Charles University in Prague

Faculty of Sciences

Immunology

Bc. Veronika Švubová

Inhibitory NK cell receptors and possibilities of manipulation of cytotoxic properties

Inhibiční receptory NK buněk a možnosti manipulace cytotoxických vlastností

Master´s thesis

Supervised by Mgr. Jan Frič, Ph.D.

Consulted with Mgr. Tereza Feglarová, Ph.D.

Prague, 2023

I hereby declare that my thesis represents my own original research work. Wherever the contribution of others is involved, every effort is made to indicate this clearly including reference to the literature. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma.

Prague, 17.4. 2023 Bc. Veronika Švubová

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Contents

Abstract

Acute myeloid leukemia makes up for 18 % of all leukemias among pediatric and young adult patients. The complete remission rate (80-90 %) and the overall survival (70 %) of the patients is relatively high, nevertheless, the relapse rate is still almost at 50 % and the prognosis remains extremely bad. The relapse treatment is rather challenging because the persisting leukemic clones might in fact start to be refractory to chemotherapy. Lately, NK cells are being perceived as an attractive therapeutical tool for treatment of the relapses. NK cells are a subpopulation of innate lymphoid cells, possessing the ability to eliminate dysfunctional cells through cytotoxic activities and further perpetuate the immune response. One of the advantages of NK cells is their functional independency of specific antigens. In the light of growing evidence about the role of leukemic stem cells in context of acute myeloid leukemia, NK cells seem to offer a new perspective in therapeutical efforts to eliminate them via several cytotoxic mechanisms. Yet despite optimistic preliminary results, treating this disease has proved to be rather challenging and the NK cell-based immunotherapy is still facing several limitations. Transforming growth factor β is partially responsible for maintenance of leukemic stem cell populations and impairment of NK cell-mediated immune response. The overall negative effect of transforming growth factor β on NK cells was confirmed by functional cytotoxic assays, as well as analysis of expression. In time-dependent manner, transforming growth factor β exposure led to decrease of cytotoxic potential of NK cells and caused dysregulation in expression of several key molecules, such as activating receptors, metabolic regulators and other molecules related to NK cell migration. The effects of long-term transforming growth factor β-induced suppression were reversed by inhibitors, suggesting that administration of the right medication following the adoptive transfer could protect the NK cells in the patient.

Key words: acute myeloid leukemia, AML, bone marrow, NK cell, suppressive microenvironment, transforming growth factor β, TGF-β, cytotoxicity

Abstrakt

Akutní myeloidní leukémie tvoří 18 % všech leukémií u dětských a mladých dospělých pacientů. V první vlně léčby remise dosahuje přibližně 80-90 % pacientů a přežití se blíží 70 %, nicméně k relapsům dochází až v 50 % případů a prognóza těchto pacientů je extrémně špatná. Léčba relapsu je pak velmi náročná, protože přetrvávající leukemické klony mohou být zcela rezistentní k chemoterapii. V poslední době začaly být jako atraktivní terapeutický nástroj pro léčbu relapsů vnímány NK buňky. NK buňky jsou jednou ze subpopulací lymfoidních buněk, které mají schopnost eliminovat dysfunkční buňky prostřednictvím cytotoxických aktivit a následně dále indukovat imunitní odpověď. Jednou z výhod NK buněk je jejich funkční nezávislost na specifických antigenech. Ve světle rostoucích důkazů o úloze leukemických kmenových buněk v kontextu akutní myeloidní leukémie se zdá, že NK buňky nabízejí novou perspektivu v terapeutickém úsilí o jejich eliminaci prostřednictvím několika cytotoxických mechanismů. I přes optimistické předběžné výsledky se však léčba tohoto onemocnění ukázala jako poměrně náročná a imunoterapie založená na NK buňkách stále naráží na několik omezení. Transformující růstový faktor β je částečně zodpovědný za udržování populací leukemických kmenových buněk a narušení imunitní odpovědi zprostředkované NK buňkami. Celkový negativní účinek transformačního růstového faktoru β na NK buňky byl potvrzen funkčními cytotoxickými testy a také analýzou exprese. Vystavení NK buněk tomuto cytokinu vedla ke snížení jejich cytotoxického potenciálu a způsobila dysregulaci exprese několika klíčových molekul, jako jsou aktivační receptory, metabolické regulátory a další molekuly související s migrací NK buněk. Účinky dlouhodobé suprese vyvolané transformujícím růstovým faktorem β byly zvráceny inhibitory, což naznačuje, že volba správné medikace po adoptivním transferu by mohla NK buňky před těmito efekty ochránit.

Klíčová slova: akutní myeloidní leukémie, AML, kostní dřeň, NK buňka, supresivní mikroprostředí, transformující růstový faktor β, TGF-β, cytotoxicita

Abbreviations

ADCC	*antibody-dependent cell cytotoxicity*
AML	*acute myeloid leukemia*
BM	*bone marrow*
BMSC	*bone marrow stem cell*
CAF	*cancer associated fibroblast*
CAR	*chimeric antigen receptor*
DNAM-1	*DNAX accessory molecule*
ECM	*extracellular matrix*
EDTA	*ethylenediaminetetraacetic acid*
ERK	*extracellular signal-regulated kinase*
ESAM	*endothelial cell-selective adhesion molecule*
Fas	*FS-7 associated surface antigen*
FcγR	*Fc fragment of IgG low affinity receptor*
GARP	*Glycoprotein A repetitions predominant protein*
GvHD	*graft versus host disease*
Gzm	*granzyme*
HSC	*hematopoietic stem cell*
(allo-HSCT)	*allogeneic HSC transplantation*
ICAM	*intracellular adhesive molecule*
IFN	*interferon*
IL	*interleukin*
irK562	*irradiated K562 cells*
JAK	*Janus kinase*
JNK	*c-Jun N-terminal kinase*
LAP	*latency-associated protein*
LSC	*leukemic stem cell*
LTBP	*latent TGF-β-binding protein*
MSC	*mesenchymal stromal cell*
mTORc1	*mammalian target of rapamycin complex 1*
NCR	*natural cytotoxicity triggering receptor*
NKG2D/2A	*natural killer group 2D/2A receptor*
PBMC	*peripheral blood mononuclear cell*
PBS	*phosphate-buffered saline*
Prf	*perforin*
Ser/Thr	*serine/threonine*
Smad	*(small) mother against decapentaplegic*
SREBP	*sterol regulatory element binding protein*
T-bet	*T-box expressed in T cells*
TGF-β	*transforming growth factor β*
TGFBR	*TGF-β receptor*
TME	*tumor microenvironment*
TNFα	*tumor necrosis factor α*
TRAIL	*tumor-related apoptosis inducing ligand*

1. Introduction

1.1. Acute myeloid leukemia

Acute myeloid leukemia (AML) is a hemato-oncological malignancy defined by accumulation of immature, functionally impaired myeloblasts in the bone marrow (BM), from which they can gradually spread to peripheral blood, secondary lymphoid organs and more (Long et al., 2022; Luciano et al., 2022). Even though AML shares many aspects with chronic myeloid leukemia; such as increased blast frequency, aggressiveness and overall poor prognosis; the outcomes of therapeutical efforts vastly differ between these two. Whereas chronic myeloid leukemia patients reach 5-year relative survival in over 90 % of cases and the therapeutical approaches are in a constant progress, the 5-year overall survival of AML patients stagnates well below 30 % and novel therapies are lacking. One of the reasons might be the molecular basis leading to AML (Vetrie et al., 2020). As has been published before, occurrence of AML is often caused by single mutation, but there are several known mutations leading to AML. As such, AML should be perceived as rather heterogeneous group of diseases with over 24 genetic variants being taken into account. However, a common element has been proposed – the leukemic stem cells (LSCs) (Long et al., 2022; Vetrie et al., 2020). The real challenge in efforts to cure AML therefore lies in targeting LSCs. Usually, these would be defined by presence or lack of common hematopoietic markers as CD34+CD38- cells. AM-LSCs however, can be found in many variants different from this typical stem-like phenotype. This is caused by the fact that AML can in fact arise from hematopoietic stem cells (HSCs) the same way as from more differentiated mature cells that were able to once more acquire the self-renewing properties. It has been shown before, using immunocompromised mice, that primary cells from AML patients do poses rather high repopulating capacity, even in case of serial engraftments, suggesting the presence of highly competent, self-renewing stem-like cell populations (Vetrie et al., 2020). Furthermore, AML has been shown to be capable of remodelling the BM niche to make it favorable for its survival and progression. This phenomenon is dependent on direct cell-to-cell interaction as well as release of modulatory mediators. Among the most important mediators of AML-induced modulation of BM niche there are growth factors (Kaweme & Zhou, 2021). In line with findings about the role of transforming growth factor β (TGF-β) in immune surveillance evasion (Lamb et al., 2021), maintenance of LSC population (Shingai et al., 2021) and general establishment of protective leukemic niche (Long et al., 2022) we decided to choose TGF-β to be the main focus of this project.

1.1.1. Cytokine environment of AML

The immunosuppressive environment of AML does affect development and functionality of both immunocytes and non-immune cells (Kaweme & Zhou, 2021). The cytokine environment of AML is poorly described however. Most of the published data concerning cytokine levels in patients come from the peripheral blood or serum. As for the most straight-forward approaches, we could expect that cytokine

levels can be assessed in the supernatants of cell cultures, as shown by Cao et al. (2010) with multiple myeloma samples. However, cultivation of primary leukemic blasts, let alone efforts using them to simulate the leukemic BM niche conditions in vitro, is rather challenging. The blasts are generally highly dependent on the leukemic environment and do not survive in culture long enough to establish the *in vivo* situation-resembling conditions. One of the ways to describe the leukemic niche is to measure protein levels in BM plasma samples though (Kováč et al., 2014). Lately, RNA-Seq was also used to uncover the cytokine-related signalling in BM and further utilized to help assess the prognosis of patients (Bolouri et al., 2022). Alternative approach to assess production of cytokines by leukemic cells in the BM microenvironment is to observe cytokine profiles in leukemic cell line cultures *in vitro*, yet the knowledge gained from these experiments is limited and does not necessarily reflect the situation *in vivo*. Due to methodological progress of past years, we are actually able to measure and compare cytokine profiles in peripheral blood and BM plasma of leukemia patients. As seen in study by Chen et al. (2021), the measured levels only seem linearly consistent in case of few cytokines, suggesting that in future research the complex knowledge of BM niche dynamics cannot be acquired solely through analysis of peripheral blood. Levels of TGF-β were shown to be generally decreased in AML patients, however, as for other above mentioned cytokines, this data was mainly obtained through peripheral blood serum samples analysis (Luciano et al., 2022; Motta & Rumjanek, 2021; Sanchez-Correa et al., 2013). Therefore, we see a potential space for re-evaluation here, as we propose that TGF-β might actually play an important role in lowering efficiency of immunotherapeutic approaches in AML treatment.

1.1.2. Advantages of NK cell-based AML therapy

Use of poly-specific antibodies or check-point inhibitors, as well as targeting the CD33 or CD123, provide somewhat optimistic numbers of complete remission rates and median survival on its own or in combination with conventional chemotherapy. These numbers, however, still remain rather low thus leaving room for research and development of other immunotherapeutic approaches (Tabata et al., 2021). Despite preliminary success in overall cancer treatment, the chimeric antigen receptor T cell (CAR-T) therapy did not prove to be as efficient in context of AML either. Not only does administration of CAR-T cells bear risk of severe toxic effects; such as graft versus host disease (GvHD) and cytokine release syndrome; it is crucially limited by antigen escape as well (Sterner & Sterner, 2021). In comparison, the main advantage of NK cell-based therapy is the potential to become the ideal „off-the-shelf" solution for many patients at once. Adoptive transfer of NK cells bears little to no risk of cytokine storm or GvHD. NK cells are effective in killing target cells with no need for antigen presentation and their potential could be generally further increased by co-administration with antibodies leading to induction of antibody-dependent cell cytotoxicity (ADCC) (Lamb et al., 2021). NK cells utilize several mechanisms to eliminate infected and/or transformed cells. They secrete functional molecules such as perforin, granulysine and

two types of granzyme contained in their cytotoxic granules. They are able to induce apoptosis in these cells via signalling through tumor-related apoptosis inducing ligand (TRAIL) and FS-7 associated surface antigen (Fas) ligand or via secretion of tumor necrosis factor α (TNFα). Beside their direct cytotoxic function, they further perpetuate attraction and activation of both innate and adaptive effector cell types, either by production of cytokines and chemokines or direct contact (Kaweme & Zhou, 2021). That being said, NK cells present potentially highly effective solution for treatment of relapsed AML patients. In general, failure of therapy leads to succession of more aggressive stress-selected leukemic clones, which are more likely to be refractory to conventional chemotherapy, along with LSCs.

1.1.3. Limitations of NK cell-based AML therapy

Importantly enough, the NK cell repertoire is being modulated during AML progression, leading to impairment of its functionality. As established, NK cells can be divided into two main subgroups. The mature CD56dim CD16+ CD57+ population, mainly circulating in peripheral blood, exhibits enhanced cytotoxic properties (including the ability to perform ADCC via CD16 – also known as Fc receptor III FcγRIII) and higher production of granzymes (Gzm), perforins (Prf) or interferon γ (IFNγ). The second population residing in secondary lymphoid organs, defined as immature CD56bright CD16- CD57-, is mainly responsible for cytokine production and therefore it is perceived as the regulatory fraction of NK cells. In AML patients, however, the process of NK cell maturation is disturbed. Both hypomature and hypermature NK cell populations have been observed in different stages of the disease with advert effects on prognosis and survival. Generally, NK cells of AML patients do express lower levels of molecules related to their activation, such as natural killer group 2D receptor (NKG2D), DNAX accessory molecule 1 (DNAM-1) and natural cytotoxicity triggering receptors (NCR1 – NKp30 and NCR3 – NKp46), while the expression of inhibitory killer-cell immunoglobulin-like (KIR) and natural killer group 2A (NKG2A) receptors is increased (Kaweme & Zhou, 2021).

1.2. Cells of the leukemic bone marrow niche

Tumor microenvironment (TME) has been shown as an obstacle before in many therapeutical efforts in context of solid tumor. Definition of TME in hematological malignancies is somewhat difficult however. In leukemic disorders, the TME overlaps with the BM compartment and that brings up several difficulties in efforts to describe and observe the actual state of the leukemic niche. Leukemic TME does not only consist of leukemic cells. It is rather complex system of many cell types affecting one another via various pathways. BM niche of leukemia patients is a very specific microenvironment. It has to accommodate the hematopoiesis, yet at the same time it poses as the hotspot of leukemic proliferation. Leukemic cells modulate activity of neighbouring cell types to support the malignant processes and even shelter remaining blasts leading to incomplete remission after therapy as illustrated by **figure 1** (Reikvam et al., 2015).

1.2.1. Leukemic stem cells

In the past years, research of the common leukemic progenitor gained attention of many, leading to establishment of the concept of LSCs. It was believed that LSCs arise directly from HSCs. As has been shown, however, the leukemic proliferation might take place on the level of multipotent progenitor cells as well, based on the HSC-restricted CD90 expression (Majeti et al., 2007). LSCs are certainly an important topic for the AML therapy research, because they do interact with BM stroma and even in small numbers compete with normal hematopoiesis. These cells use a broad spectrum of signalling factors and adhesion molecules to reshape the BM niche in their advantage (Long et al., 2022). Negative effects of exceeding proliferative potential of LSCs, such as exhaustion, is being compensated by switch to slow cycling or reversible quiescence. Taken into account that chemotherapy can induce DNA damage and increased the reactive oxygen species burden in prevailing AML cells, dealing with relapsed AML poses a real challenge. Therapy-driven selective pressure can lead to acquirement of new mutations and accelerated diversification of more resistant relapse clones (Vetrie et al., 2020). Interestingly enough, according to Shingai et al. (2021), AML (represented by KG1a cell line) maintains the heterogeneity of its LSCs with auto/paracrine production of TGF-β. Despite low levels of TGF-β being produced by these cells, blockage of TGF-β signalling did lead to decrease in phenotypical variability and induction of apoptosis in leukemic cells (KG1a) in a time-dependent manner. In experiments with primary AML cells from patients, the effects were diverse but a similar trend was observed. This supports therapeutical approach involving targeting of the TGF-β pathway as a way of dealing with poor-prognosis relapse situations and prevailing chemoresistant LSC populations.

1.2.2. Non-leukemic cell types

We believe that the overall protective effects of BM-TME on AML cells are partially mediated by mesenchymal stromal cells (MSCs) beside other cell types in the niche. As summarized by Raffaghello et al. (2015), MSCs seem to be involved in formation of protective niche shielding the leukemic cells and LSCs in the BM. Variable genetic instability was shown to be present upon MSCs in different types of hematological malignancies. BM-MSCs from both acute lymphoid and myeloid leukemia patients do promote survival, growth and chemoresistance of leukemic cells, and are therefore closely connected to development and progression of the diseases with heterogenous effects (Raffaghello et al., 2015). Co-cultivation of AML cell lines (HL-60, OCI-AML3, HEK293T) as well as primary patient blasts with MSCs leads to cell growth and acquirement of stem-like phenotype related to increased activity of aldehyde dehydrogenase in the leukemic cells. It has been proposed that the co-culture induces activation of TGF-β-dependent genes through the non-canonical pathway in AML cells. Inhibition of the aldehyde dehydrogenase activity then makes the AML cells more susceptible to chemotherapy. Therefore the endurance of AML cells and maintenance of LSCs in the BM niche might both be mediated by TGF-β (Yuan et al., 2020).

Beside establishment of the leukemic niche mentioned above, MSCs are also potential precursors of cancer associated fibroblasts (CAFs). CAFs do generally secrete high levels of collagens, elastins or fibronectin, but most importantly, they have been previously described as the main producers of TGF-β-1 and -3 in solid tumors. In this context, TGF-β plays a crucial role as tumor progression promoter by inducing the epithelial-to-mesenchymal transition and overall immune evasion (Batlle & Massagué, 2019; Huynh et al., 2019). As shown by Pan et al. (2020) using the primary B cell AML cells in a co-culture, the leukemic microenvironment also drives the conversion of bone marrow stem cells (BMSCs) into CAFs. Interestingly enough, this shift is proposed to be mediated via TGF-β-dependent mechanism (Pan et al., 2020). In line with the information above, Yoshida (2020) previously proposed the concept of self-perpetuating loop of CAF generation in breast cancer, where TGF-β secreted by CAFs already present in TME helps maintain the existing population and induces conversion of more cells into this cell type in autocrine and paracrine manner.

To elaborate on what was already said, TGF-β is produced by several cell types seen in the BM niche, such as BMSCs, myeloid-derived suppressor cells and stromal cells, as well as some leukemic cells lines. As an immunosuppressive cytokine, unsurprisingly, it induces overall quiescence of cells in the BM. Yet under increased selective pressure, tumor cells are capable of decoupling components of the effector cascade and therefore, as in the case of some AML variants, these cells develop resistance to effects of TGF-β. Taken into account that increased TGF-β serum levels were observed in other hematological malignancy patients before, we propose that TGF-β might in fact be an important immunomodulatory factor in the context of leukemic BM niche (Batlle & Massagué, 2019; Kaweme & Zhou, 2021; Otegbeye et al., 2018).

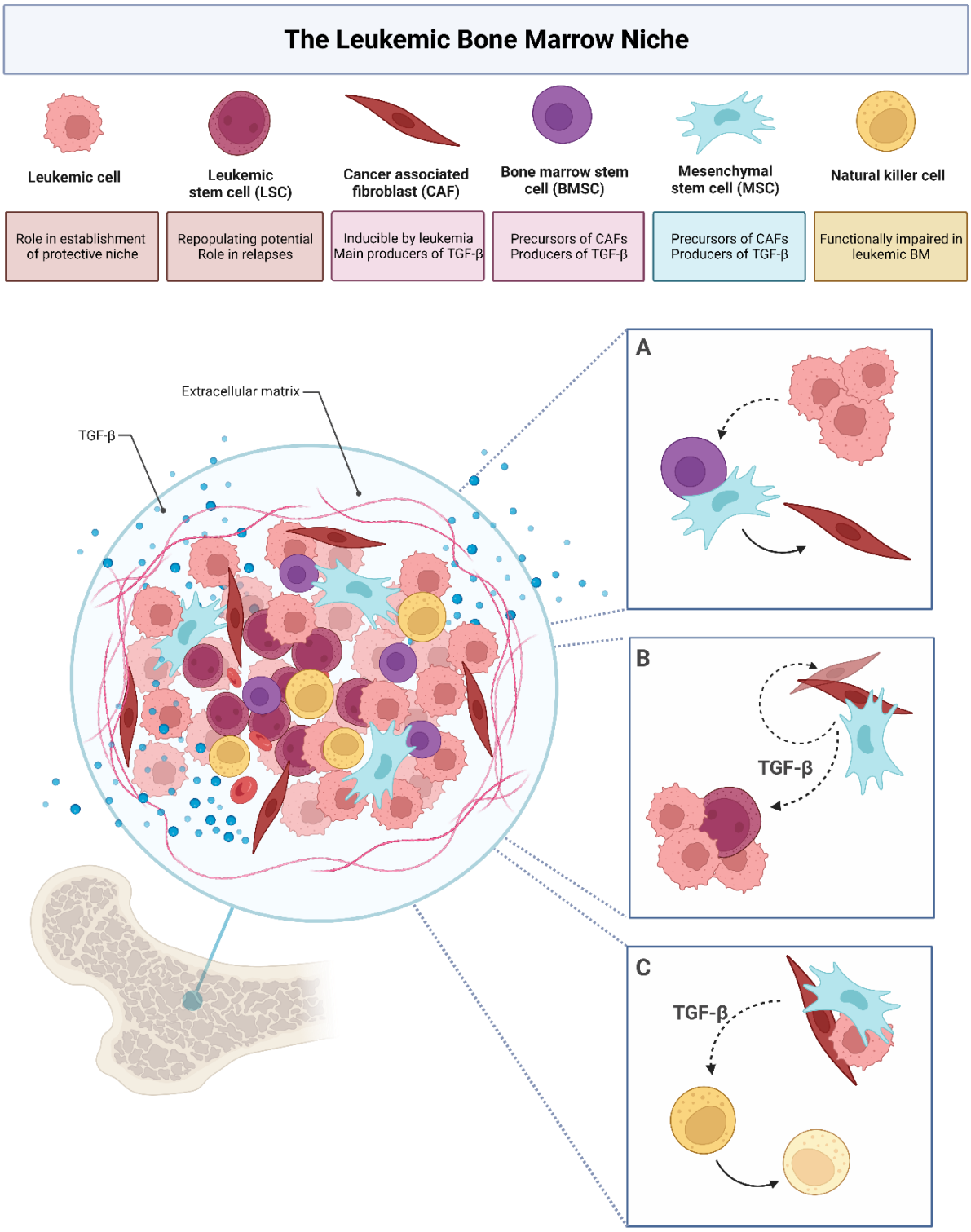


Figure 1 - Dynamics and cell interactions within bone marrow niche of AML patients
Leukemic cells are capable of reshaping of the bone marrow niche via secretion of modulatory mediators as well as direct cell-to-cell interactions. (A) In presence of leukemic cells, bone marrow stem cells (BMSCs) and mesenchymal cells (MSCs) acquire the phenotype of cancer associated fibroblasts (CAFs). (B) Both MSCs and CAFs have been described as potent producers of TGF-β. Conversely, development of CAFs was also shown to be supported by TGF-β. In context of hematological malignancies, TGF-β was associated with maintenance of leukemic stem cell (LSC) populations. (C) We propose that therapeutically administered NK cells are challenged by presence of TGF-β after entering the leukemic niche.

1.3. Transforming growth factor β

In normal conditions, TGF-β is an antiproliferative factor, blocking progression through cell cycle and pushing cells into differentiation (Dong & Blobe, 2006). It also plays a critical role in the dynamic homeostasis of BM niche, having specific effects on commitment to distinct hematopoietic lineages, generally promoting the myeloid direction (Bataller et al., 2019). In solid tumors, TGF-β is believed to be one of the most effective promoters of metastasis (Xie et al., 2018; Yoshida, 2020). In context of hemato-oncological malignancies, TGF-β has been shown to mediate impaired hematopoiesis, leukemogenesis and immune evasion. Non-standard production of TGF-β has lately been observed in acute lymphoid leukemia and lymphomas (Lamb et al., 2021) and major dysregulation of TGF-β-related signalling has been described in both T-cell and B-cell leukemia in the past (Lagneaux et al., 1998; Niitsu et al., 1988). Available publications regarding role of TGF-β in AML are often contradictory though. According to Binder et al. (2018), TGF-β levels were found to be decreased in AML patients. It has also been stated that TGF-β impairs proliferation of AML blasts in 40 % of cases, as it decreases the self-renewal abilities of blast populations. On the other hand, Ma et al. (2019) points out that, based on Cancer cell line encyclopaedia, mRNA levels for TGF-β-1 and the Glycoprotein A repetitions predominant protein (GARP) are increased in AML cell lines in comparison to different types of malignancies. Overall, it is right to say that effects of TGF-β are highly context-dependent and can be both pro- or anti-proliferative. It is interesting to consider that AML patients might have imbalanced T-cell subset ratios in peripheral blood with possible accumulation of Tregs leading to enhanced suppression via interleukin 10 (IL-10) and TGF-β. Study by Sun et al. (2014) focused on the T-cell dysregulation in this context, deals with comparison of newly diagnosed patients with those in complete remission and controls. TGF-β was found to be generally lowered in newly diagnosed patients, however the levels were high in patients with acute monocytic leukemia, making up to 37,5 % among the cohort. As already stated, effects of TGF-β can be evaded in some leukemia subtypes. Due to various mutations, leukemic cells are able to acquire several mechanisms by which they do so. Among these, there is blockage of multimerization of proteins of the mother against decapentaplegic family (Smad2/3) by fusion proteins, mutation of Smad4 gene and dysregulation of TGF-β receptors (TGFBR I and II) expression (Binder et al., 2018). Altered expression of TGFBRII isotypes observed in AML was directly correlated with clinical prognosis of patients (Wu et al., 2017).

1.3.1. Activation and action of TGF-β

The whole process of TGF-β production, secretion and utilization is quite complex (**figure 2**). TGF-β is being expressed in three isoforms as 112 amino acid chain of 25 kDa (Y. Chen et al., 2019). The cytokine itself only forms the C-terminal portion of the primary product, as it is synthetised in coupling with latency-associated protein (LAP) on the N-terminal portion. In the endoplasmic reticulum, newly

translated polypeptide is linked by disulphide bonds between TGF-β fragments and LAPs. Furin, an endoprotease, is needed for cleavage and release of active TGF-β. TGF-β is then secreted, still as a dimeric protein associated with conjugated LAPs in the form of small latent complex. Via interaction of LAPs with latent TGF-β binding proteins (LTBPs), large latent complex can be formed and as such the TGF-β can be stored in extracellular matrix (ECM). Several mechanisms of TGF-β activation have been proposed, including interaction with thrombospondin 1, plasmin, cathepsin D, metalloproteases, and others. The most immunologically relevant mechanism for TGF-β1 and β3 activation is mediated through integrins. Expression of these molecules is restricted to epithelial cells in inflammatory conditions as an immuno-regulatory process. Namely, integrins αvβ6 and αvβ8 have been proven to play a crucial role in TGF-β activation. Secreted TGF-β molecules are held together by non-covalent association with the LAP homodimer. This bond can be mechanically disrupted by integrins binding to specific sequences of LAPs. As the latent complex interacts with the integrin ectodomains interlinked with their respective cytoplasmic domains, the cytoskeleton allows for actin-myosin contraction and causes cleavage of the complex (Travis & Sheppard, n.d.). Interestingly enough, granzyme B was also shown to contribute to release of ECM-bound TGF-β by cleavage of peptidoglycans, such as decorin, or TGFBR III. NK cells could therefore be perpetuating their own suppression via secretion of this protease (Boivin et al., 2012).

Beside LTBPs, GARP is another protein utilized in TGF-β activation. It is a transmembrane docking receptor capable of binding latent TGF-β to the cell surface, playing role in extracellular deposition and cleavage – affecting overall availability of the cytokine. This protein also mediates secretion of TGF-β in Tregs and platelets. Increased production of GARP was observed in cancer cells, leading to lowered TGF-β pool, allowing for more aggressive course of the disease. Its expression was also positively correlated with production of TGF-β1 in AML cell lines (MV4-aa and AML193) (Ma et al., 2019; Metelli et al., 2018)

There are three TGF-β receptors (TGFBR I-III). TGFBR I and II are transmembrane serine/threonine (Ser/Thr) kinases with N-terminal hydrophilic ligand-binding domain and C-terminal kinase domain. TGFBR I is recruited at the site of TGF-β binding to TGFBR II. Subsequent signalling is then mediated through canonical Smad pathway or via non-canonical kinases (extracellular signal-regulated kinase – ERK, p38, c-Jun N-terminal kinase – JNK, Janus kinase – JAK) as seen in **figure 3**. TGFBR II contains the preferential binding site for TGF-β and beside the Ser/Thr kinase activity it is also a tyrosine kinase (also allowing for non-canonical downstream signalling). Independently of ligand, it is constitutively active and it is capable of phosphorylating TGFBR I as well as itself and other receptors. TGFBR III (or betaglycan) is has no kinase activity, yet it is the most abundant TGF-β receptor. It is expressed as a membrane-bound coreceptor molecule, but the ectodomain can be cleaved and released as a soluble receptor. Due to its high affinity for TGF-β, the soluble form is a crucial factor affecting availability of TGF-β (Vander Ark et al., 2018).

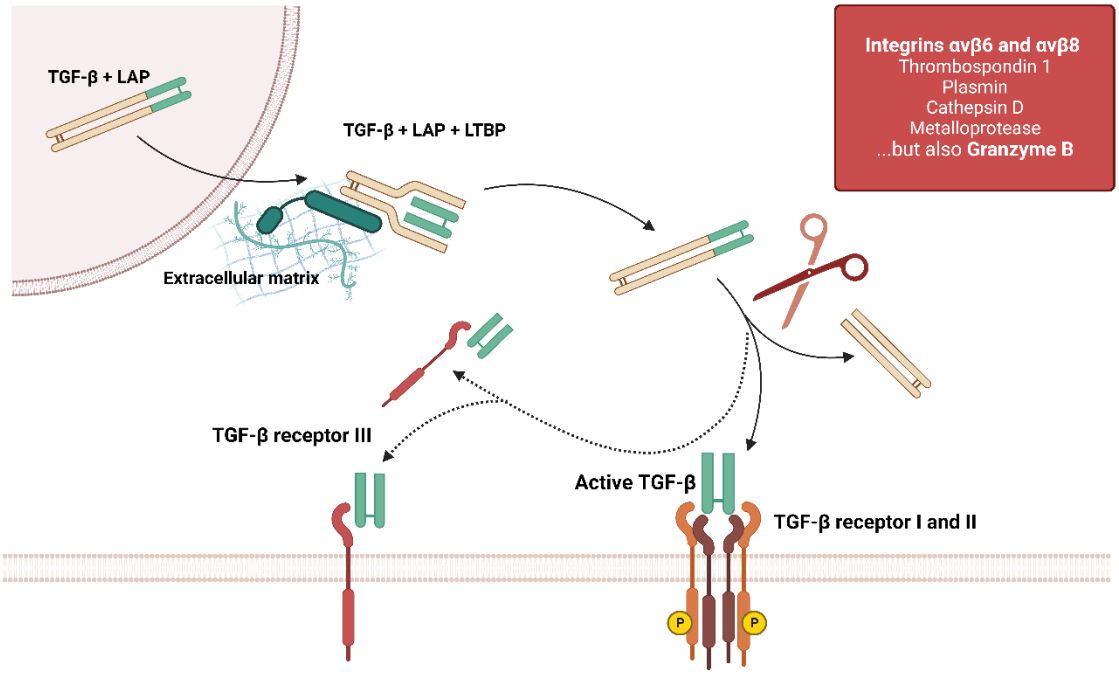


Figure 2 - Different stages and activation of TGF-β
TGF-β is being expressed in a form of small latent complex. Coupled with latency associated protein (LAP), it is secreted and usually associates with extracellular matrix via interactions with latent TGF-β-binding proteins (LTBPs). Latent TGF-β can also be deposited in the extracellular space or as a membrane-bound particle due to association with TGF-β receptor III. Activation of this cytokine requires cleavage mediated by integrins, plasmin or other proteases. Interestingly, TGF-β can also be cleaved by granzyme B.

1.3.2. Effects of TGF-β in context of NK cell-based therapy

Most deaths of AML patients are related to failure of therapy and relapse after the HSC transplantation (HSCT). In these relapses, dysfunction of NK cells was observed. According to Wang et al. (2022), functional impairment of BM-derived NK cells is correlated with high levels of active TGF-β1 in BM of early relapse AML patients compared to not-relapsed patients. TGF-β strongly regulates expression and thus affects the overall epigenetic setting of NK cells as well as their direct function (**figure 3**). NK cell metabolism, closely related to effector function of these immunocytes, is affected by TGF-β on several levels. It was proposed that TGF-β causes suppression of mammalian target of rapamycin complex 1 (mTORC1) activity, dysregulates sterol regulatory element binding protein (SREBP), c-Myc and T-box expressed in T cells (T-bet). As such, TGF-β affects the process of oxidative phosphorylation in mitochondria, promotes enhanced proliferation and impairs the overall cytotoxic properties (Regis et al., 2020). Rossi et al. (2020) even states that the TGF-β-mediated metabolic reprogramming within TME ultimately leads to transformation of said NK cells into type 1 innate lymphoid cells. The functional suppression of NK cells was shown to be mainly mediated through the Smad cascade. Overall, TGF-β-affected NK cells adopt less aggressive phenotype with downregulated activating receptors **NKG2D** (receptor for inducible surface markers), **NKp30** and **NKp46** (activatory receptors for non-MHC molecules), intracellular adhesive molecule 1 (**ICAM-1**), **DNAM-1** or **TRAIL**, and increased expression of

NKG2A. NK cell chemotaxis is also impaired by TGF-β, as it negatively regulates expression of **CXCR3**, **CXCR4** and **CX3CR1** chemokine receptors. Furthermore, TGF-β causes decrease in expression of key adaptor protein **DAP12**, **IFNγ** and **CD16**. On top of that, TGF-β was shown to downregulate important NK cell ligand **CD48** in target cells. These NK cells also differ in expression of molecules involved in the TGF-β signalling cascade compared with NK cells from healthy donors (Huang et al., 2019; Lamb et al., 2021; Regis et al., 2020).

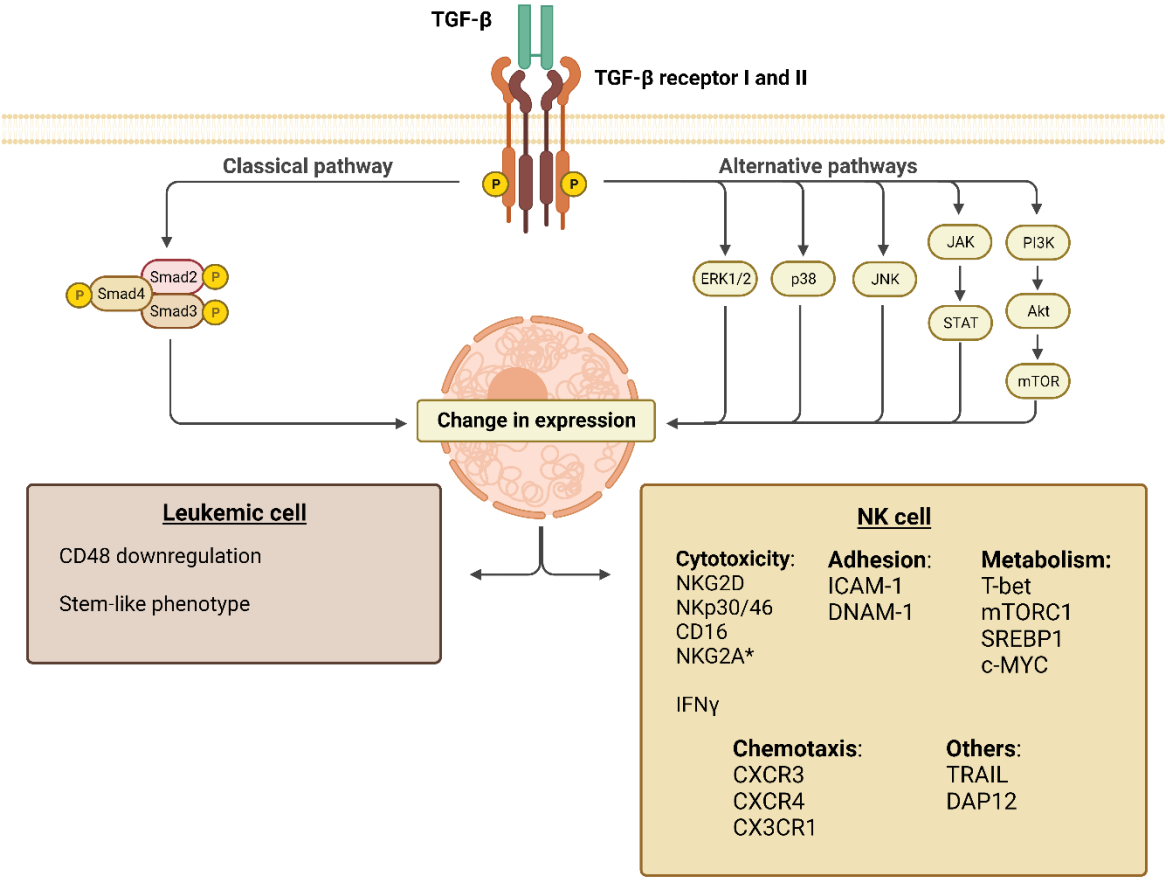


Figure 3 – Downstream TGF-β-induced signalling and its effects on NK cells and leukemic cells
Effects of TGF-β are mediated via two surface receptors. Both of them are transmembrane serine/threonine kinases. TGF-β binds to TGFBR II and TGFBR I is recruited. Then the cascade continues either in canonical pathway, employing group of Smad proteins, or through the alternative pathways, employing several MAP kinases. TGF-β has been shown to support immune evasion in leukemic cells via downregulation of CD48. Furthermore, a role in maintenance of stemness of LSCs was also proposed. In NK cells, TGF-β was shown to alter expression of key molecules related to cytotoxic properties, adhesion, metabolism, chemotaxis and intracellular signalling.

1.3.3. Reversibility of TGF-β-related effects

There are several approaches used in order to counteract TGF-β-related effects. To name a few, mRNA-targeting antisense molecules, anti-TGF-β antibodies, soluble TGF-β receptors or kinase inhibitors are already available or currently being developed (Y. Chen et al., 2019). In context of therapies dealing with leukemias, blockage of TGF-β-mediated effects by inhibitors has been previously shown to increase the efficiency of several treatment approaches – in example tyrosine kinase inhibitors in chronic myeloid

leukemia (Shingai et al., 2021). Similarly, the apoptosis-inducing effects of cytarabine are enhanced by inhibition of TGF-β signalling in AML cell lines (MV4.11, U937 and THP-1) in normoxic as well as hypoxic conditions (Tabe et al., 2013). Generally, most of the TGF-β-antagonizing inhibitors are small molecules targeting the adenosine triphosphate-binding domain of TGF-β receptor kinases (Huynh et al., 2019). Many of these were developed in past 20 years. One of the most utilised TGFBR inhibitors is **galunisertib**, already tested for solid tumor treatment in phase 2 clinical trials (Huynh et al., 2019). It is specifically targeted at abrogation of Smad2 phosphorylation within the canonical TGF-β signalling pathway. Its advantages are often explored in combination with checkpoint inhibitors, such as nivolumab or durvalumab, and it was previously shown to positively affect the anti-tumor activity in mouse model of leukemia xenograft (Holmgaard et al., 2018; Wang et al., 2022). Similarly, **LY2109761** also blocks the phosphorylation of Smad2 and, furthermore, it was previously shown to successfully inhibit proliferation of malignant cells (namely in an in vivo model of hepatocellular carcinoma) (He et al., 2018). Another inhibitor used to reverse effects of TGF-β is **SB431542**, acting as an adenosine tryphosphate-binding competitor to TGFBR I. Beside ameliorating the suppression of cytotoxic potential in effector cells, this inhibitor was shown to induce maturation of dendritic cells and to enhance the overall anti-tumor activity (Tanaka, 2010). In context of solid tumors, the efficiency of TGFBR inhibitors has been evaluated many times before, in context of hematological malignancies not as much. Yet there are few publications interested in this topic. In a study performed by Otegbeye et al. (2018), LY2157299 inhibitor was shown to preserve the cytotoxic phenotype of NK cells in a model of AML along with a mouse model of colon cancer. Interestingly enough, homoharringtonine, drug already used in AML treatment, also targets a component of TGF-β cascade, as it blocks phosphorylation of Smad3. Therefore, beside inhibiting the progression of AML itself, its use also seems beneficial in regard to enhancement of the anti-leukemic activity of cytotoxic effector cells otherwise suppressed by TGF-β (J. Chen et al., 2017).

1.4. Aims

The main challenge in the AML therapy field is rather high occurrence rate of relapses and poor prognosis of survival in relapsed patients. So far, the efforts to treat these relapsed patients have not been very successful. Based on recent publications, the problem might lie within the suppressive environment of leukemic BM niche and, subsequently, the presence of LSCs. Theory behind this thesis is that the evasion of immune system-mediated elimination and overall resistance of AML cells is partially mediated by production of immunomodulatory factors by leukemic blasts and other cell types affected by the presence of LSCs. The common factor connecting these issues together might be TGF-β. Both the establishment of the leukemic niche and maintenance of LSC population seem to be mediated through TGF-β-dependent mechanisms. Utilizing cell biology as well as molecular biology methods, this project intends to assess whether TGF-β has any significant impact on therapeutically administered NK cells in the context of AML.

First part of the project is focused on describing what does the short-term and long-term exposure of NK cells to TGF-β cause on expressional level and how does it affect the overall performance of NK cells in terms of cytotoxicity.

Second part is then focused on reversing the TGF-β-mediated suppression by inhibitors blocking the TGF-β receptors and further signalling, and assessing whether these inhibitors affect the functionality of NK cells in any way.

In the end, this project should support the efforts to improve the quality of therapeutically administered NK cells and help to better understand the dynamics within the leukemic niche.

2. Methods

2. Cell cultures

2.1.1. NK cell isolation

Complete peripheral blood mononuclear cells (PBMCs) are isolated from blood buffy coat donated by healthy individuals. Content of blood bag (30-50 ml) is transferred directly into 200ml flask and diluted to total of 120 ml with phosphate-buffered saline with ethylenediaminetetraacetic acid (PBS-EDTA) (Lonza; BE02-017F/12). Diluted blood (30 ml per falcon tube) is then carefully layered on top of 15 ml of Ficoll (Scintilla; 7861) in 50ml falcon tube and centrifuged for 30 minutes (800 g, 20 °C, acceleration 5, deceleration 0). Layer of PBMCs is then transferred into a new 50ml falcon tube, diluted to total of 50 ml with PBS-EDTA and centrifuged for 10 minutes (300 g, 20 °C, acc. max, dec. max). Pelleted cells are further resuspended and washed with PBS-EDTA twice by centrifugation (20 min, 200 g, acceleration 9, deceleration 9). PBMCs are then resuspended in PBS and pooled for counting by Automated Cell Counter TC20 (BioRad) using trypan blue dye (Trypan Blue Solution 20x, Merck Life Science; T8154-20ML).

NK cells are acquired as the untouched population via automatized magnetic separation using the Human NK Cell Isolation Kit (Miltenyi Biotec, 130-092-657). First, the PBMCs are resuspended in buffer (10^7 cells/40 µl), labelled with cocktail of biotin-conjugated antibodies (10^7 cells/10 µl) and incubated for 5 minutes in 2-8 °C. Then buffer (10^7 cells/30 µl) and magnetic beads (10^7 cells/20 µl) are added. NK cells are then separated using the AutoMACS (Miltenyi Biotec) after 10-minute incubation in 2 °C. The negative population of NK cells is then centrifuged, resuspended in complete X-VIVO 20 media (EastPort, BE04-448Q/12) and seeded.

2.1.2. NK cell culture

Freshly isolated NK cells were standardly seeded into 6-well plates combined with irradiated K562 (irK562) cells as feeders in 1:5 ratio. The seeding concentration used was 10^5 NK cells and $5x10^5$ K562 cells per 1 ml of media in total of 2 ml per well. The standard expansion took 14 days. X-VIVO 20 (EastPort, BE04-448Q/12) medium supplemented with gentamicin (Thermofisher; 15710064) and 5 % human serum (EastPort, HUM-3B) is used for the whole cultivation. Cytokines are administered at the start of the expansion and with every passage – 5 µl of IL-2 (200 UI/µl, RnD; 202-IL-500) and 20 µl of IL-15 (1 ng/µl, RnD; 247-ILB-025) per 1 ml of media. Cells were passaged as shown in **table 1**.

Table 1 - Cytokine administration schedule

No.	Media taken [ml]	Media added [ml]	Cytokines added in media	Vol. [ml]
1	-	-	IL-2 + IL-15 per total volume	2
2	-	1	IL-2 + IL-15 per 1 ml	3
3	1	2	IL-2 + IL-15 per 2 ml	4
4	2	2	IL-2 + IL-15 per 2 ml	4
5	2	3	IL-2 + IL-15 per 3 ml	5

2.1.3. Treatment with TGF-β and inhibitors

NK cells were treated with TGF-β, inhibitors or both according to passaging regime. Treatment of NK cells with TGF-β was performed using the Recombinant Human TGF-beta1 (BioTech; 7754-BH-025/CF) diluted to working concentration of 10 ng/µl. All NK cell cultures were treated with 2.5 µl of aliquoted TGF-β per 1 ml of media (Huang et al., 2019; Otegbeye et al., 2018). For all assays, inhibitors were added as follows: 1µl of Galunisertib (5 mM, **Gal**, Holmgaard et al., 2018, SigmaAldrich; SML2851-5MG), 0.25 µl of LY2109761 (20 mM, **LY**, He et al., 2018, SigmaAldrich; SML2051-5MG) and 1.25 µl of SB431542 (4 mM, **SB**, Tanaka, 2010, SigmaAldrich; S4317-5MG) per 1 ml of media in culture. Cells were kept in 24-well plates.

2.1.4. Leukemic cell cultures

Primary blast cells were acquired in form of cryostored aliquots through clinical department of Institute of Hematology and Blood Transfusion. Leukemic cell lines (listed in **table 2**) were bought from American Type Culture Collection. RPMI 1640 medium (Thermofisher; A1049101) was used for leukemic cell mono-cultures as well as co-cultures with MSCs.

Table 2 – Leukemic cell line overview

Cell line	Type	Cell line	Type
K562	*Chronic erythroid leukemia*	**MOLM13**	*Acute monocytic leukemia*
THP-1	*Acute monocytic leukemia*	**U937**	*Pro-monocytic leukemia*

2.2. Molecular biology methods

2.2.1. RNA isolation

RNA was isolated from minimum of $5x10^7$ NK cells using the RNeasy Plus Mini Kit (50) (BioTech; 74104). Cells were centrifuged (300 g, 5 min, RT) in 1.5ml Eppendorf tubes. Pellets were resuspended in RLT buffer (350 µl) and stored at -80 °C or processed immediately. For purification, the gDNA Eliminator Spin Columns were used. Flow-through supernatant was then processed according to the manufacturer´s instructions. Before elution, RNeasy MinElute Spin Columns were further centrifuged in a new collection tube for 2 additional minutes with the lid open to get rid of the excess buffer. RNA was then eluted with 20-40 µl of RNase free water (Carolina; 10977015) and measured using NanoDrop One (ThermoFisher).

2.2.2. Reverse transcription

Reverse transcription to cDNA was performed using 500 ng of RNA in 0.2 ml Eppendorf tubes. Template RNA samples were diluted to maximal total volume of 11 µl per reaction. In the first step, 1 µl of 50 µM Oligo d(T) (Fisher Scientific; 18418020), 1 µl of 10 mM dNTP mix (Fisher Scientific; 10297018) and RNase-free water was added to the total volume of 13 µl per reaction. RNA-primer mixes were then heated at 65 °C for 5 minutes on a Thermal Cycler (Biometra) and then placed onto a frozen cooler rack. In the second step, 4 µl of 5x SSIV Buffer (Fisher Scientific), 1 µl of 100 mM DTT (Fisher Scientific), 1 µl of

RNase OUT Recombinant RNase Inhibitor (ThermoFisher; 10777019) and 1 µl of SuperScript IV Reverse Transcriptase (200 U/µl, Fisher Scientific; 18090200) were added per reaction. The mix was incubated at 55 °C for 10 minutes and then heated at 80 °C for another 10 minutes. Acquired samples were stored at -20 °C or used for qPCR immediately.

2.2.3. qPCR

The quantitative polymerase chain reaction was performed using the 384-well plates (Roche). For every gene, premix was made in 1.5 ml Eppendorf tubes using 2.5 µl of PoweUp SYBR Green Master Mix (2x) (Fisher Scientific; A25918) and 0.25 µl of forward and reverse primer (10 µM, listed in **table 3**) per reaction. Samples of cDNA were diluted to concentration 4.5 ng/µl in total of 2 µl per reaction. The qPCR itself was then performed in LightCycler480 System (Roche). The plate was first incubated at 95 °C for 5 minutes. Subsequently 40 cycles of polymerisation were performer at 95–60–72 °C in 10 second intervals. Plates were then left to cool down to 40 °C. Acquired data were processed via delta-deltaCT method: Fold change in expression is calculated using the $2^{-\Delta\Delta Ct}$ formula, where ΔΔCt = (ΔCt-sample – ΔCt-control), in which ΔCt = (Ct-gene of interest – Ct-housekeeping gene). Visualization was done in GraphPad Prism9.

Table 3 - List of primers used for qPCR

GENE	SEQUENCE (FORWARD/REVERSE)	MANUFACTURER
GAPDH	*5´CTC CTC CTG TTC GAC AGT CA-* *5´CCC AAT ACG ACC AAA TCC GTT G-*	KRD
TGFBRI	*5´CAC AGA GTG GGA ACA AAA GG T-* *5´CCA ATG GAA CAT CGT CGA GCA-*	KRD
TGFBRII	*5´AAG ATG ACC GCT CTG ACA TCA-* *5´CTT ATA GAC CTC AGC AAA GCG AC-*	KRD
IFNG (Interferon γ)	*5´TCG GTA ACT GAC TTG AAT GTC CA-* *5´TCG CTT CCC TGT TTT AGC TGC-*	KRD
TNFA (TNFα)	*5´ATG TTG TAG CAA ACC CTC AAG C-* *5´TGA TGG CAG AGA GGA GGT TG-*	KRD
GZMB (Granzyme B)	*5´CCC TGG GAA AAC ACT CAC ACA-* *5´GCA CAA CTC AAT GGT ACT GTC G-*	KRD
PRF1 (Perforin 1)	*5´GAC TGC CTG ACT GTC GAG G-* *5´TCC CGG TAG GTT TGG TGG AA-*	KRD
SMAD3	*5´GTC GCA TGA CGC AAG ACC T-* *5´GAG CTG ACA CCT GAT AGG GG-*	KRD
KIR3DL	*5´GGT TCT GTT ACT CAC ACC C-* *5´TTT CTC ATA TGG ACC TGT GAC-*	KRD
NCR1 (NKp30)	*5´CCC CTG AGA TTC GTA CCC TG-* *5´CTC CAC TCT GCA CAC GTA GAT-*	KRD
NCR2 (NKp44)	*5´CCT CTC GAT TCA CAA TCT GGG-* *5´AGT TGT CAG AAG GGC GGT AGA-*	KRD
NCR3 (NKp46)	*5´CCA CCG AGG GAC ATA CCG AT-* *5´GTG CAA GGC TGG TGT TCT CA-*	KRD
NKG2D	*5´GAT GGG ACT AGT ACA CAT TCC-* *5´ATT GTT AGT AGG TTG GGT GAG-*	KRD
TBX21 (T-bet)	*5´GGT TGC GGA GAC ATG CTG-* *5´GTA GGC GTA GGC TCC AAG G-*	KRD
CRTC (mTORc1)	*5´GCC ATG CTT AGT GAC ACC GA-* *5´GGA CTG GAG GTA GTA CCG C-*	KRD

2.2.4. Library preparation and RNA sequencing

All total RNA samples were first put through ribodepletion using the RiboCop V1.3 rRNA depletion kit (TATAA Biocenter; 144.24). All steps were performed according to manufacturer´s instructions. First, the rRNA contained in samples was hybridized with Probe Mix via temperature-induced denaturalization (5 min, 75 °C) and subsequent renaturalization (30 min, 60 °C). Depletion beads were then prepared through two washing steps using the Depletion solution and magnetic rack. Afterwards, the hybridized samples were incubated with the beads (15 min, 60 °C) and rRNA was separated using the magnetic rack. The rRNA-free supernatants were recovered and RNA was further purified using the Purification beads. Bead-bound RNA was washed two times before being eluted with Elution buffer. Purified RNA was then further processed for sequencing using the Ultra II Directional RNA Library kit (BioTech, E7760S). All steps were performed according to manufacturer´s instructions. The RNA was fragmented and primed using the NEBNext First strand Synthesis Reaction Buffer and Random Primers via incubation at 94 °C for 15 minutes. First cDNA strand was synthetised using the NEBNext Strand Specifity Reagent and NEBNext First Strand Synthesis Enzyme Mix. The mixture was incubated at 25 °C (10 min), 42 °C (15 min) and 70 °C (15 min). The samples were then left to cool down at 4 °C and kept on ice. The second cDNA strand was synthetised via 1 hour-long incubation at 16 °C using the NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix, NEBNext Second Strand Synthesis Enzyme Mix and nuclease-free water. Double stranded cDNA was then purified via NEBNext Sample Purification Beads and magnetic rack. Freshly purified cDNA was eluted with the 0.1X TE Buffer. Samples were immediately processed for library preparation using the NEBNext Ultra II End Prep Reaction Buffer and Enzyme Mix via incubation at 20 °C (30 min) and at 65 °C (30 min). NEBNExt adaptors were mixed with Adaptor Dilution Buffer and added to samples mixed with NEBNExt Ligation Enhancer and NEBNext Ultra II Ligation Master Mix. The mixture was then incubated at 20 °C for 15 minutes before USER Enzyme was added and the samples were incubated once again at 37 °C for 15 minutes. Purification was again performed with the NEBNext Sample Purification Beads. Samples eluted with 0.1X TE were then send to different facility for the sequencing and subsequent data analysis was performed. Raw reads in form of FASTQ files were trimmed and filtered using trimmomatic 0.39 and their quality was assessed using FastQC 0.11.8. Filtered reads were then mapped to human genome GRCh38.p13 using STAR 2.7.2b. Post-mapping quality of alignments was verified using RNA-SeQC 2.3.5 software. Quantification of gene expression was then performed by StringTie2 software 1.3.6. For statistical analysis and visualization of expression data, several packages in R software 4.0.2 (e.g. edgeR 3.30.3, ggplot 3.3.2, pcaMethods 1.84.0, M3C 1.14.0) were used. Subsequently, data resulting from differential expression analysis (DEA) were used in Gene Ontology database to perform functional enrichment analysis.

2.3. Cytotoxic assay

K562 target cells were stained using Calce in AM cell-permanent dye (Invitrogen; 1CH2-002113). The dye was diluted according to manufacturer´s instructions. Cells were left to incubate in dark at 37 °C for 30 minutes. Target cells were then washed two times with complete media and left to rest for another 30 minutes. In the meantime, effector NK cells were serially diluted into 96-well round-bottom plate in triplicates to total amount of $45x10^5$, $15x10^5$, $5x10^5$ and $1,6x10^5$ cells per well. Target cells were then added and left in the assay at 37 °C for 3 hours. In the control wells, complete media was used as a blank, target cells without the addition of effectors were used as a spontaneous cell death control and for the maximal control target cells were heat killed on a thermo block at 70 °C for 10 minutes. After 3 hours 60 µl of supernatant was transferred into a new 96-well flat-bottom plate by multichannel pipette and the fluorescence was measured using Spark reader (Tecan, excitation 488 nm, emission 520 nm). Data were processed via excel and GraphPad Prism9.

2.4. Other methods

2.4.1. ELISA

Detection of TGF-β in cell culture supernatants was done in 96-well plates using the Human TGF-beta 1 DuoSet ELISA kit (BioTech; DY240) according to manufacturer´s instructions. Samples were kept in -20 °C prior to being processed without dilution. Day prior to assay, plates were coated with Capture Antibody (at room temperature as all subsequent steps). The next day the plates were washed with Wash buffer (0.05% Tween in PBS), blocked with Blocking buffer (5% Tween in PBS) for an hour and washed again right before sample processing. Loaded with samples and standard dilution series, plates were incubated for 2 hours, then washed and left to incubate with Detection Antibody for another 2 hours. After repeating the washing steps, Streptavidin-HRP B was added and placed in dark for 20 minutes. Once washed for the last time, the plates were left to incubate with Substrate Solution until the standard wells showed observable change in colour. Reaction was then quickly stopped by addition of Stop Solution. Optical density was then measured on Spark reader (Tecan, set to 450 nm).

2.4.2. Flow cytometry

To validate purity freshly isolated and/or expanded NK cell samples were processed on spectral flow cytometry system Aurora (Cytek) using the antibody panel described in **table 4**. TGF-β production in co-cultures of K562 or primary AML blasts with MSCs was then assessed using the panel in **table 5**.

Table 4 - Antibody panel for flow cytometry I

MARKER	ANTIBODY	MANUFACTURER	CATALOG NUMBER
LIVE/DEAD	*Blue*	Invitrogen	L34962
CD3	*SB 550*	BioLegend	BZ-344852
CD14	*AF 594*	BioLegend	325630
CD16	*BV 650*	Biomedica	740639
CD19	*BV 750*	BD	746867
CD45	*AF 647*	Exbio	304018
CD56	*APC-fire 750*	BioLegend	BZ-362554

Table 5 - Antibody panel for flow cytometry II

MARKER	ANTIBODY	MANUFACTURER	CATALOG NUMBER
LIVE/DEAD	*Blue*	Invitrogen	L34962
TGF-β	*PerCP-Cy5.5*	BD	562423
CD73	*BV 605*	BioLegend	344024
CD105	*PE-Cy7*	BioLegend	25-1057-42

For surface marker staining, the cells were transferred in polystyrene tubes and centrifuged (5 min, 600 g, RT, acceleration 9, deceleration 9). Pellets were washed in PBS twice to get rid of serum from media. PBS was drained and premix of antibodies in flow cytometry staining buffer (PBS, 10% FBS and 0.1% NaN_5 in distilled water) was then added. Cells were briefly vortexed and left to incubate in dark for 25 minutes. When needed, stained cells were fixed with IC Fixation buffer (BD; 00-8222-49) for 20 minutes. Prior to measuring, cells were washed and resuspended in Cell Wash (BD; 349524). Data were processed in FlowJo.

For intracellular staining, each sample was washed in FACS buffer and incubated with 100 µl of IC Fixation Buffer for 20 minutes. Cells were then washed with 1 ml of Permeabilization Buffer (BD) two times before being incubated in solution of 3% bovine serum albumin (Promega; R3961) in PBS for 15 minutes. Once washed in Permeabilization Buffer, the cells were left to get stained with intracellular antibodies for 1 hour. Last two washing steps were performed with Permeabilization Buffer and Cell Wash (respectively). Cells were then resuspended in Cell Wash and measured.

All schemes were created in BioRender.

3. Results

3.1. NK cell expansion purity evaluation and subset ratio assessment

Purity of NK cells after isolation and after expansion was evaluated via flow cytometry. Average purity was 99 % on the day of isolation and 92 % on day 14 of *in vitro* cell cultures. Typical profile of freshly isolated NK cells is depicted in **figure 4b** (complete gating strategy for NK cell purity evaluation provided in **supplement – figure S1**). The most represented subpopulation of NK cells is the CD56dim CD16+ fraction, which has been associated mainly with cytotoxic response towards cells destined to be eliminated. The CD56bright CD16- subpopulation lacks the CD16 surface receptor taking part in ADCC and therefore these cells are mainly perceived as regulatory. In the course of 14-day expansion, the percentage of CD56bright NK cells in the culture increases from 1-5 % to 20-30 %. To enhance the expansion of NK cells, irK562 cells were utilized as feeders. Following the initial NK cell purity assessment after isolation, the NK cells were seeded with the feeders in a 1:5 ratio. In order to understand the role of TGF-β in expansion and polarization of NK cells we administered with 2.5 ng/ml of TGF-β to the expansion cultures. Administration of TGF-β started at different time points throughout the 14-day expansion (illustrated by **figure 4a**). The changes in cell subset composition were analysed in time-dependent manner. Cells receiving TGF-β since the start of the cell culture (D-14d) exhibited significantly higher portion of the regulatory CD56bright CD16- cells compared to untreated NK cells (**figures 4c and 4e**). In order to confirm the presence of TGF-β in NK cell cultures on day 9 of the expansion, ELISA was performed (**figure 4d**; details in **supplement – figure S3**). For the validation, the NK cells were set in three conditions. Control cells (Ctrl) only received irK562 cells as feeders on the start of the expansion and no TGF-β was administered. TGF-β-treated NK cells (TGFb) were also stimulated with irK562 in the beginning and then received TGF-β on days of passages. The irK562-restimulated NK cells (K562) were initially stimulated with feeder cells as well and later they were re-administered with feeders on day 7 of the expansion. Therefore, the NK cell culture supernatants were taken on the 9th day from all three conditions to be compared. As shown in **figure 4d** we have observed TGF-β production in control cell cultures. Presence of TGF-β in supernatants of control cells is most likely caused by the initial co-cultivation with TGF-β-producing irK562 feeders which are able to persist for 6-8 days. Similarly, cultures enriched with irK562 on day 7 also contained detectable levels of TGF-β – up to 400 pg/ml. Measurements of TGF-β-treated NK cell cultures confirmed that recombinant TGF-β is still present in high concentrations in the cultures two days after the last administration, ensuring long-term exposure of NK cells to this cytokine.

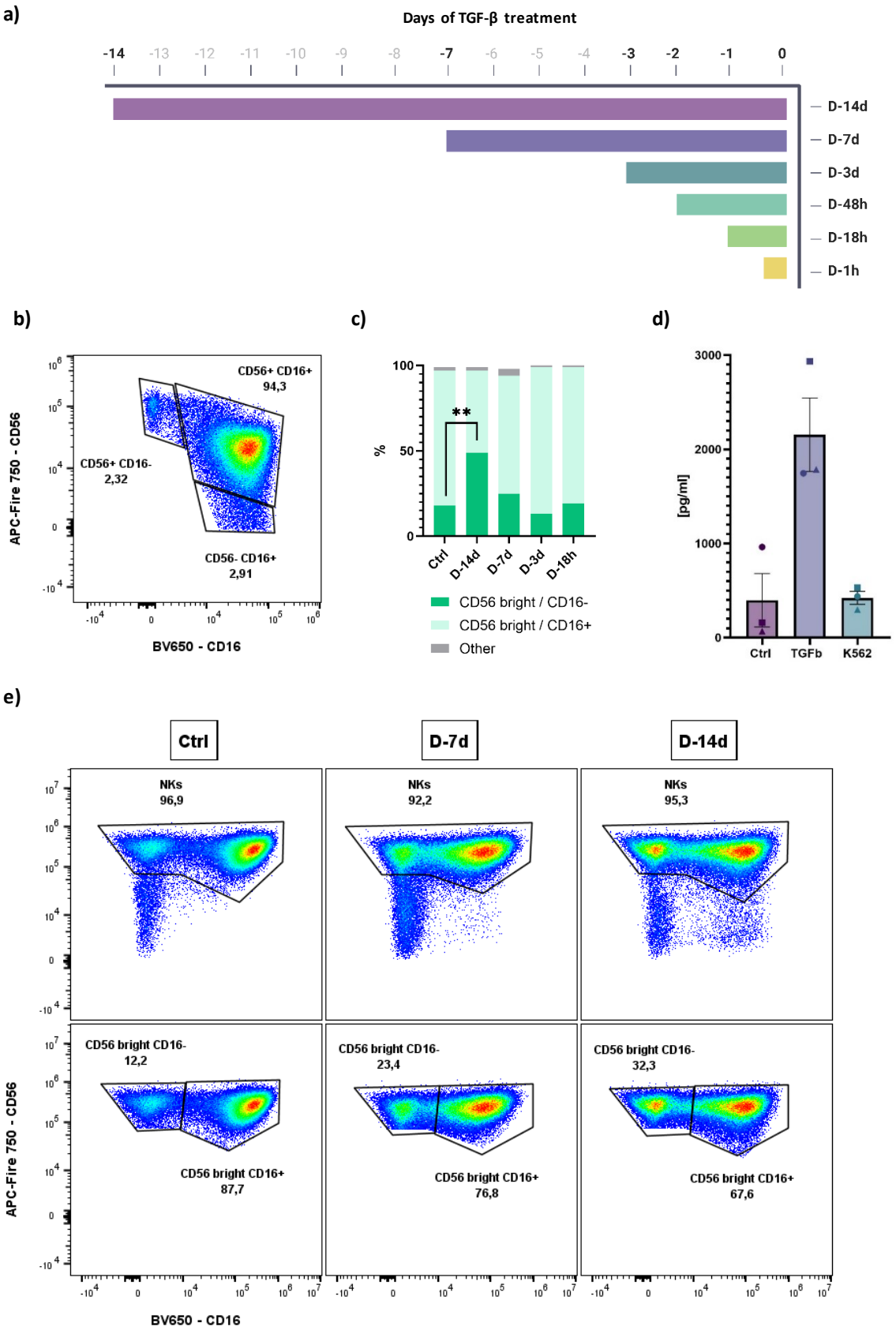

a)
Days of TGF-β treatment
-14 -13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0
D-14d
D-7d
D-3d
D-48h
D-18h
D-1h
b)
CD56+ CD16+
94,3
CD56+ CD16-
2,32
CD56- CD16+
2,91
APC-Fire 750 - CD56
BV650 - CD16
c)
**
%
Ctrl
D-14d
D-7d
D-3d
D-18h
CD56 bright / CD16-
CD56 bright / CD16+
Other
d)
[pg/ml]
Ctrl
TGFb
K562
e)
Ctrl
D-7d
D-14d
NKs
96,9
NKs
92,2
NKs
95,3
CD56 bright CD16-
12,2
CD56 bright CD16+
87,7
CD56 bright CD16-
23,4
CD56 bright CD16+
76,8
CD56 bright CD16-
32,3
CD56 bright CD16+
67,6
APC-Fire 750 - CD56
BV650 - CD16

Figure 4 – Exposure to TGF-β favors expansion of regulatory CD16- NK cells
*All NK cells used in experiments were expanded for 14 days in total (a) TGF-β administration scheme: NK cells were cultured with TGF-β for n days prior to day of experiments D (D-nd). In following results, the individual conditions are illustrated by color-coding shown above. (b) Representative cytometric profile of freshly isolated NK cells. (c) Shift in NK cell subset ratios induced by exposure to TGF-β. Subset ratios were assessed on day D via flow cytometry. Average percentages of CD56bright CD16- and CD56bright CD16+ cells in all NK cells are shown for individual conditions. 14-day long treatment with TGF-β causes significant shift towards the CD16- phenoytpe. (n=6). (d) Direct detection of TGF-β1 in NK cell culture supernatants confirmed presence of the cytokine on 9th of the expansion. Measured TGF-β concentrations correspond with administered doses and levels produced by K562 feeder cells. (n=3) (e) Representative cytometric profiles of expanded NK cells acquired on day D after 14-day cultivation. We show the TGF-β-induced shift in NK cell subset ratios for individual conditions already described in figure C. Significance was proved by paired t-test (ns P > 0.05; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001)*

3.2. TGF-β production in leukemic cell lines

In order to assess the production of TGF-β in hemato-oncologic disorders we decided to first analyse its production in leukemic cell lines. To analyse the production in selected cell lines (MOLM13, K562, THP-1 and U937) we chose to use qPCR (**figure 5a**), to assess the mRNA expression, and later validated our results with ELISA (**figure 5b**; details in **supplement - figure S4**), directly detecting TGF-β in cell culture supernatants. Results were acquired from untreated cells as well as cells stimulated with phytohemagglutinin (PHA). Expression of TGF-β1 mRNA was shown to be rather variable among the used cell lines and further detection of TGF-β1 by ELISA confirmed this observation. Both methods also showed that K562 cell line produces the highest levels of TGF-β independently of stimulation**.**

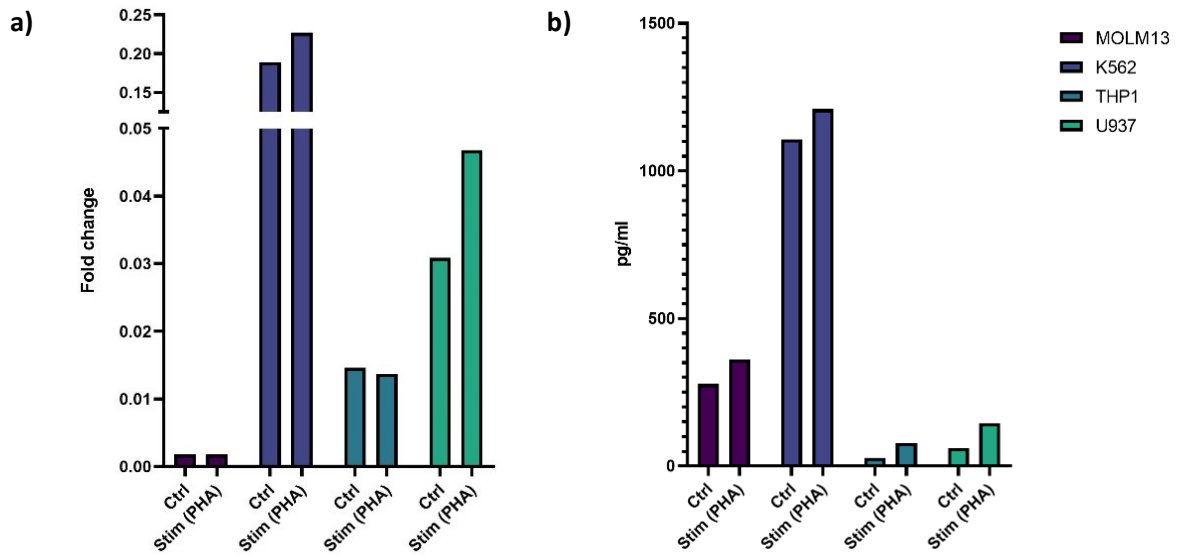


Figure 5 – Leukemic cells lines exhibit variable expression of TGF-β
(a) TGF-β1 mRNA expression was assessed in leukemic cell lines via qPCR. GAPDH was used as a house-keeping gene for delta-deltaCT analysis. Expression in leukemic cells was compared to PBMCs. (b) Direct TGF-β1 detection in cell line culture supernatants was performed using ELISA. Both methods revealed variable production of TGF-β in used cell lines and showed that K562 cells are most potent producers among the group. (n=1)

3.3. TGF-β content in co-cultures of leukemic cells with MSCs

Later we used a simplified BM niche model to illustrate how the presence of leukemic cells affects other sources of TGF-β. We assessed the cytokine production in co-cultures of leukemic cells (K562 and primary patient´s blasts) with MSCs. Preliminary ELISA results of experiment with blasts (**figure 6a**; details in **supplement – figure S5**) showed that the primary leukemic cells acquired from a patient did produce detectable levels of TGF-β. Control mono-culture of MSCs then contained even higher concentration of this cytokine. On the contrary to literature, the 24-hour co-cultivation did not induce significant increase of TGF-β production in MSCs.

To validate our results, the experiment was repeated using the K562 cells in a 48-hour co-cultivation. To distinguish the sources of TGF-β, the production was analysed via intracellular staining of the cytokine. As shown in **figure 6b**, MSCs produce substantial levels of TGF-β and the production is further enhanced in presence of K562 cells. (Complete gating strategy for this experiment provided in **supplement – figure S2**)

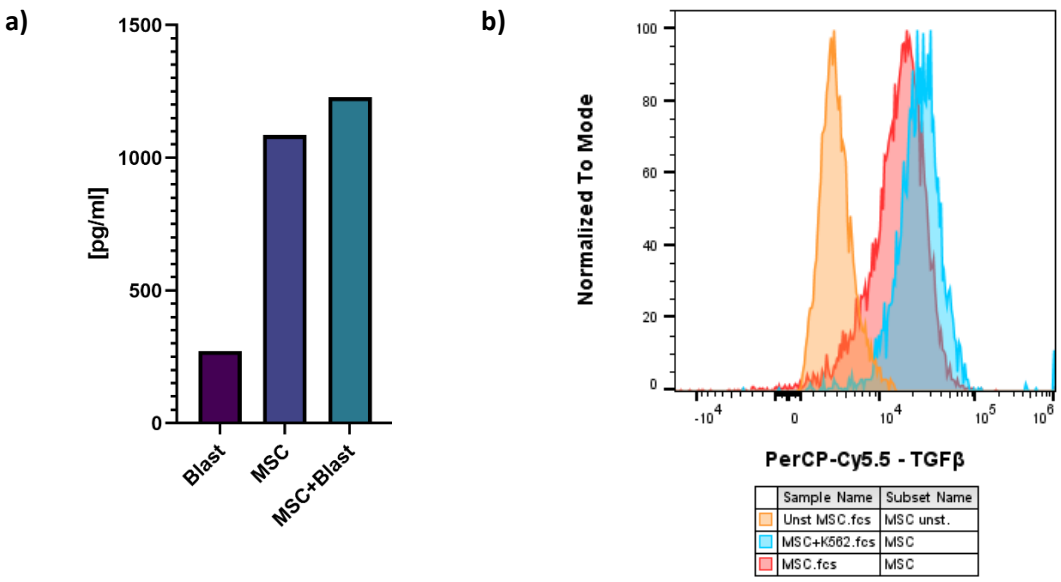


Figure 6 – Detectable TGF-β production in MSCs, K562 cells and primary blasts
(a) Direct TGF-β1 detection using ELISA was performed in supernatants of cultures of MSCs and primary blasts, as well as co-culture of the two cell types. Mono-cultures contained 10^6 cells each, co-culture was established in 1:1 ratio with $2x10^6$ cells in total. Cells were cultivated for 24 hours. (b) TGF-β production was assessed via intracellular staining of TGF-β in MSCs and MSCs co-cultured with K562 cells in 1:1 ratio. Cells were cultured for 48 hours. MSCs co-cultured with K562 cells contain more intracellular TGF-β in comparison to MSCs cultivated on their own. (n=1)

3.4. TGF-β-induced functional impairment of NK cells and restoration of their cytotoxic properties with TGF-β-antagonizing inhibitors

To determine the effects of TGF-β and inhibitors on NK cell functionality, cytotoxicity tests were performed on day 14 of the expansion. Conditions regarding the TGF-β were established in line with previously shown administration scheme illustrated in **figure 4a**. The K562 cells were chosen as targets for NK cells in these experiments. Due to the lack of MHC expression, these cells strongly activate the NK cells and, as such, any functional impairment induced by TGF-β or inhibitors would be observable.

3.4.1. TGF-β affects the cytotoxic properties of NK cells in time-dependent manner

As shown in **figure 7**, the overall cytotoxic properties of NK cells were affected by TGF-β in time-dependent manner. As expected, cells receiving TGF-β for full 14 days (D-14d) show the most significant impairment, whereas the activity of cells treated for shorter periods of time was not significantly altered in comparison with cells cultivated without TGF-β (Ctrl).

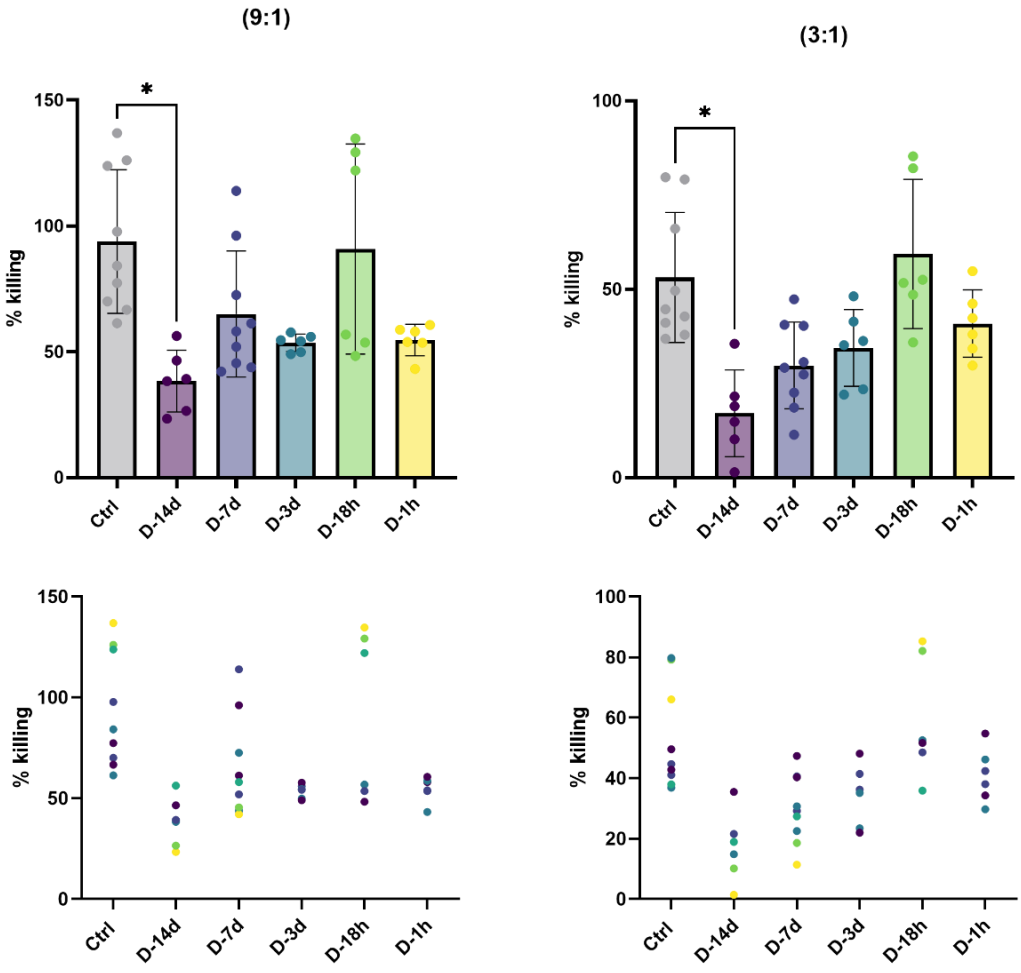


Figure 7 – Long-term exposure of NK cells to TGF-β leads to impaired cytotoxic abilities
*Calcein release assay was performed with NK cells as effectors and K562 cells as targets in 9:1 and 3:1 ratio. NK cells were treated with TGF-β for n days prior to assay on day D (D-n). The most significant decrease in cytotoxic killing is seen in condition D-14d in both ratios (9:1 and 3:1). Significance was proved by paired t-test (ns P > 0.05; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001) (n=6-9).*

3.4.2. Effect of inhibitors on NK cell viability

Second part of cytotoxicity assays was dedicated to exploration of reversibility of TGF-β-induced effects via TGF-β receptor inhibitors – galunisertib (Gal), LY2109761 (LY) and SB431542 (SB). To validate whether chosen dosage of inhibitors (adapted from He et al. (2018); Holmgaard et al. (2018); Tanaka (2010)) is not interfering with NK cell viability, flow cytometry assessment was performed 1 h (**figure 8a**) and 48 h (**figure 8b**) after administration of said inhibitors. NK cells treated with SB exhibited slightly decreased viability, yet no significant effect was observed that could be correlated to changed cytotoxic properties in further experiments.

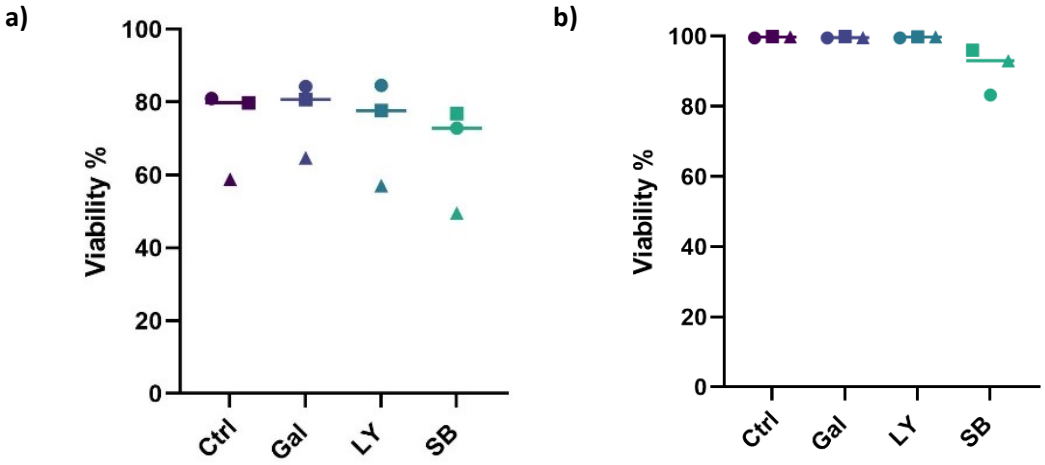


Figure 8 – Chosen inhibitors of TGF-β signalling do not significantly decrease the NK cell viability
Numbers of live NK cells were assessed after administration of inhibitors of TGF- β signalling (Galunisertib – Gal; LY2109761 – LY; SB431542 – SB) via flow cytometry. The viability of NK cells was calculated as a fraction of Live/dead blue- cells in CD45+ CD3/CD19/CD14- CD56+ population. Inhibitor-treated cells were then compared to untreated control cells. (a) Inhibitors were administered 1 hour prior to cytometric measurement. (b) Inhibitors were administered 48 hours prior to cytometric measurement. No significant decrease in viability was observed after inhibitor treatment. (n=3)

3.4.3. Reversibility the TGF-β-mediated cytotoxic impairment of NK cells by inhibitors

In all experiments, we can clearly see that in higher effector to target ratios the NK cells are more successful in eliminating the target cells due to overall redundance. Effects of different treatments are therefore generally more visible in lower ratios.

Experiment represented by **figure 9** was performed in order to describe the immediate effects of short-term inhibitor pre-conditioning. NK cells were pre-treated with inhibitors 30 minutes prior to receiving the TGF-β. The assay was then performed 1 hour later. Interestingly, on a contrary to expectations, effects of TGF-β itself were not as pronounced and the cytotoxic properties of NK cells were shown to be affected rather by inhibitors than the TGF-β treatment. Gal and SB caused a significant decrease in cytotoxicity compared to both untreated and TGF-β-treated NK cells.

Figure 10 illustrates a set of experiments performed in order to simulate the delayed migration of inhibitor-pre-conditioned NK cells to the leukemic BM niche. The NK cells were pre-treated with inhibitors 18 hours prior to start of the experiment. In this case, TGF-β was administered to NK cultures 1 hour before the target cells were added into the assay. Similarly to the previous experiment, the TGF-β-induced effects were not observed. The 18-hour cultivation with inhibitors, however, led to highly variable outcomes in terms of cytotoxic potential of NK cells from individual donors. Both LY and SB negatively affected the functionality of NK cells from 3 out of 6 donors.

The third experiment (**figure 11**), illustrates the cytotoxic potential of NK cells that entered the BM niche, but have not been activated immediately after. As such, these NK cells would possibly be exposed to both TGF-β and inhibitors prior to being activated. The inhibitors were administered 1 h prior to first addition of TGF-β on the 12th day of the expansion. The cytotoxic assay was then performed 2 days later. In comparison to the previous two experiments, the NK cells recovered and no impairment induced by TGF-β, Gal or LY could be observed. In case of SB, however, there was a noticeable decrease of cytotoxicity in higher ratios.

The last set of experiments (**figure 12**) was performed in order to simulate how effective would the inhibitors be in reversing the TGF-β-mediated suppression of patient´s NK cells already present in the BM niche. TGF-β was administered with every passage throughout the 14-day expansion. The inhibitors were then added 18 hours prior to the assay. The significant functional impairment of NK cells caused by long-term exposure to TGF-β was indeed shown to be reversed by the inhibitors.

Overall, the tested inhibitors were shown to exhibit temporary negative effect on the cytotoxicity of NK cells shortly exposed to TGF-β. When administered in advance to TGF-β treatment and the subsequent cytotoxic assay, the inhibitors cause variable shift is cytotoxicity among individual donors. Impact of short TGF-β and inhibitor pre-conditioning does not prevail and the NK cells recover in course of 2 days. Gal and LY were then shown to improve the cytotoxic potential impaired by long exposure TGF-β.

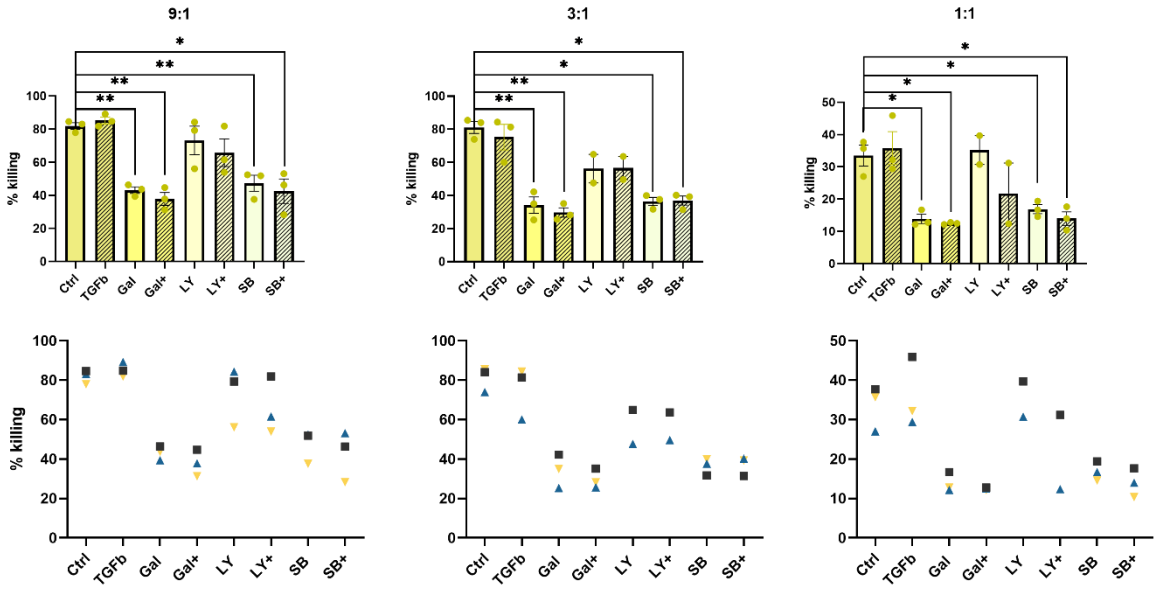


Figure 9 – Immediate effects of inhibitor pre-treatment are stronger than those of TGF-β
*Above we show the results of Calcein release assay. NK cells were pre-treated with inhibitors (Galunisertib – Gal; LY2109761 – LY; SB431542 – SB) and TGF-β (+) was administered 30 minutes later. The K562 target cells were added 1 hour later. Cytotoxic abilities were tested in 9:1, 3:1 and 1:1 ratio of NK cells to targets in all conditions mentioned. Gal and SB significantly impair cytotoxic activity of NK cells shortly after administration. Significance was proved by paired t-test (ns P > 0.05; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001) (n=3).*

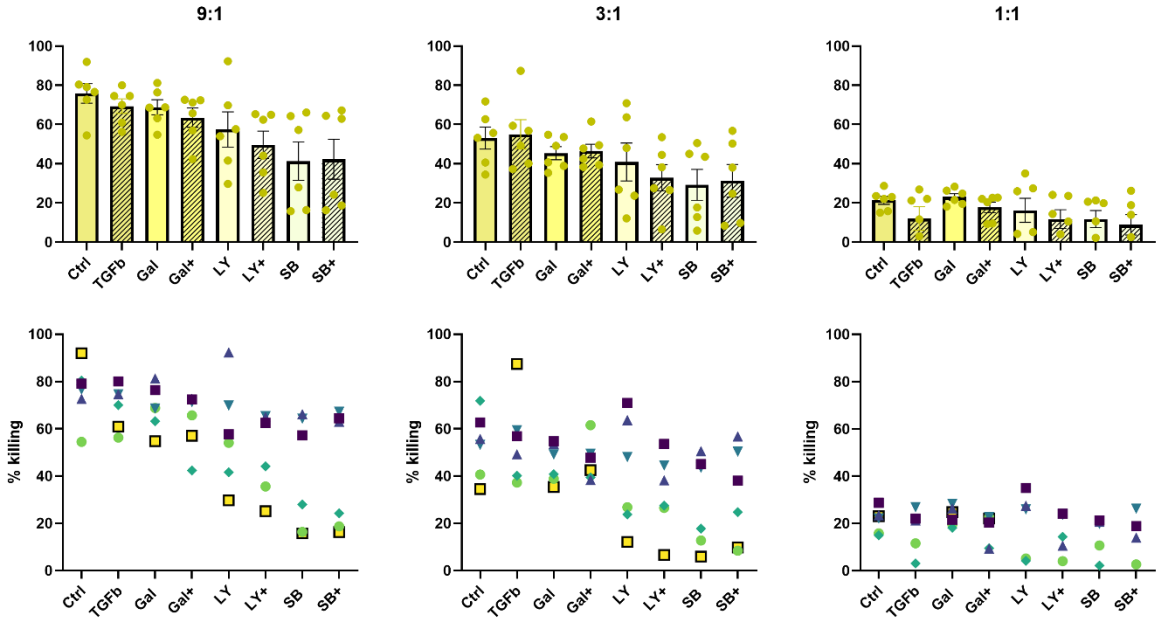


Figure 10 – Variability of effects induced by inhibitors and TGF-β among individual donors
Above we show the results of Calcein release assay. NK cells were pre-treated with inhibitors (Galunisertib – Gal; LY2109761 – LY; SB431542 – SB) 18 hours prior to start of the assay. TGF-β (+) was administered 1 hour before the target cell were added. Cytotoxic abilities were tested in 9:1, 3:1 and 1:1 ratio of NK cells to targets in all conditions mentioned. Effects of both inhibitor pre-treatment and TGF-β are highly variable among individual donors. (n=6)

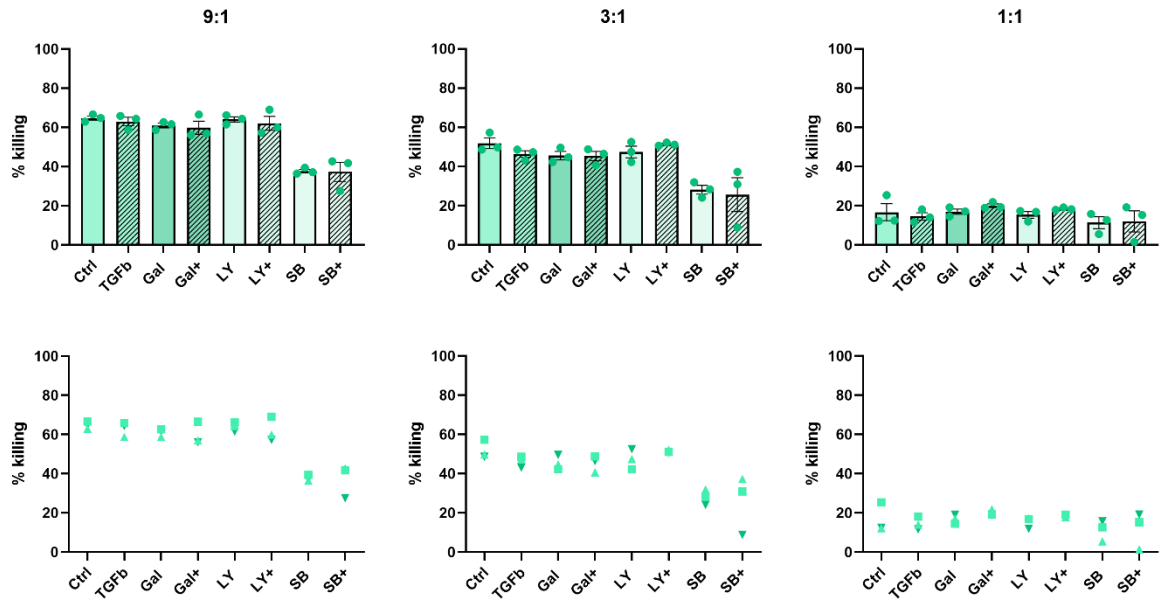


Figure 11 – Effects induced by inhibitors and TGF-β after 2-day resting period

Above we show the results of Calcein release assay. NK cells were cultured with inhibitors (Galunisertib – Gal; LY2109761 – LY; SB431542 – SB) and TGF-β (+) for 48 hours. TGF-β was administered 1 hour after the inhibitors. Cytotoxic abilities were tested in 9:1, 3:1 and 1:1 ratio of NK cells to targets in all conditions mentioned. (n=3)

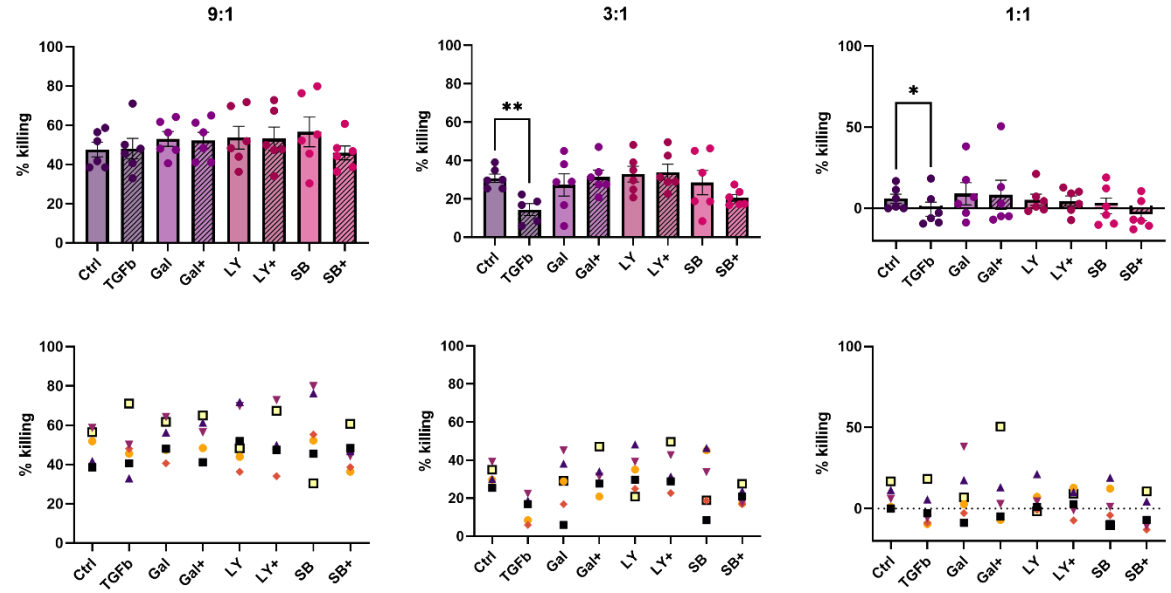


Figure 12 - Cytotoxicity of D-14d NK cells after 18h inhibitor of TGF-β treatment

*Above we show the results of Calcein release assay. NK cells were cultivated with TGF-β (+) for 14 days of the expansion. Inhibitors (Galunisertib – Gal; LY2109761 – LY; SB431542 – SB) were administered 18 hours prior to start of the assay. Cytotoxic abilities were tested in 9:1, 3:1 and 1:1 ratio of NK cells to targets in all conditions mentioned. TGF-β administration caused significant decrease in killing abilities of NK cells seen in 3:1 and 1:1 ratio. Gal and LY were shown to reverse the TGF-β-mediated suppression. Significance was proved by paired t-test (ns $P > 0.05$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$) (n=6).*

3.5. Impact of TGF-β and inhibitors on expression of key molecules in NK cells

In order to understand the TGF-β-induced functional impairment of NK cells, observed in cytotoxic assays, the expression of several key genes was assessed via qPCR. As seen in **figure 13**, the 18-hour (D-18h) TGF-β treatment caused upregulation of TGFRI, NKG2D, mTORC1, T-bet and TNFα. The long-term 14-day (D-14d) TGF-β treatment led to significant downregulation of NKG2D. Overall, there was observable trend of time-dependent downregulation also in case of NKp30, Smad3 and T-bet induced by exposure to TGF-β. Interestingly, NKp44 was slightly but significantly downregulated in D-3d NK cells. Overall, the responsiveness to TGF-β in terms of expression of TGFBR I, NKp30, Smad3, granzyme B, perforin 1 and TNFα was rather variable among donors. NKp30, NKp44, NKp46 and NKG2D are all activating receptors of NK cells inducing the immune response. Downregulation of these molecules therefore directly impacts the cytotoxic activity of NK cells (as confirmed in **figure 7**). The time-dependent downregulation of Smad3 marks a negative regulation of the TGF-β-induced signalling through the classical pathway. Strong effects of long-term TGF-β exposure could be observed nevertheless, suggesting that these effects are most likely mediated via alternative cascades.

The second part of cytotoxic tests was performed using inhibitors. Therefore, it was necessary to assess whether these inhibitors prevent the expressional changes induced by TGF-β and/or whether they alter the NK cells expression in any way. Expressional changes were analysed in NK cells cultivated with inhibitors, TGF-β and combination of both for 18 hours. In this experiment, the inhibitors were added 1 hour prior to TGF-β administration. As illustrated by **figure 14**, the inhibitors exhibited strong effects on expression of several genes. SB administration enhanced the TGF-β-induced upregulation of TGFBR I and caused TGF-β-independent upregulation of TNFα, NKp30 and granzyme B. NKp44 was then significantly downregulated by both Gal and SB, independently of TGF-β. Gal was shown to successfully reverse the TGF-β-induced changes in expression of TGFRI, TNFα, NKp46 and IFNγ. High variability in response to inhibitors among individual donors could be observed in case of TGFBR II, NKp30, perforin 1, KIR3DL and T-bet, suggesting that the donor heterogeneity is an important factor to be taken into consideration.

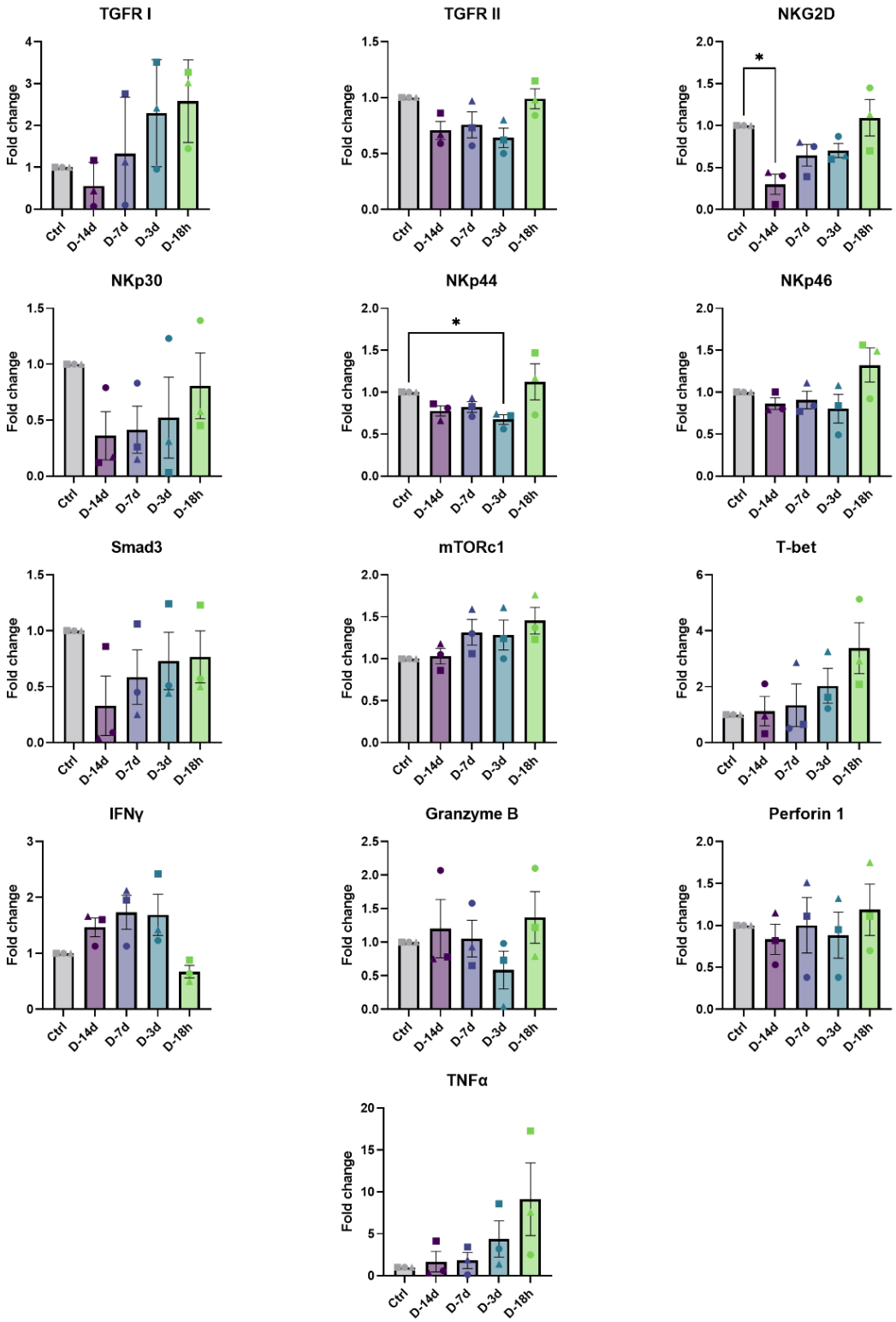


Figure 13 – Both short and long times of exposure to TGF-β have strong overall effects on NK cell expression
*Relative expression of mRNA of genes of interest was assessed via qPCR. TGF-β-treated (D-n; n days of treatment) NK cells are compared to untreated (Ctrl) cells. Long-term TGF-β treatment induces upregulation of TGFBR I, mTORc1, T-bet and TNFα. Long-term exposure then leads to downregulation of NKG2D, NKp30 and Smad3. Delta-deltaCT analysis was performed using GAPDH as a house-keeping gene. Significance was proved by paired t-test (ns P > 0.05; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001) (n=3).*

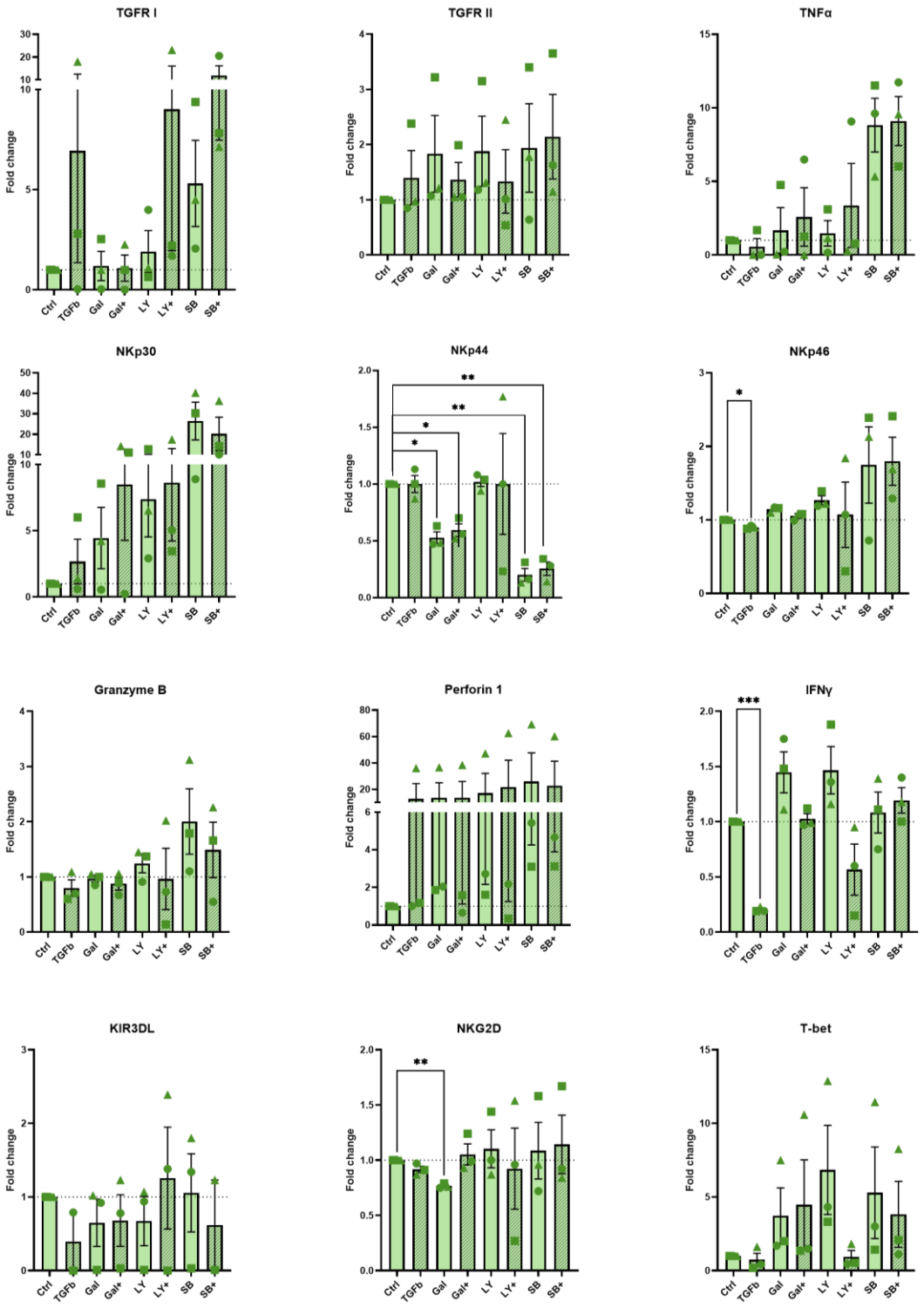


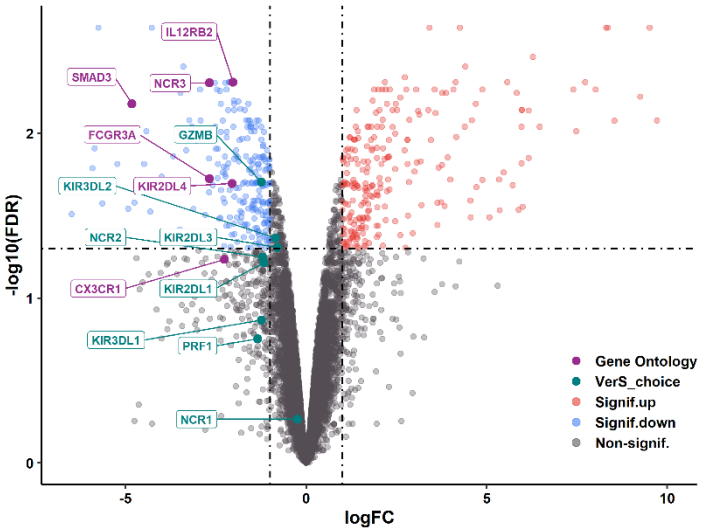
Figure 14 – Short-term inhibitor treatment-induced changes in expression profiles of NK cells

*Relative expression of mRNA of genes of interest was assessed via qPCR. The panel depicts expression of NK cells treated with inhibitors (Galunisertib – Gal; LY2109761 – LY; SB431542 – SB) and TGF-β (+) as well as their combinations compared to untreated (Ctrl) NK cells. Inhibitors and TGF-β were administered 18 hours prior to RNA isolation, respectively, with 1-hour time gap. NKp44 is significantly downregulated by Gal and SB, independently of TGF-β. SB caused upregulation of NKp30, NKp46 and TNFα. In case of TGFRII, NKp30, perforin 1, KIR3DL and T-bet expression, high variability in effects can be observed among individual donors. Delta-deltaCT analysis was performed using GAPDH as a house-keeping gene. Significance was proved by paired t-test (ns P > 0.05; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001) (n=3).*

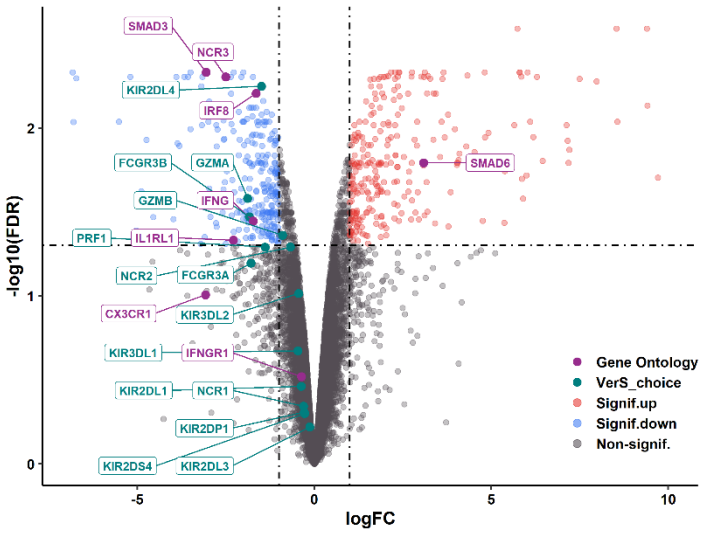
3.6. TGF-β-induced downregulation of cytotoxicity-related genes in NK cells analysed by RNA sequencing

In order to better understand the overall influence of TGF-β on NK cells we decided to compare the effects of different exposure intervals of NK cells to TGF-β via RNA sequencing. We chose to compare D-14d, D-7d and D-18h NK cells to control NK cells, that were cultivated for 14 days completely without TGF-β. A total of 657 genes for D-14d (318 upregulated; 339 downregulated), 798 genes for D-7d (403 upregulated; 395 downregulated) and 974 genes for D-18h (580 upregulated; 394 downregulated) were found to be significantly differentially expressed. The dataset of these genes was then narrowed-down based on fold change (logFC > 1) and false discovery rate (FDR < 0.05) for each comparison as depicted in **figures 15a (D-14d), 15b (D-7d) and 15c (D-18h)**. (List of top 100 differentially expressed genes provided in **supplement – tables S1-S6**) Pathway enrichment analysis was then performed with the remaining data using Gene Ontology. Chosen cytotoxicity-related genes revealed by the enrichment are depicted in purple. Furthermore, genes we expected to be expressionaly affected based on literature are highlighted in green. The highlighted genes belong to groups of effector molecules (GZMA, GZMB, IFNG, PRF1), intracellular signalling molecules (IRF8, SMAD3, SMAD6), activating receptors (FCGR3A, FCGR3B, KIR2DS4, NCR1, NCR2, NCR3), inhibitory receptors (KIR2DL1, KIR2DL3, KIR2DL4, KIR3DL1, KIR3DL2, KIR2DP1) or other surface receptors related to NK cell function in immune responses (CX3CR1, IFNGR1, IL12RB2, IL1RL1). Overall, in all three conditions (D-14d, D-7d, D-18h), majority of our genes of interest are downregulated by TGF-β treatment and among the most significantly dysregulated genes we identified Smad3 (SMAD3), NKp30 (NCR3) and KIR2DL4. The D-14d and D-7d cells significantly changed expression of granzymes (GZMB and GZMA) and CD16 subunits (FCGR3A and FCGR3B) respectively. The D-18h along with D-7d NK cells then show downregulation of IFNγ. The only uncovered upregulation of gene of interest was observed in case of Smad6 in the D-7d cells.

a) Differentially expressed genes in Ctrl vs D-14d



b) Differentially expressed genes in Ctrl vs D-7d



c) Differentially expressed genes in Ctrl vs D-18h

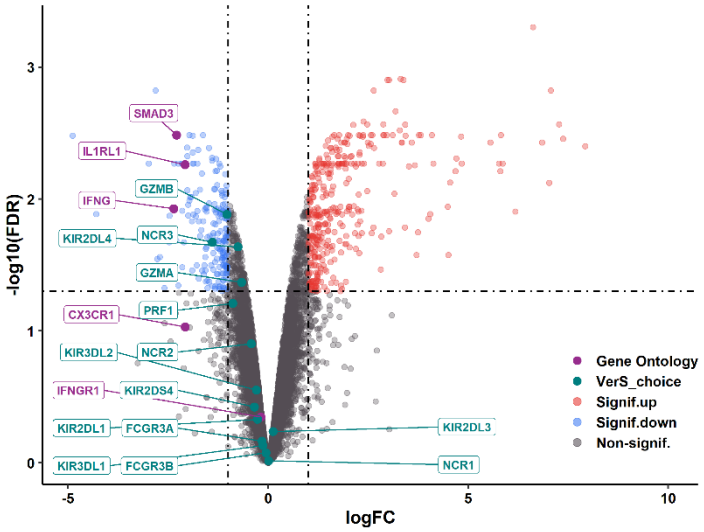


Figure 5 – Differentially expressed genes in NK cells induced by different time of exposure to TGF-β
Differential expression analysis was performed with data acquired via RNA sequencing of TGF-β-treated (D-14d; D-7d, D-18h) and untreated (Ctrl) NK cells (n=3). ***Figures a-c*** *depict differentially expressed genes for individual conditions compared to controls. Cytotoxicity-related genes of interest are highlighted. Data were filtered based on: FDR < 0.05 and logFC > 1. R software was used for statistical analysis and visualization of the data (e.g. edgeR 3.30.3, ggplot 3.3.2, pcaMethods 1.84.0, M3C 1.14.0). Subsequently, functional enrichment analysis was performed using the Gene Ontology database.*

4. Discussion

Relapsed AML patients frequently fail to respond to the conventional therapy. Intensive research is in process to develop much needed novel therapies to deal with this phenomenon. In 2010, Rubnitz et al. already showed that adoptive transfer of haploidentical NK cells in combination with low-dose immunosuppression leads to successful engraftment in pediatric AML patients. All patients remained in remission for 2 years afterwards and no significant toxicity or GvHD was observed. Recent clinical trials proven that NK cells are an efficient tool in post-HSCT relapse treatment in pediatric and young adult leukemia patients. Bednarski et al. (2022) stated that *ex vivo* cultured donor-derived NK cells do maintain enhanced anti-leukemic responsiveness. After infusion, these NK cells did expand substantially, persisted in the patient and were able to maintain the anti-leukemic activity in absence of exogenous cytokines, while no significant toxicity was observed. Four out of eight patients receiving the NK cells in this particular study did reach complete remission in 28 days post-infusion. Two of them maintained a remission for up to 3 months and one was found in remission 2 years later. One of the biggest challenges in cell-based AML therapy is the effective targeting of the leukemic cells. It is known that AML is not easily defined by specific surface markers due to rather high clonal heterogeneity. The absence of general target antigen delays the use of CAR constructs and might lead to off-target effects accompanied by escape of minor leukemic clones, increasing the probability of relapse occurrence (Haubner et al., 2019). Obvious advantages of NK cells as a therapeutical tool in AML treatment is their independency of such leukemia-specific markers. NK cells acquired from allogeneic donor also bear only low risk of GvHD or cytokine release syndrome. Yet the NK cell-based therapy faces several obstacles presented by the leukemic environment. The origins of malignant leukemic proliferation and relapse hot-spots are localized in the protective environment of BM (Shafat et al., 2017). Presence of leukemic cells in the hematopoietic niche leads to alterations in hematopoiesis and general dynamics within the BM. Through selective pressure induced by therapy and enhanced mutational rate, the malignant cell populations gain broad spectrum of advantages supporting their ability to evade immune surveillance and modulate their surroundings. These cells then reshape the BM environment through several mechanisms. The modulatory processes are mediated via cell-to-cell interactions, along with direct secretion or induced production of cytokines, chemokines or growth factors (Motta & Rumjanek, 2021). Among many modulatory factors, we chose to look closely into the role and dynamics of TGF-β.

TGF-β is an important regulatory cytokine with distinct context-dependent effects on cell cycle progression, proliferation, effector function and immune response polarization. The general consensus about the role of TGF-β in leukemia is not well established however. The plasma levels of TGF-β have previously been shown as decreased in AML patients (Sanchez-Correa et al., 2013), leading to a general idea of TGF-β being rather anti-leukemic factor, as stated by Dong & Blobe (2006). TGF-β has suppressive

properties in terms of proliferation and, according to Schelker et al. (2018), blocking its activity might lead to increased proliferation of AML cells. At the same time, Schelker et al. (2018) also stated that antagonizing TGF-β makes the leukemic cells more susceptible to therapy. TGF-β has also been described as a potent protective factor regulating survival in HSCs. This effect has also been observed in AML cell lines (MV4;11, THP-1 and U937). It was shown by Tabe et al. (2013) that TGF-β shields the leukemic cells from cytarabine-induced apoptosis. Furthermore, it has been previously observed that for some leukemic clones it is common to develop resistance to effects of this cytokine (Dong & Blobe; 2006). The role of TGF-β in leukemic progression is therefore not as easily defined.

As partially illustrated by our results (**figures 5a, 5b and 6a**) as well as previous publications (Ma et al., 2019), direct production of TGF-β is highly variable among both primary blasts and leukemic cell lines . Based on direct protein detection in cell culture supernatants we assessed that cells of the K562 and MOLM13 leukemic cell lines produce substantial levels of TGF-β independently of stimuli, whereas THP-1 or U937 cells do not exhibit strong production. In our pilot experiment, primary AML blasts isolated from patients via leukapheresis (**figure 6a**) did show a moderate production. Based on growing body of evidence, we believe that TGF-β is one of the major factors favoring the leukemic progression and immune surveillance evasion. As proposed by Borella et al. (2021) and Pan et al. (2020), presence of leukemic cells in the BM induces transformation of BMSCs and MSCs into CAFs – generally described as the major producers of TGF-β in tumor microenvironment (Huynh et al., 2019). Furthermore, development of CAFs was also shown to be enhanced by TGF-β in a self-perpetuating loop (Pan et al., 2020). Our preliminary data from intracellular cytokine staining in MSCs co-cultured with K562 cells (**figure 6b**) illustrate how the presence of leukemic cells induces upregulation of TGF-β production in the surrounding cell types. In case of ELISA measurements of supernatants from MSCs co-cultivated with primary blasts (**figure 6a**) the results have not been as straightforward and we intend to repeat the experiment and utilize the intracellular staining to validate our data.

Beside the immunomodulatory role, TGF-β has also been shown to support the LSC population within the leukemic BM niche. Shingai et al. (2021) used the endothelial cell-selective adhesion molecule (ESAM) as a marker to describe two LSC subpopulations in regard to TGF-β sensitivity and production. ESAM- LSCs (skewed towards the lymphoid lineage based on co-expression of CD33) express high levels of TGFBRII which leads to induction of TGF-β-cascade signalling even in extremely low concentrations of TGF-β. ESAM+ LSCs (polarized in the myeloid direction) on the other hand were shown to produce TGF-β while not being highly sensitive to its effects. This difference in production and responsiveness to TGF-β might actually partially explain how TGF-β, despite being generally suppressive, might favor the progression of myeloid malignancies. Targeting TGF-β might therefore be beneficial also in this regard.

To better understand the dynamics of TGF-β-mediated suppression in context of NK cell-based AML therapy, several conditions regarding TGF-β administration were established to resemble possible *in vivo* situations. We were interested in comparison of NK cells exposed to TGF-β in either short-term or long-term manner to untreated controls. As stated before, TGF-β causes changes in expression of broad spectrum of NK cell receptors and other molecules directly involved in their anti-leukemic activity. As a result, these NK cells were shown to be polarized in the direction of less cytotoxic phenotype, which we were able to observe via flow cytometry (**figure 4c and 4e**). TGF-β was shown to favor the expansion of the CD56bright CD16- NK cells in time-dependent manner.

Next step was to analyse the expression levels of key molecules crucial for NK cells function in context of TGF-β treatment. NK cell cytotoxic potential is determined by their ability to produce effector molecules as well as expression of surface receptors. Therefore, following group of molecules was chosen to assess the effects of TGF-β: granzyme B, perforin 1, IFNγ, TNFα, KIR3DL, NKp30, NKp44, NKp46 and NKG2D (Montaldo et al., 2013). Expression of TGF-β receptors (TGF-BR I and II), along with Smad3 as a key signalling molecule in TGF-β-induced cascade, was also analysed to see whether there are differences in responsiveness to TGF-β after the treatment (Regis et al., 2020). Lastly, T-bet was chosen as a major transcription factor of IFNγ-producing cells (Fang et al., 2022) and mTORc1 as a marker of metabolic fitness (Poznanski & Ashkar, 2019). GAPDH was then chosen as a house-keeping gene (Bustin, 2000).

As shown by qPCR expression analysis (**figure 13**), short-term TGF-β treatment leads to quick upregulation of TGFRI, NKp46, mTORc1, T-bet and TNFα, whereas IFNγ is slightly downregulated. In a time-dependent manner, however, longer exposure to TGF-β causes downregulation of TGFRI, NKG2D, NKp30 and Smad3. In case of NKp44, subtle but significant decrease in expression can be observed after short-term 3-day exposure. Partially on contrary to our data, (Foltz et al., 2018) claims that long-term combined TGF-β and IL-2 pre-treatment leads to induction of more pro-inflammatory NK cells producing higher levels of IFNγ and TNFα in comparison to NK cells shortly exposed to TGF-β. However, in this case, the NK cells were weekly restimulated with irradiated K562 feeder cells expressing membrane-bound IL-15 or 21 and, as such, the NK cells were exposed to strong activating stimuli throughout the experiment.

In order to better understand the impact of TGF-β on NK cells, three inhibitors were chosen to reverse its effects. Galunisertib, LY2109761 and SB431542 are all small molecules inhibiting the kinase activity of TGF-BR I and subsequently the phosphorylation of Smad2. All three inhibitors were shown to be efficient in blocking the effects of TGF-β. Galunisertib was shown to improve the responsiveness of T cells towards tumors in mice by Holmgaard et al. (2018). The second inhibitor chosen, SB431542, was also shown to enhance the anti-tumor responses. Furthermore, it supported maturation of dendritic cells, which were then highly effective in activating NK cells (Tanaka, 2010). Lastly, it was confirmed that

LY2109761 inhibits tumor cell growth, counteracting the protective effects of TGF-β produced in TME (He et al., 2018). The main idea was whether different times of exposure to TGF-β change the NK cell responsiveness to these inhibitors. If the effectivity of inhibitors proved to be sufficient in protecting the NK cells and remained unchanged in different TGF-β-related conditions, co-administration of such TGF-β-antagonizing medication could improve the overall efficiency of NK-cell based therapy in the future.

First, it had to be addressed whether the inhibitors we chose interfere with the NK cell viability or alter the gene expression in any way. The viability was assessed via flow cytometry 1 hour and 48 hours after administration of the inhibitors (**figure 8**). Slightly lowered viability was observed in NK cells treated with SB431542, yet the average decrease in viability did not exceed 8 % and. The qPCR was then performed with NK cells treated with both inhibitors and TGF-β respectively (with 1 h time gap) 18 h prior to RNA isolation. Changes in NK cell expression induced by TGF-β, inhibitors and combination of both were observed (**figure 14**). NKp44 was significantly downregulated by galunisertib and SB431542. Galunisertib was also shown to significantly downregulate NKG2D, yet, interestingly, the effect was diminished in the presence of TGF-β. In case of TNFα, NKp30, NKp46 and granzyme B there was an overall trend of enhanced expression induced by SB431542. In terms of IFNγ expression, the inhibitors all exhibited a protective effect, counteracting the TGF-β-mediated downregulation. Overall, the response to inhibitors on expressional level was highly variable among donors.

To gain more insight into TGF-β-mediated changes of NK cell function, RNA sequencing was employed. NK cells were exposed to 14 days (D-14d, **figure 15a**), 7 days (D-7d, **figure 15b**) or 18 hours (D-18h, **figure 15c**) of TGF-β treatment and compared to untreated controls. Preliminary analysis of data from this experiment confirmed that TGF-β strongly affects the overall gene expression of NK cells. Several genes associated with cytotoxicity of NK cells were significantly downregulated by the TGF-β treatment. NKp46 (NCR3) was significantly downregulated in all three conditions, which is in line with the general knowledge (Lamb et al., 2021). IFNγ was significantly downregulated in D-7d and D-18h conditions, but not in the case of D-18h NK cells. Expression of granzyme A and B was then decreased in D-7d and D-14d NK cells respectively. The D-14d NK cells also exhibited significantly lowered expression of CD16a (FCGR3A) compared to untreated NK cells. Also, significant downregulation of Smad3 could be observed in all three assessed conditions, suggesting that the TGF-β cascade is being regulated through negative feedback loop since early exposure to this cytokine. Surprisingly, other molecules, such as NKp30 (NCR1), NKp44 (NCR2) or other KIRs, employed in NK cell cytotoxic activity were not dysregulated as it could be anticipated. These results of gene expression analysis under the influence of TGF-β are generally in line with previous publications. Downregulation of NKG2D, along with other surface cytotoxicity-related receptors of NK cells is often described as the main effects of TGF-β-mediated NK cell suppression. Beside impaired production of granzymes, perforin or IFNγ, Yang et al. (2021) states that

TGF-β also negatively impacts the cascade leading to degranulation in NK cells. In broader context, Regis et al. (2020) pointed out that exposure of NK cells to TGF-β generally leads to epigenetically induced conversion to type 1 innate lymphoid cells with poor cytotoxic properties.

Interestingly, beside the cytotoxicity-related genes, the RNA sequencing also revealed that the TGF-β treatment significantly affected expression of molecules involved in adhesion, chemotaxis and cellular metabolism, hinting a bigger role of TGF-β in mobility and metabolic reprogramming of NK cells (list of differentially expressed genes provided in **supplement – table S1-S6**). Further validation with more donors would be beneficial though, because the heterogeneity in response to both TGF-β and inhibitors is rather high in some cases, making it difficult to observe any significance and get to a general conclusion.

Inspecting closely the cytotoxic response towards target leukemic cells (K562), we were able to show that the suppressive effects of TGF-β on NK cells are time-dependent (**figure 7**) and short-term exposure does not significantly alter their cytotoxic properties (**figures 9-11**). Fast-acting NK cells are therefore most likely not affected after entering the leukemic BM niche. On the other hand, the long-term co-cultivation of NK cells with TGF-β did lead to enhanced expansion of less cytotoxic NK cells over the course of the cultivation (**figures 7 and 12**). TGF-β potentially produced and deposited in the leukemic niche could therefore be an issue in regard to more long-lived NK cell populations. In this case, the long-term TGF-β exposure could pose as a major suppressive factor, negatively affecting the clearance of malignant cells. Yet these adverse effects could be reversed by inhibitors blocking the activity of TGF-β receptors, as shown in **figure 12**.

In comparison to the tissue-resident NK cells, conventional NK cells are generally seen as short-lived immunocytes exhibiting effective, yet rather rapid immune response, requiring constant renewal in periphery (Tarannum & Romee, 2021). Two main subsets of NK cells are distinguished based on CD56 and CD16 expression. The more abundant population, CD56dim CD16+, is accepted as the more cytotoxic one. Approximately 10 % of peripheral NK cells, described as CD56rbight CD16-, are then seen as regulatory due to lower cytotoxicity and high production of IFNγ, TNFα, IL-10, IL-13 or GM-CSF. Yet, both subsets co-express T-bet and Eome and secrete cytotoxic granules. However, recently NK cells are starting to be perceived as a heterogeneous population of many different subtypes with distinct functions (Tarannum & Romee, 2021; Zhang & Tian, 2017). The heterogeneity should further be addressed using additional methods such se single-cell RNA sequencing, or include cell sorting as cell isolation step into the expansion protocols.

Lately, the memory-like NK cells have gained attention in terms of their potential in therapy. Memory-like or adaptive NK cells rise from populations exposed to widely spread viral infections (i.e. cytomegalovirus, Epstein-Barr virus, hepatitis C) and, as such, these cells acquire adaptive traits like

longevity and ability of oligoclonal expansion (Sivori et al., 2021). These cells have been proposed to be highly effective in elimination of resident leukemic cells in the BM, while being able to persist in tissues for much longer time periods than conventional NK cells (Zhang & Tian, 2017). According to Tarannum & Romee (2021), memory-like NK cells express higher levels of CD16, CD25, NKG2C, NKp46 and IFNγ, while expression of KIRs and TGF-BRs is decreased. They also state that these cells are able to maintain the co-expression of T-bet and Eomes after adoptive transfer, unlike conventional NK cells, and as such they continue to exhibit increased proliferative potential and cytotoxic properties. According to Leong et al. (2014), the memory-like phenotype of NK cells shows after (16-hour) pre-activation with IL-2, IL-15 and IL-18. Taken into account that the prevalence of cytomegalovirus among the caucasian population reaches 50-80 % (Cannon et al., 2010), it can be hypothesised that utilization of IL-2 and IL-15 in NK cell cultures could also lead to preferential expansion of the memory-like population. Interestingly, induction of the memory-like phenotype in our NK cells could partially explain some of the unexpected results shown above. Bednarski et al. (2022) shows that the memory-like NK cells produce higher levels of IFNγ and exhibit improved anti-leukemic function. Tumino et al. (2022) states that IL-15 reverses the metabolic modulation of NK cells in TME, leading to restoration of anti-tumor activity and, according to Kaweme & Zhou (2021), IL-15 was also previously associated with increased NK cell proliferation. Song et al. (2006) states that cultivation of NK cells in the presence of IL-2 and IL-18 prevents the TGF-β-mediated downregulation of NKG2D in NK cells and consequently reverses their functional suppression.

Therefore, the absence of observable cytotoxicity impairment in NK cells shortly exposed to TGF-β (**figures 7, 9-11**) might actually be a result of IL-2-mediated functional rescue. Furthermore, low dose IL-2 administration (combined with TGF-β neutralization) was shown to accelerate reconstitution of NK cell pool after HSCT with stronger anti-tumor potential (Alvarez et al., 2020). In general, cytokine-induced memory-like NK cells are believed to be highly resistant to immune suppression mediated by MSCs or Tregs (Tarannum & Romee, 2021), while possessing stronger anti-leukemic responsiveness regardless of KIR-mediated inhibition (Kaweme & Zhou, 2021). Due to the long-term cultivation with IL-2 and IL-15 some of the TGF-β-mediated effects might have also been ameliorated on expressional level. The shift of NK cells towards the memory-like phenotype could explain the variability of responsiveness to TGF-β treatment among different donors (**figure 7**). Furthermore, it would be interesting to see whether this potential shift-induction also affects the way the NK cells respond to inhibitors (**figure 10**), or if the observed variability depends solely on the donors themselves.

It would certainly be beneficial explore the cytokines and inhibitors mentioned above as individual factors in terms of sensitivity to TGF-β and overall performance in cytotoxic tests. RNA sequencing could be utilized to assess whether using our cultivation protocol leads to preferential expansion of cytokine-

induced memory-like NK cells. Furthermore, cytomegalovirus screening among donors and selective expansion of positive NK cells could be an interesting direction for research of therapeutical possibilities.

5. Conclusion

In regards to TGF-β in the leukemic niche, the experiments confirmed that leukemic cells produce variable levels of the cytokine and, furthermore, that these cells are able to induce its production in other cell types present in the bone marrow niche as well. In terms of NK cell functionality, TGF-β was shown to affect expression of several key molecules related to cytotoxicity of NK cells. There were different outcomes associated with short-term and long-term TGF-β treatment, such as rapid upregulation TNFα induced by short exposure, and significant downregulation of NKG2D induced by longer exposure. The cytotoxic activity of NK cells was impaired by TGF-β in time-dependent manner, reaching a significant decrease after long-term exposure to this cytokine. Several tested inhibitors were shown to ameliorate the long-term adverse effects of TGF-β in terms of cytotoxicity without significant side effects of their own. Donor heterogeneity did however lead to variable outcomes of inhibitor pre-treatment in case of NK cells shortly exposed to TGF-β. Among dysregulation of genes directly related to cytotoxic activity of NK cells, RNA sequencing revealed that TGF-β also impacts NK cell metabolism and migration.

In broader context, our data suggest that potential TGF-β-mediated suppression after adoptive transfer to patients would not significantly affect the conventional therapeutically administered NK cells. Yet the long-term effects of TGF-β produced in the leukemic BM niche could in fact play a role in suppression and reprogramming of more long-lived NK cell populations and patient´s own developing NK cells. The long-term suppression was, however, shown to be reversible by inhibitors. Therefore, with the right pre-conditioning and/or administration of inhibitors and the right combination of cytokines, NK cells could be used as an ideal tool for dealing with residual blasts as well as LSCs shielded by the leukemic niche.

6. Literature

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