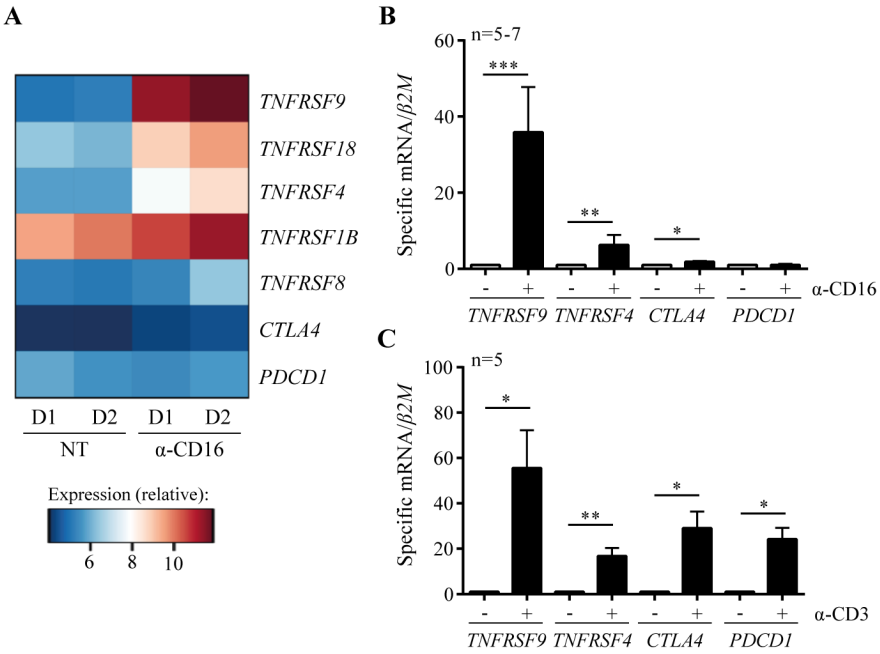

Figure 1. CD16-dependent activation triggered stimulatory rather than inhibitory co-receptor mRNA expression in NK cells. (A, B) NK cells were activated by plate-bound α-CD16 antibody and analyzed for mRNA expression. **(A)** Microarray analyses of gene expression at 12h. Heatmap represents relative expression of *TNFRSF9, TNFRSF18, TNFRSF4, TNFRSF1B, CTLA4* and *PD1* in two different donors (D1, D2). NT: non-treated **(B)** mRNA expression of *TNFRSF9, TNFRSF4, CTLA4, PDCD1* relative to β2-microglobulin determined by reverse transcription quantitative PCR (RT-qPCR) after O/N activation. **(C)** Relative mRNA expression of *TNFRSF9, TNFRSF4, CTLA4, PDCD1 in* PBMC after O/N activation by plate-bound α-CD3 antibody. Graphs represent mean±SEM of data from 5-7 individuals in independent experiments. Statistical significance was calculated using the Mann-Whitney U test (* ≤ .05, ** ≤ .01, *** ≤ .001).

In order to validate the induction of CD137 expression in NK cells upon CD16-activation, we set up an *in vitro* experimental system by coculturing purified primary NK cells with the HER2+ breast cancer cell line SKBR3 in the presence of anti-HER2 therapeutic antibody trastuzumab. Coculture with SKBR3 and trastuzumab induced a potent NK cell degranulation as analyzed by CD107a mobilization assay and TNF-α production, leading to target cell apoptosis, as measured by activation of caspase 3 by flow cytometry (**Figure 2**).

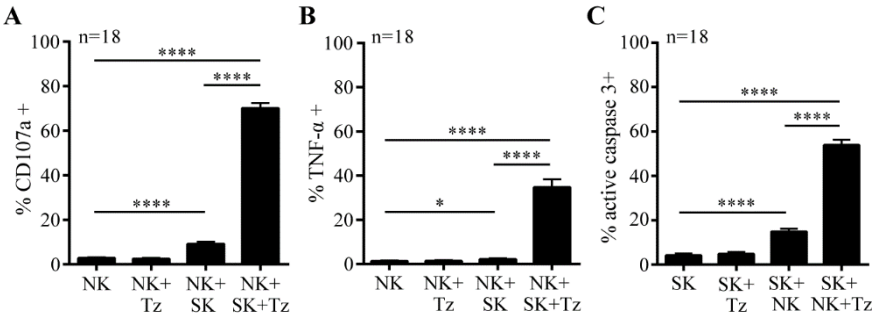

Figure 2. Trastuzumab-triggered NK cell-mediated ADCC against HER2+ breast cancer cells. Purified NK cells were cocultured with SKBR3 (SK) cells in the presence of trastuzumab (Tz, 210 ng/ml). NK cell degranulation (CD107a mobilization assay) and TNF-α production (E:T 4:1), as well as active caspase 3 in SKBR3 cells (E:T 1:2) were checked in parallel assays at 4h by flow cytometry. **(A-C)** Graphs represent mean±SEM of data obtained using NK cells from 18 donors in multiple independent experiments. Statistical significance was calculated using the Mann-Whitney U test (* ≤ .05, **** ≤ .0001).

In agreement with mRNA expression, NK cell coculture with SKBR3 and trastuzumab resulted in the up-regulation of CD137 and OX40 surface expression, but not PD-1, as checked by flow cytometry at 24 hours post-activation (**Figure 3A, B**). CD137 and OX40 induction was mainly observed in $CD56^{dim}$ NK cells, and paralleled surface CD16 down-regulation (**Figure 3A**). Time-course experiments showed that CD137

was up-regulated with early kinetics (i.e. 8 hours), large magnitude and sustained persistence, whereas OX40 expression peaked at 24 hours and markedly decreased at 48 hours post-activation (**Figure 3C**). Hence, our results pointed to CD137 as a targetable receptor induced upon NK cell-mediated ADCC against breast cancer cells.

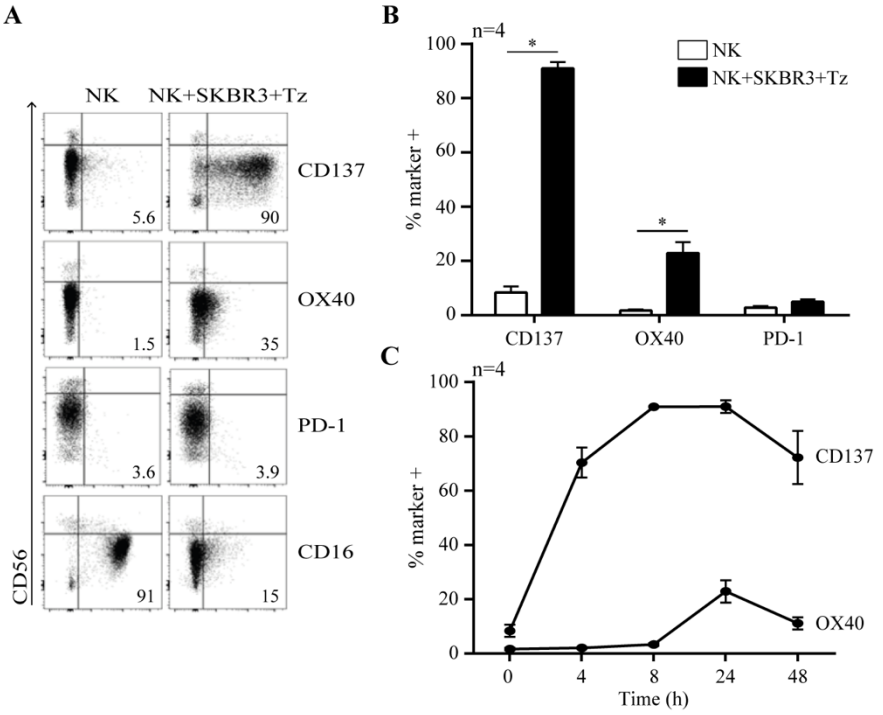

Figure 3. ADCC induced the expression of CD137 and OX40 expression, concomitant to CD16 downregulation in NK cells. NK cells were cocultured with SKBR3 cells in the presence of trastuzumab (Tz, 210 ng/ml). **(A, B)** Expression of CD137, OX40, PD-1 and CD16 in NK cells at 24h by flow cytometry. **(A)** Dot plots from a representative donor are shown. Inset numbers indicate proportions of marker+ NK cells. **(B)** Mean±SEM of data from marker+ $CD56^{dim}$ NK cells from 4 donors in independent experiments. Statistical significance was calculated using the Mann-Whitney U test (* ≤ .05). **(C)** Kinetics of CD137 and OX40 expression in $CD56^{dim}$ NK cells along coculture with trastuzumab-coated SKBR3 cells.

1.2. Cooperation between activating receptors and autocrine TNF-α regulates CD137 and OX40 expression in NK cells.

We next addressed whether other activating stimuli could also induce CD137 expression in NK cells. NK cell stimulation by IL-2 and IL-15 cytokines or by redirected activation through NK cell receptors (NKR) coupled to tyrosine kinase-mediated signaling pathways (i.e. CD16, NKp30, NKp46) induced surface CD137 up-regulation in a variable proportion of cells (**Figure 4A, B**). Noteworthy, the frequency of NK cells up-regulating CD137 was proportional to the percentage of degranulation upon redirected activation through distinct NKR, likely reflecting the magnitude of NK cell activation (**Figure 4B**). Indeed, experiments of NK cell redirected activation by increasing doses of anti-CD16 agonist mAb showed that CD137 surface expression was proportional to the intensity of signal through the activating NKR (**Figure 4C**).

As members of the TNF receptor superfamily, CD137 and OX40 have been shown to be up-regulated by TNF-α in regulatory T cells (206,207). Based on these observations, we aimed at elucidating whether autocrine TNF-α produced along ADCC was also regulating the expression of both receptors in activated NK cells. We used the TNF-α neutralizing mAb infliximab, and supplementation with recombinant TNF-α in NK-SKBR3-trastuzumab cocultures. Addition of infliximab reduced the expression of CD137 and OX40 in NK cells upon ADCC (**Figure 5A, B**). On the other hand, recombinant TNF-α triggered low CD137 and OX40 expression in resting NK cells and enhanced the expression of both molecules upon ADCC (**Figure 5A, B**). Time-course experiments showed that treatment with infliximab effectively blocked TNF-α signaling through NF-κB along ADCC as evidenced by decreased phosphorylation of p65 NF-κB in NK cells at 30 minutes and 24 hours post-activation (**Figure 5C**).

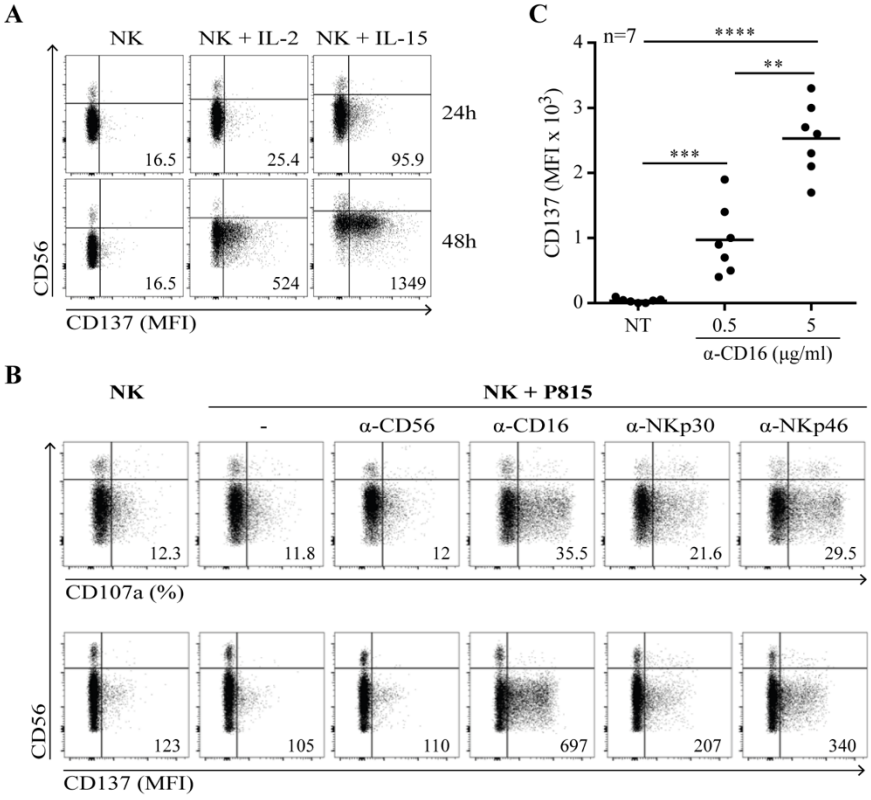

Figure 4. Stimulatory cytokines and activating NKR regulated CD137 expression in NK cells. **(A)** NK cells were stimulated with IL-2 (200 U/ml) or IL-15 (10 ng/ml) for 24 or 48h. **(B)** P815 cells were incubated with α-CD56, -CD16, -NKp30 or –NKp46 antibodies prior to co-culture with NK cells (ratio 1:1). NK cell degranulation and CD137 expression were checked by flow cytometry at 4 and 24h, respectively. **(A, B)** Dot plots showing CD107a and CD137 expression in NK cells from representative donors are shown. Inset numbers indicate CD137 mean fluorescence intensity (MFI) and percentage of CD107+ NK cells. **(C)** NK cells were activated by plate-bound α-CD16 antibody and analyzed for surface CD137 expression at 24h by flow cytometry. Graph represents CD137 MFI on NK cells from 7 donors in independent experiments. NT: non-treated. Statistical significance was calculated using the Mann-Whitney U test (** ≤ .01).

Despite effective signaling blockade, presence of infliximab did not affect CD137 expression at early time-points yet resulted in a reduction of CD137 levels at 24 and 48 hours post NK cell activation (**Figure 5D, E**). In contrast, trastuzumab-induced up-regulation of surface OX40 expression in NK cells was virtually inhibited in the presence of infliximab and significantly enhanced by the addition of rTNF-α (**Figure 5F**).

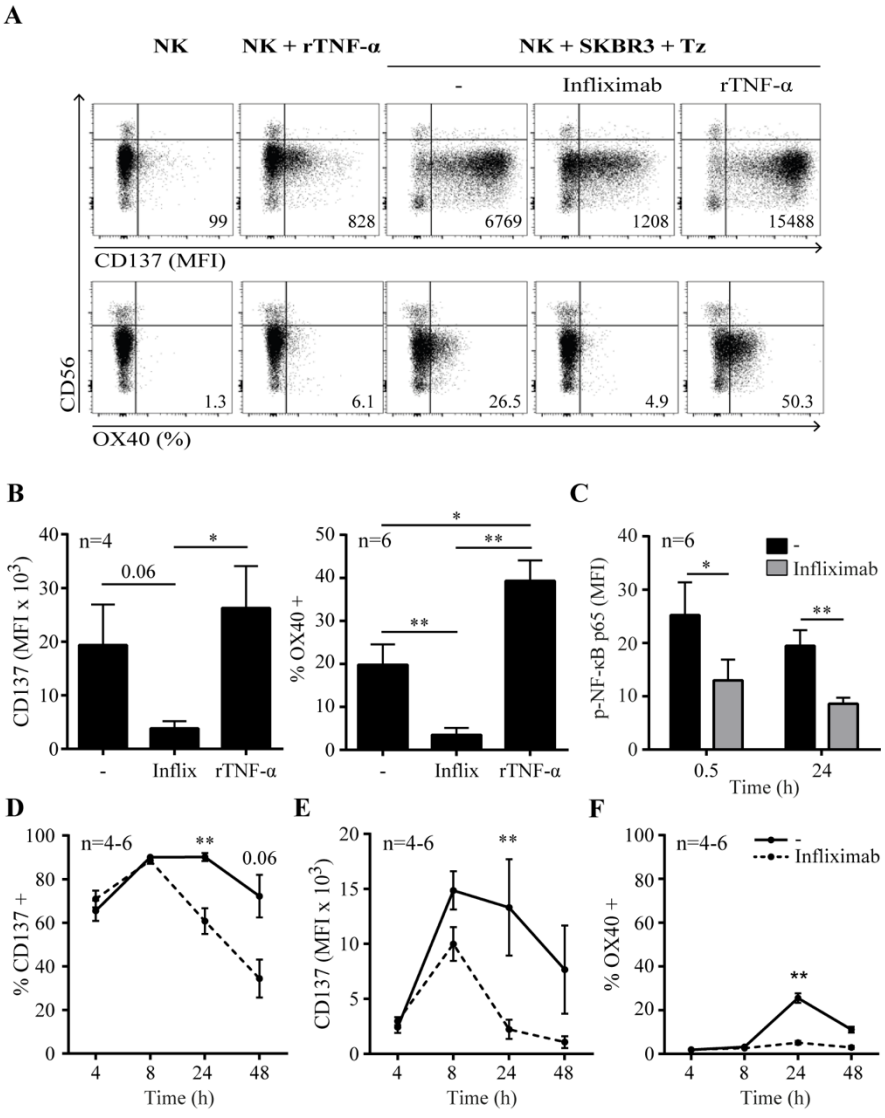

Figure 5. Regulation of CD137 and OX40 expression in activated NK cells by autocrine TNF-α. NK cells were cocultured with SKBR3 (ratio 1:1) and trastuzumab (Tz, 210 ng/ml) in the presence or absence (-) of the anti-TNF-α mAb, infliximab (Inflix, 50 μg/ml), or recombinant human TNF-α (rTNF-α, 10 ng/ml). **(A)** Dot plots showing CD137 and OX40 expression at 24h upon ADCC including TNF-α blockade or rTNF-α supplementation in NK cells from a representative donor. Inset numbers indicate CD137 mean fluorescence intensity (MFI) and percentage of OX40+ NK cells. **(B)** Mean±SEM of CD137 and OX40 expression in $CD56^{dim}$ NK cells from 4-6 donors in independent experiments. **(C)** Phospho-NF-κB p65 in $CD56^{dim}$ NK cells at 30min and 24h after trastuzumab-induced ADCC in the presence or absence (-) of infliximab. **(D-F)** Kinetics of CD137 and OX40 expression in $CD56^{dim}$ NK cells upon trastuzumab-induced ADCC in the presence or absence (-) of infliximab. Graphs represent mean±SEM of data from 4-6 independent experiments. Statistical significance was calculated using the Mann-Whitney U test (* ≤ .05, ** ≤ .01).

TNF-α signals through TNF-RI (*TNFRSF1A*) and TNF-RII (*TNFRSF1B*) (208). Expression of TNF-RI and TNF-RII was detected by flow cytometry in resting NK cells and TNF-RII expression was enhanced upon trastuzumab-induced ADCC. Of note, levels of TNF-RI were remarkably low as compared to TNF-RII and did not change upon activation (**Figure 6A**). Blocking antibodies specific for TNF-RI or –RII showed that dual blockade was required for abrogating TNF-α-dependent CD137 and OX40 expression in activated NK cells, whereas single blockade of TNF-RI or TNF-RII only resulted in a partial inhibition (**Figure 6B-D**). Neither the addition of infliximab nor TNF-R blocking antibodies induced changes in NK cell degranulation along coculture with SKBR3 and trastuzumab, excluding an effect of these antibodies on NK cell activation (**Figure 7A, B**). Moreover, the blocking capacity of anti-TNF-RI and –RII antibodies was confirmed by the abrogation of TNF-α-induced phospho-p65 NF-κB, as measured by intracellular flow cytometry (**Figure 7C**).

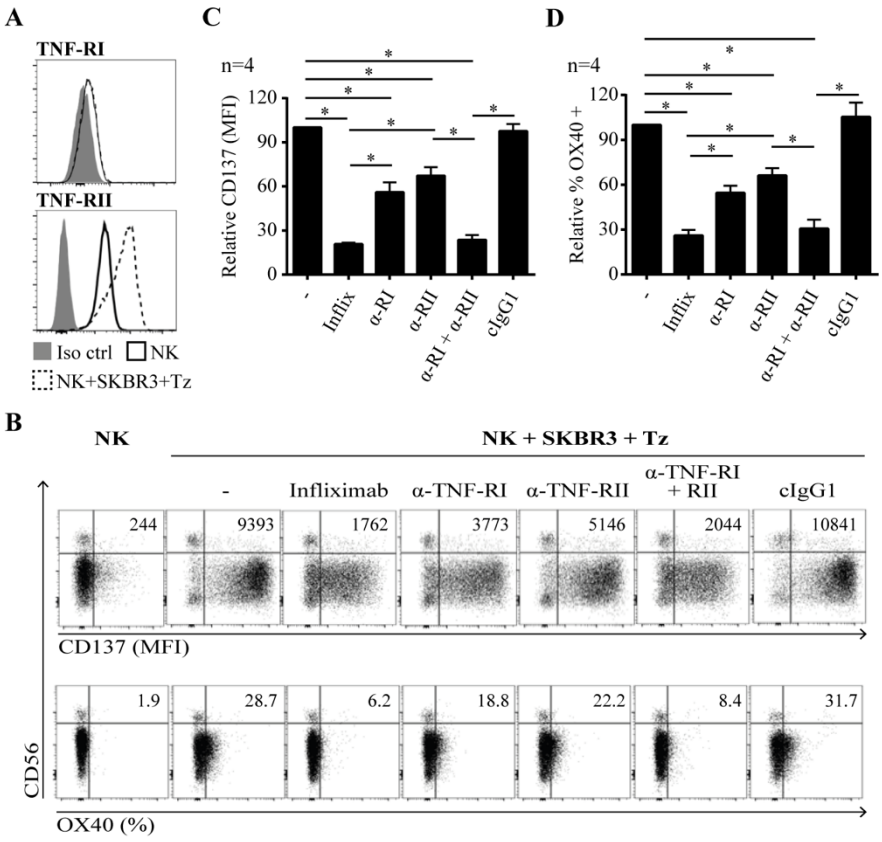

Figure 6. Signaling through TNF-RI and –RII regulates TNF-α dependent expression of CD137 and OX40 in NK cells upon ADCC. NK cells were cocultured with SKBR3 cells (ratio 1:1) and trastuzumab (Tz, 210 ng/ml) in the presence or absence of infliximab (50 µg/ml), anti-TNFRI (α-RI, 10 µg/ml), anti-TNFRII (α-RII, 10 µg/ml) blocking antibodies or control mouse IgG1 antibody (cIgG1, 10 µg/ml) for 24h. CD137 and OX40 expression in NK cells was analyzed by flow cytometry. **(A)** Histograms depicting TNF-RI and TNF-RII staining in resting (NK, solid line) and trastuzumab-activated NK cells (NK+SKBR3+Tz, dashed line). Control staining (Iso ctrl, filled histogram) is shown. **(B)** Dot plots showing CD137 and OX40 expression in NK cells from two representative donors. Inset numbers indicate CD137 mean fluorescence intensity (MFI) and percentage of OX40+ NK cells. **(C, D)** Mean±SEM of CD137 and OX40 expression in $CD56^{dim}$ NK cells from 4 independent donors normalized to trastuzumab-activated NK cells in the absence of blocking antibodies (-). Statistical significance was calculated using the Mann-Whitney U test (* ≤ .05).

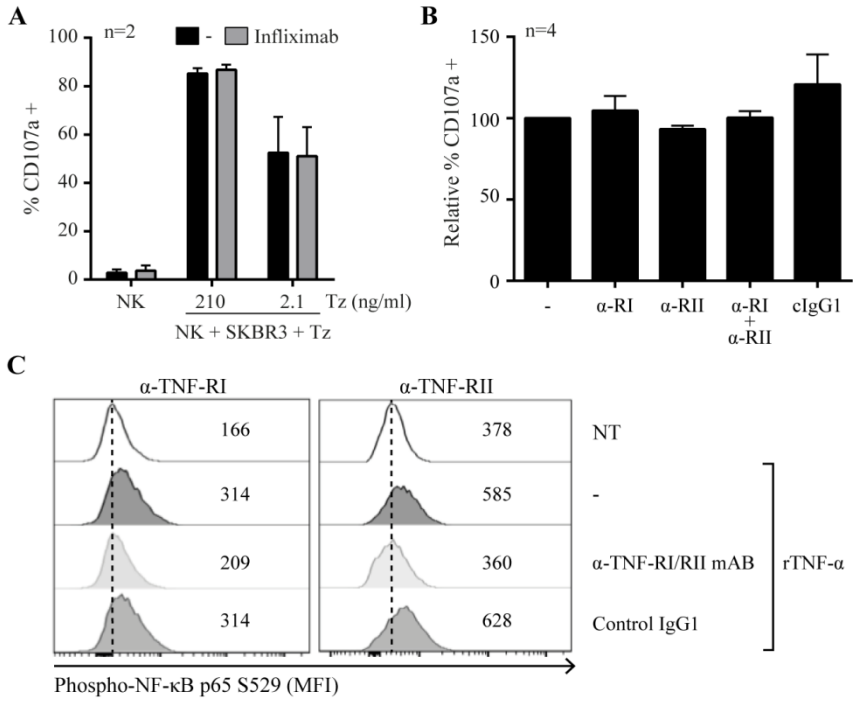

Figure 7. Effect of anti-TNF-α (infliximab), anti-TNF-RI and anti-TNF-RII blocking antibodies on NK cell degranulation and phosphorylation of p65 NF-κB. (A) NK cells were incubated with 10 μg/ml of anti-TNF-RI (α-RI), anti-TNF-RII (α-RII), or control mouse IgG1 antibody (cIgG1) and co-cultured with SKBR3 (ratio 1:1) and trastuzumab (210 or 2.1 ng/ml). NK cell degranulation (CD107a) was analyzed cells by flow cytometry at 4h. **(A, B)** Graphs represent mean±SEM of data in $CD56^{dim}$ NK cells from 2-4 individuals in independent experiments. In **(B)**, data is normalized to trastuzumab-activated NK cells in the absence of blocking antibodies (-). Statistical significance was calculated using the Mann-Whitney U test. **(C)** NK cells were incubated with 10 μg/ml of α-TNF-RI/-RII or control IgG1 for 20 minutes and subsequently treated with rTNF-α (10 ng/ml) for 10 minutes. Phospho-NF-κB p65 S529 was checked by intracellular flow cytometry. Inset numbers indicate phospho-NF-κB p65 mean fluorescence intensity (MFI).

Altogether, these results point towards a cooperation between signaling through CD16 and autocrine TNF-α in the regulation of CD137 expression in NK cells after ADCC, whereas OX40 expression was dependent on TNF-α produced along NK cell activation.

1.3. Induction of CD137 is enhanced in adaptive NKG2C+ NK cells upon ADCC

Among different NK cell subpopulations, adaptive NKG2C+ NK cells have been shown to be proficient on ADCC and TNF-α production (78,79). To evaluate whether presence of adaptive NKG2C+ NK cells influenced ADCC-induced expression of CD137 and OX40 in NK cells, donors with NKG2C^{bright} (i.e. >30% NKG2C+ NK cells) or NKG2C^{dim} (i.e. <5% NKG2C+ NK cells) NK cell profile paired by their CD16A F/V genotype were selected for ADCC experiments (**Figure 8A**). NK cells from individuals harboring NKG2C+ NK cells expansions showed higher percentage of FcεR γ-chain deficient cells and increased production of TNF-α after ADCC, in comparison to NK cell samples lacking adaptive expansions (NKG2C^{dim}) (**Figure 8B**). Initial ADCC-induced CD137 up-regulation was comparable in all NK cell samples, however, NK cells from NKG2C^{bright} donors maintained sustained CD137 expression whereas in samples from donors lacking adaptive NK cells CD137 expression decreased at 24 hours post-activation (**Figure 8C**). Indeed, higher CD137 expression was detected in NKG2C+ as compared to NKG2C- NK cells in NKG2C^{bright} donors (**Figure 8D**). On the contrary, no differences were observed in OX40 expression on activated NK cells between donors with different NKG2C profile (**Figure 8E**).

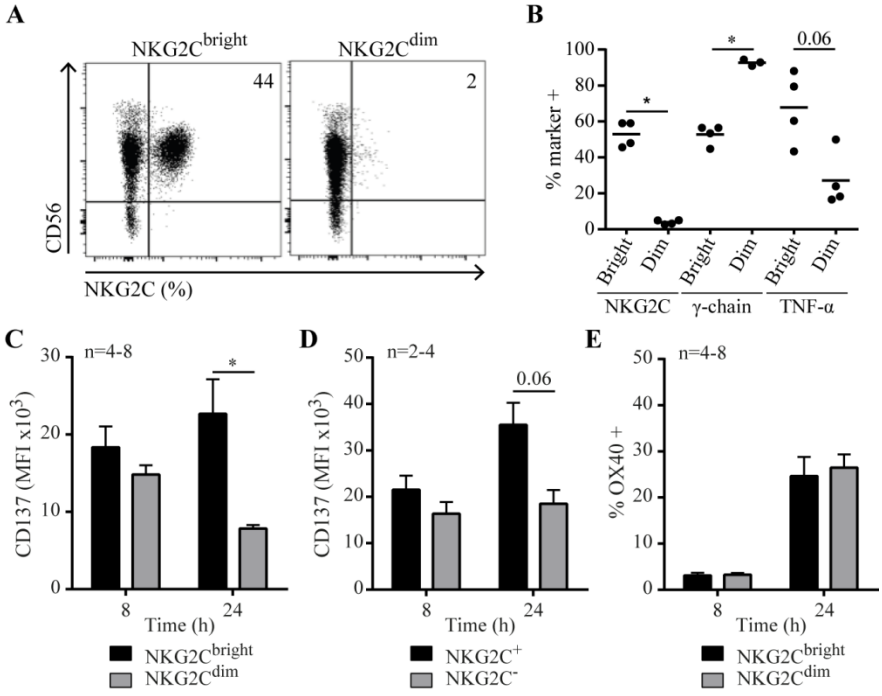

Figure 8. CD16-induced CD137, but not OX40 expression, is higher in NK cells from individuals with adaptive NKG2C+ NK cells expansions. **(A)** Dot plots showing proportions of NKG2C+ cells in NK cell samples from two representative donors with distinct NKG2C profile. Inset numbers represent percentage of NKG2C+ NK cells. **(B)** NK cells from donors with NKG2C^{bright} or NKG2C^{dim} profile were characterized for the expression of NKG2C, γ-chain and trastuzumab-induced TNF-α production (4h-coculture with SKBR3 and trastuzumab (210 ng/ml), E:T 4:1). **(C-E)** CD137 and OX40 expression in CD56^{dim} NK cells at 8 or 24h coculture with SKBR3 (E:T 1:1) and trastuzumab (210 ng/ml). **(C, E)** Graphs represent mean±SEM of data from 4-8 individuals paired by their CD16A F/V genotype and with NKG2C profile, tested in 2-4 independent experiments (minimum n, t=8h). **(D)** Graph represent mean±SEM of data from 2-4 individuals with NKG2C^{bright} profile tested in 2-4 independent experiments (minimum n, t=8h). Statistical significance was calculated using the Mann-Whitney U test (* ≤ .05).

1.4. Anti-HER2 antibody treatment induced CD137 up-regulation in tumor-associated NK cells

We next interrogated whether CD137 could be induced by trastuzumab in the tumor microenvironment. For this purpose, cellular suspensions were obtained by mechanical and enzymatic digestion of treatment-naïve breast carcinoma specimens and unsorted multicellular fractions were cultured overnight in the presence of IL-2 and trastuzumab. After overnight culture, CD137 expression was analyzed by flow cytometry in tumor-associated lymphocytes. In line with previous results, trastuzumab induced the expression of CD137 but not PD-1 on tumor-associated CD16+ NK cells (**Figure 9A, B**). Remarkably, tumor-associated CD8+ and CD4+ T cells, expressing variable levels of PD-1 depending on the tumor sample, did not up-regulate CD137 after trastuzumab treatment (**Figure 9B**). Thus, in whole primary tumor cultures, trastuzumab treatment promoted the up-regulation of CD137 expression in CD16+ tumor-associated NK cells.

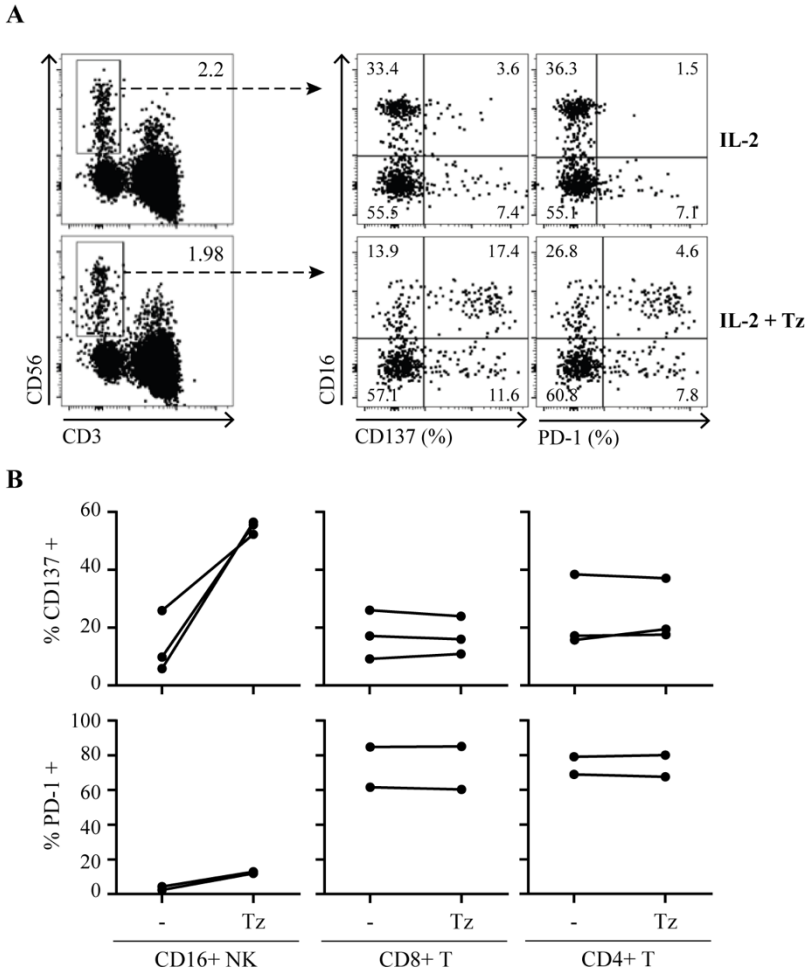

Figure 9. Trastuzumab treatment of breast carcinoma-derived multicellular culture induced CD137 expression in CD16+ tumor-associated NK cells. Unsorted multicellular suspensions obtained by enzymatic and mechanical digestion of fresh breast carcinoma specimens were cultured in complete medium with IL-2 (200 U/ml) in the presence or absence (-) of trastuzumab (Tz, 210 ng/ml) for 24h. CD137 and PD-1 expression in tumor-associated lymphocytes was analyzed by flow cytometry. **(A)** Dot plots showing CD137, PD-1 and CD16 expression in tumor-associated NK cells from one representative culture. Inset numbers indicate the percentage of CD137+ or PD-1+ NK cells. **(B)** CD137 and PD-1 expression in tumor associated CD16+ NK, CD8+ and CD4+ T cells. Graphs represent data from 3 different whole tumor-derived cultures.

2. Functional impact of the anti-CD137 agonistic antibody urelumab in the antitumor activity of NK cells

Engagement of CD137 by agonistic antibodies has been shown to enhance NK cell proliferation and survival, as well as IFN-γ production *in vitro* (94,95,209). However, the effect of CD137 costimulation on NK cell-mediated ADCC remains unclear. On the other hand, TGF-β, a major immunosuppressive factor in the tumor microenvironment, has been shown to inhibit NK cell proliferation, as well as the expression of activating receptors and effector molecules (e.g. NKp30, NKG2D, granzymes, perforin and IFN-γ) (158–161), altogether impairing direct and antibody-mediated killing of target cells in *in vitro* experiments (159,160).

Based on these premises we next evaluated whether an anti-CD137 agonistic antibody urelumab (210) could enhance NK cell-mediated antitumor responses in a TGF-β1-rich microenvironment.

2.1. CD137 ligation by an agonistic antibody enhances CD16-induced NK cell proliferation and survival in response to IL-2, overcoming TGF-β1 inhibition

Initial experiments were aimed at assessing whether CD137 crosslinking with the agonistic mAb urelumab could enhance activation-induced proliferation in NK cells. CFSE-labeled primary NK cells were activated through plate-bound anti-CD16 antibody in the presence of IL-2. Subsequently, urelumab and TGF-β1 were added after 8 hours, and proliferation was assessed by CFSE dilution at 6 days, together with the analysis of CD25 (IL-2Rα) expression by flow cytometry. Agreeing with previous reports (85), CD16-induced IL-2-dependent NK cell proliferation was almost abrogated by TGF-β1. Crosslinking of CD137 enhanced

CD16-induced proliferation at basal conditions and bypassed TGF-β1 inhibition, restoring NK cell proliferation in a dose-dependent manner (**Figure 10A, B**). Accordingly, expression of CD25 in activated NK cells was inhibited by TGF-β1 and rescued by CD137 costimulation (**Figure 10C, D**). As a consequence, addition of urelumab resulted in an increased recovery of total and CD16+ NK cells at 6 days post-activation in a TGF-β1-rich milieu (**Figure 10E, F).** Control human IgG4 (cIgG4) had no effect in any of the monitored readouts.

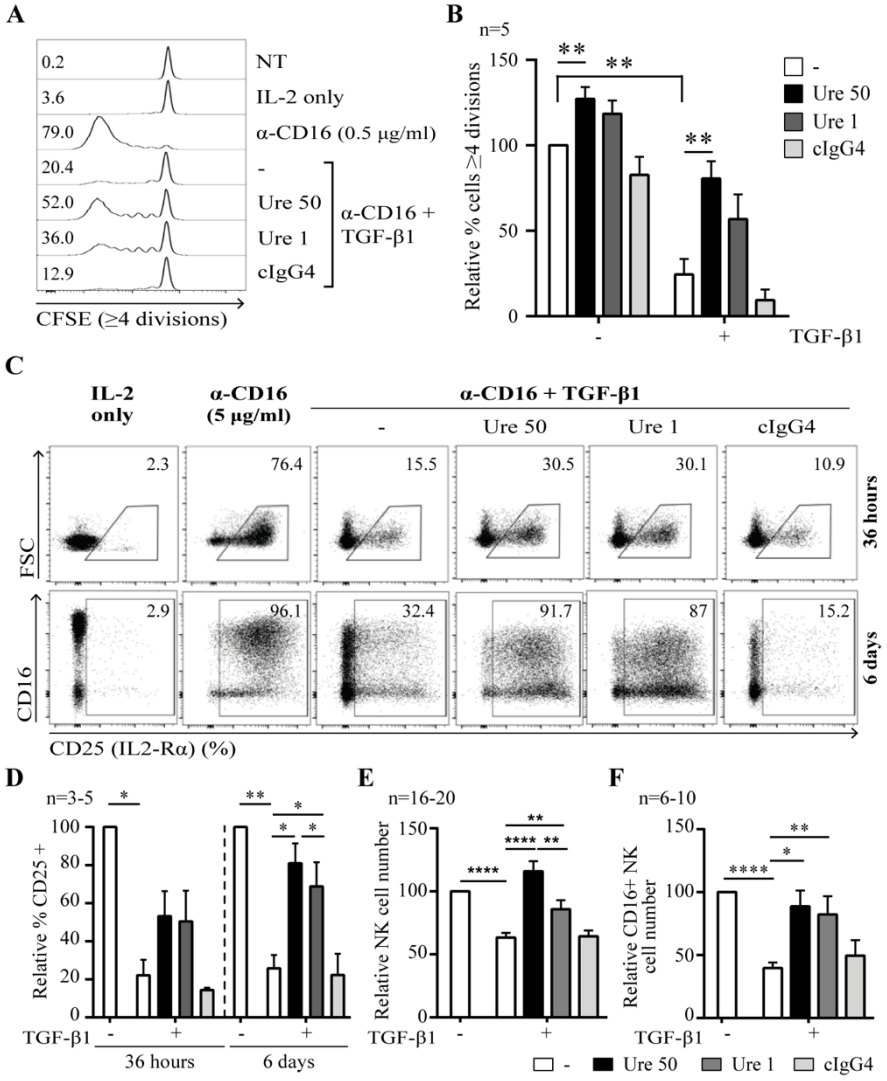

Figure 10. CD137 co-stimulation in CD16-activated NK cells counteracted TGF-β1-mediated inhibition, restoring CD25 expression, IL-2-dependent proliferation, and CD16+ NK cell numbers. CFSE-labeled NK cells were activated with α-CD16 and IL-2 (200 U/ml) in the presence or absence of TGF-β1 (10 ng/ml), urelumab (Ure, 50 or 1 μg/ml) and control human IgG4 (cIgG4, 50 μg/ml). **(A, B)** Proliferation was assessed by CFSE dilution by flow cytometry at 6 days after activation with α-CD16 (0.5 μg/ml). **(C, D)** CD25 (IL2Rα) expression was checked at 36h and 6 days after α-CD16 (5μg/ml) by flow cytometry. **(E, F)** Total and CD16+ NK cell numbers at 6 days. **(A, C)** Histograms and dot plots from data of representative experiments are shown. Inset numbers indicate proportions of NK cells divided 4 times or more **(A)** and percentage of CD25+ cells **(C)**. **(B, D-F)** Bar graphs show mean±SEM of data from multiple independent experiments normalized to data in CD16-activated NK cells. Statistical significance was calculated using the Mann-Whitney U test (* ≤ .05, ** ≤ .01, **** ≤ .0001). NT: non-treated.

Due to changes in CD56 expression upon CD16 activation in the presence of IL-2, combined staining of surface CD56 and CD117 (c-Kit) was used for distinguishing $CD56^{bright}$ from $CD56^{dim}$ NK cell subsets in 6-day cultures (60) (**Figure 11A**). Numbers of $CD56^{bright}$ $c\text{-}Kit^{bright}$ ($CD56^{bright}$) cells remained relatively stable in all conditions whereas numbers of $CD56^{dim/-}$ NK cells decreased upon CD16-activation in the presence of TGF-β1, and recovered in cultures with urelumab (**Figure 11B, C).** Proliferation experiments comparing simultaneous (t=0h) or sequential (t=8h) treatment with α-CD16 and TGF-β1 showed comparable results (**Figure 12**).

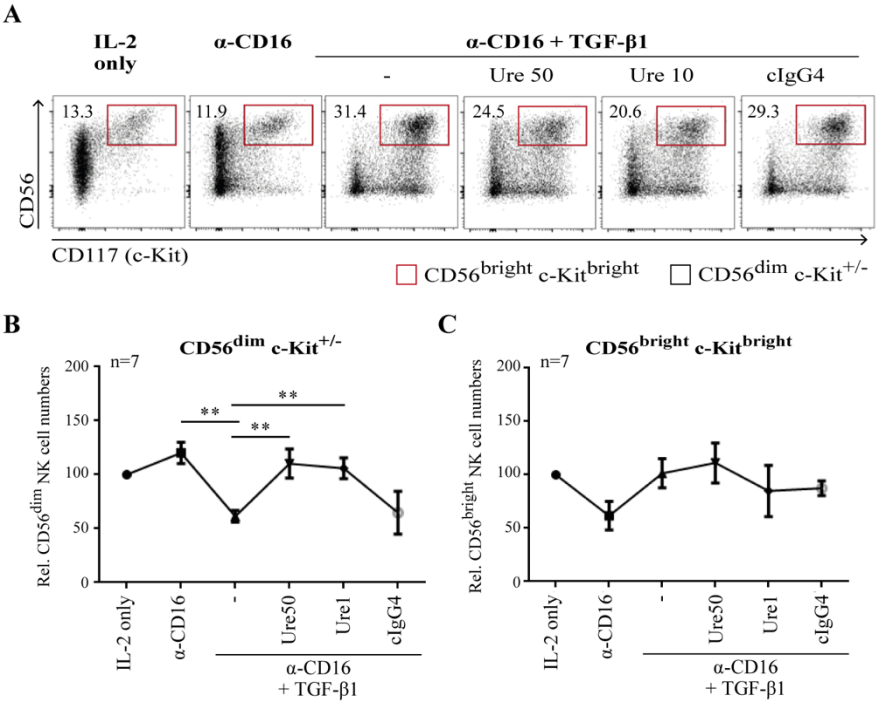

Figure 11. Proliferation of CD56^{bright} cKit^{bright} and CD56$^{dim/-}$ cKit$^{+/-}$ NK cell numbers in different culture conditions. NK cells were activated with α-CD16 (5 µg/ml) and IL-2 (200 U/ml) in the presence or absence of TGF-β1 (10 ng/ml), urelumab (Ure, 50 or 1 µg/ml) and control human IgG4 (cIgG4, 50 µg/ml). **(A)** CD56^{bright} (CD56^{bright} cKit^{bright}) and CD56^{dim} (CD56^{dim} cKit$^{+/-}$) were gated by CD56 and cKit expression at 6 days. Dot plots from a representative donor are shown. Inset numbers represent percentage of CD56^{bright} c-Kit^{bright} NK cells. **(B, C)** Graphs represent mean±SEM of CD56^{bright} cKit^{bright} and CD56^{dim} cKit$^{+/-}$ NK cell numbers from 7 donors normalized to IL-2 only at 6 days post-activation. Statistical significance was calculated using the Mann-Whitney U test

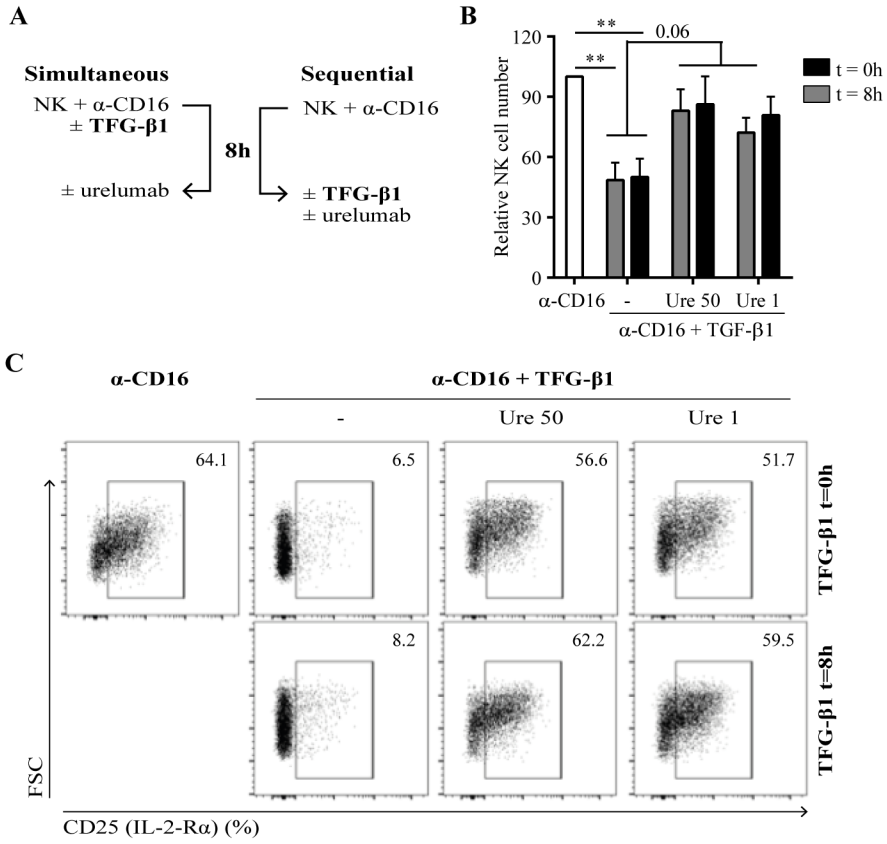

Figure 12. Influence of TGF-β1 and urelumab on NK cell numbers and CD25 expression were equally observed regardless of TGF-β1 timing. NK cells were activated through CD16 (5 µg/ml) in the presence of IL-2 (200 U/ml), and simultaneously (t=0h) or subsequently (t=8h) treated with TGF-β1 (10 ng/ml). Urelumab (50 µg/ml) was added at 8h. NK cell numbers and CD25 expression were analyzed at 6 days. **(A)** Scheme on the experimental design. **(B)** Graph of mean±SEM numbers of NK cells recovered in 5 independent experiments normalized to α-CD16. Statistical significance was calculated using the Mann-Whitney U test (** ≤ .01). **(C)** Dot plots from a representative experiment out of 2 performed showing percentage of CD25+ $CD56^{dim}$ NK cells in each condition (inset numbers).

In order to characterize global changes in the transcriptome of CD16-activated NK cells in the presence of TGF-β1 and urelumab, we performed microarray analysis at 5 days post-activation. 2477 genes were differentially expressed in CD16-activated versus IL-2-treated NK cells (i.e. p-value<0.05 and a |log fold-change|>0.585), 1877 genes were differentially expressed in CD16-activated NK cells in the presence or absence of TGF-β1, and 85 genes in urelumab-treated versus non-treated CD16-activated NK cells in the presence of TGF-β1 (**Figure 13A**). Unsupervised analysis of the 85 genes differentially expressed between TGF-β1-treated CD16-activated NK cells in the presence or absence of CD137 costimulation by Ingenuity Pathway Analysis (IPA) identified "Cellular growth and proliferation" as the biological pathway most significantly affected by CD137-costimulation followed by "Cell death and survival" and "Cell movement", among others (**Figure 13B**). Analysis of upstream regulators of differentially expressed genes predicted the activation of TNF (p-value 0.003, z-score 2.5) and NF-κB complex (p-value ≤0.001, z-score 2.2), linked to lymphocyte proliferation through changes in *VCAM1, CD80, LTA, SDC4, CSF2* and *IL13* mRNA expression (**Figure 13C**). A supervised analysis confirmed that CD137 costimulation: (i) increased expression of genes involved in CD137 signaling (i.e. *TRAF1, TAB1, NFKBIA, NFKBIB, NFKB1, NFKB2*); (ii) up-regulated mRNA levels of *MYC*, *CCNB1*, *CCDNB2*, *CDK4* or *CDK6,* related to NK cell proliferation; and (iii) increased mRNA expression of anti-apoptotic molecules *BCL2A1* and *BCL2L1* (Bcl-xL) (**Figure 13D-F**).

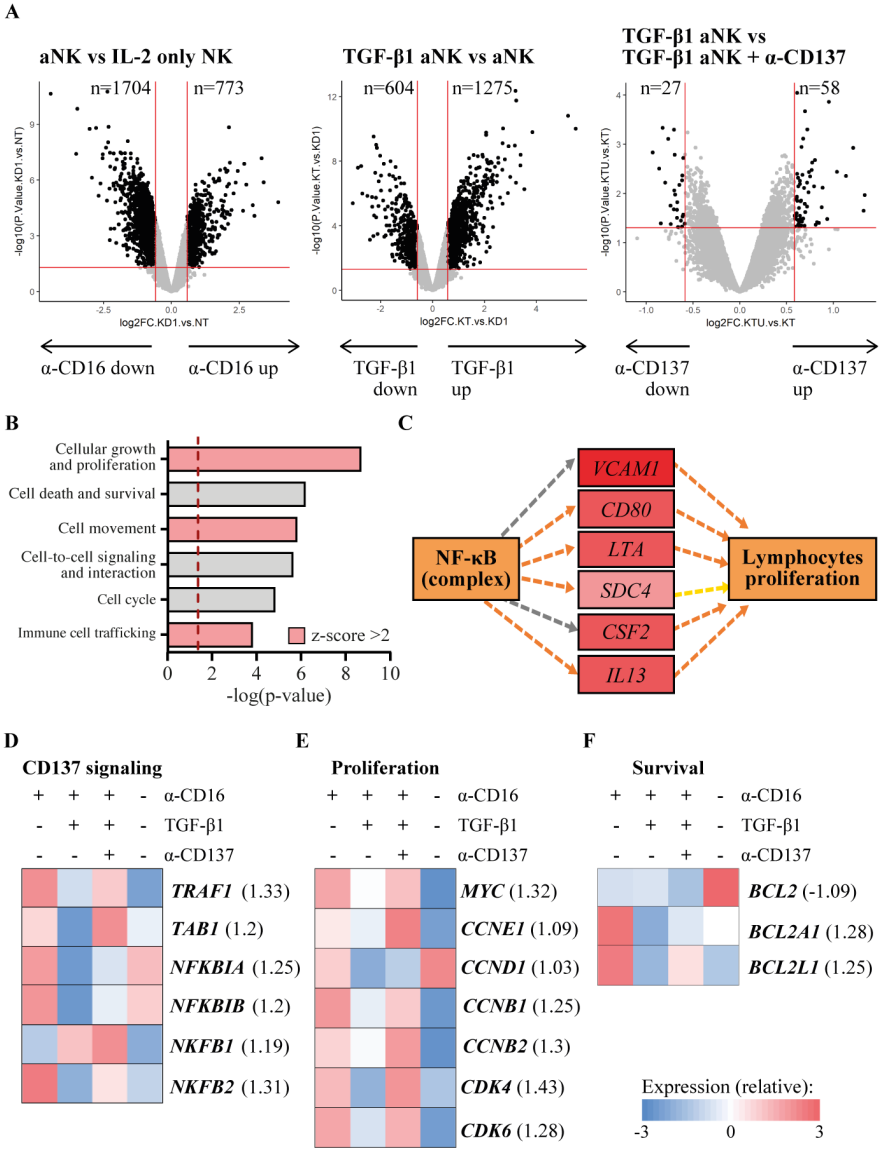

Figure 13. Influence of TGF-β1 and urelumab in the transcriptomic profile of CD16-activated NK cells. NK cells were activated with α-CD16 (5 µg/ml) and IL-2 (200 U/ml) in the presence or absence of TGF-β1 (10 ng/ml) and urelumab (50 µg/ml). Total RNA was isolated for microarray analysis at 5 days. **(A)** Volcano plots of genes differentially expressed between CD16-activated (aNK) and non-activated NK cells (IL-2 only), CD16-activated NK cells in the presence or absence of TGF-β1, and CD16-activated NK cells in the presence of

TGF-β1 with or without CD137 costimulation. Log fold-change cut-off: $log_2$1.5; -log (p-value) cut-off: -log_{10}0.05. Inset number indicate differentially expressed genes. **(B)** Selection of relevant biological functions over-represented in CD137 costimulated NK cells upon activation in a TGF-β1-rich milieu with p-value ≤0.001. Red line corresponds to –log(p-value)=1.3. Biological functions with a z-score >2 are colored in pink. **(C)** "NF-κB complex-proliferation of lymphocytes" network identified in differently expressed genes using IPA. Orange boxes: predicted activation; red to pink boxes: upregulation level in the dataset; orange arrows: activation; yellow arrow: inconsistent findings; grey arrow: effect not predicted. **(D-F)** Heatmaps of mean relative expression of genes involved in CD137 signaling **(D)**, cell proliferation **(E)** and cell survival **(F)**. Fold-change in gene expression between TGF-β1-treated cells ± α-CD137 is indicated next to each gene.

2.2. CD137 and TGF-β1 combined signaling promotes the differentiation of NK cells with tissue (tumor)-resident features which partially preserve cytotoxic potential

Gene set enrichment analysis (GSEA) of microarray data evidenced that CD16-activated NK cells cultured with TGF-β1 for 5 days presented a gene expression profile reminiscent to that of liver- and intestine-isolated ILC1 (203). On the contrary, CD137 costimulation triggered a gene program more alike to that of IL-2- and IL-15-activated NK cells, counteracting the effect of TGF-β1 (**Figure 14A, B**). CD16-mediated NK cell activation induced enhanced mRNA expression of *TNF* (TNF-α), *IFNG* (IFN-γ), *GZMB* (granzyme B), or *TNFRSF9* (CD137), and down-regulated *FCGR3A* (CD16) mRNA levels, among other changes in gene expression (**Figure 14C-H**). In CD16-activated NK cells, treatment with TGF-β1 induced a reduction in the expression of genes encoding for activating NKR and signaling adaptors [*NCR3* (NKp30), *CD226* (DNAM-1), *HCST* (DAP10) or *FCER1G* (FcRγ)] (**Figure 14C**), effector molecules

[granzymes (*GZMA, GZMB, GZMH, GZMK*), *PRF1* (perforin 1), *FASLG* (Fas ligand) and *TNFSF10* (TRAIL), *TNF* (TNF-α), *IFNG* (IFN-γ), *CFS2* (GM-CSF), *LTA* (lymphotoxin-α), *XCL1* or *CCL2* (MCP-1)] (**Figure 14D**), costimulatory receptors such as *ICOS* and members of the TNFRSF [*TNFRSF1B* (TNFR-II), *TNFRSF4* (OX40), *TNFRSF9* (CD137), *TNFRSF18* (GITR)] (**Figure 14E**), and adhesion molecules such as *ICAM1* or *VCAM1* (**Figure 14F**). On the other hand, presence of TGF-β1 was also associated to the mRNA expression of CD103 (*ITGAE*) and chemokine receptors such as *CCR4, CXCR3, CCR7* or *CXCR4* (**Figure 14F**). Indeed, down-regulation of transcription factors involved in NK cell differentiation and function [EOMES (eomesodermin), TBX21 (T-bet), NFIL3 (E4bp4) and PRDM1 (Blimp-1)] (211–214), and the up-regulation of *RUNX3* or *ZNF683* (Hobit), associated to tissue-resident lymphocytes (215–217), could underlie the gene expression program triggered by TGF-β1 in activated NK cells (**Figure 14G**). In this context, CD137 crosslinking prevented, in part, the down-regulation of some activating receptors, effector and adhesion molecules mRNA levels (i.e. *NCR3, CD226, GZMA, GZMK, TNF, IFNG, CSF2*, *LTA, ICOS, TNFRSF4, TNFRSF9, ICAM1, VCAM1)* while sparing or further emphasizing the tissue residency gene expression program triggered by TGF-β1 (**Figure 14C-F**). Expression of exhaustion markers such as *CTLA4, PDCD1* (PD-1) or *TIGIT*, which were up-regulated after CD16-activation and down-regulated in the presence of TGF-β1, were mostly unaffected by CD137 costimulation. As an exception, an increase in *TIGIT* mRNA levels was observed after treatment with urelumab (**Figure 14H**).

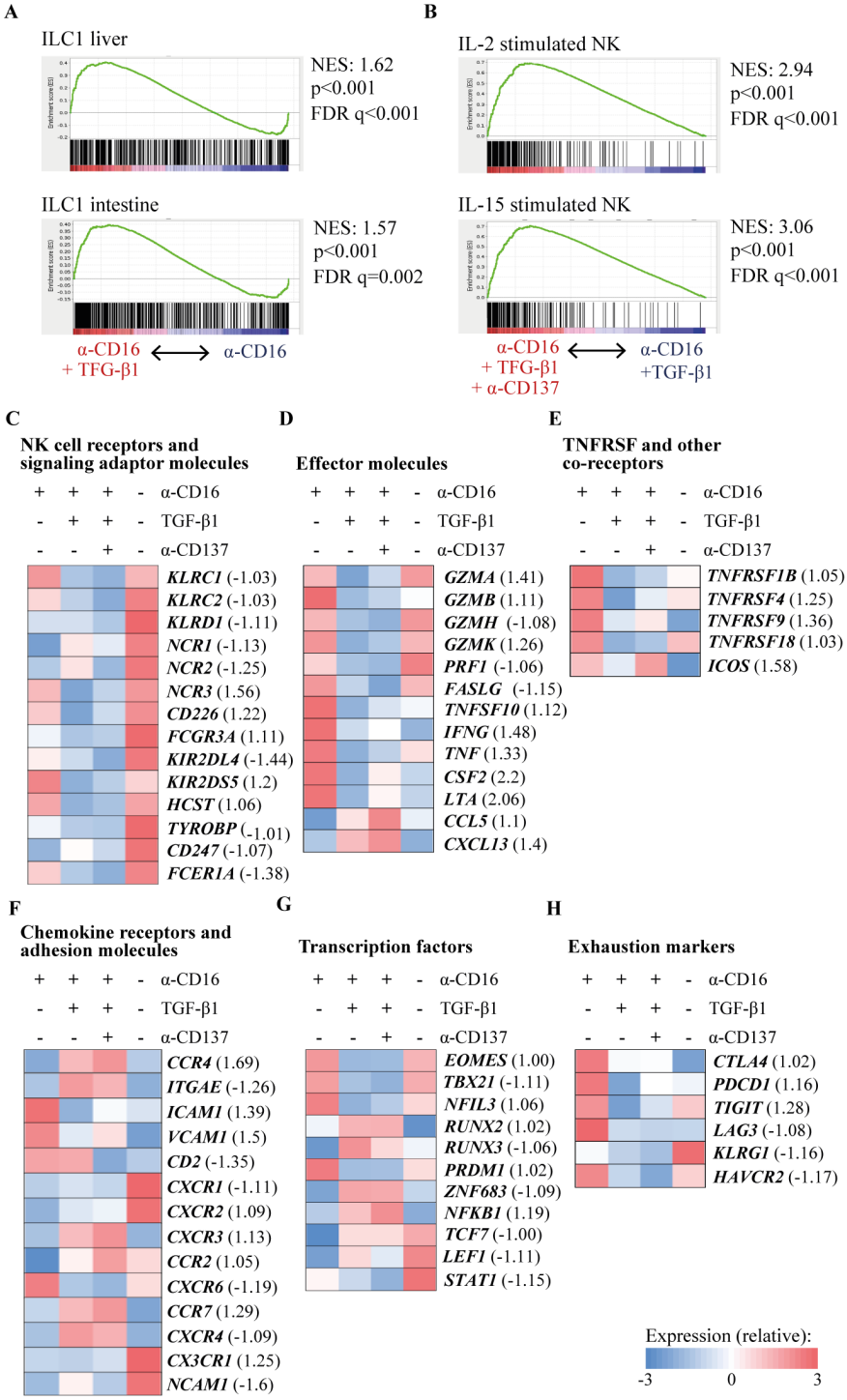
A
ILC1 liver
NES: 1.62
p<0.001
FDR q<0.001
ILC1 intestine
NES: 1.57
p<0.001
FDR q=0.002
α-CD16 + TFG-β1
α-CD16
B
IL-2 stimulated NK
NES: 2.94
p<0.001
FDR q<0.001
IL-15 stimulated NK
NES: 3.06
p<0.001
FDR q<0.001
α-CD16 + TFG-β1 + α-CD137
α-CD16 +TGF-β1
C
NK cell receptors and signaling adaptor molecules
+ + + - α-CD16
- + + - TGF-β1
- - + - α-CD137
KLRC1 (-1.03)
KLRC2 (-1.03)
KLRD1 (-1.11)
NCR1 (-1.13)
NCR2 (-1.25)
NCR3 (1.56)
CD226 (1.22)
FCGR3A (1.11)
KIR2DL4 (-1.44)
KIR2DS5 (1.2)
HCST (1.06)
TYROBP (-1.01)
CD247 (-1.07)
FCER1A (-1.38)
D
Effector molecules
+ + + - α-CD16
- + + - TGF-β1
- - + - α-CD137
GZMA (1.41)
GZMB (1.11)
GZMH (-1.08)
GZMK (1.26)
PRF1 (-1.06)
FASLG (-1.15)
TNFSF10 (1.12)
IFNG (1.48)
TNF (1.33)
CSF2 (2.2)
LTA (2.06)
CCL5 (1.1)
CXCL13 (1.4)
E
TNFRSF and other co-receptors
+ + + - α-CD16
- + + - TGF-β1
- - + - α-CD137
TNFRSF1B (1.05)
TNFRSF4 (1.25)
TNFRSF9 (1.36)
TNFRSF18 (1.03)
ICOS (1.58)
F
Chemokine receptors and adhesion molecules
+ + + - α-CD16
- + + - TGF-β1
- - + - α-CD137
CCR4 (1.69)
ITGAE (-1.26)
ICAM1 (1.39)
VCAM1 (1.5)
CD2 (-1.35)
CXCR1 (-1.11)
CXCR2 (1.09)
CXCR3 (1.13)
CCR2 (1.05)
CXCR6 (-1.19)
CCR7 (1.29)
CXCR4 (-1.09)
CX3CR1 (1.25)
NCAM1 (-1.6)
G
Transcription factors
+ + + - α-CD16
- + + - TGF-β1
- - + - α-CD137
EOMES (1.00)
TBX21 (-1.11)
NFIL3 (1.06)
RUNX2 (1.02)
RUNX3 (-1.06)
PRDM1 (1.02)
ZNF683 (-1.09)
NFKB1 (1.19)
TCF7 (-1.00)
LEF1 (-1.11)
STAT1 (-1.15)
H
Exhaustion markers
+ + + - α-CD16
- + + - TGF-β1
- - + - α-CD137
CTLA4 (1.02)
PDCD1 (1.16)
TIGIT (1.28)
LAG3 (-1.08)
KLRG1 (-1.16)
HAVCR2 (-1.17)
Expression (relative):
-3
0
3

Figure 14. CD137 signaling in CD16-activated NK cells preserved TGF-β1-induced tissue-residency gene profile while partially maintaining features of activated NK cells. NK cells were activated with α-CD16 (5 μg/ml) and IL-2 (200 U/ml) in the presence or absence of TGF-β1 (10 ng/ml) and urelumab (α-CD137, 50 μg/ml). mRNA was isolated for microarray analysis at 5 days. **(A, B)** Gene set enrichment analysis (GSEA) against liver and intestine ILC1: GSE37448; IL-2 and IL-15 stimulated NK cells: GSE22886. NES: normalized enrichment score. FDR: false discovery rate. **(C-H)** Heatmaps show mean relative expression of genes related to NK cell biology and function. Fold-change in gene expression between TGF-β1-treated cells ± α-CD137 is indicated next to each gene.

In order to confirm the information obtained in the microarray, expression of NKG2D, CD16, granzyme B, CD103, c-Kit, CCR7, CXCR3 and NKp46 was analyzed by multiparametric flow cytometry in NK cells at 6 days post CD16-activation in the presence of TGF-β1 with or without urelumab. After CD16-activation, NK cells up-regulated surface NKG2D (**Figure 15A, 16**) and decreased surface CD16 (**Figure 15B, 16**). Treatment with TGF-β1 significantly reduced CD16-induced NKG2D up-regulation and granzyme B expression in activated NK cells, an effect partially prevented by CD137 costimulation (**Figure 15A and C, 16**). In agreement with mRNA data, TGF-β1 treatment triggered increased expression of CD103 and c-Kit, as well as CCR7 and CXCR3 in activated NK cells (**Figure 15D-G, 16**). CD137 costimulation further increased CXCR3 levels and the proportion of CCR7+ NK cells (**Figure 15F-H, 16**). Surface NKp46 expression was comparable in all conditions (**Figure 15I**). Control IgG4 had no impact in any of the readouts analyzed (**Figure 15**).

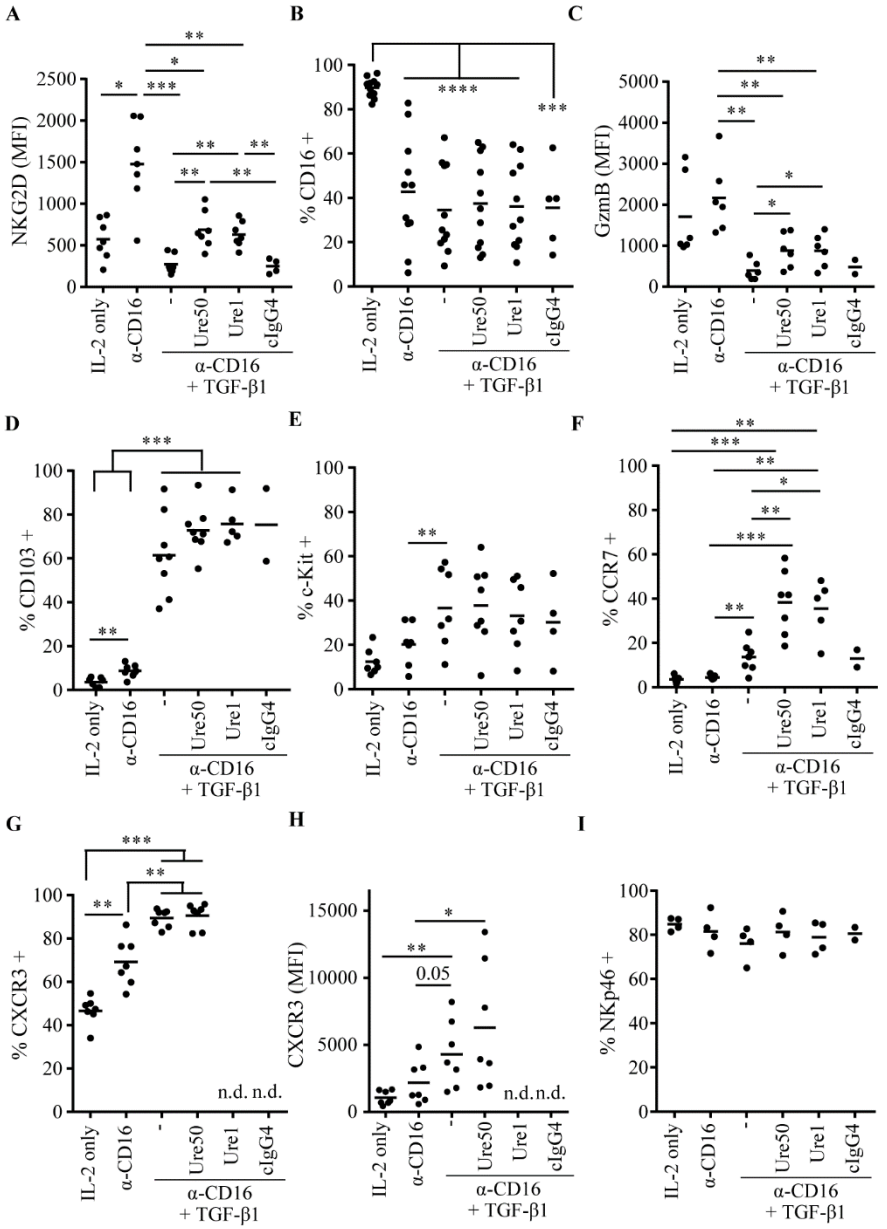

Figure 15. Changes in the NK cell phenotype upon activation in the presence of TGF-β1 and urelumab. NK cells were phenotyped by flow cytometry 6 days post-activation with anti-CD16 antibody (5 µg/ml), IL-2 (200 U/ml), TGF-β1 (10 ng/ml), urelumab (Ure, 50 or 1 µg/ml) and/or cIgG4 (50 µg/ml). Expression of different markers was analyzed by flow cytometry. Graphs depict the expression

levels of each marker in CD56^{dim} NK cells. Statistical significance was calculated using the Mann-Whitney U test (* ≤ .05, ** ≤ .01, *** ≤ .001, **** ≤ .0001). MFI: mean fluorescence intensity. n.d.: no data. GzmB: granzyme B.

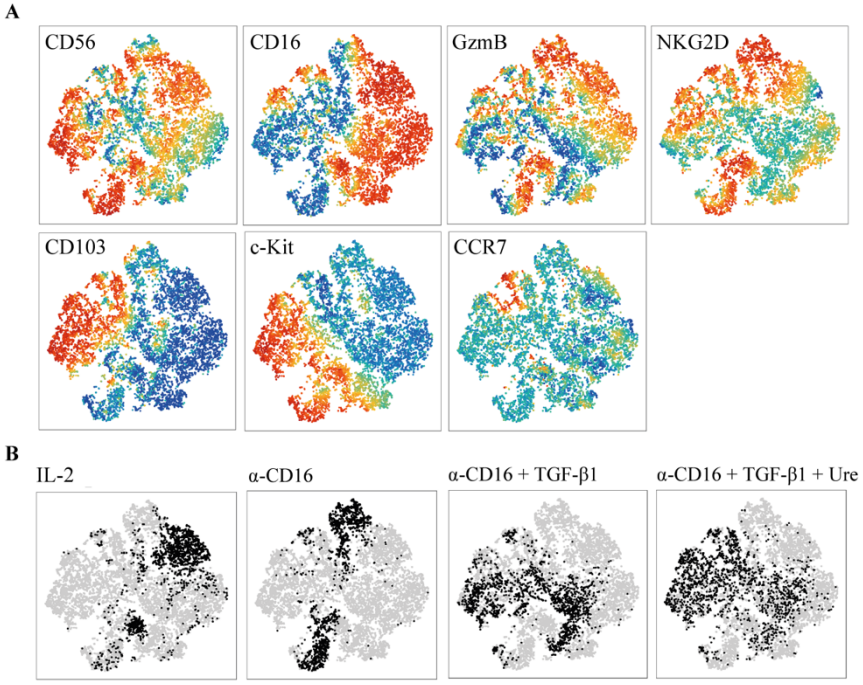

Figure 16. Impact of TGF-β1 treatment and CD137 costimulation in the phenotype of CD16-activated NK cells. 6 days CD16-activated NK cells (5 µg/ml) in the presence of TGF-β1 (10 ng/ml) and urelumab (α-CD137, 50 µg/ml) were analyzed for the expression of different markers by multiparametric flow cytometry. Data from one representative donor was analyzed using the t-SNE algorithm. **(A)** Plots show expression of different markers in the t-SNE field. Color codes indicate the expression intensity from the lowest (blue) to the highest (red). GzmB: granzyme B. **(B)** Cell distribution in the t-SNE field of NK cells from different culture conditions.

Overall, data from transcriptomic and phenotypic analysis indicated that TGF-β1 promoted the differentiation of CD16-activated NK cells towards an ILC1-like gene expression profile, whereas CD137 costimulation altered the effect of TGF-β1 by partially preserving the expression of some activating receptors and cytotoxic effector molecules while maintaining tissue-residency features.

2.3. Configuration of the NK cell repertoire after CD16-activation in the presence of TGF-β1 and urelumab

We next analyzed the impact of TGF-β1 and CD137 costimulation upon CD16-activation in NK cell subsets at distinct maturation/differentiation stages. Expression of NKG2A, KIR, CD57, NKG2C and PD-1 was monitored in CD16-activated NK cells after 6-day culture in the presence of TGF-β1 and urelumab. Percentage of NKG2A+ $CD56^{dim}$ NK cells increased after CD16-activation (**Figure 17A**), whereas proportions of CD57+ $CD56^{dim}$ NK cells markedly decreased (**Figure 17B**). A slight reduction in the percentage of KIR+ and NKG2C+ $CD56^{dim}$ NK cells was also observed in CD16-activated NK cells as compared to cells cultured in the presence of IL-2 only (**Figure 17C**). Treatment with TGF-β1 precluded NKG2A up-regulation and partially prevented CD57 down-regulation (**Figure 17A, B**). KIR and NKG2C expression were unaffected by TGF-β1 (**Figure 17C, D**). Costimulation through CD137 in TGF-β1-treated NK cells increased the percentage of cells expressing NKG2A (**Figure 17A**), whereas no changes were observed in CD57, KIR or NKG2C expression (**Figure 17B-D**). No expression of PD-1 could be detected in NK cells in any of the conditions tested (**Figure 17E**).

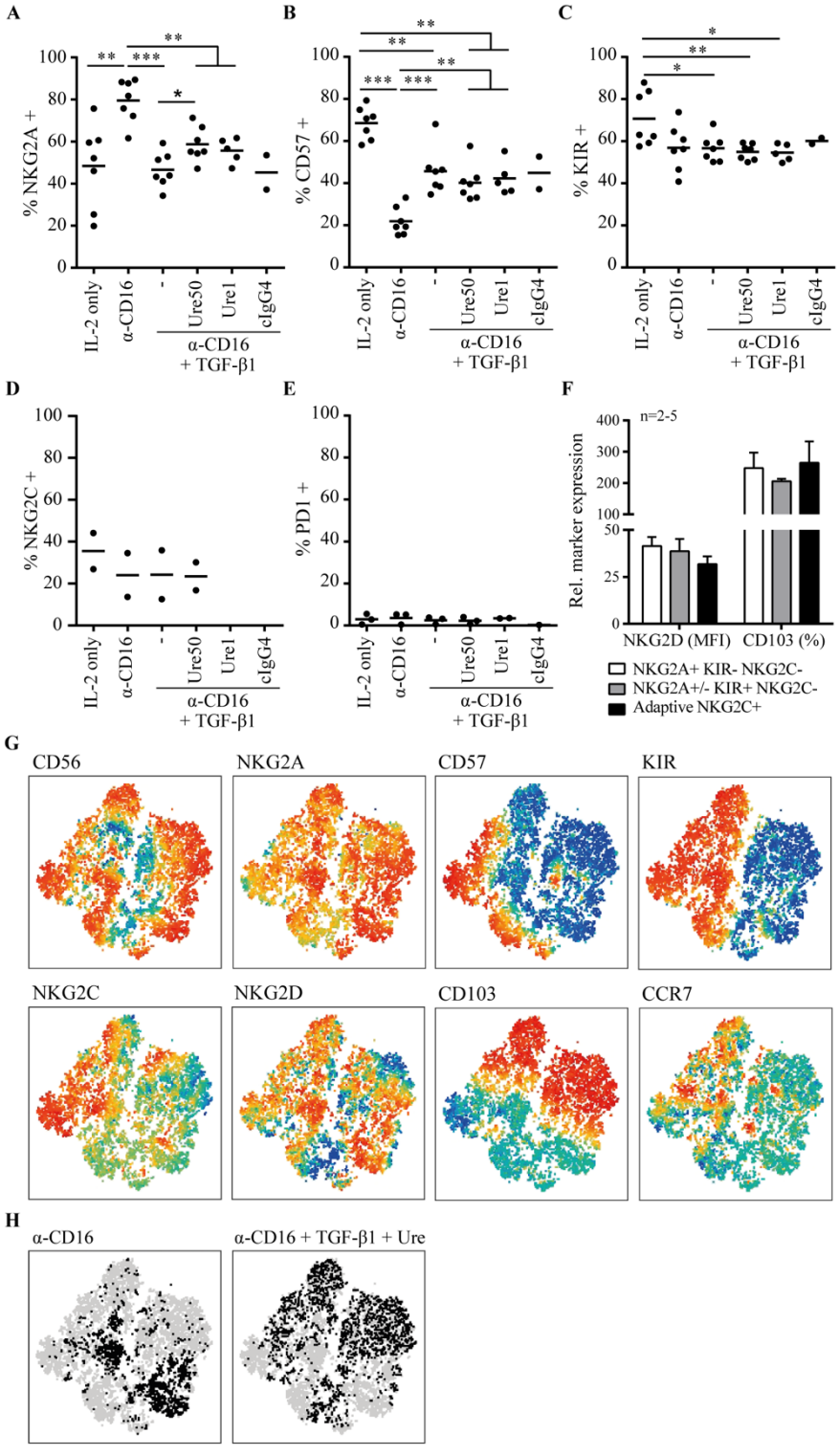
A
B
C
% NKG2A +
% CD57 +
% KIR +
IL-2 only
α-CD16
-
Ure50
Ure1
cIgG4
α-CD16
+ TGF-β1
D
E
F
% NKG2C +
% PD1 +
Rel. marker expression
n=2-5
NKG2D (MFI)
CD103 (%)
NKG2A+ KIR- NKG2C-
NKG2A+/- KIR+ NKG2C-
Adaptive NKG2C+
G
CD56
NKG2A
CD57
KIR
NKG2C
NKG2D
CD103
CCR7
H
α-CD16
α-CD16 + TGF-β1 + Ure

Figure 17. NK cell phenotype in CD16-activated NK cells cultured in the presence of TGF-β1 and urelumab. NK cells were phenotyped by flow cytometry after 6 days activation in the presence of anti-CD16 antibody (5 μg/ml), IL-2 (200 U/ml), TGF-β1 (10 ng/ml), urelumab (Ure, 50 or 1 μg/ml) and/or cIgG4 (50 μg/ml). **(A-E)** Expression of different markers in $CD56^{dim}$ NK cells was analyzed by flow cytometry. Graphs depict the expression levels of each marker in $CD56^{dim}$ NK cells. Statistical significance was calculated using the Mann-Whitney U test (* ≤ .05, ** ≤ .01, *** ≤ .001). **(F)** Graph of mean±SEM NKG2D and CD103 expression in different NK cells subsets from 2 to 5 individuals upon CD137 costimulation normalized to α-CD16 (minimum n: adaptive NKG2C+). MFI: mean fluorescence intensity. **(G, H)** Data from one representative donor was analyzed using the t-SNE algorithm. **(G)** Plots show expression of different markers in the t-SNE field. Color codes indicate the expression intensity from the lowest (blue) to the highest (red). **(H)** Cell distribution in the t-SNE field of NK cells from different culture conditions.

The effect of TGF-β1 and CD137 costimulation on NKG2D and CD103 expression were comparable in all NK cell subpopulations (i.e. NKG2A+ KIR- NKG2C- cells, NKG2A± KIR+ NKG2C- cells, and adaptive NKG2A± KIR+ NKG2C+ cells) (**Figure 17F-H**).

Altogether, different NK cells from early to terminally-differentiated subsets were similarly susceptible to CD137 costimulation and TGF-β1.

2.4. CD137 costimulation by urelumab partially prevents TGF-β1-impairment of NK cell function enabling subsequent responses against transformed targets

We next evaluated whether CD137 costimulation in TGF-β1-treated cells could enable subsequent NK cell effector function (**Figure 18A**). NK cells activated for 6 days in distinct conditions including or not TGF-β1 and urelumab were harvested and restimulated in 4-hour-coculture experiments with trastuzumab-coated SKBR3 cells (**Figure 18B, C**). NK

cell ADCC was assessed by monitoring active caspase 3 in target cells by flow cytometry. Activated NK cells cultured for 6 days with TGF-β1 presented impaired ADCC capacity. Conversely, CD137-costimulation restored ADCC in NK cells cultured with TGF-β1, to that of CD16-activated NK cells in the absence of TGF-β1 (**Figure 18B, C**). Degranulation against the erythroleukemic cell line K562 upon restimulation was also reduced in NK cells which had been cultured in the presence of TGF-β1, and rescued by CD137 costimulation (**Figure 18D, E**).

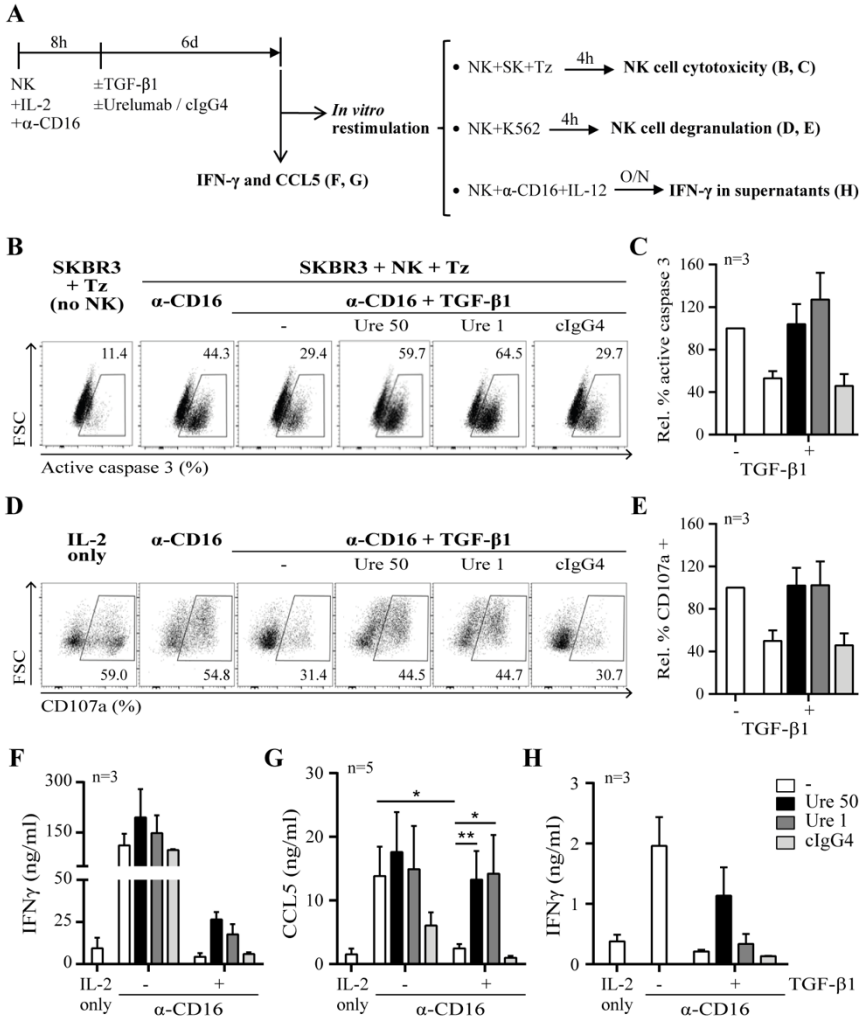

Figure 18. CD137 co-stimulation in CD16-activated NK cells reestablished TGF-β1-impaired anti-tumor function *in vitro*. **(A)** Scheme of the experimental design. In detail, after 6 days activation by α-CD16 (5 μg/ml) in the presence of TGF-β1 (10 ng/ml) and urelumab (Ure, 50 or 1 μg/ml) or cIgG4 (50 μg/ml), cell-free supernatants were collected and NK cells used for restimulation. **(B)** Dot plots showing activate caspase 3 in SKBR3 cells after 4-hour-coculture with restimulated NK cells and trastuzumab (Tz, 210 ng/ml) at an E:T ratio 1:2. Data from a representative experiment. **(C)** Bar graphs represent mean±SEM active caspase 3+ SKBR3 in each condition. Data from 3 independent experiments normalized to α-CD16. **(D)** Dot plots showing CD107a expression (inset numbers) in NK cells restimulated with K562 at an E:T 4:1. Data from a representative experiment. **(E)** Bar graphs represent mean±SEM CD107a+ NK cells in each condition. Data from 3 independent experiments normalized to α-CD16. **(F, G)** 6-day cell-free culture supernatants were collected and analyzed for IFN-γ **(F)** and CCL5 production **(G)** by ELISA. IFN-γ production was also analyzed after overnight NK cell reestimulation by plate-bound α-CD16 antibody (5 μg/ml) and IL-12 (10 ng/ml) **(H)**. **(F-H)** Graphs represent mean±SEM of data from 3-5 independent experiments. Statistical significance was calculated using the Mann-Whitney U test (* ≤ .05, ** ≤ .01).

On the other hand, secretion of IFN-γ and CCL5 was analyzed in conditioned cell-free supernatants obtained from the 6-day cultures by ELISA (**Figure 18F, G**). Activation through CD16 induced the production of both IFN-γ and CCL5, which was clearly dampened by TGF-β1 treatment. CD137-costimulation slightly increased IFN-γ and CCL5 secretion in CD16-activated NK cells in the absence of TGF-β1, and partially or almost completely prevented the inhibition of IFN-γ and CCL5 production in TGF-β1-treated cells, respectively (**Figure 18F, G**). Moreover, IFN-γ production after NK cell restimulation with anti-CD16 agonist antibody and IL-12 was as well reduced in TGF-β1-treated NK

cells and partially rescued in NK cells which had been costimulated with high doses of urelumab (i.e. 50 µg/ml) (**Figure 18H**).

Of note, costimulation through CD137 did not enhance initial ADCC (**Figure 19**), yet rescued subsequent killing by promoting the proliferation and persistence of NK cells with cytotoxic potential in the context of TGF-β1 immunosuppression.

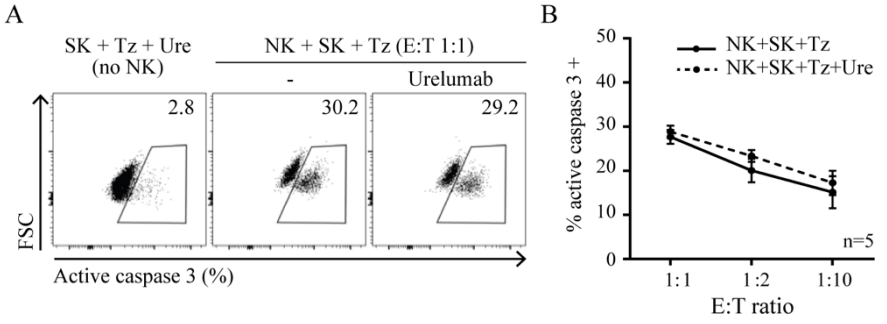

Figure 19. CD137 co-stimulation has no impact on initial trastuzumab-induced NK cell-mediated ADCC. NK cells were cocultured with SKBR3 cells (SK) and trastuzumab (Tz, 210 ng/ml) at different E:T ratios in the presence or absence urelumab (Ure, 50 µg/ml). Active caspase 3 was analyzed in SKBR3 cells at 24h by flow cytometry. **(A)** Dot plots from representative donors are shown. Percentage of active caspase 3+ SKBR3 cells is indicated (inset numbers). **(B)** Graphs represent mean±SEM of data from 5 independent donors. Levels of active caspase 3 in SKBR3 cells in the absence of NK cells are subtracted.

As a whole, CD137 costimulation reversed TGF-β1 inhibition, partially preserving the cytotoxic and helper potential of previously activated NK cells for subsequent antitumor activity.

2.5. Tumor-infiltrating NK cells from treatment-naïve patients show features of TGF-β imprinting and are sensitive to CD137 costimulation

Cellular suspension was obtained by mechanical and enzymatic digestion of a treatment-naïve breast tumor specimen. Unsorted multicellular fraction was cultured in the presence of IL-2, trastuzumab and urelumab for 6 days, and tumor-associated NK cells (i.e. DAPI- CD45+ CD3- CD56+) were analyzed by flow cytometry for the expression of CD16, CD137, NKG2D, CD103 and PD-1 (**Figure 20**). Addition of trastuzumab

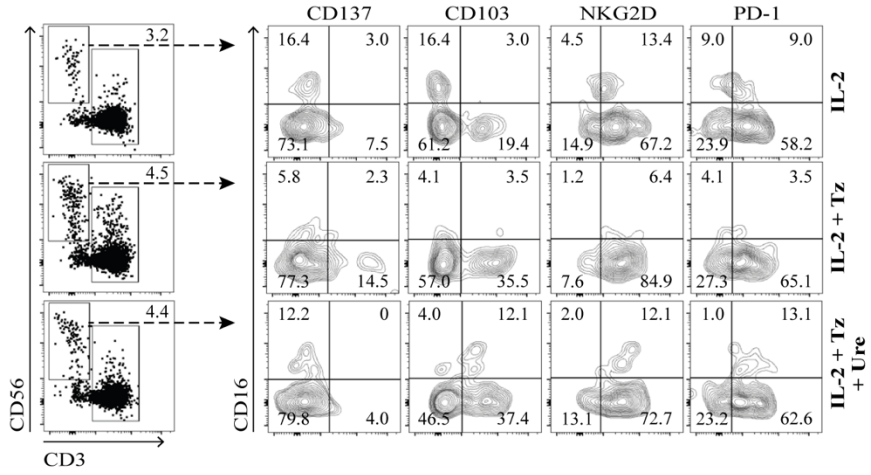

Figure 20. Urelumab and trastuzumab treatment of breast carcinoma-derived multicellular culture promotes the recovery of CD16+ NK cells with TGF-β-related features. Unsorted multicellular suspension derived from fresh breast carcinoma specimen were cultured in complete medium with IL-2 (200 U/ml) in the presence or absence (-) of trastuzumab (Tz, 210 ng/ml) and urelumab (Ure, 50 μg/ml) for 6 days. CD16, CD137, NKG2D, CD103 and PD-1 expression in tumor-associated lymphocytes was analyzed by flow cytometry. Contour dot plot show expression of different markers in NK cells (i.e. DAPI- CD45+ CD3- CD56+ cells) are shown.

in the culture induced the up-regulation of CD137 and NKG2D, and the down-regulation of CD16 in NK cells. Remarkably, CD16+ NK cells were recovered in CD137 costimulated cultures at 6 days. Moreover, CD103 expression was detected in a fraction of tumor-associated NK cells, suggestive of TGF-β imprinting and increased upon trastuzumab and urelumab treatment. PD-1 expression was also observed in a significant proportion of CD16- cells, and increased after treatment with trastuzumab and urelumab. Of note, after CD137 costimulation CD16 expression was mostly detected in NK cells co-expressing CD103 and PD-1 (**Figure 20**).

2.6. CD137-costimulation associates with decreased TGF-β-RII expression in CD16-activated NK cells and mainly curtails Smad4-dependent TGF-β1 targets

We next attempted to gain insights into the molecular mechanisms underlying the crosstalk between CD137 and TGF-β1 signaling pathways in CD16-activated NK cells. In order to elucidate whether modulation of the NK cell phenotype by TGF-β1 was dependent on canonical signaling through TGF-β-RI, we treated NK cells with SB-431542, a specific inhibitor of TGF-β-RI kinase activity (218), along CD16-activation in the presence of TGF-β1 and urelumab. After 6-day treatment, expression of CD25, NKG2D, granzyme B, c-Kit, CD103 and CCR7 was analyzed by flow cytometry, and NK cell numbers were calculated. Presence of SB-431542 precluded the inhibitory effect of TGF-β1 on CD16-activated NK cell proliferation (**Figure 21A)** and abolished all phenotypic changes associated to TGF-β1 in activated NK cells (**Figure 21B-G**), resembling the effect of urelumab.

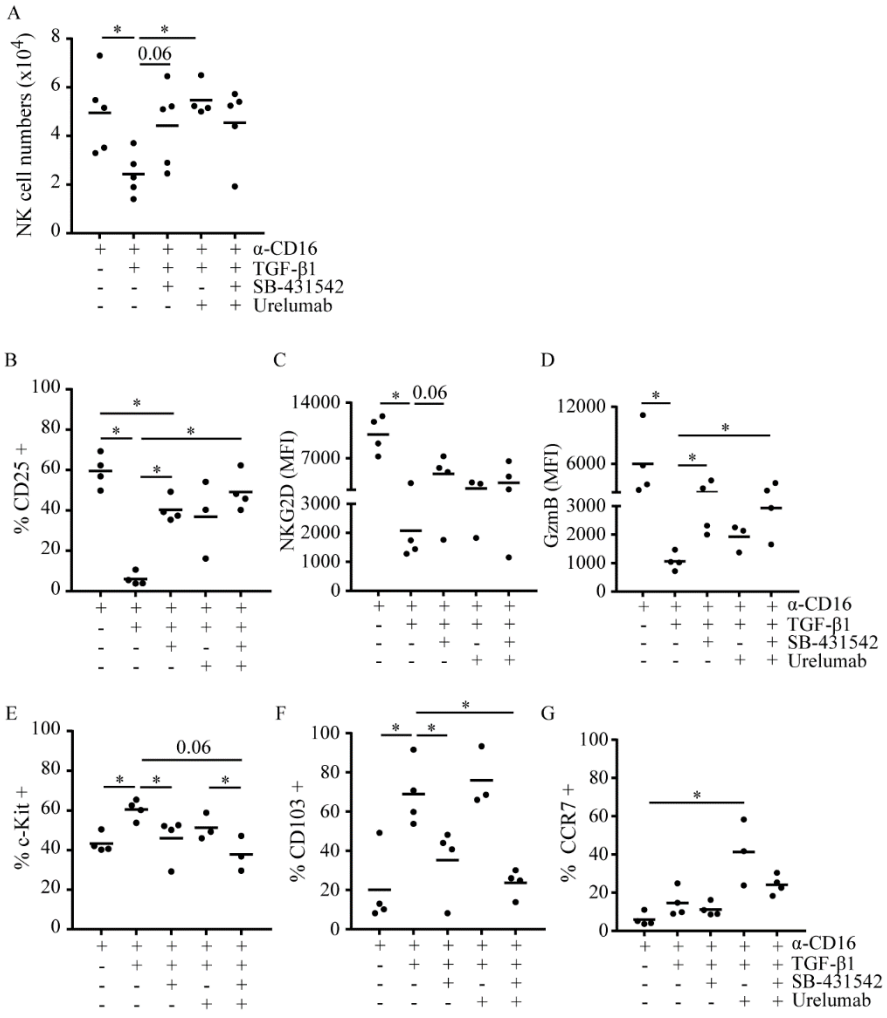

Figure 21. Effect of TGF-β-RI inhibitor SB-431542 on TGF-β1-induced phenotypic changes in activated NK cells. NK cells were incubated with the TGF-β superfamily ALK receptors inhibitor SB-431542 (20 μM) prior and during CD16-activation (5 μg/ml) with TGF-β1 (10 ng/ml) and urelumab (50 μg/ml) for 6 days. **(A)** Graph display $CD56^{dim}$ NK cell numbers recovered after 6-day culture in each condition. **(B-G)** Expression of CD25, NKG2D, GzmB, cKit, CD103, CCR7 was analyzed by flow cytometry. Graph indicate proportions or mean fluorescence intensity (MFI) for the analyzed markers in $CD56^{dim}$ NK cells on the different culture conditions. Statistical significance was calculated using the Mann-Whitney U test (* ≤ .05).

Down-stream of canonical TGF-β-RI signaling through pSmad2/3 Smad4-dependent and –independent (e.g. involving TIF-1γ) transcriptional programs have been described in distinct cell types (148,149,219). Heterozygous germline mutations in *SMAD4* are responsible for some cases of juvenile polyposis syndrome (JPS), an autosomal dominant disease characterized by the development of intestinal polyps and increased risk of gastrointestinal cancer (220,221). We could study the impact of TGF-β1 and urelumab on NK cells from a Smad4 haploinsufficient patient. Decreased expression of Smad4 in PBMC from JPS patient was confirmed by western blot analysis (**Figure 22A**). PBMC from the JPS patient and a healthy control were activated with anti-CD16 agonistic mAb with or without TGF-β1 and urelumab. At 6 days post-activation cells were counted and their phenotype analyzed by multiparametric flow cytometry. In contrast to NK cells from the healthy donor, numbers of Smad4+/- NK cells recovered at 6 days post-activation were not reduced by treatment with TGF-β1. Nonetheless, urelumab increased NK cell recovery both in the healthy donor and JPS patient-derived PBMC (**Figure 22B**). CD16-activated Smad4+/- NK cells appeared less sensitive to TGF-β1-mediated down-regulation of NKG2D and granzyme B, whereas showed an apparently enhanced acquisition of CD103 and CCR7 upon activation in the presence of TGF-β1 (**Figure 22C**). Remarkably, CD137 costimulation resulted in almost complete recovery of NKG2D and granzyme B levels in these conditions (**Figure 22C**). Thus, these results indicate that CD137 costimulation mostly prevented Smad4-dependent transcriptional programs in activated NK cells (i.e. proliferation, granzyme B and NKG2D expression) sparing or synergizing with Smad4-independent transcriptional programs for the expression of CD103 and CCR7, respectively.

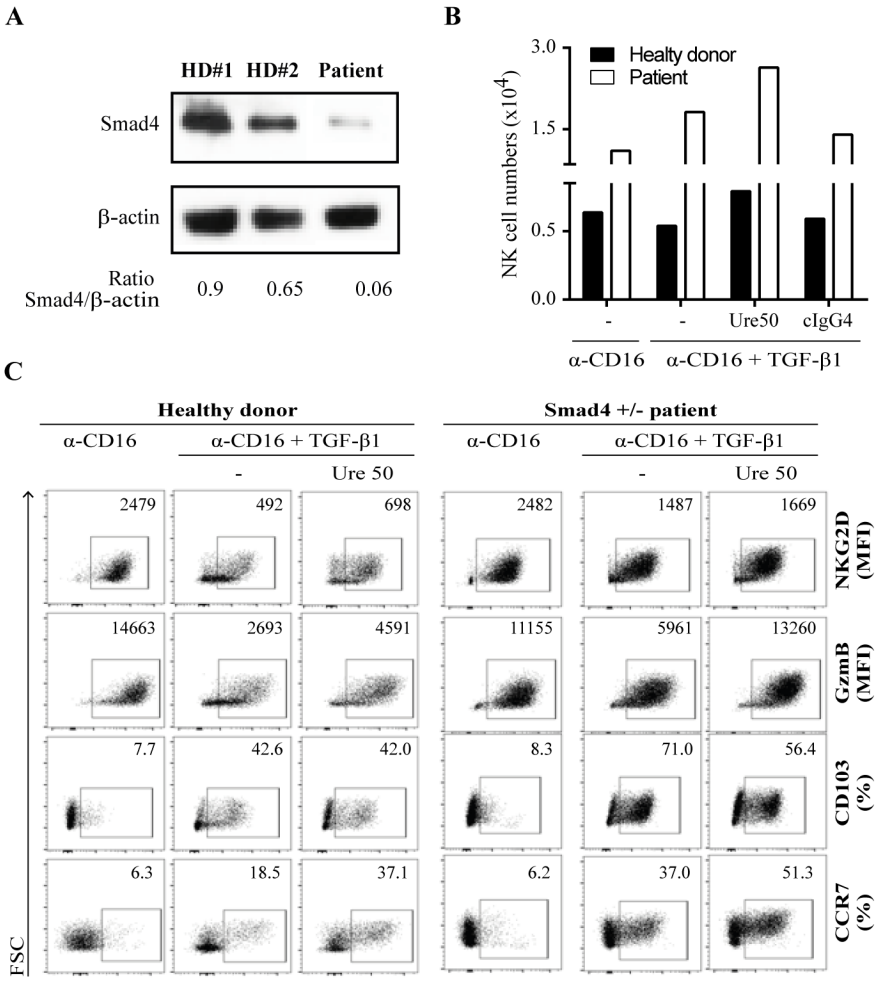

Figure 22. Urelumab and TGF-β1 influence in the activation and differentiation of Smad4 haploinsufficient NK cells. (A) Smad4 and β-actin expression in resting PBMC from a patient with juvenile polyposis syndrome and two healthy donors (HD#1 and HD#2) by western blot. **(B, C)** PBMC were cultured O/N with IL-2 (200 U/ml) and subsequently treated with plate-bound α-CD16 (5 μg/ml) and fresh IL-2 (200 U/ml) in the presence or absence of TGF-β1 (10 ng/ml) and urelumab (Ure, 50 μg/ml) for 6 days. **(B)** $CD56^{dim}$ NK cell numbers at day 6. **(C)** Dot plots showing NKG2D, granzyme B (GzmB), CD103 and CCR7 on Smad4+/+ and Smad4+/- $CD56^{dim}$ NK cells in each condition. Inset numbers indicate mean fluorescence intensity (MFI) or percentage of positive cells.

Signaling through NF-κB has been shown to modulate TGF-β canonical signaling either by: (i) promoting the expression of the inhibitory Smad7 (222), or (ii) decreasing TGF-β-RII expression (223). Further experiments were aimed at analyzing the effect of CD137 costimulation in both TGF-β signaling mediators. Overnight treatment with urelumab limited the up-regulation of TGF-β-RII induced by TGF-β1 in CD16-activated NK cells, whereas no changes were detected on Smad7 levels (**Figure 23A, B**). Analysis of microarray data at 5 days showed that TGF-β1 treatment up-regulated *SMAD2, SMAD4, SMAD7* and *TRIM33* (TIF-1γ) mRNA in activated NK cells whereas urelumab treatment in these conditions associated to decreased expression of *TGFBR2, SMAD3* and *SMAD7* mRNA (**Figure 23C**).

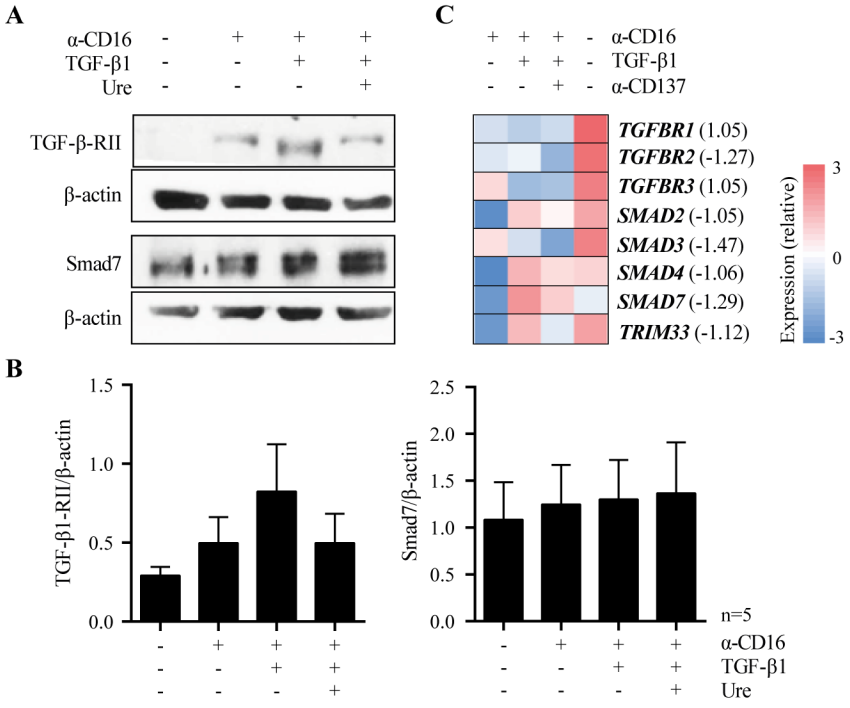

Figure 23. CD137-costimulation associated with diminished TGF-β-RII expression. NK cells were activated with α-CD16 Ab (5 μg/ml) and IL-2 (200 U/ml) in the presence or absence of TGF-β1 (10 ng/ml) and urelumab (Ure, 50 μg/ml). **(A, B)** TGF-β-RII and Smad7 were checked by western blot after overnight activation. **(A)** Western blot images from a representative experiment. **(B)** Graphs represent mean±SEM of TGFβ-RII and Smad7/β-actin ratio in NK cells from 5 donors in multiple independent experiments. **(C)** Heatmaps showing mean relative expression of genes involved in TGF-β1 canonical signaling at 5 days in microarray expression data including 3 donors. Fold-change in gene expression between TGF-β1-treated cells ± α-CD137 is indicated.

Overall, these data indicate that CD137 costimulation associates to reduced TGF-β-RII expression in activated NK cells and modulates the transcriptional program down-stream of TGF-β-RI-dependent canonical signaling by reducing Smad4-dependent while preserving Smad4-independent transcriptional targets.

2.7. CD137-costimulation in a TGF-β1-rich microenvironment promotes a Th9-like gene signature and triggers secretion of IL-9 in a fraction of CD16-activated NK cells

Further analysis of gene expression microarray data revealed that activated NK cells treated with TGF-β1 in the presence of urelumab up-regulated several genes associated to Th9 helper profile in CD4+ T cells (i.e. *IL-9, IL-5, IL-13, CCR4, ICOS, BATF3, IRF4*) (224–226) (**Figure 24A, B**). Indeed, TGF-β signaling in combination with TNFRSF-dependent costimulation has been shown to promote the differentiation of CD4+ T cells with a Th9 functional profile (225,227–229). Since IL-9 is the hallmark cytokine of Th9 helper profile, we analyzed its presence on conditioned supernatants derived from CD16-activated NK cells after 6 day culture in the presence of TGF-β1 and urelumab by ELISA. IL-9 was almost exclusively detected in supernatants from NK cells which had been

cultured in the presence of TGF-β1 and urelumab, both at high and suboptimal doses (**Figure 24C**). In addition, IL-9 production in a fraction of NK cells upon activation in the presence of TGF-β1 and CD137 costimulation was corroborated by intracellular flow cytometry with and without restimulation with PMA/ionomycin (**Figure 24D, E**). Overall, CD137 costimulation in the presence of TGF-β1 can promote the differentiation of NK cells secreting IL-9 and with a Th9-like gene expression profile.

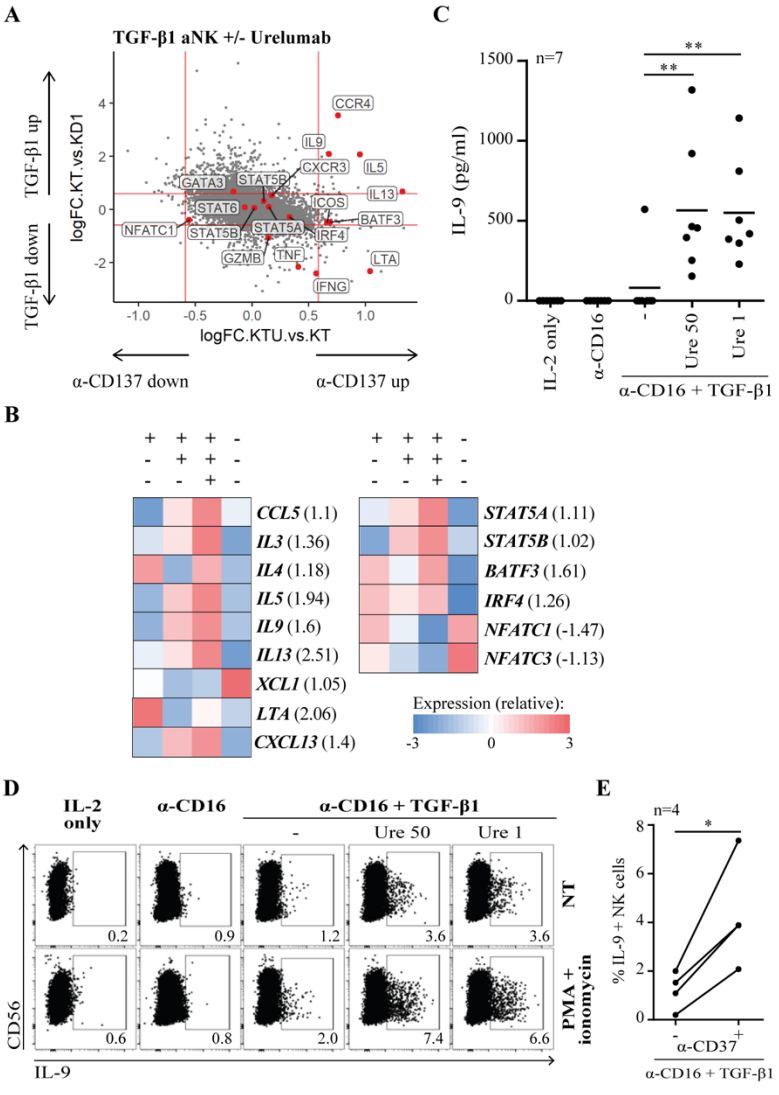

Figure 24. CD137-costimulation in a TGF-β1-rich microenvironment promoted a Th9-like functional profile in a fraction of CD16-activated NK cells. NK cells were activated with α-CD16 Ab (5 μg/ml) and cultured in the presence or absence of TGF-β1 (10 ng/ml) and urelumab (Ure, 50 μg/ml) for 5-6 days. **(A, B)** Heatmaps show mean relative expression of genes related to Th9 functional profile in microarray data at 5 days. Fold-change in gene expression between TGF-β1-treated cells ±α-CD137 is indicated next to each gene. **(C)** IL-9 production was analyzed in 6-day cell-free supernatants by ELISA. **(D, E)** IL-9 expression by intracellular flow cytometry in 6-day activated NK cells reestimulated or not with PMA/ionomycin (10 ng/ml, 1 μg/ml). **(D)** Dot plots from a representative experiment. **(E)** Graph showing percentage of IL-9+ cells in NK cells reestimulated with PMA/ionomycin upon 6-day treatment with TGF-β1 in the presence or absence of α-CD137 (urelumab 50 μg/ml).

DISCUSSION

The incorporation of HER2-targeted antibodies into the clinics brought a significant improvement in the clinical outcome of HER2+ breast cancer patients. However, poor clinical responses are still observed in some individuals due to *de novo* or acquired resistance, highlighting the need of implementing additional therapeutic strategies. Strengthening NK cell-mediated ADCC responses by targeting activating (i.e. TNFRSF members such as CD137 or OX40) or inhibitory (i.e. PD-1, CTLA-4) inducible receptors in the tumor microenvironment is envisaged as a relevant option for enhancing the clinical efficacy of anti-HER2 antibodies. However, although different antibodies targeting the aforementioned receptors are in development or have been approved for the treatment of a variety of tumors, the expression and role of inducible receptors in NK cells remains rather unexplored (204,205).

In the present study we have shown that CD16-redirected NK cell activation or anti-HER2 antibody-dependent ADCC against breast cancer cells induces the up-regulation of CD137 in peripheral blood NK cells. Trastuzumab-induced CD137 expression was also ascertained in tumor-associated NK cells in multicellular breast carcinoma-derived cultures, yet differences in concomitant PD-1 up-regulation were observed among the experimental systems. It has been reported that PD-1 is up-regulated after prolonged stimulation with anti-NKp30 and –NKp46 antibodies *in vitro*, but not after CD16-mediated NK cell activation (109). Differences between CD16 and NKp30/46 may rely on the intensity and persistence of the activating signal. Whereas activation through CD16 induces a potent yet transitory stimulation due to CD16 shedding from cell surface by the metalloprotease ADAM17 (230), ligation of NKp30 and/or NKp46 may trigger a lower intensity but persistent stimulation in NK cells. In breast cancer, expression of NKp30 ligands has been detected in different cell lines and in tumor specimens (193,231,232), and could be one of the

factors, among others, contributing to PD-1 expression in tumor-infiltrating as compared to CD16-activated NK cells. PD-1 expression has been reported to inhibit NK cell effector function in human and mice, impairing NK cell-mediated antitumor responses (113,114). In this line, although trastuzumab-induced CD137 expression in tumor-infiltrating NK cells enabled their sensitivity to CD137 costimulation, as shown by the recovery of CD16 expression, it is likely that simultaneous PD-1 up-regulation could limit the effect of CD137 agonists *in vivo*. Indeed, dual targeting of CD137 and PD-1 has been reported to enhance the antitumor activity of human T lymphocytes adoptively transferred into immunodeficient mice (233) and could similarly coregulate NK cell antitumor activity. In this regard, anti-CD137 agonistic antibodies urelumab and utomilumab are being tested in phase I/II clinical trials in combination with anti-PD-1/PD-L1 blocking antibodies for the treatment of different neoplasms including breast cancer (NCT03414658) showing good safety and promising efficacy in a phase Ib study (234). The relative contribution of NK cell antitumor function on the efficacy of CD137 agonists in combination with PD-1/PD-L1 immunocheckpoints could be of relevance, particularly on the surveillance of HLA class I-deficient tumors.

TNFRSF9 (encoding for CD137 or 4-1BB) resides in a compact locus within the chromosomal region 1p36 together with *TNFRSF4, TNFRSF18* and *TNFRSF1B,* encoding for OX40, GITR and TNF-RII, respectively, what could suggest a coordinated regulation of various co-stimulatory members of the TNFRSF (91). Indeed, expression of CD137, OX40 and TNF-RII was coordinately up-regulated in NK cells upon ADCC. Both NF-κB and AP-1 are involved in TCR stimulation-dependent transcriptional regulation of the CD137 promoter upon T cell activation and CD137 mRNA has a relatively short half-life in lymphocytes

(206,207,235,236). Here we described that in NK cells, TNF-α alone is not sufficient to significantly up-regulate CD137 and OX40 expression, yet contributes to their sustained levels upon CD16-triggered expression by signaling through TNF-RI and TNF-RII. Our results would support a model in which TNF-RI acts synergistically with TNF-RII for down-stream NF-κB activation, supporting CD137 and OX40 sustained transcription upon AP-1 activation downstream of CD16 signaling via FcRγ and CD3ζ heterodimers, as previously described in T cells (235). Our results also indicate that despite showing coordinated expression, the dependency for AP-1 and NF-κB might differ for CD137 and OX40 promoter activity. TNF-α neutralizing antibodies almost completely abrogated OX40 expression yet only partially reduced CD137 levels pointing towards a greater dependency of OX40 as compared to CD137 on TNF-α for its effective expression. In addition, trastuzumab-induced ADCC triggered higher CD137 yet comparable OX40 levels in adaptive NKG2C+ NK cells displaying enhanced CD16 signaling by the preferential coupling of the activating receptor to CD3ζ adaptor (78–80), further supporting different dependency on CD16-derived or TNF-derived signaling for CD137 and OX40 expression in activated NK cells.

In NK cells, TGF-β has been shown to inhibit proliferation, expression of activating receptors and effector molecules (85,159–161,237). Moreover, it has been associated with diminished NK cell-mediated ADCC capacity in cancer patients and related to disease progression in the metastatic setting (193). As previously described in mice (21), we have shown that TGF-β promotes the differentiation of NK cells towards an ILC1-like tissue-resident profile with acquired expression of tissue-resident features such as *ZNF683* (Hobit), CD103 and CXCR3, yet reduced effector molecules expression (i.e. NKG2D, granzyme B) and impaired cytotoxic capacity. Upon binding of TGF-β, TGF-β-RII phosphorylates TGF-β-RI

triggering its activation and downstream signaling through canonical Smad-dependent and non-canonical Smad-independent pathways (147). Treatment with SB-431542, a specific inhibitor of the kinase activity of TGF-β-RI, disclosed that both the impairment of NK cell proliferation and function, and the promotion of tissue-residency features by TGF-β were mostly dependent on canonical signaling through TGF-β-RI. On the other hand, data from Smad4+/- NK cells evidenced that TGF-β-mediated inhibition of proliferation, granzyme B and NKG2D appeared mostly driven by Smad4-dependent transcriptional program, while up-regulation of tissue-associated marker CD103 and CCR7 were Smad4-independent, in agreement with previous studies in mouse NK cells (163,219,238). In hematopoietic stem cells, association of Smad2/3 with Smad4 has been related to inhibition of proliferation, yet alternative binding to the transcriptional intermediary factor 1 gamma (TIF-1γ) has been shown to enhance erythroid differentiation (148). Based on these observations, it is tempting to speculate that promotion of a tissue-residency-like profile in TGF-β-stimulated NK cells may occur through association of Smad2/3 with TIF1-γ, a factor showing increased expression in transcriptional analysis of CD16-activated NK cells treated with TGF-β.

Crosstalk between canonical TGF-β and NF-κB signaling has been reported at two different levels: through the downregulation of TGF-β-RII and through the upregulation of inhibitory Smad7 (222,223). We have observed that CD137 co-stimulation in TGF-β-treated activated NK cells associates to reduced TGF-β-RII expression already at early time-points, as well as decreased *TGFBR2, SMAD3* and *SMAD7* gene expression after sustained CD137 costimulation at 5 days. However, urelumab mostly prevented Smad4-dependent transcriptional programs in activated NK cells but spared or even enhanced Smad4-independent targets such as CD103 and CCR7, which were shown to be as well dependent on

signaling through TGF-β-RI, and should be likewise impaired by TGF-β-RII down-regulation. Although further investigation is required in order to understand the putative mechanism by which CD137 costimulation overturns TGF-β signaling, higher affinity of TIF1-γ or other cofactors as compared to Smad4 for lower amounts of pSmad2/3 due to reduced TGF-β-RII signaling could preserve the expression of Smad4-independent targets concomitant to reduced Smad4-dependent effects. On the other hand, ligation of CD137 and activation of NF-κB may, directly or indirectly, contribute to restored expression of TGF-β-inhibited targets (i.e. IFN-γ, granzyme B, CCL5, CD25, Myc) (239–244). Of note, it is worth mentioning that CD137 and autocrine TNF-α could synergize for NF-κB activation on activated NK cells, further enhancing the transcription of NF-κB-dependent genes. Experiments including TNF-α neutralizing antibodies could be performed in order to assess the role of TNF-α in the crosstalk between CD137 and TGF-β in CD16-activated NK cells.

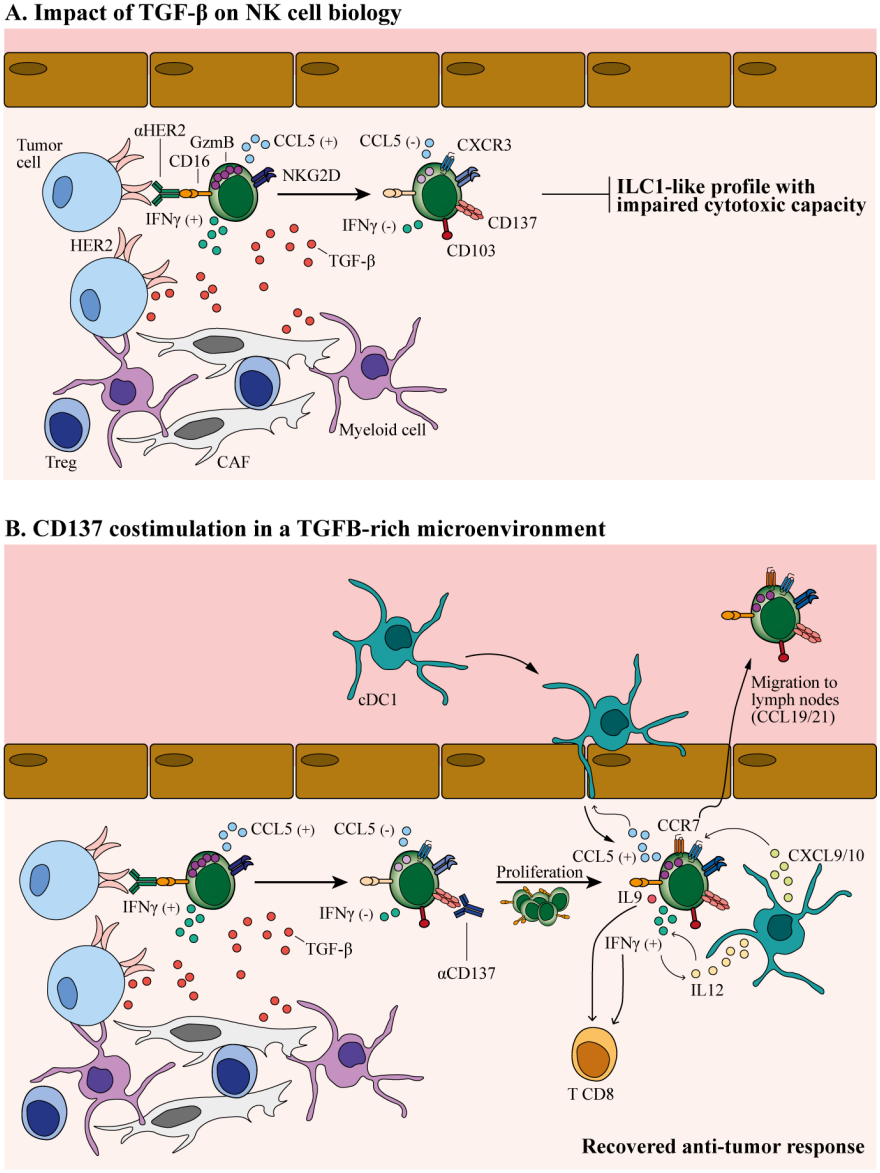

Figure 5. Impact of CD137 costimulation in a TGF-β-rich mileu. GzmB: granzyme B; Treg: T regulatory cells; CAF: cancer-associated fibroblast; cDC1: conventional dendritic cells type I.

In the recent years, a role for NK cells in the regulation of efficient antitumor responses has been emphasized. Conserved TGF-β-induced tissue-associated features (e.g. CD103, CXCR3, CCR7) could potentially contribute to NK cell tumor immunosurveillance, by increasing presence and/or persistence of NK cells in the tumor niche and epithelial cell recognition through the CD103-E-cadherin axis, while promoting migration to lymph nodes of the NK cell fraction acquiring CCR7 (9,245,246). Indeed, high proportions of activated $CD56^{bright}$ CD16+ NK cells with high expression of NKG2A and CXCR3 have been reported in draining lymph nodes from breast cancer patients (247). A previous report suggested that TGF-β may impair NK cell tumor homing by increasing CXCR4 expression, while down-regulating CX3CR1 in resting NK cells *in vitro* (248). Whether CD137 costimulation in CD16-activated NK cells also modulates CXCR4 and CX3CR1 warrants attention to further understand NK cell trafficking. In mouse models NK cells mediate the recruitment of conventional type I dendritic cells (cDC1s, also known as stimulatory dendritic cells) into the tumor niche by the secretion of the chemoattractants CCL5 (RANTES) and XLC1 (26). In human, infiltration of NK cells within the tumor has been associated to presence of cDCs and CD8+ T cells (27,132). Once in the tumor, cDC1s may produce IL-12, which has been proposed to be essential for the antitumor activity of NK cells by promoting IFN-γ secretion, which reciprocally, sustains IL-12 production by cDC1, creating a positive feedback loop (26,249–251). Secretion of IFN-γ-induced CXCL9 and CXCL10 by cDC1 may further contribute to colocalization of NK cells and cDC1s in the tumor niche, as has been observed in murine models, through the attraction of NK cells via CXCR3 (245). Our analyses indicate that in a TGF-β-rich microenvironment such as the tumor niche, CD137 costimulated NK cells may present higher cDC1 chemoattractive capacity due to their sustained CCL5 production, and enhanced CXCL9/10 sensing capacity by increased

CXCR3 expression, altogether favoring colocalization of NK cells and DC within the tumor. Interaction between NK cells and DC in the presence of CD137 agonists may then result in increased DC maturation, as reported in previous studies (209). Besides, higher IFN-γ production by CD137 co-stimulated NK cells suggests greater ability of these cells to promote Th1 responses *in vitro* (252). This ability could be of relevance in the tumor as well as upon migration of CCR7+ NK cells to regional lymph nodes, as previously reported for IL18-induced CCR7+/CD25+ NK cells (252). Altogether, our results suggest that treatment with anti-CD137 antibodies may result in NK cells exhibiting an increased potential for boosting and maintaining cDC1-dependent adaptive anti-tumor responses. We have also uncovered the capacity of a proportion of NK cells for secreting IL-9 when co-stimulated through CD137 in a TGF-β-rich milieu. Costimulation through other TNFRSF members such as OX40, GITR and TNFR-II in the presence of TGF-β has been reported to contribute and/or amplify CD4+ T cell differentiation towards a Th9 IL-9-producing phenotype (225,227–229). A constellation of transcription factors including Smad2/3, Stat5, Irf4, Batf3 and NF-κB have been shown to be required for Th9 induction and IL-9 expression in different contexts, yet a single master transcription factor has not been identified (253,254). In our microarray data, *STAT5B* was up-regulated in TGF-β-treated NK cells, and increased mRNA expression of *IRF4* and *BATF3* was observed after combined treatment with TGF-β and urelumab, pointing to a role of these transcription factors for the expression of IL-9 by a fraction of CD137-costimulated NK cells. Paralleling *IL9* gene expression pattern, *IL3, IL4, IL5* and *IL13* mRNA expression was up-regulated in TGF-β-treated NK cells after CD137-costimulation. IL-9 has been shown to enhance antitumor responses by cytotoxic lymphocytes (229,255–257), at least in part, because of its role as a T cell proproliferative factor (258). In this sense, IL-9-producing NK cells may be relevant for the development of

tumor-specific CD8+ T cell responses. According to our microarray analysis, *IL9R* (IL-9 receptor) mRNA expression is as well upregulated in CD16-activated NK cells treated with TGF-β, suggesting that IL-9 may also promote NK cell proliferation in an autocrine/paracrine manner. Although the release of Th2-related cytokines into the tumor niche by CD137-costimulated NK cells could be detrimental for efficient antitumor responses, enhanced *IL5* and *IL13* gene expression has also been described in TNF-α-induced Th9 cells in mice, yet TNF-α-Th9 cells still exhibited greater antitumor effects than conventional Th9 cells after adoptive transfer to *in vivo* models (225). Whether CD137-costimulated NK cells do indeed secrete IL-3, IL-4, IL-5 and/or IL-13 and their exact role in the efficacy of HER2-targeted antibodies against breast cancer would require additional studies in *in vivo* experimental models.

Addition of exogenous IL-2 to our experimental setting raises the question of whether our observations may be dependent on the presence of IL-2 or the expression of CD25 (IL-2Rα) by NK cells, which is indeed one of the earliest readouts affected by crosslinking of CD137. IL-2 is mainly produced by T cells, implying that presence of activated T cells within the tumor niche may be needed for efficient CD137 co-stimulation. Whether IL-15, produced by macrophages and dendritic cells would mimic the effects of IL-2 has not been addressed in our experiments, yet recent results described the synergism of IL-15 and CD137 in triggering NK cell proliferation (259). Hence, it is reasonable that CD137 costimulation in the presence of IL-15 would parallel the effects observed upon culture with IL-2.

The NK cell compartment comprises NK cell subpopulations with clonal NK cell receptor distribution at distinct maturation and differentiation stages which may be differentially affected by stimuli from the microenvironment (61). In this sense, distinct epigenetic profiles along

NK cell differentiation appear to dictate chromatin accessibility to specific transcription factors and could influence their responses upon stimulation/costimulation with CD137 in the presence of TGF-β (260,261). Indeed, putative binding sites for the TCF–LEF and NF-κB transcription factor family members, *NFKB1* and *BCL2LL* loci appeared less accessible whereas cytotoxic effectors such as the *PRF1* locus displayed increased accessibility in adaptive as compared to conventional murine NK cells by ATAC-seq (261). Therefore, intra- but also interpersonal heterogeneity of the NK cell *repertoire* may result in certain variability on TGF-β- and/or CD137-mediated effects and more or less restrained functional plasticity upon CD16 activation. We have reported that persistence of CD16-induced CD137 expression was greater in individuals harboring adaptive NKG2C+ NK cell expansions yet this NK cell subpopulation did not appear to be particularly enhanced upon urelumab-restored NK cell proliferation. On the other hand, a marked decrease in the percentage of $CD57^{+}$ NK cells after sustained CD16-mediated activation *in vitro* indicates that this highly differentiated NK cell subset might display low capacity for adapting and surviving upon activation. Given the fact that adaptive NKG2C+ subset contains large proportions of CD57+ cells it is likely that low proliferation and persistence of the CD57+ adaptive NK cell fraction hinders the putative advantage of enhanced CD137 expression in these cells. This observation fits with the results from the first part of the thesis in which we showed that CD57+ NK cells exhibit enhanced ADCC yet reduced proliferative capacity, as well as decreased CXCR3 expression, what could underlie reduced presence of these cells in the tumor niche as compared to peripheral blood.

The anti-CD137 agonistic antibody used in this study, urelumab, is one of the two antibodies against CD137 in clinical evaluation for the treatment

of cancer patients. Urelumab (BMS-663513, clone 10C7, Bristol-Myers Squibb) is a fully human IgG4, while utomilumab (PF-05082566, Pfizer) is a fully human IgG2, with much weaker agonistic strength relying on crosslinking by Fcγ receptors to activate CD137 (210,262). Currently, both CD137 agonists are in phase I/II clinical trials in combination with chemotherapy, anti-PD-1/PD-L1 blocking antibodies, and/or tumor-antigen specific mAb such as rituximab (anti-CD20), trastuzumab and T-DM1 (anti-HER2) for the treatment of different malignancies (https://clinicaltrials.gov). Structurally, utomilumab binds to adjacent domains to that of CD137L, interfering with CD137-CD137L interaction, whereas urelumab binds to the very N-terminus distant from CD137L binding site, allowing simultaneous CD137-CD137L ligation and forming tertiary structures inducing enhanced clustering and potential increased signaling downstream of the receptor (263). Presence or absence of CD137L in the system, as well as the use of distinct anti-CD137 antibodies with different agonistic activity and interfering or not with CD137-CD137L signaling may underlie controversial data from different studies regarding the role of CD137 costimulation on NK cell biology. In this regard, the effect of CD137 costimulation in enhancing antitumor ADCC responses could only be observed upon NK cell restimulation after activation-induced proliferation but not along initial activation, according to previous reports (94,95).

Our results would support the suitability of combinatorial strategies including CD137-targeted agonists and tumor antigen-specific antibodies for the treatment of cancer patients. However, a better understanding of CD137 biology and signaling is required to identify the best approach for targeting CD137 in the context of solid tumors. Considerable efforts have been devoted in the recent years to the development of alternative drug formats to target CD137 that would provide improved targeting to the

tumor, enhanced efficacy and limited toxicity (264,265). Strategies include CD137L transfection into tumor cells, modified forms of recombinant CD137L, and dual targeting of CD137 and tumor-associated antigens or proteins broadly expressed in the tumor microenvironment (i.e. vascular endothelial growth factor (VEGF) or fibroblast activation protein (FAP)) by aptamers or antibodies (266–273). However, intricacies of CD137 signaling should be exhaustively studied so as to guide the development of suitable CD137-targeted therapies.

Lastly, the results presented in this thesis may be further relevant in other contexts within tumor immunotherapy. TNF-α production by activated NK cells in the tumor niche may not only regulate CD137 expression in an autocrine manner but also modulate CD137 expression by other immune cell types, such as tumor-specific CD8+ T cells or regulatory T cells. The effect of CD137 costimulation in the latter remains unclear, with data going from enhanced proliferation upon ligation of CD137 to reprogramming of regulatory T cells into cytotoxic CD4+ T cells by CD137 agonistic antibodies (274,275). On the other hand, crosslinking of CD137 in CD8+ T cells has been shown to promote survival through the up-regulation of antiapoptotic molecules such as Bcl-xL, and to enhance IFN-γ production and tumor-specific responses in *in vivo* models (264). Moreover, CD137 costimulation in naïve CD8+ T cells curtailed TGF-β1 suppression of differentiation towards a cytotoxic T cell phenotype (276). Finally, cell therapy by chimeric antigen receptor T-cells (CAR T-cells) has been recently approved for the treatment of patients with hematological malignancies (277). Although first generation CARs only comprised the ζ chain of the TCR/CD3 complex, costimulatory domains of other receptors, such as CD137, have been added to new generation CAR T cells with the aim of achieving enhanced responses (278). Indeed, CD137 CARs exhibited superior efficacy than CD28 CARs in patients

with acute lymphoblastic leukemia (279,280). Differences are attributed to increased persistence and reduced exhaustion of CD137 CAR T cells as compared to CD28 CAR T cells (280–282). In this sense, our observations provide hints of the potential impact of CD137 costimulation in the proliferative capacity, tumor persistence, and effector function of CAR T cells, further supporting the use of CD137 costimulatory domains in the design of these therapies.

CONCLUSIONS

1. Baseline circulating CD57+ NK-cell numbers may be used as a systemic biomarker for identifying breast cancer patients with primary resistance to HER2-specific therapeutic antibody-based neoadjuvant treatment.

2. Aging may influence NK cell antitumor function by reducing their tumor-homing, proliferation and persistence upon ADCC.

3. TNFRSF stimulatory receptors are induced in NK cells upon antibody-dependent activation, becoming actionable targets for enhancing subsequent NK cell function.

4. CD137 expression in NK cells is induced by the ligation of activating receptors coupled to tyrosine kinase signaling pathways and sustained by autocrine TNF-α signaling through NF-κB.

5. CD137 costimulation by the agonist antibody urelumab enhances CD16-induced NK cell proliferation and survival in response to IL-2, regardless of TGF-β inhibition.

6. CD137 and TGF-β combined signaling promote the differentiation of NK cells with tumor-resident features (CD103 and CXCR3) which partially preserve their cytotoxic potential (NKG2D, Granzyme B) and NKR *repertoire*, enabling subsequent ADCC responses against breast cancer cells.

7. Ligation of CD137 enhances NK cell regulatory function in a TGF-β-rich environment by preserving CCL5 and IFN-γ production and cooperating with TGF-β for the secretion of IL-9; all cytokines involved in the development of effective adaptive anti-tumor responses.

8. CD137 costimulation interplays with canonical TGF-β signaling pathway by curtailing Smad4-dependent while preserving or enhancing Smad4-independent transcriptional programs in NK cells.

9. Breast carcinoma-derived multicellular *in vitro* cultures are suitable experimental systems for exploring the translational relevance of the effects of therapeutic drugs on autologous tumor-infiltrating NK cells.

10. Treatment with urelumab and trastuzumab preserves CD16, NKG2D and enhances PD-1 and CD103 expression in breast carcinoma-infiltrating NK cells and may promote sensitivity to PD-1 blockade, as directly ascertained in patient samples.

11. Our results reveal the capacity of CD137 costimulation for enhancing NK cell anti-tumor function by counteracting TGF-β inhibition and provide the rational for including CD137 agonists in therapeutic regimens aimed at broadening the efficacy of anti-HER2 therapeutic antibodies in breast cancer.

REFERENCES

1. Kiessling R, Klein E, Pross H, Wigzell H. „Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. Eur J Immunol. 1975 Feb;5(2):117–21.

2. Herberman RB, Nunn ME, Holden HT, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. Int J Cancer. 1975 Aug 15;16(2):230–9.

3. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol. 2008 May 18;9(5):503–10.

4. Cooper MA, Fehniger TA, Turner SC, Chen KS, Ghaheri BA, Ghayur T, et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood. 2001 May 15;97(10):3146–51.

5. Michel T, Poli A, Cuapio A, Briquemont B, Iserentant G, Ollert M, et al. Human CD56 ^{bright} NK Cells: An Update. J Immunol. 2016 Apr 1;196(7):2923–31.

6. Moretta L. Dissecting CD56dim human NK cells. Blood. 2010 Nov 11;116(19):3689–91.

7. Fauriat C, Long EO, Ljunggren H-G, Bryceson YT. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. Blood. 2010 Mar 18;115(11):2167–76.

8. Castriconi R, Carrega P, Dondero A, Bellora F, Casu B, Regis S, et al. Molecular Mechanisms Directing Migration and Retention of Natural Killer Cells in Human Tissues. Front Immunol. 2018 Oct 11;9:2324.

9. Grégoire C, Chasson L, Luci C, Tomasello E, Geissmann F, Vivier E, et al. The trafficking of natural killer cells. Immunol Rev. 2007 Dec;220(1):169–82.

10. Eberl G, Colonna M, Di Santo JP, McKenzie ANJ. Innate lymphoid cells: A new paradigm in immunology. Science (80-). 2015 May 22;348(6237):aaa6566-aaa6566.

11. Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate Lymphoid Cells: 10 Years On. Cell. 2018 Aug 23;174(5):1054–66.

12. Diefenbach A, Colonna M, Koyasu S. Development, Differentiation, and Diversity of Innate Lymphoid Cells. Immunity. 2014 Sep 18;41(3):354–65.

13. Artis D, Spits H. The biology of innate lymphoid cells. Nature. 2015 Jan 14;517(7534):293–301.

14. Montaldo E, Vacca P, Vitale C, Moretta F, Locatelli F, Mingari MC, et al. Human innate lymphoid cells. Immunol Lett. 2016 Nov;179:2–8.

15. Wagner M, Moro K, Koyasu S. Plastic Heterogeneity of Innate Lymphoid Cells in Cancer. Trends in cancer. 2017 May;3(5):326–35.

16. Colonna M. Innate Lymphoid Cells: Diversity, Plasticity, and Unique Functions in Immunity. Immunity. 2018 Jun;48(6):1104–17.

17. Lim AI, Menegatti S, Bustamante J, Le Bourhis L, Allez M, Rogge L, et al. IL-12 drives functional plasticity of human group 2 innate lymphoid cells. J Exp Med. 2016 Apr 4;213(4):569–83.

18. Ohne Y, Silver JS, Thompson-Snipes L, Collet MA, Blanck JP, Cantarel BL, et al. IL-1 is a critical regulator of group 2 innate lymphoid cell function and plasticity. Nat Immunol. 2016 Jun 25;17(6):646–55.

19. Bernink JH, Krabbendam L, Germar K, de Jong E, Gronke K, Kofoed-Nielsen M, et al. Interleukin-12 and -23 Control Plasticity of CD127+ Group 1 and Group 3 Innate Lymphoid Cells in the Intestinal Lamina Propria. Immunity. 2015 Jul 21;43(1):146–60.

20. Cella M, Otero K, Colonna M. Expansion of human NK-22 cells with IL-7, IL-2, and IL-1 reveals intrinsic functional plasticity. Proc Natl Acad Sci. 2010 Jun 15;107(24):10961–6.

21. Gao Y, Souza-Fonseca-Guimaraes F, Bald T, Ng SS, Young A, Ngiow SF, et al. Tumor immunoevasion by the conversion of effector NK cells into type 1 innate lymphoid cells. Nat Immunol. 2017 Sep;18(9):1004–15.

22. Cortez VS, Ulland TK, Cervantes-Barragan L, Bando JK, Robinette ML, Wang Q, et al. SMAD4 impedes the conversion of NK cells into ILC1-like cells by curtailing non-canonical TGF-β signaling. Nat Immunol. 2017 Sep;18(9):995–1003.

23. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. Nat Immunol. 2008 May 18;9(5):495–502.

24. Parham P. Taking license with natural killer cell maturation and repertoire development. Immunol Rev. 2006 Dec;214(1):155–60.

25. Smyth MJ, Cretney E, Kelly JM, Westwood JA, Street SEA, Yagita H, et al. Activation of NK cell cytotoxicity. Mol Immunol. 2005 Feb;42(4):501–10.

26. Böttcher JP, Bonavita E, Chakravarty P, Blees H, Cabeza-Cabrerizo M, Sammicheli S, et al. NK Cells Stimulate Recruitment of cDC1 into the Tumor Microenvironment Promoting Cancer Immune Control. Cell. 2018 Feb 22;172(5):1022–1037.e14.

27. Barry KC, Hsu J, Broz ML, Cueto FJ, Binnewies M, Combes AJ, et al. A natural killer-dendritic cell axis defines checkpoint therapy-responsive tumor microenvironments. Nat Med. 2018 Aug 25;24(8):1178–91.

28. Martín-Fontecha A, Thomsen LL, Brett S, Gerard C, Lipp M, Lanzavecchia A, et al. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. Nat Immunol. 2004 Dec 7;5(12):1260–5.

29. Moretta A, Marcenaro E, Sivori S, Chiesa M Della, Vitale M, Moretta L. Early liaisons between cells of the innate immune system in inflamed peripheral tissues. Trends Immunol. 2005 Dec;26(12):668–75.

30. Moretta A, Bottino C, Vitale M, Pende D, Biassoni R, Mingari MC, et al. Receptors for HLA class-I molecules in human natural killer cells. Annu Rev Immunol. 1996 Apr;14(1):619–48.

31. Vilches C, Parham P. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. Annu Rev Immunol. 2002 Apr;20(1):217–51.

32. López-Botet M, Pérez-Villar JJ, Carretero M, Rodríguez A, Melero I, Bellón T, et al. Structure and function of the CD94 C-type lectin receptor complex involved in recognition of HLA class I molecules. Immunol Rev. 1997 Feb;155:165–74.

33. Colonna M, Navarro F, Bellón T, Llano M, García P, Samaridis J, et al. A Common Inhibitory Receptor for Major Histocompatibility Complex Class I Molecules on Human Lymphoid and Myelomonocytic Cells. J Exp Med. 1997 Dec 1;186(11):1809–18.

34. Baía D, Pou J, Jones D, Mandelboim O, Trowsdale J, Muntasell A, et al. Interaction of the LILRB1 inhibitory receptor with HLA class Ia dimers. Eur J Immunol. 2016 Jul;46(7):1681–90.

35. Parham P. MHC class I molecules and KIRs in human history, health and survival. Nat Rev Immunol. 2005 Mar 18;5(3):201–14.

36. Ivarsson MA, MichaÃ«lsson J, Fauriat C. Activating Killer Cell Ig-Like Receptors in Health and Disease. Front Immunol. 2014 Apr 22;5.

37. Stanietsky N, Rovis TL, Glasner A, Seidel E, Tsukerman P, Yamin R, et al. Mouse TIGIT inhibits NK-cell cytotoxicity upon interaction with PVR. Eur J Immunol. 2013 Aug;43(8):2138–50.

38. Bernhardt G. TACTILE becomes tangible: CD96 discloses its inhibitory peculiarities. Nat Immunol. 2014 May 18;15(5):406–8.

39. Martinet L, Ferrari De Andrade L, Guillerey C, Lee JS, Liu J, Souza-Fonseca-Guimaraes F, et al. DNAM-1 Expression Marks an Alternative Program of NK Cell Maturation. Cell Rep. 2015 Apr 7;11(1):85–97.

40. Wang PL, O'Farrell S, Clayberger C, Krensky AM. Identification and molecular cloning of tactile. A novel human T cell activation antigen that is a member of the Ig gene superfamily. J Immunol. 1992 Apr 15;148(8):2600–8.

41. Iguchi-Manaka A, Kai H, Yamashita Y, Shibata K, Tahara-Hanaoka S, Honda S, et al. Accelerated tumor growth in mice deficient in DNAM-1 receptor. J Exp Med. 2008 Dec 22;205(13):2959–64.

42. Stanietsky N, Simic H, Arapovic J, Toporik A, Levy O, Novik A, et al. The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity. Proc Natl Acad Sci U S A. 2009 Oct 20;106(42):17858–63.

43. Chan CJ, Martinet L, Gilfillan S, Souza-Fonseca-Guimaraes F, Chow MT, Town L, et al. The receptors CD96 and CD226 oppose each other in the regulation of natural killer cell functions. Nat Immunol. 2014 May 23;15(5):431–8.

44. Long EO. Negative signaling by inhibitory receptors: the NK cell paradigm. Immunol Rev. 2008 Aug;224(1):70–84.

45. Vitale M, Bottino C, Sivori S, Sanseverino L, Castriconi R, Marcenaro E, et al. NKp44, a Novel Triggering Surface Molecule Specifically Expressed by Activated Natural Killer Cells, Is Involved in Non–Major Histocompatibility Complex–restricted Tumor Cell Lysis. J Exp Med. 1998 Jun 15;187(12):2065–72.

46. Bryceson YT, March ME, Ljunggren H-G, Long EO. Activation, coactivation, and costimulation of resting human natural killer cells. Immunol Rev. 2006 Dec;214(1):73–91.

47. Lanier LL. Natural killer cell receptor signaling. Curr Opin Immunol. 2003 Jun;15(3):308–14.

48. Vivier E, Nunès JA, Vély F. Natural killer cell signaling pathways. Science. 2004 Nov 26;306(5701):1517–9.

49. Lanier LL. DAP10- and DAP12-associated receptors in innate immunity. Immunol Rev. 2009 Jan;227(1):150–60.

50. Watzl C, Long EO. Signal Transduction During Activation and Inhibition of Natural Killer Cells. In: Current Protocols in Immunology. Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2010. p. Unit 11.9B.

51. Pende D, Rivera P, Marcenaro S, Chang C-C, Biassoni R, Conte R, et al. Major histocompatibility complex class I-related chain A and UL16-binding protein expression on tumor cell lines of different histotypes: analysis of tumor susceptibility to NKG2D-dependent natural killer cell cytotoxicity. Cancer Res. 2002 Nov 1;62(21):6178–86.

52. Cerboni C, Fionda C, Soriani A, Zingoni A, Doria M, Cippitelli M, et al. The DNA Damage Response: A Common Pathway in the Regulation of NKG2D and DNAM-1 Ligand Expression in Normal, Infected, and Cancer Cells. Front Immunol. 2014 Jan 7;4:508.

53. Pogge von Strandmann E, Simhadri VR, von Tresckow B, Sasse S, Reiners KS, Hansen HP, et al. Human leukocyte antigen-B-associated transcript 3 is released from tumor cells and engages the NKp30 receptor on natural killer cells. Immunity. 2007 Dec;27(6):965–74.

54. Baychelier F, Sennepin A, Ermonval M, Dorgham K, Debre P, Vieillard V. Identification of a cellular ligand for the natural cytotoxicity receptor NKp44. Blood. 2013 Oct 24;122(17):2935–42.

55. Brandt CS, Baratin M, Yi EC, Kennedy J, Gao Z, Fox B, et al. The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. J Exp Med. 2009 Jul 6;206(7):1495–503.

56. Gumá M, Angulo A, Vilches C, Gómez-Lozano N, Malats N, López-Botet M, et al. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. Blood. 2004 Dec 1;104(12):3664–71.

57. Bottino C, Castriconi R, Pende D, Rivera P, Nanni M, Carnemolla B, et al. Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. J Exp Med. 2003 Aug 18;198(4):557–67.

58. Bruhns P, Iannascoli B, England P, Mancardi DA, Fernandez N, Jorieux S, et al. Specificity and affinity of human Fc receptors and their polymorphic variants for human IgG subclasses. Blood. 2009 Apr 16;113(16):3716–25.

59. Koene HR, Kleijer M, Algra J, Roos D, von dem Borne AE, de Haas M. Fc gammaRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell Fc gammaRIIIa, independently of the Fc gammaRIIIa-48L/R/H phenotype. Blood. 1997 Aug 1;90(3):1109–14.

60. Matos ME, Schnier GS, Beecher MS, Ashman LK, William DE, Caligiuri MA. Expression of a functional c-kit receptor on a subset of natural killer cells. J Exp Med. 1993 Sep 1;178(3):1079–84.

61. López-Botet M, Vilches C, Redondo-Pachón D, Muntasell A, Pupuleku A, Yélamos J, et al. Dual Role of Natural Killer Cells on Graft Rejection and Control of Cytomegalovirus Infection in Renal Transplantation. Front Immunol. 2017 Feb 16;8:166.

62. Bjorkstrom NK, Riese P, Heuts F, Andersson S, Fauriat C, Ivarsson MA, et al. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. Blood. 2010 Nov 11;116(19):3853–64.

63. Parham P, Moffett A. Variable NK cell receptors and their MHC class I ligands in immunity, reproduction and human evolution. Nat Rev Immunol. 2013 Feb 21;13(2):133–44.

64. Chan H-W, Kurago ZB, Stewart CA, Wilson MJ, Martin MP, Mace BE, et al. DNA Methylation Maintains Allele-specific *KIR* Gene Expression in Human Natural Killer Cells. J Exp Med. 2003 Jan 20;197(2):245–55.

65. Anfossi N, André P, Guia S, Falk CS, Roetynck S, Stewart CA, et al. Human NK cell education by inhibitory receptors for MHC class I. Immunity. 2006 Aug;25(2):331–42.

66. Lanier LL, Le AM, Phillips JH, Warner NL, Babcock GF. Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. J Immunol. 1983 Oct;131(4):1789–96.

67. Abo T, Miller CA, Balch CM. Characterization of human granular lymphocyte subpopulations expressing HNK-1 (Leu-7) and Leu-11 antigens in the blood and lymphoid tissues from fetuses, neonates and adults. Eur J Immunol. 1984 Jul;14(7):616–23.

68. Krishnaraj R, Svanborg A. Preferential accumulation of mature NK cells during human immunosenescence. J Cell Biochem. 1992 Dec;50(4):386–91.

69. Le Garff-Tavernier M, Béziat V, Decocq J, Siguret V, Gandjbakhch F, Pautas E, et al. Human NK cells display major phenotypic and functional changes over the life span. Aging Cell. 2010 Apr 29;9(4):527–35.

70. Ong E, Yeh J-C, Ding Y, Hindsgaul O, Pedersen LC, Negishi M, et al. Structure and Function of HNK-1 Sulfotransferase. J Biol Chem. 1999 Sep 3;274(36):25608–12.

71. Lopez-Verges S, Milush JM, Pandey S, York VA, Arakawa-Hoyt J, Pircher H, et al. CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. Blood. 2010 Nov 11;116(19):3865–74.

72. Lopez-Vergès S, Milush JM, Schwartz BS, Pando MJ, Jarjoura J, York VA, et al. Expansion of a unique CD57^{+}NKG2Chi natural killer cell subset during acute human cytomegalovirus infection. Proc Natl Acad Sci U S A. 2011 Sep 6;108(36):14725–32.

73. Beziat V, Liu LL, Malmberg J-A, Ivarsson MA, Sohlberg E, Bjorklund AT, et al. NK cell responses to cytomegalovirus infection lead to stable imprints in the human KIR repertoire and involve activating KIRs. Blood. 2013 Apr 4;121(14):2678–88.

74. Muntasell A, López-Montañés M, Vera A, Heredia G, Romo N, Peñafiel J, et al. NKG2C zygosity influences CD94/NKG2C receptor function and the NK-cell compartment redistribution in response to human cytomegalovirus. Eur J Immunol. 2013;

75. López-Botet M, Muntasell A, Vilches C. The CD94/NKG2C+ NK-cell subset on the edge of innate and adaptive immunity to human cytomegalovirus infection. Semin Immunol. 2014 Apr;26(2):145–51.

76. Lee J, Zhang T, Hwang I, Kim A, Nitschke L, Kim M, et al. Epigenetic modification and antibody-dependent expansion of memory-like NK cells in human cytomegalovirus-infected individuals. Immunity. 2015 Mar 17;42(3):431–42.

77. Schlums H, Cichocki F, Tesi B, Theorell J, Beziat V, Holmes TD, et al. Cytomegalovirus Infection Drives Adaptive Epigenetic Diversification of NK Cells with Altered Signaling and Effector Function. Immunity. 2015 Mar 17;42(3):443–56.

78. Zhang T, Scott JM, Hwang I, Kim S. Cutting Edge: Antibody-Dependent Memory-like NK Cells Distinguished by FcR Deficiency. J Immunol. 2013;190(4):1402–6.

79. Costa-Garcia M, Vera A, Moraru M, Vilches C, López-Botet M, Muntasell A. Antibody-Mediated Response of NKG2C ^{bright} NK Cells against Human Cytomegalovirus. J Immunol. 2015 Mar 15;194(6):2715–24.

80. Wu Z, Sinzger C, Frascaroli G, Reichel J, Bayer C, Wang L, et al. Human cytomegalovirus-induced NKG2C(hi) CD57(hi) natural killer cells are effectors dependent on humoral antiviral immunity. J Virol. 2013 Jul 1;87(13):7717–25.

81. Waldmann TA. The Shared and Contrasting Roles of IL2 and IL15 in the Life and Death of Normal and Neoplastic Lymphocytes: Implications for Cancer Therapy. Cancer Immunol Res. 2015 Mar 1;3(3):219–27.

82. Okamura H, Kashiwamura S, Tsutsui H, Yoshimoto T, Nakanishi K. Regulation of interferon-gamma production by IL-12 and IL-18. Curr Opin Immunol. 1998 Jun;10(3):259–64.

83. Smyth MJ, Taniguchi M, Street SEA. The Anti-Tumor Activity of IL-12: Mechanisms of Innate Immunity That Are Model and Dose Dependent. J Immunol. 2000 Sep 1;165(5):2665–70.

84. Müller L, Aigner P, Stoiber D. Type I Interferons and Natural Killer Cell Regulation in Cancer. Front Immunol. 2017 Mar 31;8:304.

85. Viel S, Marçais A, Guimaraes FS-F, Loftus R, Rabilloud J, Grau M, et al. TGF-β inhibits the activation and functions of NK cells by repressing the mTOR pathway. Sci Signal. 2016 Feb 16;9(415):ra19.

86. Melero I, Johnston J V., Shufford WW, Mittler RS, Chen L. NK1.1 Cells Express 4-1BB (CDw137) Costimulatory Molecule and Are Required for Tumor Immunity Elicited by Anti-4-1BB Monoclonal Antibodies. Cell Immunol. 1998 Dec;190(2):167–72.

87. Lin W, Voskens CJ, Zhang X, Schindler DG, Wood A, Burch E, et al. Fc-dependent expression of CD137 on human NK cells: insights into "agonistic" effects of anti-CD137 monoclonal antibodies. Blood. 2008 Aug 1;112(3):699–707.

88. Turaj AH, Cox KL, Penfold CA, French RR, Mockridge CI, Willoughby JE, et al. Augmentation of CD134 (OX40)-dependent NK anti-tumour activity is dependent on antibody cross-linking. Sci Rep. 2018 Dec 2;8(1):2278.

89. Hanabuchi S. Human plasmacytoid predendritic cells activate NK cells through glucocorticoid-induced tumor necrosis factor receptor-ligand (GITRL). Blood. 2006 May 1;107(9):3617–23.

90. Sanmamed MF, Pastor F, Rodriguez A, Perez-Gracia JL, Rodriguez-Ruiz ME, Jure-Kunkel M, et al. Agonists of Co-stimulation in Cancer Immunotherapy Directed Against CD137, OX40, GITR, CD27, CD28, and ICOS. Semin Oncol. 2015 Aug;42(4):640–55.

91. Ward-Kavanagh LK, Lin WW, Šedý JR, Ware CF. The TNF Receptor Superfamily in Co-stimulating and Co-inhibitory Responses. Immunity. 2016 May;44(5):1005–19.

92. Croft M, Duan W, Choi H, Eun S-Y, Madireddi S, Mehta A. TNF superfamily in inflammatory disease: translating basic insights. Trends Immunol. 2012 Mar;33(3):144–52.

93. Baessler T, Charton JE, Schmiedel BJ, Grünebach F, Krusch M, Wacker A, et al. CD137 ligand mediates opposite effects in human and mouse NK cells and impairs NK-cell reactivity against human acute myeloid leukemia cells. Blood. 2010 Apr 15;115(15):3058–69.

94. Wilcox RA, Tamada K, Strome SE, Chen L. Signaling Through NK Cell-Associated CD137 Promotes Both Helper Function for CD8 + Cytolytic T Cells and Responsiveness to IL-2 But Not Cytolytic Activity. J Immunol. 2002 Oct 15;169(8):4230–6.

95. Ochoa MC, Perez-Ruiz E, Minute L, Oñate C, Perez G, Rodriguez I, et al. Daratumumab in combination with urelumab to potentiate anti-myeloma activity in lymphocyte-deficient mice reconstituted with human NK cells. Oncoimmunology. 2019 Jul 3;8(7):1599636.

96. Buechele C, Baessler T, Schmiedel BJ, Schumacher CE, Grosse-Hovest L, Rittig K, et al. 4-1BB ligand modulates direct and Rituximab-induced NK-cell reactivity in chronic lymphocytic leukemia. Eur J Immunol. 2012 Mar;42(3):737–48.

97. Liu C, Lou Y, Lizée G, Qin H, Liu S, Rabinovich B, et al. Plasmacytoid dendritic cells induce NK cell–dependent, tumor antigen–specific T cell cross-priming and tumor regression in mice. J Clin Invest. 2008 Feb 1;118(3):1165–75.

98. Baltz KM, Krusch M, Bringmann A, Brossart P, Mayer F, Kloss M, et al. Cancer immunoediting by GITR (glucocorticoid-induced TNF-related protein) ligand in humans: NK cell/tumor cell interactions. FASEB J. 2007 Aug;21(10):2442–54.

99. Baltz KM, Krusch M, Baessler T, Schmiedel BJ, Bringmann A, Brossart P, et al. Neutralization of tumor-derived soluble Glucocorticoid-Induced TNFR-Related Protein ligand increases NK cell anti-tumor reactivity. Blood. 2008 Nov 1;112(9):3735–43.

100. Sanchez-Paulete AR, Labiano S, Rodriguez-Ruiz ME, Azpilikueta A, Etxeberria I, Bolaños E, et al. Deciphering CD137 (4-1BB) signaling in T-cell costimulation for translation into successful cancer immunotherapy. Eur J Immunol. 2016 Mar;46(3):513–22.

101. Chen ZJ. Ubiquitination in signaling to and activation of IKK. Immunol Rev. 2012 Mar;246(1):95–106.

102. Sabbagh L, Pulle G, Liu Y, Tsitsikov EN, Watts TH. ERK-Dependent Bim Modulation Downstream of the 4-1BB-TRAF1 Signaling Axis Is a Critical Mediator of CD8 T Cell Survival In Vivo. J Immunol. 2008 Jun 15;180(12):8093–101.

103. Cannons JL, Choi Y, Watts TH. Role of TNF receptor-associated factor 2 and p38 mitogen-activated protein kinase activation during 4-1BB-dependent immune response. J Immunol. 2000 Dec 1;165(11):6193–204.

104. Hutloff A, Dittrich AM, Beier KC, Eljaschewitsch B, Kraft R, Anagnostopoulos I, et al. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. Nature. 1999 Jan 21;397(6716):263–6.

105. Ogasawara K, Yoshinaga SK, Lanier LL. Inducible Costimulator Costimulates Cytotoxic Activity and IFN-γ Production in Activated Murine NK Cells. J Immunol. 2002 Oct 1;169(7):3676–85.

106. Senju H, Kumagai A, Nakamura Y, Yamaguchi H, Nakatomi K, Fukami S, et al. Effect of IL-18 on the Expansion and Phenotype of Human Natural Killer Cells: Application to Cancer Immunotherapy. Int J Biol Sci. 2018;14(3):331–40.

107. Swallow MM, Wallin JJ, Sha WC. B7h, a novel costimulatory homolog of B7.1 and B7.2, is induced by TNFalpha. Immunity. 1999 Oct;11(4):423–32.

108. Qian X, Agematsu K, Freeman GJ, Tagawa Y, Sugane K, Hayashi T. The ICOS-ligand B7-H2, expressed on human type II alveolar epithelial cells, plays a role in the pulmonary host defense system. Eur J Immunol. 2006 Apr;36(4):906–18.

109. Beldi-Ferchiou A, Caillat-Zucman S. Control of NK Cell Activation by Immune Checkpoint Molecules. Int J Mol Sci. 2017 Oct 12;18(10):2129.

110. Benson DM, Bakan CE, Mishra A, Hofmeister CC, Efebera Y, Becknell B, et al. The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. Blood. 2010 Sep 30;116(13):2286–94.

111. MacFarlane AW, Jillab M, Plimack ER, Hudes GR, Uzzo RG, Litwin S, et al. PD-1 expression on peripheral blood cells increases with stage in renal cell carcinoma patients and is rapidly reduced after surgical tumor resection. Cancer Immunol Res. 2014 Apr 1;2(4):320–31.

112. Pesce S, Greppi M, Tabellini G, Rampinelli F, Parolini S, Olive D, et al. Identification of a subset of human natural killer cells expressing high levels of programmed death 1: A phenotypic and functional characterization. J Allergy Clin Immunol. 2016 Jan;4(1):335–346.e3.

113. Hsu J, Hodgins JJ, Marathe M, Nicolai CJ, Bourgeois-Daigneault M-C, Trevino TN, et al. Contribution of NK cells to immunotherapy mediated by PD-1/PD-L1 blockade. J Clin Investig. 2018 Sep 10;128(10):4654–68.

114. Liu Y, Cheng Y, Xu Y, Wang Z, Du X, Li C, et al. Increased expression of programmed cell death protein 1 on NK cells inhibits NK-cell-mediated anti-tumor function and indicates poor prognosis in digestive cancers. Oncogene. 2017 Nov 2;36(44):6143–53.

115. Stojanovic A, Fiegler N, Brunner-Weinzierl M, Cerwenka A. CTLA-4 Is Expressed by Activated Mouse NK Cells and Inhibits NK Cell IFN-γ Production in Response to Mature Dendritic Cells. J Immunol. 2014 May 1;192(9):4184–91.

116. Terme M, Ullrich E, Aymeric L, Meinhardt K, Coudert JD, Desbois M, et al. Cancer-Induced Immunosuppression: IL-18-Elicited Immunoablative NK Cells. Cancer Res. 2012 Jun 1;72(11):2757–67.

117. Talmadge JE, Meyers KM, Prieur DJ, Starkey JR. Role of natural killer cells in tumor growth and metastasis: C57BL/6 normal and beige mice. J Natl Cancer Inst. 1980 Nov;65(5):929–35.

118. Ljunggren HG, Kärre K. Host resistance directed selectively against H-2-deficient lymphoma variants. Analysis of the mechanism. J Exp Med. 1985 Dec 1;162(6):1745–59.

119. Seaman WE, Sleisenger M, Eriksson E, Koo GC. Depletion of natural killer cells in mice by monoclonal antibody to NK-1.1. Reduction in host defense against malignancy without loss of cellular or humoral immunity. J Immunol. 1987 Jun 15;138(12):4539–44.

120. Diefenbach A, Jensen ER, Jamieson AM, Raulet DH. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. Nature. 2001 Sep 13;413(6852):165–71.

121. Wu J, Lanier LL. Natural killer cells and cancer. Adv Cancer Res. 2003;90:127–56.

122. Street SEA, Hayakawa Y, Zhan Y, Lew AM, MacGregor D, Jamieson AM, et al. Innate Immune Surveillance of Spontaneous B Cell Lymphomas by Natural Killer Cells and γδ T Cells. J Exp Med. 2004 Mar 15;199(6):879–84.

123. Marcus A, Gowen BG, Thompson TW, Iannello A, Ardolino M, Deng W, et al. Recognition of tumors by the innate immune system and natural killer cells. Adv Immunol. 2014;122:91–128.

124. Morvan MG, Lanier LL. NK cells and cancer: you can teach innate cells new tricks. Nat Rev Cancer. 2015 Jan 23;16(1):7–19.

125. Imai K, Matsuyama S, Miyake S, Suga K, Nakachi K. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. Lancet. 2000 Nov;356(9244):1795–9.

126. Krasnova Y, Putz EM, Smyth MJ, Souza-Fonseca-Guimaraes F. Bench to bedside: NK cells and control of metastasis. Clin Immunol. 2017 Apr;177:50–9.

127. Ishigami S, Natsugoe S, Tokuda K, Nakajo A, Xiangming C, Iwashige H, et al. Clinical impact of intratumoral natural killer cell and dendritic cell infiltration in gastric cancer. Cancer Lett. 2000 Oct 16;159(1):103–8.

128. Türkseven MR, Oygür T. Evaluation of natural killer cell defense in oral squamous cell carcinoma. Oral Oncol. 2010 May;46(5):e34–7.

129. Delahaye NF, Rusakiewicz S, Martins I, Ménard C, Roux S, Lyonnet L, et al. Alternatively spliced NKp30 isoforms affect the prognosis of gastrointestinal stromal tumors. Nat Med. 2011 Jun 8;17(6):700–7.

130. Remark R, Alifano M, Cremer I, Lupo A, Dieu-Nosjean M-C, Riquet M, et al. Characteristics and Clinical Impacts of the Immune Environments in Colorectal and Renal Cell Carcinoma Lung Metastases: Influence of Tumor Origin. Clin Cancer Res. 2013 Aug 1;19(15):4079–91.

131. Tian W, Wang L, Yuan L, Duan W, Zhao W, Wang S, et al. A prognostic risk model for patients with triple negative breast cancer based on stromal natural killer cells, tumor-associated macrophages and growth-arrest specific protein 6. Cancer Sci. 2016 Jul;107(7):882–9.

132. Muntasell A, Rojo F, Servitja S, Rubio-Perez C, Cabo M, Tamborero D, et al. NK Cell Infiltrates and HLA Class I Expression in Primary HER2+ Breast Cancer Predict and Uncouple Pathological Response and Disease-free Survival. Clin Cancer Res. 2019 Mar 1;25(5):1535–45.

133. Schmiedel D, Mandelboim O. NKG2D Ligands–Critical Targets for Cancer Immune Escape and Therapy. Front Immunol. 2018 Sep 11;9:2040.

134. Holdenrieder S, Stieber P, Peterfi A, Nagel D, Steinle A, Salih HR. Soluble MICA in malignant diseases. Int J Cancer. 2006 Feb 1;118(3):684–7.

135. Palmisano GL, Contardi E, Morabito A, Gargaglione V, Ferrara GB, Pistillo MP. HLA-E surface expression is independent of the availability of HLA class I signal sequence-derived peptides in human tumor cell lines. Hum Immunol. 2005 Jan;66(1):1–12.

136. de Kruijf EM, Sajet A, van Nes JGH, Natanov R, Putter H, Smit VTHBM, et al. HLA-E and HLA-G Expression in Classical HLA Class I-Negative Tumors Is of Prognostic Value for Clinical Outcome of Early Breast Cancer Patients. J Immunol. 2010 Dec 15;185(12):7452–9.

137. Reimer T, Koczan D, Müller H, Friese K, Thiesen H-J, Gerber B. Tumour Fas ligand:Fas ratio greater than 1 is an independent marker of relative resistance to tamoxifen therapy in hormone receptor positive breast cancer. Breast Cancer Res. 2002;4(5):R9.

138. Seitz S, Wassmuth P, Fischer J, Nothnagel A, Jandrig B, Schlag PM, et al. Mutation analysis and mRNA expression of trail-receptors in human breast cancer. Int J Cancer. 2002 Nov 10;102(2):117–28.

139. Ding Q, He X, Xia W, Hsu J-M, Chen C-T, Li L-Y, et al. Myeloid cell leukemia-1 inversely correlates with glycogen synthase kinase-3beta activity and associates with poor prognosis in human breast cancer. Cancer Res. 2007 May 15;67(10):4564–71.

140. Booy EP, Henson ES, Gibson SB. Epidermal growth factor regulates Mcl-1 expression through the MAPK-Elk-1 signalling pathway contributing to cell survival in breast cancer. Oncogene. 2011 May 19;30(20):2367–78.

141. Holt DM, Ma X, Kundu N, Collin PD, Fulton AM. Modulation of Host Natural Killer Cell Functions in Breast Cancer via Prostaglandin E2 Receptors EP2 and EP4. J Immunother. 2012;35(2):179–88.

142. Chiesa MD, Carlomagno S, Frumento G, Balsamo M, Cantoni C, Conte R, et al. The tryptophan catabolite L-kynurenine inhibits the surface expression of NKp46- and NKG2D-activating receptors and regulates NK-cell function. Blood. 2006 Dec 15;108(13):4118–25.

143. Pietra G, Vitale M, Moretta L, Mingari MC. How melanoma cells inactivate NK cells. Oncoimmunology. 2012 Sep 1;1(6):974–5.

144. Batlle E, Massagué J. Transforming Growth Factor-β Signaling in Immunity and Cancer. Immunity. 2019 Apr 16;50(4):924–40.

145. Kubiczkova L, Sedlarikova L, Hajek R, Sevcikova S. TGF-β – an excellent servant but a bad master. J Transl Med. 2012 Sep 3;10(1):183.

146. López-Casillas F, Wrana JL, Massagué J. Betaglycan presents ligand to the TGFβ signaling receptor. Cell. 1993 Jul;73(7):1435–44.

147. David CJ, Massagué J. Contextual determinants of TGFβ action in development, immunity and cancer. Nat Rev Mol Cell Biol. 2018 Jul 11;19(7):419–35.

148. He W, Dorn DC, Erdjument-Bromage H, Tempst P, Moore MAS, Massagué J. Hematopoiesis Controlled by Distinct TIF1γ and Smad4 Branches of the TGFβ Pathway. Cell. 2006 Jun 2;125(5):929–41.

149. Doisne J-M, Bartholin L, Yan K-P, Garcia CN, Duarte N, Le Luduec J-B, et al. iNKT cell development is orchestrated by different branches of TGF-beta signaling. J Exp Med. 2009 Jun 8;206(6):1365–78.

150. Zhang Y, Alexander PB, Wang X-F. TGF-β Family Signaling in the Control of Cell Proliferation and Survival. Cold Spring Harb Perspect Biol. 2017 Apr 3;9(4):a022145.

151. Yan X, Liu Z, Chen Y. Regulation of TGF- signaling by Smad7. Acta Biochim Biophys Sin (Shanghai). 2009 Mar 4;41(4):263–72.

152. Derynck R, Akhurst RJ, Balmain A. TGF-β signaling in tumor suppression and cancer progression. Nat Genet. 2001 Oct;29(2):117–29.

153. Narai S, Watanabe M, Hasegawa H, Nishibori H, Endo T, Kubota T, et al. Significance of transforming growth factor ?1 as a new tumor marker for colorectal cancer. Int J Cancer. 2002 Feb 1;97(4):508–11.

154. Friese MA, Wischhusen J, Wick W, Weiler M, Eisele G, Steinle A, et al. RNA Interference Targeting Transforming Growth Factor-β Enhances NKG2D-Mediated Antiglioma Immune Response, Inhibits Glioma Cell Migration and Invasiveness, and Abrogates Tumorigenicity ***In vivo*** . Cancer Res. 2004 Oct 15;64(20):7596–603.

155. Lee J-C, Lee K-M, Kim D-W, Heo DS. Elevated TGF-β1 Secretion and Down-Modulation of NKG2D Underlies Impaired NK Cytotoxicity in Cancer Patients. J Immunol. 2004 Jun 15;172(12):7335–40.

156. Dong M, Blobe GC. Role of transforming growth factor-beta in hematologic malignancies. Blood. 2006 Jun 15;107(12):4589–96.

157. Ikushima H, Miyazono K. TGFβ signalling: a complex web in cancer progression. Nat Rev Cancer. 2010 Jun;10(6):415–24.

158. Yu J, Wei M, Becknell B, Trotta R, Liu S, Boyd Z, et al. Pro- and Antiinflammatory Cytokine Signaling: Reciprocal Antagonism Regulates Interferon-gamma Production by Human Natural Killer Cells. Immunity. 2006 May;24(5):575–90.

159. Trotta R, Dal Col J, Yu J, Ciarlariello D, Thomas B, Zhang X, et al. TGF-beta utilizes SMAD3 to inhibit CD16-mediated IFN-gamma production and antibody-dependent cellular cytotoxicity in human NK cells. J Immunol. 2008 Sep 15;181(6):3784–92.

160. Castriconi R, Cantoni C, Della Chiesa M, Vitale M, Marcenaro E, Conte R, et al. Transforming growth factor 1 inhibits expression of NKp30 and NKG2D receptors: Consequences for the NK-mediated killing of dendritic cells. Proc Natl Acad Sci. 2003 Apr 1;100(7):4120–5.

161. Wilson EB, El-Jawhari JJ, Neilson AL, Hall GD, Melcher AA, Meade JL, et al. Human Tumour Immune Evasion via TGF-β Blocks NK Cell Activation but Not Survival Allowing Therapeutic Restoration of Anti-Tumour Activity. Zimmer J, editor. PLoS One. 2011 Sep 6;6(9):e22842.

162. Keskin DB, Allan DSJ, Rybalov B, Andzelm MM, Stern JNH, Kopcow HD, et al. TGFbeta promotes conversion of CD16+ peripheral blood NK cells into CD16- NK cells with similarities to decidual NK cells. Proc Natl Acad Sci. 2007 Feb 27;104(9):3378–83.

163. Cortez VS, Cervantes-Barragan L, Robinette ML, Bando JK, Wang Y, Geiger TL, et al. Transforming Growth Factor-β Signaling Guides the Differentiation of Innate Lymphoid Cells in Salivary Glands. Immunity. 2016 May 17;44(5):1127–39.

164. Koboldt DC, Fulton RS, McLellan MD, Schmidt H, Kalicki-Veizer J, McMichael JF, Fulton LL, Dooling DJ, Ding L, Mardis ER, Wilson RK, Ally A, Balasundaram M, Butterfield YS, Carlsen R, Carter C, Chu A, Chuah E, Chun HJ, Coope RJ, Dhalla N, Guin R, Hirst C, PJ, Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. Nature. 2012 Oct 4;490(7418):61–70.

165. Polyak K. Heterogeneity in breast cancer. J Clin Invest. 2011 Oct 3;121(10):3786–8.

166. Weigelt B, Geyer FC, Reis-Filho JS. Histological types of breast cancer: how special are they? Mol Oncol. 2010 Jun;4(3):192–208.

167. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature. 2000 Aug 17;406(6797):747–52.

168. Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, et al. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res. 2010 Oct 2;12(5):R68.

169. Waks AG, Winer EP. Breast Cancer Treatment. JAMA. 2019 Jan 22;321(3):288.

170. Baselga J, Bradbury I, Eidtmann H, Di Cosimo S, de Azambuja E, Aura C, et al. Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): a randomised, open-label, multicentre, phase 3 trial. Lancet (London, England). 2012 Feb 18;379(9816):633–40.

171. Gianni L, Pienkowski T, Im Y-H, Roman L, Tseng L-M, Liu M-C, et al. Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial. Lancet Oncol. 2012 Jan;13(1):25–32.

172. Diéras V, Miles D, Verma S, Pegram M, Welslau M, Baselga J, et al. Trastuzumab emtansine versus capecitabine plus lapatinib in patients with previously treated HER2-positive advanced breast cancer (EMILIA): a descriptive analysis of final overall survival results from a randomised, open-label, phase 3 trial. Lancet Oncol. 2017 Jun;18(6):732–42.

173. Blackwell KL, Burstein HJ, Storniolo AM, Rugo H, Sledge G, Koehler M, et al. Randomized Study of Lapatinib Alone or in Combination With Trastuzumab in Women With ErbB2-Positive, Trastuzumab-Refractory Metastatic Breast Cancer. J Clin Oncol. 2010 Mar 1;28(7):1124–30.

174. Cameron D, Casey M, Press M, Lindquist D, Pienkowski T, Romieu CG, et al. A phase III randomized comparison of lapatinib plus capecitabine versus capecitabine alone in women with advanced breast cancer that has progressed on trastuzumab: updated efficacy and biomarker analyses. Breast Cancer Res Treat. 2008 Dec 11;112(3):533–43.

175. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science. 1987 Jan 9;235(4785):177–82.

176. Hudis CA. Trastuzumab — Mechanism of Action and Use in Clinical Practice. N Engl J Med. 2007 Jul 5;357(1):39–51.

177. Pohlmann PR, Mayer IA, Mernaugh R. Resistance to Trastuzumab in Breast Cancer. Clin Cancer Res. 2009 Dec 15;15(24):7479–91.

178. Clynes RA, Towers TL, Presta LG, Ravetch J V. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. Nat Med. 2000 Apr;6(4):443–6.

179. Spiridon CI. A Comparison of the in Vitro and in Vivo Activities of IgG and F(ab')2 Fragments of a Mixture of Three Monoclonal Anti-Her-2 Antibodies. Clin Cancer Res. 2004 May 15;10(10):3542–51.

180. Park S, Jiang Z, Mortenson ED, Deng L, Radkevich-Brown O, Yang X, et al. The therapeutic effect of anti-HER2/neu antibody depends on both innate and adaptive immunity. Cancer Cell. 2010 Aug 9;18(2):160–70.

181. Stagg J, Loi S, Divisekera U, Ngiow SF, Duret H, Yagita H, et al. Anti-ErbB-2 mAb therapy requires type I and II interferons and synergizes with anti-PD-1 or anti-CD137 mAb therapy. Proc Natl Acad Sci U S A. 2011 Apr 26;108(17):7142–7.

182. Gennari R. Pilot Study of the Mechanism of Action of Preoperative Trastuzumab in Patients with Primary Operable Breast Tumors Overexpressing HER2. Clin Cancer Res. 2004 Sep 1;10(17):5650–5.

183. Arnould L, Gelly M, Penault-Llorca F, Benoit L, Bonnetain F, Migeon C, et al. Trastuzumab-based treatment of HER2-positive breast cancer: an antibody-dependent cellular cytotoxicity mechanism? Br J Cancer. 2006 Jan 10;94(2):259–67.

184. Müller P, Kreuzaler M, Khan T, Thommen DS, Martin K, Glatz K, et al. Trastuzumab emtansine (T-DM1) renders HER2+ breast cancer highly susceptible to CTLA-4/PD-1 blockade. Sci Transl Med. 2015 Nov 25;7(315):315ra188.

185. Loi S, Michiels S, Salgado R, Sirtaine N, Jose V, Fumagalli D, et al. Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial. Ann Oncol. 2014 Aug;25(8):1544–50.

186. Salgado R, Denkert C, Campbell C, Savas P, Nuciforo P, Aura C, et al. Tumor-Infiltrating Lymphocytes and Associations With Pathological Complete Response and Event-Free Survival in HER2-Positive Early-Stage Breast Cancer Treated With Lapatinib and Trastuzumab. JAMA Oncol. 2015 Jul 1;1(4):448.

187. Ingold Heppner B, Untch M, Denkert C, Pfitzner BM, Lederer B, Schmitt W, et al. Tumor-Infiltrating Lymphocytes: A Predictive and Prognostic Biomarker in Neoadjuvant-Treated HER2-Positive Breast Cancer. Clin Cancer Res. 2016 Dec 1;22(23):5747–54.

188. Solinas C, Ceppi M, Lambertini M, Scartozzi M, Buisseret L, Garaud S, et al. Tumor-infiltrating lymphocytes in patients with HER2-positive breast cancer treated with neoadjuvant chemotherapy plus trastuzumab, lapatinib or their combination: A meta-analysis of randomized controlled trials. Cancer Treat Rev. 2017 Jun;57:8–15.

189. Denkert C, von Minckwitz G, Darb-Esfahani S, Lederer B, Heppner BI, Weber KE, et al. Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. Lancet Oncol. 2018 Jan;19(1):40–50.

190. Varchetta S, Gibelli N, Oliviero B, Nardini E, Gennari R, Gatti G, et al. Elements related to heterogeneity of antibody-dependent cell cytotoxicity in patients under trastuzumab therapy for primary operable breast cancer overexpressing Her2. Cancer Res. 2007 Dec 15;67(24):11991–9.

191. Beano A, Signorino E, Evangelista A, Brusa D, Mistrangelo M, Polimeni MA, et al. Correlation between NK function and response to trastuzumab in metastatic breast cancer patients. J Transl Med. 2008 May 16;6(1):25.

192. Taylor RJ, Saloura V, Jain A, Goloubeva O, Wong S, Kronsberg S, et al. *Ex Vivo* Antibody-Dependent Cellular Cytotoxicity Inducibility Predicts Efficacy of Cetuximab. Cancer Immunol Res. 2015 May;3(5):567–74.

193. Mamessier E, Sylvain A, Thibult M-L, Houvenaeghel G, Jacquemier J, Castellano R, et al. Human breast cancer cells enhance self tolerance by promoting evasion from NK cell antitumor immunity. J Clin Invest. 2011 Sep 1;121(9):3609–22.

194. Roberti MP, Rocca YS, Amat M, Pampena MB, Loza J, Coló F, et al. IL-2- or IL-15-activated NK cells enhance Cetuximab-mediated activity against triple-negative breast cancer in xenografts and in breast cancer patients. Breast Cancer Res Treat. 2012 Dec 14;136(3):659–71.

195. Stanton SE, Adams S, Disis ML. Variation in the Incidence and Magnitude of Tumor-Infiltrating Lymphocytes in Breast Cancer Subtypes: A Systematic Review. JAMA Oncol. 2016 Oct 1;2(10):1354–60.

196. Cimino-Mathews A, Foote JB, Emens LA. Immune targeting in breast cancer. Oncology (Williston Park). 2015 May;29(5):375–85.

197. Schmid P, Adams S, Rugo HS, Schneeweiss A, Barrios CH, Iwata H, et al. Atezolizumab and Nab-Paclitaxel in Advanced Triple-Negative Breast Cancer. N Engl J Med. 2018 Nov 29;379(22):2108–21.

198. Zacharakis N, Chinnasamy H, Black M, Xu H, Lu Y-C, Zheng Z, et al. Immune recognition of somatic mutations leading to complete durable regression in metastatic breast cancer. Nat Med. 2018 Jun 4;24(6):724–30.

199. Pyatt RE, Pilarski R, Prior TW. Mutation screening in juvenile polyposis syndrome. J Mol Diagn. 2006 Feb;8(1):84–8.

200. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, et al. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 2004;5(10):R80.

201. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics. 2003 Apr 1;4(2):249–64.

202. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015 Apr 20;43(7):e47–e47.

203. Elpek KG, Cremasco V, Shen H, Harvey CJ, Wucherpfennig KW, Goldstein DR, et al. The tumor microenvironment shapes lineage, transcriptional, and functional diversity of infiltrating myeloid cells. Cancer Immunol Res. 2014 Jul 1;2(7):655–67.

204. Vanpouille-Box C, Lhuillier C, Bezu L, Aranda F, Yamazaki T, Kepp O, et al. Trial watch: Immune checkpoint blockers for cancer therapy. Oncoimmunology. 2017 Nov 2;6(11):e1373237.

205. Cabo M, Offringa R, Zitvogel L, Kroemer G, Muntasell A, Galluzzi L. Trial Watch: Immunostimulatory monoclonal antibodies for oncological indications. Oncoimmunology. 2017 Dec 2;6(12):e1371896.

206. Hamano R, Huang J, Yoshimura T, Oppenheim JJ, Chen X. TNF optimally activatives regulatory T cells by inducing TNF receptor superfamily members TNFR2, 4-1BB and OX40. Eur J Immunol. 2011 Jul;41(7):2010–20.

207. Nagar M, Jacob-Hirsch J, Vernitsky H, Berkun Y, Ben-Horin S, Amariglio N, et al. TNF activates a NF-kappaB-regulated cellular program in human CD45RA- regulatory T cells that modulates their suppressive function. J Immunol. 2010 Apr 1;184(7):3570–81.

208. Vandenabeele P, Declercq W, Beyaert R, Fiers W. Two tumour necrosis factor receptors: structure and function. Trends Cell Biol. 1995 Oct;5(10):392–9.

209. Srivastava RM, Trivedi S, Concha-Benavente F, Gibson SP, Reeder C, Ferrone S, et al. CD137 Stimulation Enhances Cetuximab-Induced Natural Killer: Dendritic Cell Priming of Antitumor T-Cell Immunity in Patients with Head and Neck Cancer. Clin Cancer Res. 2017 Feb 1;23(3):707–16.

210. Sznol M, Hodi FS, Margolin K, McDermott DF, Ernstoff MS, Kirkwood JM, et al. Phase I study of BMS-663513, a fully human anti-CD137 agonist monoclonal antibody, in patients (pts) with advanced cancer (CA). J Clin Oncol. 2008 May 20;26(15_suppl):3007–3007.

211. Simonetta F, Pradier A, Roosnek E. T-bet and Eomesodermin in NK Cell Development, Maturation, and Function. Front Immunol. 2016 Jun 20;7:241.

212. Zhang J, Marotel M, Fauteux-Daniel S, Mathieu A-L, Viel S, Marçais A, et al. T-bet and Eomes govern differentiation and function of mouse and human NK cells and ILC1. Eur J Immunol. 2018 May;48(5):738–50.

213. Kamizono S, Duncan GS, Seidel MG, Morimoto A, Hamada K, Grosveld G, et al. Nfil3/E4bp4 is required for the development and maturation of NK cells in vivo. J Exp Med. 2009 Dec 21;206(13):2977–86.

214. Smith MA, Maurin M, Cho H Il, Becknell B, Freud AG, Yu J, et al. PRDM1/Blimp-1 Controls Effector Cytokine Production in Human NK Cells. J Immunol. 2010 Nov 15;185(10):6058–67.

215. Shin H, Iwasaki A. Tissue-resident memory T cells. Immunol Rev. 2013 Sep;255(1):165–81.

216. Milner JJ, Toma C, Yu B, Zhang K, Omilusik K, Phan AT, et al. Runx3 programs CD8+ T cell residency in non-lymphoid tissues and tumours. Nature. 2017 Dec 14;552(7684):253–7.

217. Mackay LK, Minnich M, Kragten NAM, Liao Y, Nota B, Seillet C, et al. Hobit and Blimp1 instruct a universal transcriptional program of tissue residency in lymphocytes. Science. 2016 Apr 22;352(6284):459–63.

218. Inman GJ, Nicolás FJ, Callahan JF, Harling JD, Gaster LM, Reith AD, et al. SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol Pharmacol. 2002 Jul 1;62(1):65–74.

219. Wang Y, Chu J, Yi P, Dong W, Saultz J, Wang Y, et al. SMAD4 promotes TGF-β–independent NK cell homeostasis and maturation and antitumor immunity. J Clin Invest. 2018 Oct 15;128(11):5123–36.

220. Howe JR, Roth S, Ringold JC, Summers RW, Järvinen HJ, Sistonen P, et al. Mutations in the SMAD4/DPC4 gene in juvenile polyposis. Science. 1998 May 15;280(5366):1086–8.

221. Houlston R, Bevan S, Williams A, Young J, Dunlop M, Rozen P, et al. Mutations in DPC4 (SMAD4) cause juvenile polyposis syndrome, but only account for a minority of cases. Hum Mol Genet. 1998 Nov 1;7(12):1907–12.

222. Freudlsperger C, Bian Y, Contag Wise S, Burnett J, Coupar J, Yang X, et al. TGF-β and NF-κB signal pathway cross-talk is mediated through TAK1 and SMAD7 in a subset of head and neck cancers. Oncogene. 2013 Mar 28;32(12):1549–59.

223. Yamane K, Ihn H, Asano Y, Jinnin M, Tamaki K. Antagonistic effects of TNF-alpha on TGF-beta signaling through down-regulation of TGF-beta receptor type II in human dermal fibroblasts. J Immunol. 2003 Oct 1;171(7):3855–62.

224. Jabeen R, Goswami R, Awe O, Kulkarni A, Nguyen ET, Attenasio A, et al. Th9 cell development requires a BATF-regulated transcriptional network. J Clin Invest. 2013 Nov 1;123(11):4641–53.

225. Jiang Y, Chen J, Bi E, Zhao Y, Qin T, Wang Y, et al. TNF-α enhances Th9 cell differentiation and antitumor immunity via TNFR2-dependent pathways. J Immunother cancer. 2019 Feb 4;7(1):28.

226. Micossé C, von Meyenn L, Steck O, Kipfer E, Adam C, Simillion C, et al. Human "TH9" cells are a subpopulation of PPAR-γ+ TH2 cells. Sci Immunol. 2019 Jan 18;4(31):eaat5943.

227. Xiao X, Shi X, Fan Y, Zhang X, Wu M, Lan P, et al. GITR subverts Foxp3(+) Tregs to boost Th9 immunity through regulation of histone acetylation. Nat Commun. 2015 Sep 14;6:8266.

228. Xiao X, Balasubramanian S, Liu W, Chu X, Wang H, Taparowsky EJ, et al. OX40 signaling favors the induction of T(H)9 cells and airway inflammation. Nat Immunol. 2012 Oct 29;13(10):981–90.

229. Kim I-K, Kim B-S, Koh C-H, Seok J-W, Park J-S, Shin K-S, et al. Glucocorticoid-induced tumor necrosis factor receptor–related protein co-stimulation facilitates tumor regression by inducing IL-9–producing helper T cells. Nat Med. 2015 Aug 17;21(9):1010–7.

230. Romee R, Foley B, Lenvik T, Wang Y, Zhang B, Ankarlo D, et al. NK cell CD16 surface expression and function is regulated by a disintegrin and metalloprotease-17 (ADAM17). Blood. 2013 May 2;121(18):3599–608.

231. Byrd A, Hoffmann SC, Jarahian M, Momburg F, Watzl C. Expression analysis of the ligands for the Natural Killer cell receptors NKp30 and NKp44. Khoury J El, editor. PLoS One. 2007 Dec 19;2(12):e1339.

232. Mamessier E, Sylvain A, Bertucci F, Castellano R, Finetti P, Houvenaeghel G, et al. Human Breast Tumor Cells Induce Self-Tolerance Mechanisms to Avoid NKG2D-Mediated and DNAM-Mediated NK Cell Recognition. Cancer Res. 2011 Nov 1;71(21):6621–32.

233. Sanmamed MF, Rodriguez I, Schalper KA, Onate C, Azpilikueta A, Rodriguez-Ruiz ME, et al. Nivolumab and urelumab enhance antitumor activity of human T lymphocytes engrafted in Rag2-/-IL2R null immunodeficient mice. Cancer Res. 2015 Sep 1;75(17):3466–78.

234. Tolcher AW, Sznol M, Hu-Lieskovan S, Papadopoulos KP, Patnaik A, Rasco D, et al. Phase Ib Study of Utomilumab (PF-05082566), a 4-1BB/CD137 Agonist, in Combination with Pembrolizumab (MK-3475) in Patients with Advanced Solid Tumors. Clin Cancer Res. 2017 Jun 20;clincanres.1243.2017.

235. Kim J-O, Kim HW, Baek K-M, Kang C-Y. NF-κB and AP-1 regulate activation-dependent CD137 (4-1BB) expression in T cells. FEBS Lett. 2003 Apr 24;541(1–3):163–70.

236. Schwarz H, Valbracht J, Tuckwell J, von Kempis J, Lotz M. ILA, the human 4-1BB homologue, is inducible in lymphoid and other cell lineages. Blood. 1995 Feb 15;85(4):1043–52.

237. Donatelli SS, Zhou J-M, Gilvary DL, Eksioglu EA, Chen X, Cress WD, et al. TGF-β-inducible microRNA-183 silences tumor-associated natural killer cells. Proc Natl Acad Sci U S A. 2014 Mar 18;111(11):4203–8.

238. Lim SK, Hoffmann FM. Smad4 cooperates with lymphoid enhancer-binding factor 1/T cell-specific factor to increase c-myc expression in the absence of TGF-beta signaling. Proc Natl Acad Sci U S A. 2006 Dec 5;103(49):18580–5.

239. Sica A, Tan TH, Rice N, Kretzschmar M, Ghosh P, Young HA. The c-rel protooncogene product c-Rel but not NF-kappa B binds to the intronic region of the human interferon-gamma gene at a site related to an interferon-stimulable response element. Proc Natl Acad Sci U S A. 1992 Mar 1;89(5):1740–4.

240. Sica A, Dorman L, Viggiano V, Cippitelli M, Ghosh P, Rice N, et al. Interaction of NF-kappaB and NFAT with the interferon-gamma promoter. J Biol Chem. 1997 Nov 28;272(48):30412–20.

241. Huang C, Bi E, Hu Y, Deng W, Tian Z, Dong C, et al. A Novel NF-κB Binding Site Controls Human Granzyme B Gene Transcription. J Immunol. 2006 Apr 1;176(7):4173–81.

242. Duyao MP, Buckler AJ, Sonenshein GE. Interaction of an NF-kappa B-like factor with a site upstream of the c-myc promoter. Proc Natl Acad Sci U S A. 1990 Jun 1;87(12):4727–31.

243. Wickremasinghe MI, Thomas LH, O'Kane CM, Uddin J, Friedland JS. Transcriptional Mechanisms Regulating Alveolar Epithelial Cell-specific CCL5 Secretion in Pulmonary Tuberculosis. J Biol Chem. 2004 Jun 25;279(26):27199–210.

244. Ballard DW, Böhnlein E, Lowenthal JW, Wano Y, Franza BR, Greene WC. HTLV-I tax induces cellular proteins that activate the kappa B element in the IL-2 receptor alpha gene. Science. 1988 Sep 23;241(4873):1652–5.

245. Wendel M, Galani IE, Suri-Payer E, Cerwenka A. Natural Killer Cell Accumulation in Tumors Is Dependent on IFN- and CXCR3 Ligands. Cancer Res. 2008 Oct 15;68(20):8437–45.

246. Le Floc'h A, Jalil A, Vergnon I, Le Maux Chansac B, Lazar V, Bismuth G, et al. Alpha E beta 7 integrin interaction with E-cadherin promotes antitumor CTL activity by triggering lytic granule polarization and exocytosis. J Exp Med. 2007 Mar 19;204(3):559–70.

247. Frazao A, Messaoudene M, Nunez N, Dulphy N, Roussin F, Sedlik C, et al. CD16 $^{+}$ NKG2A ^{high} Natural Killer Cells Infiltrate Breast Cancer–Draining Lymph Nodes. Cancer Immunol Res. 2019 Feb;7(2):208–18.

248. Castriconi R, Dondero A, Bellora F, Moretta L, Castellano A, Locatelli F, et al. Neuroblastoma-Derived TGF-β1 Modulates the Chemokine Receptor Repertoire of Human Resting NK Cells. J Immunol. 2013 May 15;190(10):5321–8.

249. Jaime-Ramirez AC, Mundy-Bosse BL, Kondadasula S, Jones NB, Roda JM, Mani A, et al. IL-12 Enhances the Antitumor Actions of Trastuzumab via NK Cell IFN-γ Production. J Immunol. 2011 Mar 15;186(6):3401–9.

250. Mittal D, Vijayan D, Putz EM, Aguilera AR, Markey KA, Straube J, et al. Interleukin-12 from CD103+ Batf3-Dependent Dendritic Cells Required for NK-Cell Suppression of Metastasis. Cancer Immunol Res. 2017 Dec;5(12):1098–108.

251. Alexandre YO, Ghilas S, Sanchez C, Le Bon A, Crozat K, Dalod M. XCR1+ dendritic cells promote memory CD8+ T cell recall upon secondary infections with Listeria monocytogenes or certain viruses. J Exp Med. 2016 Jan 11;213(1):75–92.

252. Mailliard RB, Alber SM, Shen H, Watkins SC, Kirkwood JM, Herberman RB, et al. IL-18-induced CD83+CCR7+ NK helper cells. J Exp Med. 2005 Oct 3;202(7):941–53.

253. Kaplan MH. The transcription factor network in Th9 cells. Semin Immunopathol. 2017 Jan 11;39(1):11–20.

254. Zhu YX, Kang LY, Luo W, Li CC, Yang L, Yang YC. Multiple transcription factors are required for activation of human interleukin 9 gene in T cells. J Biol Chem. 1996 Jun 28;271(26):15815–22.

255. Végran F, Berger H, Boidot R, Mignot G, Bruchard M, Dosset M, et al. The transcription factor IRF1 dictates the IL-21-dependent anticancer functions of TH9 cells. Nat Immunol. 2014 Aug 29;15(8):758–66.

256. Zhao Y, Chu X, Chen J, Wang Y, Gao S, Jiang Y, et al. Dectin-1-activated dendritic cells trigger potent antitumour immunity through the induction of Th9 cells. Nat Commun. 2016 Nov 5;7(1):12368.

257. Lu Y, Hong S, Li H, Park J, Hong B, Wang L, et al. Th9 cells promote antitumor immune responses in vivo. J Clin Invest. 2012 Nov 1;122(11):4160–71.

258. Uyttenhove C, Simpson RJ, Van Snick J. Functional and structural characterization of P40, a mouse glycoprotein with T-cell growth factor activity. Proc Natl Acad Sci U S A. 1988 Sep 1;85(18):6934–8.

259. Vidard L, Dureuil C, Baudhuin J, Vescovi L, Durand L, Sierra V, et al. CD137 (4-1BB) Engagement Fine-Tunes Synergistic IL-15- and IL-21-Driven NK Cell Proliferation. J Immunol. 2019 Aug 1;203(3):676–85.

260. Cichocki F, Miller JS, Anderson SK, Bryceson YT. Epigenetic regulation of NK cell differentiation and effector functions. Front Immunol. 2013;4:55.

261. Lau CM, Adams NM, Geary CD, Weizman O-E, Rapp M, Pritykin Y, et al. Epigenetic control of innate and adaptive immune memory. Nat Immunol. 2018 Sep 6;19(9):963–72.

262. Qi X, Li F, Wu Y, Cheng C, Han P, Wang J, et al. Optimization of 4-1BB antibody for cancer immunotherapy by balancing agonistic strength with FcγR affinity. Nat Commun. 2019 Dec 20;10(1):2141.

263. Chin SM, Kimberlin CR, Roe-Zurz Z, Zhang P, Xu A, Liao-Chan S, et al. Structure of the 4-1BB/4-1BBL complex and distinct binding and functional properties of utomilumab and urelumab. Nat Commun. 2018 Dec 8;9(1):4679.

264. Chester C, Sanmamed MF, Wang J, Melero I. Immunotherapy targeting 4-1BB: mechanistic rationale, clinical results, and future strategies. Blood. 2017 Nov 8;131(1):blood-2017-06-741041.

265. Sanmamed MF, Etxeberría I, Otano I, Melero I. Twists and turns to translating 4-1BB cancer immunotherapy. Sci Transl Med. 2019 Jun 12;11(496):eaax4738.

266. Hinner MJ, Aiba R-SB, Wiedenmann A, Schlosser C, Allersdorfer A, Matschiner G, et al. Costimulatory T cell engagement via a novel bispecific anti-CD137 /anti-HER2 protein. J Immunother Cancer. 2015 Dec 4;3(S2):P187.

267. Claus C, Ferrara C, Xu W, Sam J, Lang S, Uhlenbrock F, et al. Tumor-targeted 4-1BB agonists for combination with T cell bispecific antibodies as off-the-shelf therapy. Sci Transl Med. 2019 Jun 12;11(496):eaav5989.

268. Compte M, Harwood SL, Muñoz IG, Navarro R, Zonca M, Perez-Chacon G, et al. A tumor-targeted trimeric 4-1BB-agonistic antibody induces potent anti-tumor immunity without systemic toxicity. Nat Commun. 2018 Dec 15;9(1):4809.

269. Yang Y, Yang S, Ye Z, Jaffar J, Zhou Y, Cutter E, et al. Tumor cells expressing anti-CD137 scFv induce a tumor-destructive environment. Cancer Res. 2007 Mar 1;67(5):2339–44.

270. Ye Z, Hellström I, Hayden-Ledbetter M, Dahlin A, Ledbetter JA, Hellström KE. Gene therapy for cancer using single-chain Fv fragments specific for 4-1BB. Nat Med. 2002 Apr;8(4):343–8.

271. Barsoumian HB, Batra L, Shrestha P, Bowen WS, Zhao H, Egilmez NK, et al. A Novel Form of 4-1BBL Prevents Cancer Development via Nonspecific Activation of CD4+ T and Natural Killer Cells. Cancer Res. 2019 Feb 15;79(4):783–94.

272. Pastor F, Kolonias D, McNamara II JO, Gilboa E. Targeting 4-1BB Costimulation to Disseminated Tumor Lesions With Bi-specific Oligonucleotide Aptamers. Mol Ther. 2011 Oct;19(10):1878–86.

273. Schrand B, Berezhnoy A, Brenneman R, Williams A, Levay A, Kong L-YY, et al. Targeting 4-1BB costimulation to the tumor stroma with bispecific aptamer conjugates enhances the therapeutic index of tumor immunotherapy. Cancer Immunol Res. 2014 Sep 1;2(9):867–77.

274. Akhmetzyanova I, Zelinskyy G, Littwitz-Salomon E, Malyshkina A, Dietze KK, Streeck H, et al. CD137 Agonist Therapy Can Reprogram Regulatory T Cells into Cytotoxic CD4+ T Cells with Antitumor Activity. J Immunol. 2015 Jan 1;196(1):484–92.

275. Zheng G, Wang B, Chen A. The 4-1BB costimulation augments the proliferation of CD4+CD25+ regulatory T cells. J Immunol. 2004 Aug 15;173(4):2428–34.

276. Kim Y-J, Stringfield TM, Chen Y, Broxmeyer HE. Modulation of cord blood CD8+ T-cell effector differentiation by TGF-beta1 and 4-1BB costimulation. Blood. 2005 Jan 1;105(1):274–81.

277. Miliotou AN, Papadopoulou LC. CAR T-cell Therapy: A New Era in Cancer Immunotherapy. Curr Pharm Biotechnol. 2018 May 31;19(1):5–18.

278. Dwivedi A, Karulkar A, Ghosh S, Rafiq A, Purwar R. Lymphocytes in Cellular Therapy: Functional Regulation of CAR T Cells. Front Immunol. 2019 Jan 18;9:3180.

279. Brentjens RJ, Davila ML, Riviere I, Park J, Wang X, Cowell LG, et al. CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. Sci Transl Med. 2013 Mar 20;5(177):177ra38.

280. Lee DW, Kochenderfer JN, Stetler-Stevenson M, Cui YK, Delbrook C, Feldman SA, et al. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. Lancet (London, England). 2015 Feb 7;385(9967):517–28.

281. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. N Engl J Med. 2014 Oct 16;371(16):1507–17.

282. Long AH, Haso WM, Shern JF, Wanhainen KM, Murgai M, Ingaramo M, et al. 4-1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors. Nat Med. 2015 Jun 4;21(6):581–90.

283. Muntasell A, Cabo M, Servitja S, Tusquets I, Martínez-García M, Rovira A, et al. Interplay between Natural Killer Cells and Anti-HER2 Antibodies: Perspectives for Breast Cancer Immunotherapy. Front Immunol. 2017 Nov 13;8:1544.

ABBREVIATIONS

ADCC	Antibody-dependent cell cytotoxicity
cDC1	Conventional dendritic cells type I
HCMV	Human cytomegalovirus
HLA	Human leukocyte antigen
IgG	Immunoglobulin G
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cells
KIR	Killer immunoglobulin-like receptor
mAb	Monoclonal antibody
NCR	Natural cytotoxicity receptors
NK	Natural killer
NKR	Natural killer receptor
O/N	Overnight
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TNFRSF	Tumor necrosis factor receptors superfamily

APPENDIX

LIST OF PUBLICATIONS

Publications included in the thesis

1. Muntasell A, Servitja S, **Cabo M**, Bermejo B, Pérez-Buira S, Rojo F, et al. High Numbers of Circulating CD57 $^{+}$ NK Cells Associate with Resistance to HER2-Specific Therapeutic Antibodies in HER2+ Primary Breast Cancer. Cancer Immunol Res. 2019 Jun 12.

Additional publications

1. Muntasell A, Ochoa MC, Cordeiro L, Berraondo P, López-Díaz de Cerio A, **Cabo M**, et al. Targeting NK-cell checkpoints for cancer immunotherapy. Curr Opin Immunol. 2017 Apr;45:73–81.

2. Muntasell A, **Cabo M**, Servitja S, Tusquets I, Martínez-García M, Rovira A, et al. Interplay between Natural Killer Cells and Anti-HER2 Antibodies: Perspectives for Breast Cancer Immunotherapy. Front Immunol. 2017 Nov 13;8.

3. **Cabo M**, Offringa R, Zitvogel L, Kroemer G, Muntasell A, Galluzzi L. Trial Watch: Immunostimulatory monoclonal antibodies for oncological indications. Oncoimmunology. 2017 Dec 2;6(12):e1371896.

4. Muntasell A, Rojo F, Servitja S, Rubio-Perez C, **Cabo M**, Tamborero D, et al. NK Cell Infiltrates and HLA Class I Expression in Primary HER2+ Breast Cancer Predict and Uncouple Pathological Response and Disease-free Survival. Clin Cancer Res. 2019 Mar 1;25(5):1535–45.