T.C.
DOKUZ EYLÜL UNIVERSITY
IZMIR INTERNATIONAL
BIOMEDICINE AND
GENOME INSTITUTE

THE ROLE OF TRAIL MEDIATED SIGNALS ON THE PROLIFERATION AND FUNCTION OF HUMAN iNKT CELLS

ZEYNEP ÖZGE AYYILDIZ

MOLECULAR BIOLOGY AND GENETICS **DOCTOR OF PHILOSOPHY THESIS**

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Supervisor: Assoc. Prof. Şerif Şentürk

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LIST OF ABBREVIATIONS

αGalCer..... αGalactosylceramide

APCs..... Antigen presenting cells Bcl-2.....

B cell lymphoma 2 cFLIP..... Cellular FADD-like IL-1β converting enzyme (FLICE) inhibitory protein

DD..... Death domain

DISC..... Death inducing signalling complex

DRs..... Death receptors

DR4..... Death receptor 4

DR5..... Death receptor 5

DcR1..... Decoy receptor 1

DcR2..... Decoy receptor 2

DCs…… Dendritic cells FASL……..

Fas ligand

FADD…… Fas-associated death domain protein iNKT……..

Invariant Natural Killer T

IL-2…… Interleukin-2

IL-4…… Interleukin-4 IL-12…….

Interleukin-12

IL-12Rα..... IL-12 receptor α IL-18…….

Interleukin-18

IL-18R…… IL-18 receptor IFNɣ……..

Interferon ɣ

IFNɣ$^{-/-}$…… Interferon ɣ-deficient mice

MAPK…… Mitogen activated protein kinases

MHC…… Major histocompatibility complex

mTRAIL…… Membrane bound TRAIL NK……

Natural Killer

NF-κB..... Nuclear factor kappa B

NK1.1…… Mouse ortholog of CD161

NKG2D…… Natural killer group 2D

NCAM……Neural cell adhesion molecule

NSG…… Nod severe combined immunodeficient (SCID) gamma OPG……

Osteoprotegerin

PBMC…… Peripheral blood mononuclear cells rhTRAIL……..

Recombinant human TRAIL sTRAIL…….. Soluble TRAIL

TCR……

T cell receptor

TRAIL…… Tumor necrosis factor apoptosis inducing ligand

TRAIL$^{-/-}$…… TRAIL-deficient mice

TNFSF…… Tumor necrosis factor superfamily

TNF…… Tumor necrosis factor WT……

Wild type

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THE ROLE OF TRAIL MEDIATED SIGNALS ON THE PROLIFERATION AND FUNCTION OF HUMAN iNKT CELLS

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ABSTRACT

Invariant Natural Killer T (iNKT) cells constitute a natural memory T cell subset that respond to glycolipid antigens presented by the non-polymorphic CD1d molecule. In line with their effector characteristics, iNKT cells produce copious amounts of cytokines and exert cytolytic function after activation. iNKT cells are also involved in tumour immunosurveillance. iNKT cells can induce cell death in cancer cells via perforin/granzyme and the death ligand CD178/FASL. Tumour necrosis factor related apoptosis inducing ligand (TRAIL) is another death ligand that is a safer therapeutic alternative to CD178/FASL because of its non-toxicity towards healthy cells. TRAIL can be a tool in tumour immunosurveillance as well as a tumour escape mechanism depending on the cell type it is expressed. However, the data on TRAIL-mediated iNKT cell control of tumour are controversial. Furthermore, there is no information regarding the outcomes of retrograde TRAIL signalling on human iNKT cells. In this study, these open questions were addressed. My data show that not primary human iNKT cells but in vitro expanded iNKT cell lines express surface TRAIL. Furthermore, the TRAIL receptors CD261/DR4 and CD262/DR5 were largely expressed intracellular in iNKT cells. A second antigenic stimulation did modulate the expression of surface TRAIL but not of the receptors. Further, I show that activated iNKT cell lines are insensitive to recombinant human TRAIL (rhTRAIL), as their survival, phenotype, and function remained unchanged following chronic or acute rhTRAIL exposure. These findings suggest that iNKT cells could be an effective immunotherapeutic tool to fight $TRAIL^{+}$ tumours.

Keywords; iNKT cells, TRAIL, CD261/DR4, CD262/DR5

TRAIL ARACILIĞI İLE GERÇEKLEŞEN SİNYALLERİN İNSANİNKT

HÜCRELERİNİN PROLİFERASYONUNA VE FONKSİYONLARINA ETKİSİ

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ÖZET

İnvaryant Doğal Öldürücü T (iNKT) hücreleri, polimorfik olmayan CD1d molekülü tarafından sunulan glikolipid antijenlere yanıt verebilen bir doğal hafıza T hücre alt grubudur. Effektör karakteristik özellikleri ile uyumlu olarak, iNKT hücreleri, aktivasyonlarından sonra pek çok farklı sitokin üretebilir ve sitolitik fonksiyon sergileyebilirler. iNKT hücreleri ayrıca tümör immün takibinde de bulunurlar. iNKT hücreleri perforin/granzyme ve ölüm ligandı, CD178/FASL, aracılığı ile kanser hücrelerinde hücre ölümünü tetikleyebilirler. Tümör nekrosiz faktörü ile bağlantılı apoptoz indükleyici ligand (TRAIL) ise sağlıklı hücreler için zararlı olmayışı ile CD178/FASL'ın yerine daha güvenli törepatik bir alternatiftir. TRAIL eksprese edildiği hücre tipine göre, hücre tarafından tümör immün takibinde görev alabileceği gibi, bir tümör kaçış mekanizması olarak da kullanılabilir. Bununlar beraber, iNKT hücrelerinin TRAIL aracılığı ile gerçekleştirdiği tümör kontrolü verileri çelişkilidir. Ayrıca, iNKT hücrelerinin TRAIL sinyaline verdiği cevaba dair de bir bilgi bulunmamaktadır. Bu çalışmada, bu sorulara cevap verilmiştir. Verilerim, primer insan iNKT hücreleri yerine in vitro olarak çoğaltılmış iNKT hücre hatlarının TRAIL'i hücre yüzeyinde eksprese ettiğini göstermiştir. Buna ek olarak, iNKT hücreleri TRAIL reseptörleri, CD261/DR4 ve CD262/DR5'i büyük oranda hücre içinde eksprese etmektedir. İkincil bir antijenik stimulasyon yüzey TRAIL ekspresyonunu modüle ederken, reseptörlerin ifadelerini değiştirmemiştir. Ayrıca, aktive olmuş iNKT hücre hatları rekombinant insan TRAIL'inden (rhTRAIL) etkilenmemektedir.

Bu hücrelerin sağ kalımları, fenotipleri ve fonksiyonları kronik veya akut rhTRAIL'e maruz kaldıktan sonra değişmemiştir. Bu sonuçlar iNKT hücrelerinin $TRAIL^{+}$ tümörlere karşı kullanılabilecek etkili bir immünoterapötik olabileceğini önermektedir.

Anahtar kelimeler; iNKT hücreleri, TRAIL, CD261/DR4, CD262/DR5

1. OBJECTIVES AND AIMS

iNKT cells are innate-like T cells that combine features of both conventional T cells and NK cells. iNKT cells respond to glycolipid antigens presented on the non-polymorphic MHC I-like CD1d molecules. As iNKT cells develop as memory cells, they can produce various cytokines and exert cytolytic functions upon activation. iNKT cells have been shown to participate in cancer immunosurveillance. Aside from their indirect role in activating other immune cells and shaping the immune milieu of the tumour microenvironment, iNKT cells can directly control tumour formation and tumour load and can prevent metastasis in mice models. Besides the perforin-granzyme pathway of cytotoxicity, iNKT cells can utilize the death ligand, CD178/FASL to eliminate cancer cells. However, the role of CD178/FASL as well as other death ligands in iNKT cell - dependent cytotoxicity has not been studied in detail in mice and humans.

Tumour necrosis factor - related apoptosis inducing ligand (TRAIL) is another death ligand like CD178/FASL, that belongs to the tumour necrosis factor (TNF) super family (TNFSF). Unlike other death ligands, TRAIL is unique due to its specificity for transformed cancer cells. As TRAIL is also well tolerated after systemic administration, it became one of the most attractive immunotherapeutic approaches in cancer. However, recent findings indicated that TRAIL can also be utilized for immune escape by cancer cells. Little is known about the expression of the TRAIL receptors CD261/DR4 and CD262/DR5 on human iNKT cells and the data regarding TRAIL-dependent iNKT cell cytotoxicity against cancer are contradictory. Furthermore, whether iNKT cells can respond to TRAILmediated signalling via CD261/DR4 and CD262/DR5 is unknown. In this thesis, I aimed to address the following aspects of the CD253/TRAIL signalling on human primary iNKT cells and in vitro expanded iNKT cell lines:

(1) Determination of the expression of CD261/DR4, CD262/DR5, and CD253/TRAIL on human primary iNKT cells and in vitro expanded iNKT cell lines under resting conditions and after antigenic activation.

(2) Determination of the impact of chronic CD253/TRAIL signalling on the expansion, phenotype, and function of iNKT cell lines.

(3) Determination of the impact of acute CD253/TRAIL signalling on the phenotype and function of already established iNKT cell lines.

2. LITERATURE REVIEW

2.1 Introduction to Human iNKT Cells: Properties, General Features, and Function

Invariant Natural Killer T (iNKT) cells are CD1d-restricted unconventional T cells [1–3] . CD1d is a highly conserved non-polymorphic MHC class I like molecule expressed on antigen presenting cells (APCs) of mice and humans [1,4,5]. Unlike major histocompatibility complex (MHC) class I molecules, which present proteins to conventional T cells, CD1d presents glycolipid antigens, which are recognized by the semiinvariant T cell receptor (TCR) chain of iNKT cells [6,7]. The semi-invariant TCR chain is composed of the invariant TCR α chain, Vα14-Jα18 in mice and Vα24-Jα18 in humans, which pairs with a limited number of Vβ chains (Vβ7, Vβ8, and Vβ2 in mice, Vβ11 in humans) [1,8,9]. The frequency of iNKT cells differs based on the analysed tissue and species [8,10]. For example, the highest frequency of iNKT cells was detected in the mouse liver [9] and the human omentum (a large adipose tissue in the peritoneum of humans) [11], with lower frequencies in the spleen, lymph nodes, peripheral blood, and lung [4,8,10,11]. iNKT cells combine features of conventional memory T cells and Natural Killer (NK) cells. They lack CD62L, an adhesion molecule for lymph node recruitment, but express markers characteristic of activated-memory T cells (CD45RO, CD69, CD154/CD40L) and NK cells (CD56/NCAM, CD161/NK1.1, CD314/NKG2D) [4,7,10,12]. Furthermore, iNKT cells express higher levels of the cytokine receptors interleukin-12 receptor α (IL-12Rα) and interleukin-18 receptor (IL-18R), which allows them to respond to microenvironmental clues in the absence of the antigen[13,14] . Like conventional T cells, iNKT cells can also express the TCR co-receptors CD4 and CD8. Mice iNKT cells can only express CD4, whereas human iNKT cells can express both, although the $CD4^{-}$ $CD8^{+}$ ($CD8^{+}$) cells are the least frequent subset [15,16]. However, there are phenotypic differences with fewer of the $CD4^{+}$ iNKT cells expressing the NK cell markers CD56, CD161, and CD314/NKG2D, than the $CD4^{-}$ iNKT cells in humans [15,17–19].

There are three modes of activation for iNKT cells, (i) TCR - dependent (direct), (ii) cytokine - dependent (via e.g. IL-12 and IL-18) (indirect), and (iii) CD314/NKG2D - dependent activation [20,21]. Activation of iNKT cells rapidly induces cytokine production

and cytotoxicity, however, each mode modulates the outcome differently. TCR - dependent activation is widely studied in vivo and in vitro using the synthetic analogue of a probably bacterial-derived antigen, α-galactosylceramide (αGalCer) [22]. αGalCer - activated iNKT cells produce both Th_1 cytokines, like interferong (IFNg) and tumour necrosis factor (TNF), and Th_2 cytokines, like interleukin -4 (IL-4), and, therefore, display a Th_0 profile in mice and humans [8,9]. In humans, the Th_0 profile is typical for $CD4^+$ iNKT cells, whereas $CD4^-$ iNKT cells produce less IL-4, leading to a Th_1 profile [15,17–19,23]. In contrast, the cytokine dependent activation with e.g. IL-12 and IL-18, only induces IFNg production by iNKT cells [13]. Both direct and indirect stimulation promotes perforin expression and cytotoxic function of iNKT cells [17]. However, the CD314/NKG2D-dependent activation of iNKT cells only contributes to perforin - dependent cytotoxicity of iNKT cells, either in the absence of an antigen or after a weak antigenic stimulation [24].

2.2 iNKT cells and Cancer: What Do We Know?

2.2.1. The role of iNKT cells in cancer models in mice

aGalCer was discovered due to its anti-tumorigenic activities, which was soon linked to iNKT cells and CD1d [25,26]. Activated iNKT cells can fight tumours directly, due to their cytotoxic activity [27], or by activating NK cells [28–30]. The best therapeutic results so far have been achieved by activating iNKT cells in vivo with injected αGalCerloaded dendritic cells (DCs) [31,32] or tumour cells [33]. However, even after the tumour formed, such treatment was able to reduce tumour metastasis [33], extend survival, and induce long-term protection when given post-surgery [34].

IFNɣ is the main cytokine that promotes protection against tumour metastasis after αGalCer and/or IL-12 administration in different cancer models [28–30,35,36]. Furthermore, the long-term protection of αGalCer was also dependent on functional IFNɣ to prevent tumour formation, delay tumour onset, and tumour load [37]. However, the amount of cytokines produced by iNKT cells and their cytokine profile depends on many variables, including the characteristics of the tumour cells and their ability to present antigens to iNKT cells [38–40].

2.2.2. Cytotoxicity of iNKT cells in cancer

iNKT cells can exert cytotoxic functions via two main effector mechanisms, via perforin and granzyme containing cytotoxic vesicles [7,10] and via death ligands that induce apoptosis in the target cell [41,42]. CD178/FASL, a pro-apoptotic death ligand in the tumour necrosis factor superfamily (TNFSF) [43], can be used by iNKT cells to kill target cells [41]. However, under which conditions iNKT cells primarily use the perforingranzyme [41] or the death ligand [13,42,44] pathway to initiate apoptosis is incompletely understood.

2.3 TRAIL: Mechanism of Function

The death ligand 'tumour necrosis factor related apoptosis inducing ligand' (TRAIL, CD253) was the last member to be identified, based on its sequence homology to CD178/FASL and TNF [45,46]. Like CD178/FASL and TNF, TRAIL has an extracellular cleavage site and can be found in two forms, either as membrane-bound TRAIL (mTRAIL) or as soluble TRAIL (sTRAIL)[46–49].

The receptors for CD253/TRAIL can be categorized into three groups: (i) the functional death receptors (DRs) CD261/DR4 and CD262/DR5, (ii) the decoy receptors DcR1 and DcR2, and (iii) the soluble osteoprotegerin (OPG) receptor [45,48,50]. The two functional receptors have an intact intracellular death domain (DD), which initiates the apoptotic signalling. In contrast, the DcRs and OPG lack the DD, have no apoptotic function, and act as decoy receptors for TRAIL [46–48]. TRAIL, in its trimeric form, can induce the apoptotic (canonical) pathway after its engagement with CD261/DR4 or CD262/DR5[45,46]. Binding of mTRAIL to DRs initiates engagement of other TRAIL-DR pairs in the vicinity to form oligomeric structures for an efficient downstream signalling [49,51]. Clustered DRs recruit FADD (Fas associated death domain) to the DD [52]. FADD then recruits pro-caspase-8 or pro-caspase-10 to form the DISC (death inducing signalling complex), which can cleave the pro-caspases to release their active forms [47,52]. Caspase-8 and caspase-10 are released into the cytosol and further activate the enzymes caspase-3, -6, and -7 which execute apoptosis via the cleavage of various death substrates [46,49]. This is how TRAIL induces apoptosis in TRAIL sensitive cells, which are also called type I cells [46,47,52]. In contrast, type II cells can resist TRAIL-induced apoptosis [16,50,52,53].

Unlike TNF and CD178/FASL, whose expression is restricted, TRAIL is widely expressed in tissues like spleen, thymus, prostate, ovary, small intestine, colon, lung, kidney, and placenta [54]. Therefore, healthy cells need internal mechanisms that block

TRAIL-dependent apoptosis [55]. One of them is c-FLIP (cellular FLICE inhibitor protein), which is a homolog of pro-caspase-8 and pro-caspase-10, which lacks the catalytic subunit [50,52,53]. Therefore, c-FLIP competes with caspase-8 and caspase-10 for binding to the DISC and inhibits its formation [47,53]. Another mechanisms to block TRAIL-dependent apoptosis are the expression of anti-apoptotic proteins of the Bcl-2 and the inhibitor of apoptosis (IAP) family that can counterbalance the apoptotic signals [50,53,56].

Beside the canonical pathway, TRAIL can also induce non-apoptotic (noncanonical) pathways via the formation of a secondary signalling complex following DISC assembly [52,53]. NF-κB (nuclear factor kappa B) and MAPK (mitogen activated protein kinases) family members, like c-Jun, p38 MAPK, and ERKs, are activated downstream of the noncanonical pathways to contribute to proliferation, differentiation, and migration [47,50]. Which pathway is activated downstream of DR - signalling depends on (i) the localization of the receptors related to lipid rafts [45,47,50,51], (ii) the composition of the initial receptor trimer [48,51,52], (iii) the composition of the initial DISC complex [53], and (iv) the activation status of the caspase-8[52] .

2.4 Role of TRAIL in Cancer? Protective Against or Supportive of Cancer?

The initial studies about TRAIL tested its cytotoxicity against various human cancer cell lines of both hematopoietic and non-hematopoietic tissue showed that some cell lines were sensitive, and others were resistant to TRAIL killing [54,57,58]. However, TRAIL attracted the attention, when it was shown that recombinant human TRAIL (rhTRAIL) was non-toxic to normal cells, yet initiated apoptosis in cancer cell lines in vitro [55,59]. In vivo studies further showed that rhTRAIL protected mice against tumour formation when administrated right after the tumour challenge and controlled the tumour load even in already established tumour models [55,59] . Most importantly, systemic administration of rhTRAIL was well tolerated in mice [59] and in non-human primates [55] with no detectable change in clinical parameters.

Many studies on the role of TRAIL in cancer focus on NK cells [28,60–62]. Some hepatic NK cells constitutively express mTRAIL [28,60]. The injection of cytokines (e.g. IL-2, IL-12) or αGalCer into mice can upregulate TRAIL expression on NK cells [28]. TRAIL partially contributes to the cytotoxicity induced by NK cells in vitro [60,63] and in vivo [28,60,62]. TRAIL - mediated control of metastasis by NK cells was also confirmed via experiments in TRAIL-deficient ($TRAIL^{-/-}$) mice or with antagonistic TRAIL - antibodies

[62]. Moreover, these mice were unable to fully control tumour onset [37] and tumour load [62,64,65] of xenograft tumours, which lead to reduced survival[65] . Furthermore, TRAIL$^{-/-}$ aged mice were prone to develop spontaneous tumours [66].

Although in several in vivo studies, the control of tumour development and metastasis by TRAIL was shown, the subsequent clinical trials with TRAIL and agonistic DR-antibodies showed limited success [49]. This is best explained by particular properties of the utilized therapeutics and by mechanisms adopted by cancer cells to become TRAILinsensitive. The only FDA - approved rhTRAIL protein (Dulanermin) used in the clinical trials has a short half-life and lacks efficacy as the receptor aggregation it induces is incomplete [48,51,56]. Similarly, the agonistic aDR-antibodies tested were unable to induce efficient receptor aggregation [67] . Various alternative rhTRAIL versions and antibodies are under development to increase their stability and effectiveness [49]. Furthermore, cancer cells can gain TRAIL-insensitivity by upregulating cFLIP or other antiapoptotic proteins [50,52,56] and can strengthen the non-canonical DR-pathway to promote survival and the dissemination of the cancer [45,47,50] .

2.5 TRAIL on iNKT cells in Health and in Cancer

All studies to date [28,61], with one exception [68], reported no TRAIL expression on mouse iNKT cells but upregulation after a stimulation with IL-2 or αGalCer [68]. There is no information yet on TRAIL expression on primary human iNKT cells. For in vivo expanded iNKT cell lines, either obtained from healthy donor [69] or a cancer patient [70] restimulation with αGalCer induced TRAIL expression. However, the surface expression of TRAIL on patient-derived iNKT cell lines was only transient and declined after three days
[70].

iNKT cell cytotoxicity was shown to be TRAIL-independent in vitro [41]. Also, the adoptive transfer of TRAIL$^{-/-}$ iNKT cells to NSG (‘Nod SCID gamma) mice, which lack T, B, and NK cells, were equally effective to control the tumour formation in vivo as the WT iNKT cells [41]. Patient-derived iNKT cell lines could kill autologous cancer cells via TRAIL in vitro [70] , however, iNKT cell lines generated from healthy donor used mainly perforin and TNF for their cytotoxicity [69].

However, there is no information available to date regarding the expression of the TRAIL receptors, CD261/DR4 and CD262/DR5, on iNKT cells or how iNKT cells respond to TRAIL-signalling in healthy or cancer conditions. For this thesis, I analysed the expression and localization of CD253/TRAIL and its receptors CD261/DR4 and CD262/DR5 on primary iNKT cells ex vivo and on generated iNKT cell lines in vitro. iNKT cells lines were further analysed in detail for any potential phenotypic and functional changes after rhTRAIL exposure.

3. MATERIALS AND METHODS

3.1 Type of the Research

This research is an experimental study, in which we analysed the phenotype and function of iNKT cells from human peripheral blood mononuclear cells (PBMCs) ex vivo and after in vitro expansion.

3.2 Location and Time Frame of the Research

The ethical approval of this study was obtained from the Dokuz Eylül University (see 3.11). The funding was provided by Tübitak (1001, project number 118Z997). The sample collection started after the initiation of the 1001 project in March 2019 and the sample processing and data collection was carried out at the Izmir International Biomedicine and Genome Center (IBG) until the end of September 2022.

3.3 Research Population, Sampling, and Experimental Groups

Residual leukocytic units of healthy blood donors of both sexes were provided by the Dokuz Eylül University (DEU) Blood Bank. These units were used to isolate PBMCs. Donors with chronic diseases, autoimmune diseases, cancer, infections, abnormal blood test results, or abnormal blood counts were excluded from the study. All donors were < 40 years of age at the time of blood donation.

3.4 Research Materials

We used PBMCs isolated from residual leukocytic units of healthy blood donors. We analysed primary iNKT cells in freshly isolated PBMCs ex vivo and in vitro expanded iNKT cell lines.

3.5 **Research Variables** iNKT cells, analysed ex vivo or after in vitro expansion, are the independent variables of the study. The changes in the expression of surface molecules and functional molecules analysed in both primary iNKT cells and iNKT cell lines after CD253/TRAILtreatment are the dependent variables.

3.6 Data Collection Tools

2.6.1. Human samples

The DEU Blood Bank (Izmir, Turkey) provided the residual leukocyte units of healthy blood donors after obtaining the informed consent from all donors. All protocols performed were in accordance with the relevant guidelines and regulations for human samples.

2.6.2. Purification of PBMCs

PBMCs were isolated from the residual leukocyte units of healthy donors via density gradient centrifugation. Residual leukocyte units were processed within the first 20 hours after collection and stored at 4°C until processing. The cell suspension from the residual leukocyte units were diluted 1:1 with 1x PBS, laid over Ficoll-Paque PLUS (GE Healthcare), and centrifuged at room temperature (RT) for 30 minutes at 400 g without brakes. The PBMCs, located above the Ficoll-Paque layer, were collected and washed twice with 1x PBS at RT for 7 minutes at 400 g. Each sample were treated with 1 ml red blood cell lysis buffer (ACK Lysis buffer, Lonza) for 3 minutes and washed twice with 1x PBS for 7 minutes.

2.6.3. Generation of human iNKT cell lines

Freshly isolated PBMCs were seeded (1 x 10^6 cell/ml, 5 ml/well) with 100 ng/ml αGalCer (KRN7000, Avanti Polar Lipids) containing RPMI-T media (RPMI 1640 medium (Gibco) supplemented with 5% (v/v) human AB serum (Sigma), 1% (v/v) Penicillin/Streptomycin (Gibco), 1 mM sodium pyruvate (Lonza), 1% (v/v) non-essential amino acids (Cegrogen), 15 mM HEPES buffer (Sigma), and 55 µM 2-mercaptoethanol (AppliChem)) and cultured for 13 days at 37°C in an CO_2 (5%) incubator. Cultured cells were supplied with recombinant human IL-2 (rhIL-2, Proleukin, Novartis) every other day, starting from day two. For the first four days, 20 IU/ml rhIL-2 was added to the cultures. From day six onwards the cultured samples were supplemented with 40 IU/ml rhIL-2. Each sample was collected and counted on day 13 and the frequency of iNKT cells was

determined by flow cytometry. iNKT cells were identified as either live $CD14^-$ $CD20^-$ $CD3^+$ $iV\alpha24^+$ (6B11) cells (day 0, day 13) or as live $CD3^+$ $V\alpha24^+$ cells (day 13). The fold expansion of iNKT cells for each sample was calculated with the following equation: (total number of live cells collected on day 13 * the frequency of $CD3^+$ $6B11^+$ or $V\alpha24^+$ on day 13) / (seeded PBMC number * percentage of $CD3^+$ $6B11^+$ cells in the starting population on day zero).

2.6.4. Treatment of iNKT cell lines with recombinant human CD253/TRAIL

The optimal concentration of recombinant human TRAIL (rhTRAIL, R&D Systems) to be used was determined by a dose optimization experiment. Expanding iNKT cell lines were treated with 10 ng/ml, 50 ng/ml, 100 ng/ml, or 200 ng/ml rhTRAIL either (i) on day 8 and every other day thereafter for the chronic stimulation or (ii) on day 12 for the last 16 hours for the acute stimulation. On day 13, samples of each stimulation condition were collected, counted, and analysed directly or used for functional studies. Following the optimization experiments, 10 ng/ml rhTRAIL was chosen as the concentration used in this study.

2.6.5. In vitro stimulation of iNKT cells

For the ex vivo stimulation of iNKT cells, freshly isolated PBMCs were incubated either (i) with 100 ng/ml αGalCer for 4h (direct stimulation) or (ii) with 100 ng/ml LPS for 4h or 24h in vitro (indirect stimulation). iNKT cell lines require antigen presenting cells for the antigen (αGalCer) - specific stimulation. Therefore, on day 12 of every ongoing expansion, an aliquot of autologous PBMCs for each sample was thawed and seeded with 100 ng/ml αGalCer in a separate six well plate overnight for the antigen-loading of the monocytes. The number of PBMCs seeded to stimulate iNKT cells was based on the frequency of monocytes, as to keep the number of monocytes per well constant among samples. The iNKT cell lines were collected, counted, and added on top of their matched loaded and washed PBMCs at a monocyte to iNKT cell ratio of 1:1. The cells were incubated for 4 hours at 37°C in the presence of Brefeldin A (Golgi Plug, 0.5 µl/ml) and Monensin (Golgi stop, 0.33 µl/ml) (both BD Biosciences). PBMCs seeded without αGalCer

but cocultured with their autologous iNKT cell lines and iNKT cell lines seeded alone served as negative controls. iNKT cell lines alone treated with 25 ng/ml PMA (SigmaAldrich) and 1 µg/ml ionomycin (Sigma-Aldrich) served as positive controls.

2.6.6. Flow cytometric analysis

For the live-dead discrimination, cell suspensions were resuspended in 1x PBS containing Zombie UV (1:500) or Zombie NIR (1:2000) (both BioLegend) and incubated on ice for 10 min. The samples were washed twice with 1x PBS (5 min, 4°C, 400 g). For the surface staining, the antibodies of choices were added to FACS buffer (1X PBS, 1% (v/v) FBS, 0.01% (w/v) sodium azide (Sigma-Aldrich)) containing Human TruStain FcX (1:200) (BioLegend). Human TruStain FcX binds to Fc receptors and blocks the unspecific binding of antibodies to Fc receptors on the cells. Samples were resuspended in 50 µl of the antibody-containing solution and incubated on ice for 15 min in the dark. For experiments without intracellular staining, the samples were washed twice with 1x PBS (5 min, 4°C, 400 g) and either analysed immediately or fixed with Cytofix (BD Biosciences) on ice for 10 minutes. Fixed samples were washed twice with 1X PBS (5 min, 4°C, 400 g) and once with FACS buffer and stored at 4°C in the dark. For experiments with intracellular staining, the surface-stained samples were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) at 37°C for 10 minutes. The samples were washed twice with Perm/Wash buffer (BD Biosciences) and incubated with the antibodies of choices in Perm/Wash buffer overnight at 4°C in the dark. The next day, samples were washed twice with Perm/Wash buffer (BD Biosciences) and once with
FACS buffer and stored at 4°C in the dark. The samples were measured with a BD LSRFortessa cell analyser (BD Biosciences) and analysed with Flow Jo (Tree Star) software.

Primary iNKT cells were identified as live $CD14^-$ $CD20^-$ $CD3^+$ $iV\alpha24^+$ (6B11) cells. In vitro expanded iNKT cells were identified as live $CD3^+$ $V\alpha24^+$ cells. B cells were identified as live $CD14^-$ $CD3^-$ $CD56^-$ $CD20^+$ cells and monocytes were identified as live $CD3^-$ $CD56^-$ $CD20^-$ $CD14^+$ cells. Detailed information on the antibodies used in this study are given in **Table 1**.

Table 1. Human monoclonal antibody list					
Marker	**Clone**	**Conjugate**	**Vendor**	**Identifier**	**Dilution**
CD3	UCHT1	BUV395	BD Biosciences	Cat#563546, RRID:AB_2744387	1/400
CD4	OKT-4	BV605	BioLegend	Cat#317438, RRID:AB_11218995	1/200
CD4	RPA-T4	BV785	BioLegend	Cat#300553, RRID:AB_2564381	1/400
CD8α	RPA-T8	AF700	BioLegend	Cat#301028, RRID:AB_493745	1/800
CD14	M5E2	V500	BD Biosciences	Cat#561391, RRID:AB_10611856	1/200
CD14	M5E2	BV510	BioLegend	Cat#301842, RRID:AB_2561946	1/200
CD20	2H7	BV570	BioLegend	Cat#302332, RRID:AB_2563805	1/200
CD56	MY31	FITC	Tonbo Biosciences	Cat#35-0564-T100	1/100
CD56	HCD56	PE-CF594	BioLegend	Cat#318347, RRID:AB_2563563	1/100
CD62L	DREG56	BV650	BioLegend	Cat#304832, RRID:AB_2563821	1/100

CD95/ FAS	DX2	APC-Cy7	BioLegend	Cat#305635, RRID:AB_2566108	1/400
CD69	FN50	AF700	BioLegend	Cat#310921, RRID:AB_493774	1/100
CD161	HP-3G10	BV421	BioLegend	Cat#339914, RRID:AB_2561421	1/40
CD161	HP-3G10	Pacific Blue	BioLegend	Cat#339926, RRID:AB_2563960	1/50
CD178/ FASL	NOK1	BV421	BioLegend	Cat#306411, RRID:AB_2716104	1/800
CD253/ TRAIL	RIK-2	PE	BioLegend	Cat#308205, RRID:AB_345291	1/50
CD253/ TRAIL	RIK-2	APC	BioLegend	Cat#308209, RRID:AB_2564397	1/50
CD261/ DR4	DJR1	APC	BioLegend	Cat#307208, RRID:AB_2204667	1/50
CD261/ DR4	DJR1	PE-Cy7	BioLegend	Cat#307209, RRID:AB_2814152	1/50
CD262/ DR5	DJR2-4	APC	BioLegend	Cat#307407, RRID:AB_2204813	1/50
CD262/ DR5	DJR2-4	PE	BioLegend	Cat#307406, RRID:AB_2204926	1/50

Granzyme B	QA16A02	PerCP-Cy5.5	BioLegend	Cat#372211, RRID:AB_272837	1/50

GM-CSF	BVD2-21C11	Pacific Blue	BioLegend	Cat#502313, RRID:AB_2561838	1/800
HLA-DR	L243	BV711	BioLegend	Cat#307644, RRID:AB_2562913	1/100
HLA-DR	L243	APC-Cy7	BioLegend	Cat#307618, RRID:AB_493586	1/100
IFNɣ	4S.B3	BV785	BioLegend	Cat#502541, RRID:AB_11219192	1/100
IL-2	MQ1-17H12	BV650	BioLegend	Cat#500334, RRID:AB_2563878	1/100
IL-4	MP4-25D2	FITC	BioLegend	Cat#500807, RRID:AB_315126	1/200
IL-4	MP4-25D2	APC	BioLegend	Cat#500812, RRID:AB_315131	1/400
IFNɣ	4S.B3	PE	eBiosciences	Cat#12-7319-82, RRID:AB_1272026	1/800
iVα24	6B11	BV711	BioLegend	Cat#342922, RRID:AB_2572068	1/80
iVα24	6B11	BV785	BioLegend	Cat#342932, RRID:AB_2814264	1/200

Perforin	B-D48	FITC	BioLegend	Cat#353309, RRID:AB_2571966	1/50
TNF	Mab11	PerCP-Cy5.5	eBiosciences	Cat#45-7349-73, RRID:AB_953664	1/100
TNF	Mab11	AF700	BioLegend	Cat#502928, RRID:AB_2561315	1/200

3.7 Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). For all experiments, at least three samples were analysed per group, and all experiments were repeated at least twice with different donors at each time point. Collected data were pooled and tested using D'Agastino – Pearson Test for normal distribution. Statistical comparisons of two values were drawn for normally distributed data with a two-tailed paired Student's ttest and for non-normally distributed data with Wilcoxon matchedpairs signed rank test. Statistical analysis of non-normally distributed grouped samples was performed with Friedman's test (GraphPad Prism, GraphPad Software, USA). pvalues <0.05 were considered significant and are indicated with (*p < 0.05, **p < 0.01, ***p< 0.001). Graphs were generated with GraphPad Prism (GraphPad Software) and edited with Adobe Illustrator (Adobe).

3.8 Research plan and timeline

Table 2. Research timeline and plan

Year	2018				2019				2020				2021				2022			
Quarter	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Ethical approval		X																		
Thesis proposal						X	X	X												
Optimization of the iNKT cell line protocol							X	X	X	X										

	Determination of the expression levels of markers of interests in primary iNKT cells and expanded iNKT cell lines.											X	X	X	X	X	X				
	Evaluation of phenotype and function of primary and expanded iNKT cells. after antigenic stimulation.												X	X	X	X					
	Determination of the impact of rhTRAIL on the phenotype and function of iNKT cell lines in vitro														X	X	X	X	X	X	
Data analysis and reporting						X				X				X				X			
Thesis defence date																					

Data Collection	Determination of the expression levels of markers of interest after direct and indirect stimulation.												X	X							

3.9 Data Analysis

Raw data obtained from BD LSR-Fortessa cell analyser (BD Biosciences) device were analysed with Flow-Jo software (Tree star). Analysed data of positive frequency or geometric mean fluorescence intensity (GeoMean) were used as the input for the statistical analysis. In functional experiments, the baseline positive frequency of cytokines being analysed was subtracted from the test and positive control samples before any statistical analysis was performed. The plots that show the gating strategy used to identify iNKT cells ex vivo and the iNKT cells during the expansion were obtained from the Flow Jo software after the analysis.

3.10 Limitations of the research

There were no limitations.

3.11 Ethical committee approval

The 'Noninvasive Research Ethics Committee' of the Dokuz Eylül University approved this work with human material (Title: "The role of TRAIL mediated signals on the proliferation and function of human iNKT cells"; approval number: 2018/0627/3801GOA).

4. RESULTS

4.1 Determination of CD253/TRAIL, CD261/DR4, and CD262/DR5 expression on primary iNKT cells at the basal level

In all our experiments, we used residual leukocytic units of healthy donors that we obtained from the blood bank of DEU (Ethics approval no. 2018/06-27/3801-GOA) as the starting material to isolate PBMCs. The residual leukocytic unit is the leftover material of the processed blood which is enriched for leukocytes but poor in erythrocytes, thrombocytes, and plasma (**Figure 1**).

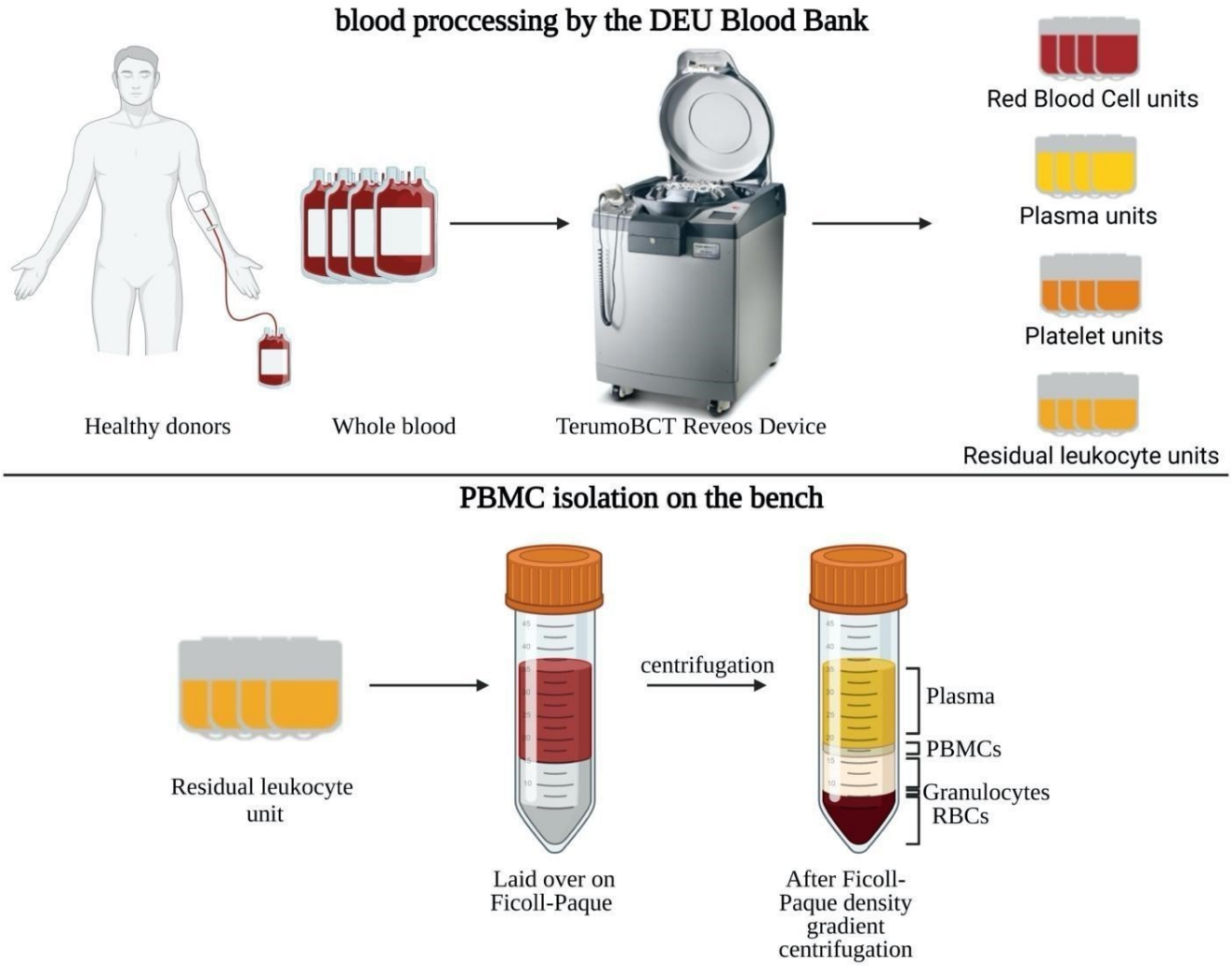


Figure 1. Isolation of PBMCs from healthy donors.

Whole blood of healthy blood donors was processed by the DEU Blood Bank with a Reveos automated blood processor in a 20-minute centrifugation step. The processed blood components are the red blood cell unit, platelet unit, plasma unit, and residual leukocyte unit (upper panel). The leukocyte enriched residual leukocyte units were used as the starting material to isolate PBMCs with Ficoll-Paque gradient centrifugation (lower panel). Each residual leukocyte unit was diluted with 1:1 with 1X PBS and laid over Ficoll-Paque gradient. The samples were centrifuged at 400 g for 30 minutes without breaks at RT. After centrifugation, the thin white layer corresponding to the PBMCs between the plasma and Ficoll-Paque was collected and used for further experiments.

We isolated PBMCs from each residual leukocytic unit via Ficoll-Paque gradient centrifugation. The iNKT cells were identified ex vivo in the isolated PBMCs with fluorophore-conjugated antibodies specific for the complementary determining region 3 (CDR3) region of the invariant Vα24-Jα18 chain (clone 6B11) and CD3, the T cell marker, within the live population. We also used antibodies to identify B cells (CD20), monocytes (CD14), and Natural Killer cells (CD56). A representation of the gating tree that we used to identify iNKT cells ex vivo is given in **figure 2**.

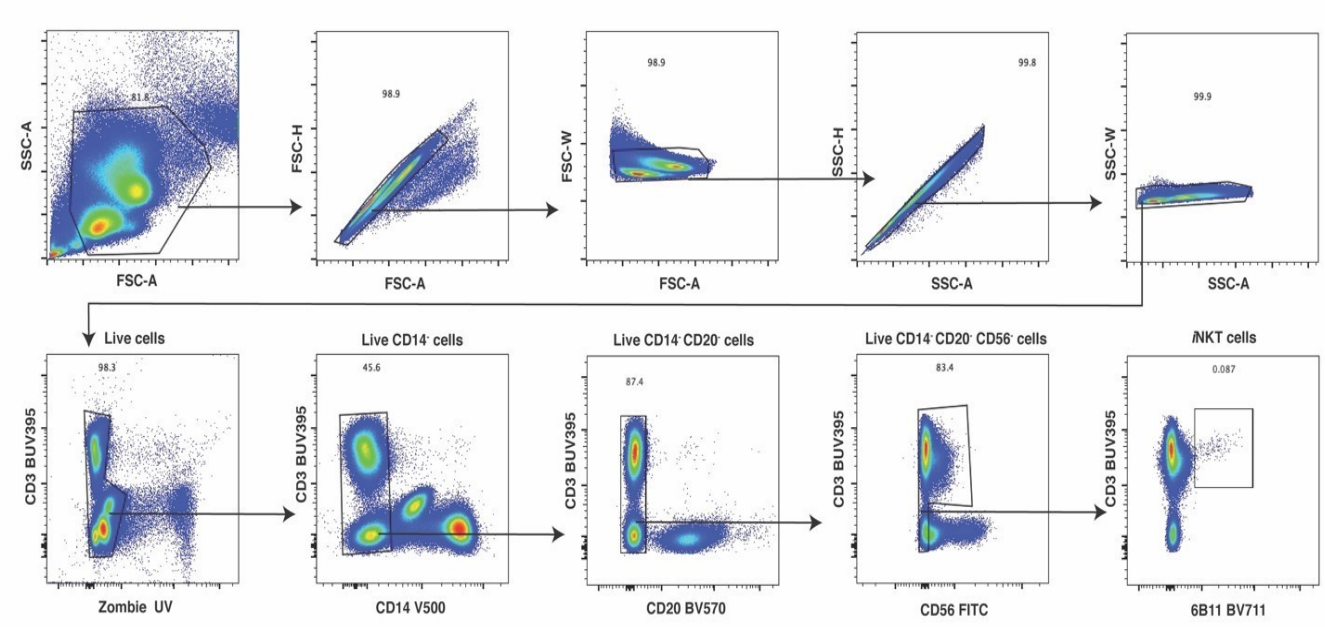


Figure 2. The gating tree for iNKT cell identification applied for the ex vivo analysis of PBMCs.

For each sample, single cell populations were identified in height (H) vs. area (A) and width (W) vs. area (A) plots of forward and side scatter. Zombie UV or NIR viability dyes were used to identify the live cells. Within the live population, we used CD14 (V500), CD20 (BV570), CD56 (FITC) as dump channels to exclude monocyte, B cells, and NK cells, respectively. iNKT cells were defined as live $CD14^-$ $CD20^-$ $CD56^-$ $CD3^+$ $6B11^+$ cells.

We evaluated the basal expression of CD253/TRAIL, CD261/DR4, and CD262/DR5 in primary iNKT cells at the basal level. B cells were used as negative control and monocytes as positive control for the expression of each marker [71]. We initially analysed intracellular and surface expression of CD253/TRAIL, CD261/DR4, and CD262/DR5 separately.

No surface CD253/TRAIL expression on primary iNKT cells, monocytes, or B cells was detected (**Figure 3A**). As expected [72,73], monocytes expressed intracellular TRAIL (**Figure 3B**). Relative to the signals that we obtained from our positive (monocytes) and negative (B cells) controls; we detect a very weak intracellular CD253/TRAIL expression on iNKT cells (**Figure 3B**). The data indicates that primary iNKT cells analysed ex vivo have no surface or intracellular CD253/TRAIL expression.

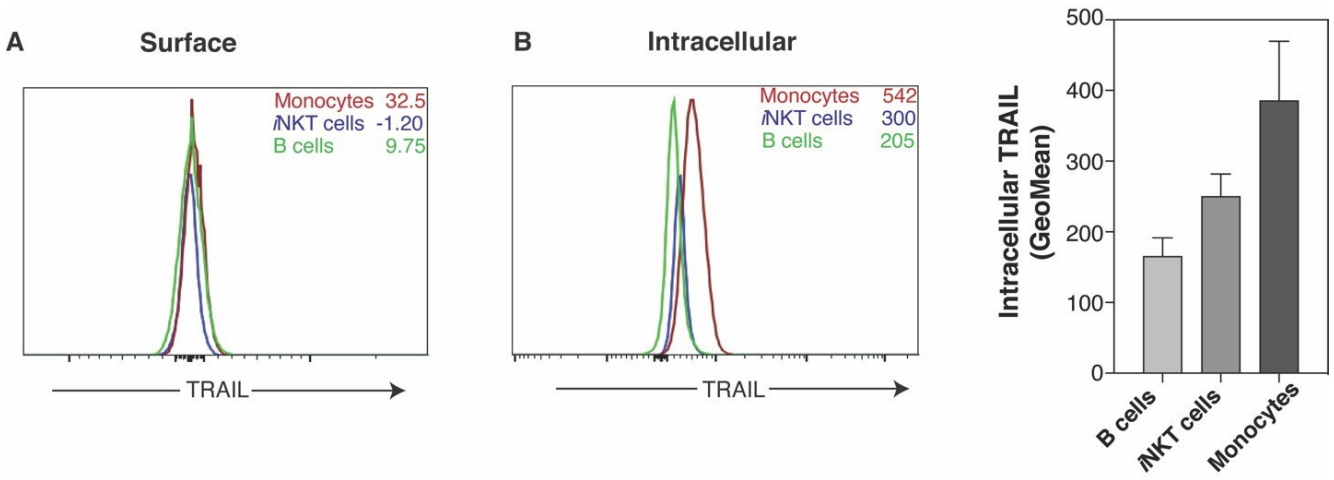


Figure 3. Surface and intracellular expression of CD253/TRAIL on primary iNKT cells.

PBMCs were isolated from healthy donors and prepared individually for flow cytometric analysis. **(A)** Surface (surface stain with αCD253/TRAIL-PE antibodies) and **(B)** intracellular (intracellular stain with αCD253/TRAIL-APC antibodies) specific CD253/TRAIL expression is shown for iNKT cells (Live $CD14^-$ $CD20^-$ $CD3^+$ $iV\alpha24^+$ (6B11) cells), B cells (Live $CD3^-$ $CD14^-$ $CD56^-$ $CD20^+$ cells), and monocytes (Live $CD3^-$ $CD20^-$ $CD56^-$ $CD14^+$ cells). Representative histograms for surface and intracellular CD253/TRAIL are given in **(A)** and **(B)**. A summary graph is given for the intracellular CD253/TRAIL expression only in **(B)**. The figure shows representative data of one of three independent experiments performed with three donors (n = 3).

We next analysed the surface and intracellular expression of CD261/DR4 and CD262/DR5 with the same approach. No surface or intracellular CD261/DR4 expression was detected in primary iNKT cells analysed ex vivo (**Figure 4**).

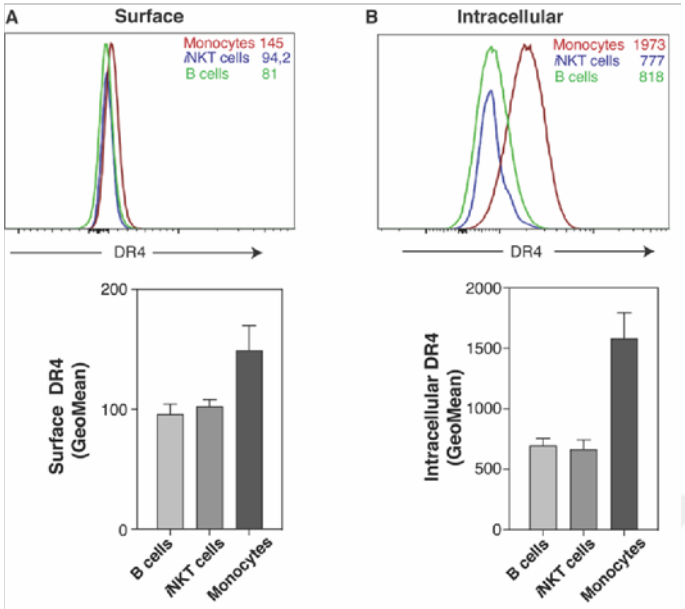


Figure 4. Surface and intracellular CD261/DR4 expression on primary iNKT cells.

PBMCs were isolated from healthy donors and prepared individually for flow cytometric analysis. **(A)** Surface (surface stain with αCD261/DR4-APC antibodies) and **(B)** intracellular (intracellular stain with αCD261/DR4PE-Cy7 antibodies) specific CD261/DR4 expression is shown for iNKT cells (Live $CD14^-$ $CD20^-$ $CD56^-$ $CD3^+$ $V\alpha24^+$ (6B11) cells), B cells (Live $CD3^-$ $CD14^-$ $CD56^-$ $CD20^+$ cells), and monocytes (Live $CD3^-$ $CD20^-$ $CD56^-$ $CD14^+$ cells). The top panels show representative histograms and the bottom panels the corresponding summary data. The figure shows representative data of one of three independent experiments performed with four different donors (n = 4).

i

For CD262/DR5, we detected no surface or intracellular expression on primary iNKT cells (**Figure 5**).

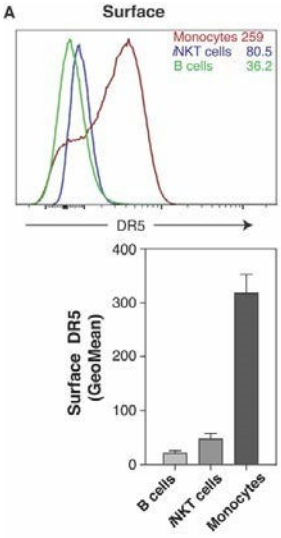


Figure 5. Surface and intracellular CD262/DR5 expression on primary iNKT cells.

PBMCs were isolated from healthy donors and prepared individually for flow cytometric analysis. **(A)** Surface (surface stain with αCD262/DR5-PE antibodies) and **(B)** intracellular (intracellular stain with αCD262/DR5APC antibodies) specific CD262/DR5 expression is shown for

NKT cells (Live $CD14^-$ $CD20^-$ $CD3^+$ $iV\alpha24^+$ (6B11) cells), B cells (Live $CD3^-$ $CD14^-$ $CD56^-$ $CD20^+$ cells), and monocytes (Live $CD3^-$ $CD20^-$ $CD56^-$ $CD14^+$ cells). The top panels show representative histograms and the bottom panels the corresponding summary data. The figure shows representative data of one of two independent experiments performed with five different donors (n = 5).

i

Therefore, primary iNKT cells did not express CD253/TRAIL or its receptors CD261/DR4 or CD262/DR5. As we focus on measuring the expression of these markers on primary iNKT cells independent of their localization, we continued our analysis with their total expression, i.e. combined surface and intracellular measurement. At the basal state, we detected no CD261/DR4 or CD262/DR5 on primary iNKT cells (**Figure 6 A, B**) however, they expressed CD95/FAS (**Figure 6C**). For the ligands of these receptors, no TRAIL (**Figure 6D**) or CD178/FASL (data not shown) was detected on the resting primary iNKT cells.

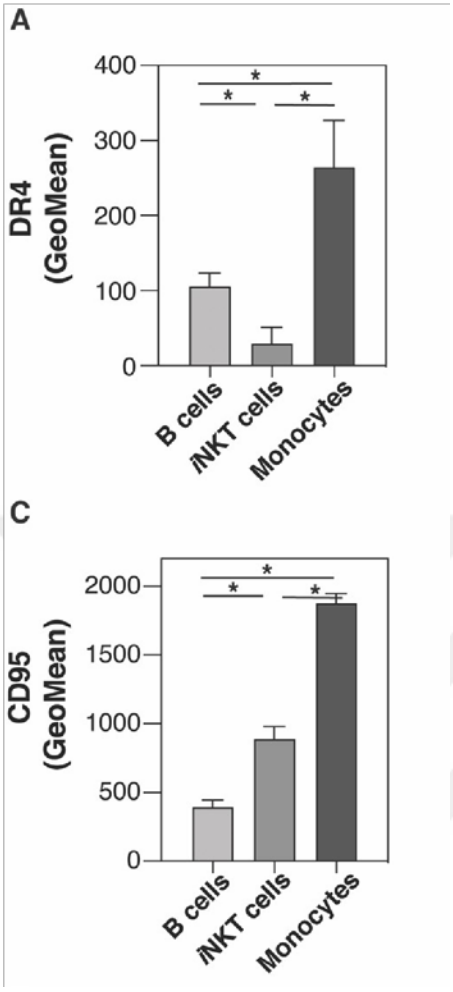


Figure 6. Total expression of CD95/FAS, CD253/TRAIL, CD261/DR4, and CD262/DR5 on primary iNKT cells.

PBMCs were isolated from healthy donors and prepared individually for flow cytometric analysis. Expression of total **(A)** CD261/DR4, **(B)** CD262/DR5, **(C)** CD95/FAS, and **(D)** CD253/TRAIL is shown for iNKT cells (Live $CD14^-$ $CD20^-$ $CD3^+$ $V\alpha24^+$ (6B11) cells), B cells (Live $CD3^-$ $CD14^-$ $CD56^-$ $CD20^+$ cells), and monocytes (Live $CD3CD20CD56^-$ $CD14^+$ cells). The figure shows representative data of one of three independent experiments with six different donors (n = 6).

i

The contribution of a small positive population within the iNKT cell gate to the GeoMean data of these markers among all iNKT cells might not be obvious. Therefore, we reanalysed the data for the frequencies of iNKT cells expressing each marker. Very low numbers of primary iNKT cells were positive for the death ligands CD253/TRAIL and CD178/FASL (**Figure 7A**). The frequency of $CD95/FAS^+$ iNKT cells (**Figure 7A**) correlated/reflected the GeoMean data of CD95/FAS on these cells (**Figure 6C**). Very few primary iNKT cells were positive for C261/DR4 or CD262/DR5 (**Figure 7A**). The cytotoxic markers perforin and granzyme B were also expressed by a small fraction of primary iNKT cells at the basal level (**Figure 7A**). Some resting iNKT cells were positive for the naïve T cell marker CD62L and for the NK cell markers CD56/NCAM and CD161/KLRB1 (**Figure 7B**). We also check the expression of the coreceptors, CD4 and CD8, on resting primary iNKT cells. Primary iNKT cell were mainly $CD4^+$ or $CD4^-$ $CD8^-$ (double negative, DN), with smaller fractions being $CD8^+$ (**Figure 7C**).

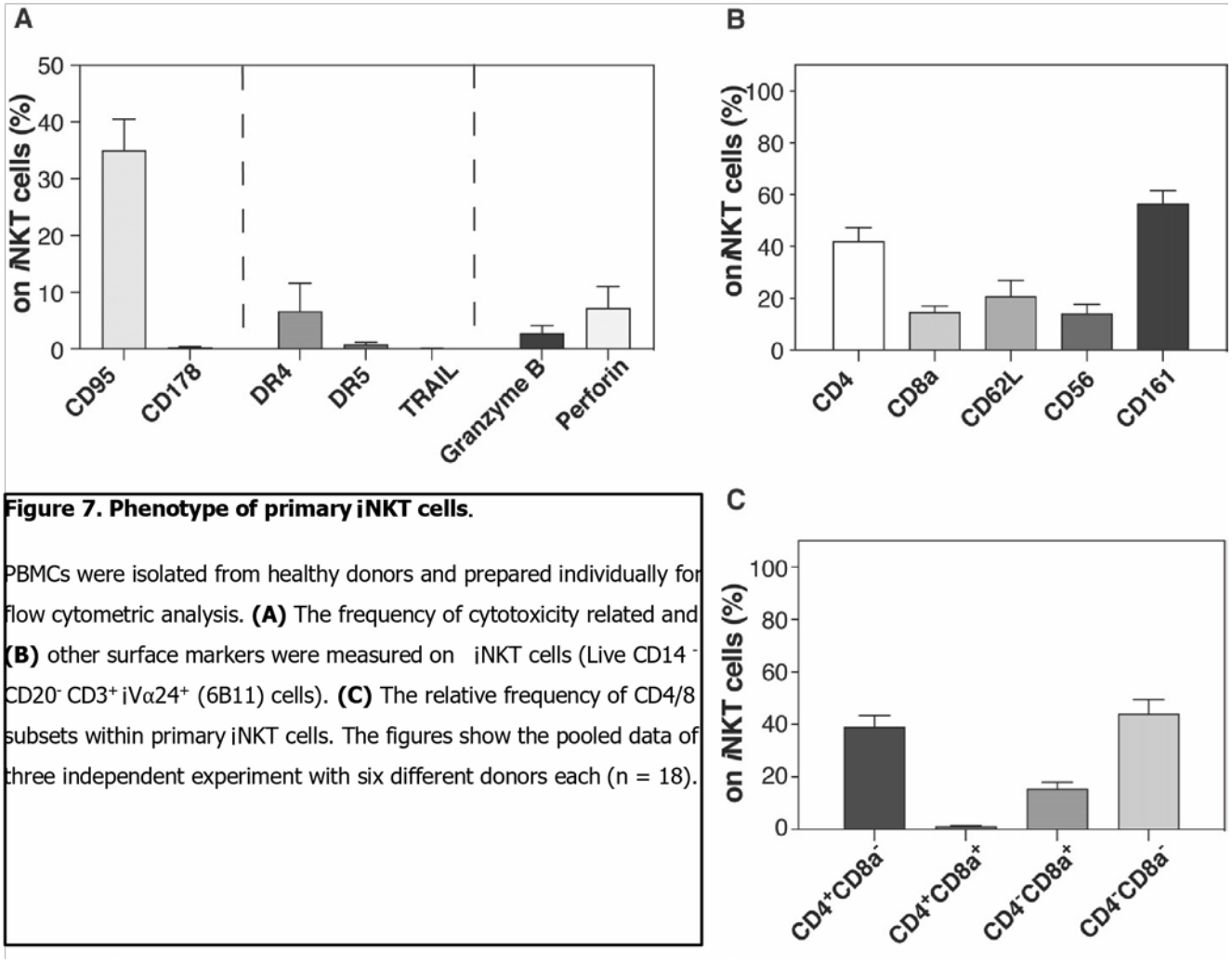


Figure 7. Phenotype of primary iNKT cells.

PBMCs were isolated from healthy donors and prepared individually for flow cytometric analysis. **(A)** The frequency of cytotoxicity related and **(B)** other surface markers were measured on iNKT cells (Live CD14$^-$ CD20$^-$ CD3$^+$ iVα24$^+$ (6B11) cells). **(C)** The relative frequency of CD4/8 subsets within primary iNKT cells. The figures show the pooled data of three independent experiment with six different donors each (n = 18).

4.2 Evaluation of CD253/TRAIL, CD261/DR4, and CD262/DR5 expression on stimulated primary iNKT cells

iNKT cells respond to αGalCer presented on CD1d. Additionally, resting iNKT cells express IL-12R and IL-18R, allowing iNKT cells to respond to IL-12 and IL-18 and getting activated in the absence of TCR-dependent stimulation [14,74]. Monocytes, activated by lipopolysaccharide (LPS) through the toll like receptor 4 (TLR-4), secretes IL-12 and IL18 [74] . Therefore, we stimulated PBMCs either with αGalCer or LPS to trigger antigendependent and -independent activation of iNKT cells.

After stimulation with αGalCer or LPS, iNKT cells upregulated expression of CD253/TRAIL (**Figure 8A**) and CD262/DR5 (**Figure 8C**) but not CD261/DR4 (**Figure 8B**). The monocytes in the co-cultures upregulated CD253/TRAIL expression too (**Figure 8 D**) but downregulated CD261/DR4 (**Figure 8E**). In contrast, the expression of CD262/DR5 on monocytes was unchanged by the stimulations (**Figure 8F**).

4.3 Expression of CD253/TRAIL, CD261/DR4, and CD262/DR5 on iNKT cell lines.

The relative frequency of iNKT cells is low in human PBMCs (0.01-0.5% of the T cell population)[4,9,10] . This limits the functional studies that can be performed with primary iNKT cells ex vivo. To overcome this, we expanded iNKT cells in vitro to generate iNKT cell lines.

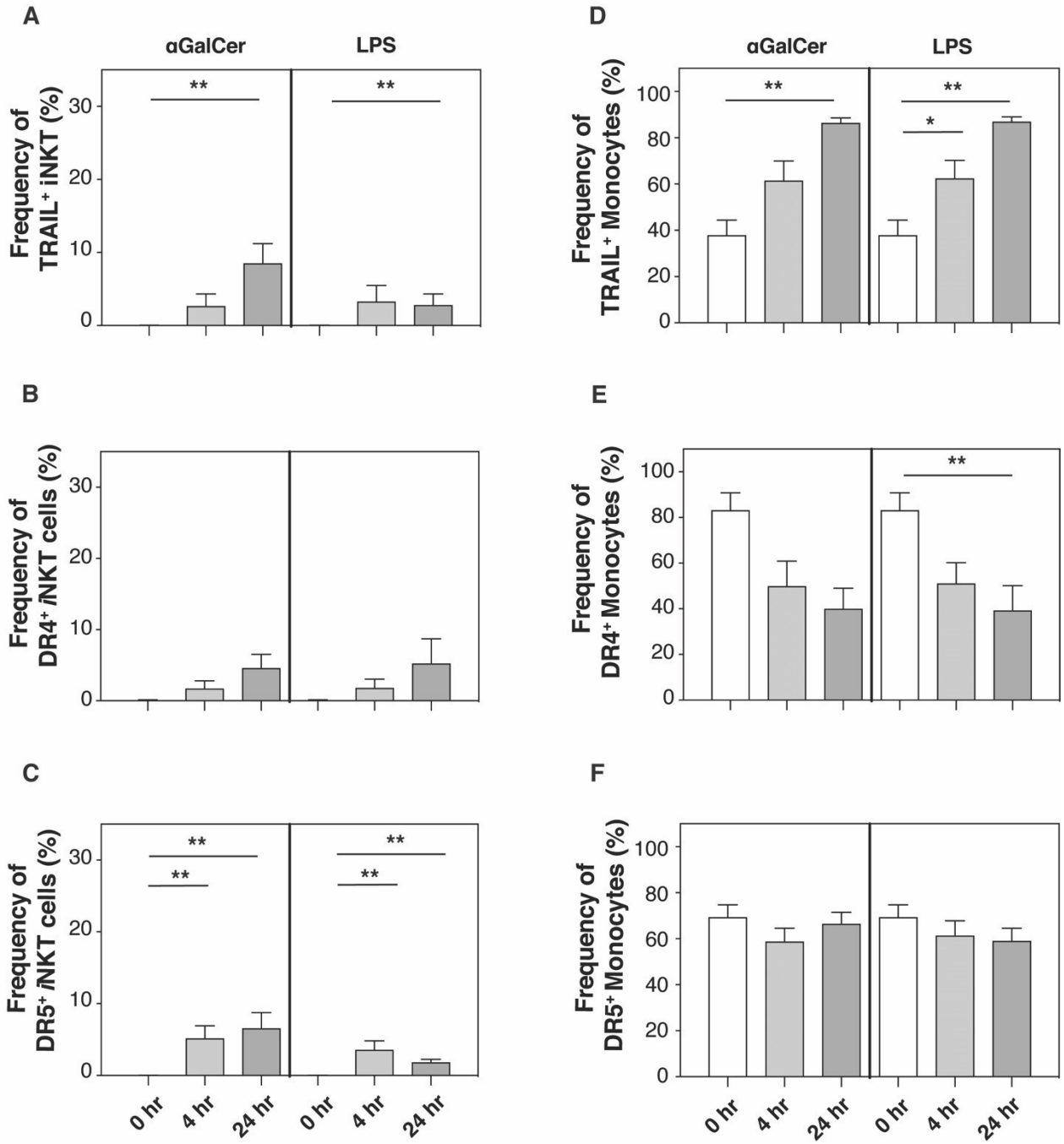


Figure 8. Impact of direct and indirect stimulation on the expression of CD253/TRAIL, CD261/DR4, and CD262/DR5 on primary iNKT cells and monocytes.

PBMCs were purified from healthy donors and stimulated in vitro with either 100 ng/ml αGalCer or with 100 ng/ml LPS for the indicated time points. The global expression (surface and intracellular) of **(A, D)** CD261/DR4, **(B, E)** CD262/DR5, and **(C, F)** CD253/ TRAIL in iNKT cells ($CD14^-$ $CD20^-$ $CD3^+$ $iV\alpha24^+$ (6B11) cells) **(A, B, C)** or monocytes ($CD3^-$ $CD20^-$ $CD14^+$ cells) (**D, E, F**) is shown. Pooled data of three independent experiments with three donors each (n = 9) are shown.

The most common method to generate iNKT cell lines is to culture PBMCs in the presence of the prototypic iNKT cell antigen αGalCer and the cytokine IL- 2 (rhIL-2) (**Figure 9A**). PBMCs contain APCs such as monocytes, dendritic cells, and B cells which

all express CD1d and can present αGalCer. PBMCs were cultured with αGalCer and rhIL2 for 13 days. **Figure 9B** shows representative data of the iNKT cell expansion for day 0, 7, 10, and 13. With this expansion protocol, about half of the cells remaining in the culture were iNKT cells on day 13 (**Figure 9C**). We also analysed the fold expansion of the iNKT cells based on the absolute cell count and the frequency of iNKT cells within the live population that we obtained from the flow analysis software (**Figure 9D**). We used day 0 iNKT cell frequency and seeded PBMC number as a reference point.

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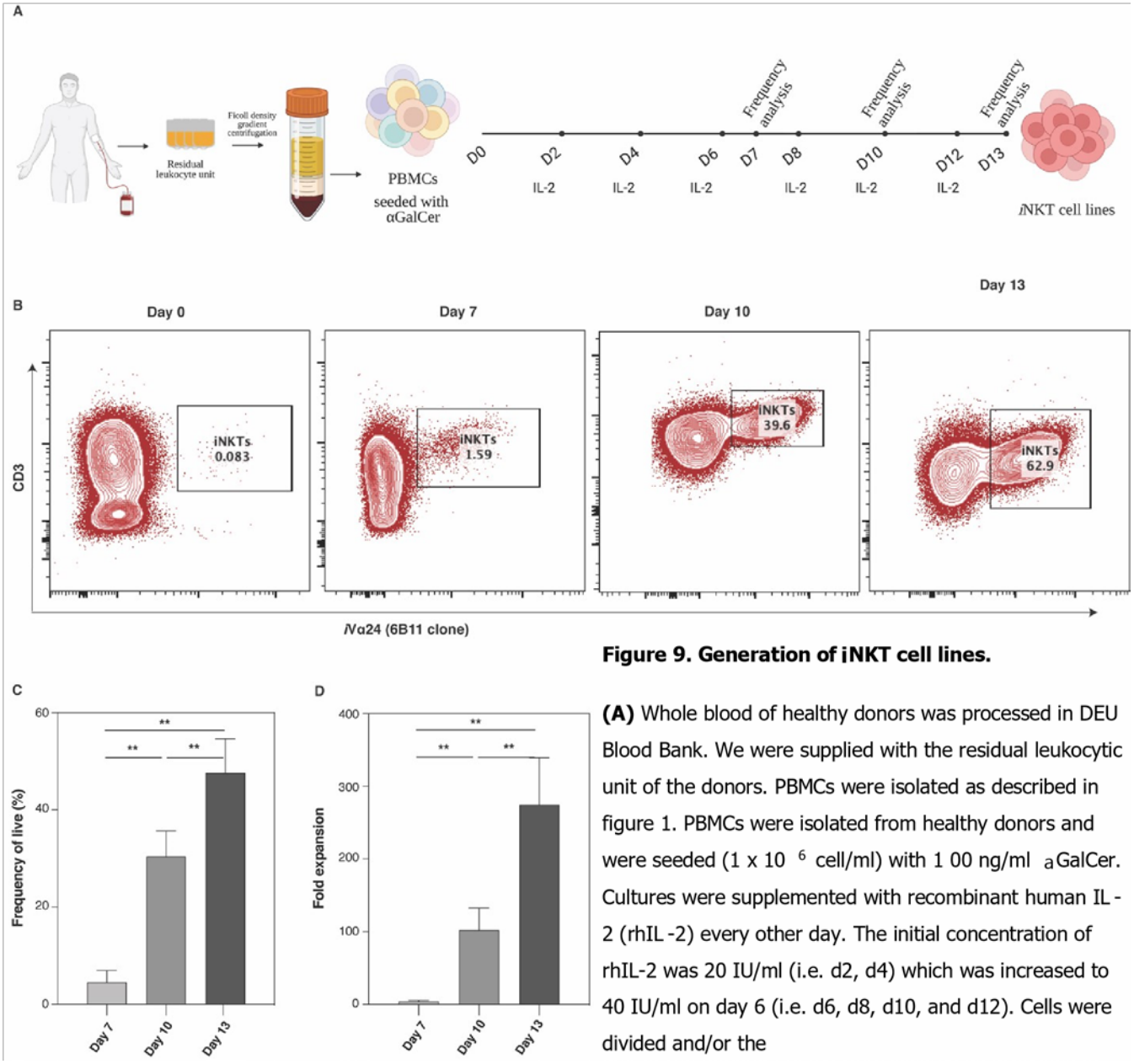


Figure 9. Generation of iNKT cell lines.

(A) Whole blood of healthy donors was processed in DEU Blood Bank. We were supplied with the residual leukocytic unit of the donors. PBMCs were isolated as described in figure 1. PBMCs were isolated from healthy donors and were seeded (1 x 10^6 cell/ml) with 1 00 ng/ml αGalCer. Cultures were supplemented with recombinant human IL-2 (rhIL-2) every other day. The initial concentration of rhIL-2 was 20 IU/ml (i.e. d2, d4) which was increased to 40 IU/ml on day 6 (i.e. d6, d8, d10, and d12). Cells were divided and/or the medium replenished when needed. On indicated days, iNKT cells (live $CD3^+$ $CD20^-$ $iV\alpha24^+$ cells) were analysed by flow cytometry. **(B)** Representative data of the expansion of iNKT cell lines from one sample. **(C)** Frequency of iNKT cells in the live population of cultured cell lines. The figures B and C show pooled data from 3 independent experiments (n = 10). **(D)** The fold expansion of iNKT cells at indicated time points. The fold expansion was determined as follows: (total number of live cells recovered on day X * percentage of $CD3^+$ $iV\alpha24^+$ cells on day X) / (total numbers of seeded PBMCs * percentage of $CD3^+$ $iV\alpha24^+$ cells in the starting population on day 0) (n = 10).

iNKT cells can change their phenotype during such an expansion [16]. Therefore, we determined the expression of several common markers on the expanded iNKT cell lines. We first checked the distribution of the CD4 and CD8 coreceptors (**Figure 10**). After the expansion, the $CD4^+$ single positive population became the predominant subpopulation in

the generated iNKT cell lines, which was accompanied with a reduced frequency of DN and $CD8^+$ iNKT cells (**Figure 10A**). Human $CD4^+$ iNKT cells differ phenotypically and functionally from $CD8^+$ and DN cells [15,16]. $CD4^+$ iNKT cells have a Th_0 profile, characterized by the production of both Th_1 (IFNγ, TNF) and Th_2 (IL-4) cytokine, whereas DN and $CD8^+$ iNKT cells have a Th_1 profile, as they produce less IL-4 [16,75,76]. Furthermore, both $CD8^+$ and DN iNKT cells are more cytotoxic than the $CD4^+$ iNKT cells, as they express more perforin and granzyme B [16,17]. Therefore, we categorized expanded iNKT cell lines based on CD4 expression alone, as $CD4^+$ or $CD4^-$ cells (**Figure 10B**). Although there was no difference between the frequency of $CD4^+$ and $CD4^-$ iNKT cells in the primary iNKT cells, in the expanded iNKT cell lines the $CD4^+$ population was dominant (**Figure 10B**).

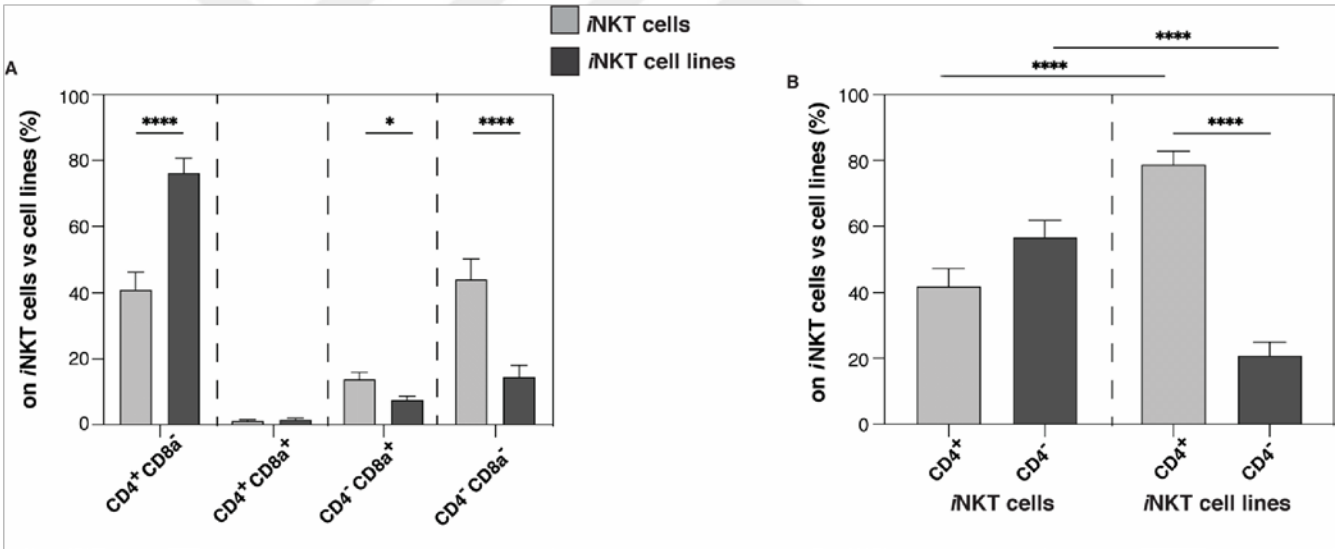


Figure 10. iNKT cell subset distribution, human primary iNKT cells vs. expanded iNKT cell lines.

Primary human iNKT cells (live $CD14^-$ $CD20^-$ $CD3^+$ $iVα24^+$ (6B11) cells) were analysed on day 0 from an aliquot of isolated PBMCs. iNKT cells were expanded from PBMCs as described in figure 9. On day 13, the iNKT cells were identified as $CD3^+$ $iVα24^+$ (6B11) cells. **(A)** CD4/CD8 subset distribution in primary human iNKT cells (light grey bars) and iNKT cell lines (dark grey bars). **(B)** Frequency $CD4^+$ (light grey bars) and $CD4^-$ (dark grey bars) cell in primary human iNKT cells (left) and iNKT cell lines (right). The figure shows pooled data from three independent experiments (n = 15).

We then examined several surface markers on the expanded $CD4^+$ and $CD4^-$ iNKT cell lines. Except CD62L, a marker for naïve T cells, fewer $CD4^+$ iNKT cells expressed perforin and the NK cell markers CD56/NCAM, CD161, and CD314/NKG2D, than $CD4^-$ iNKT cells (**Figure 11**). No difference in the granzyme B^+ frequency between $CD4^+$ and $CD4^-$ iNKT cell lines was observed (**Figure 11**).

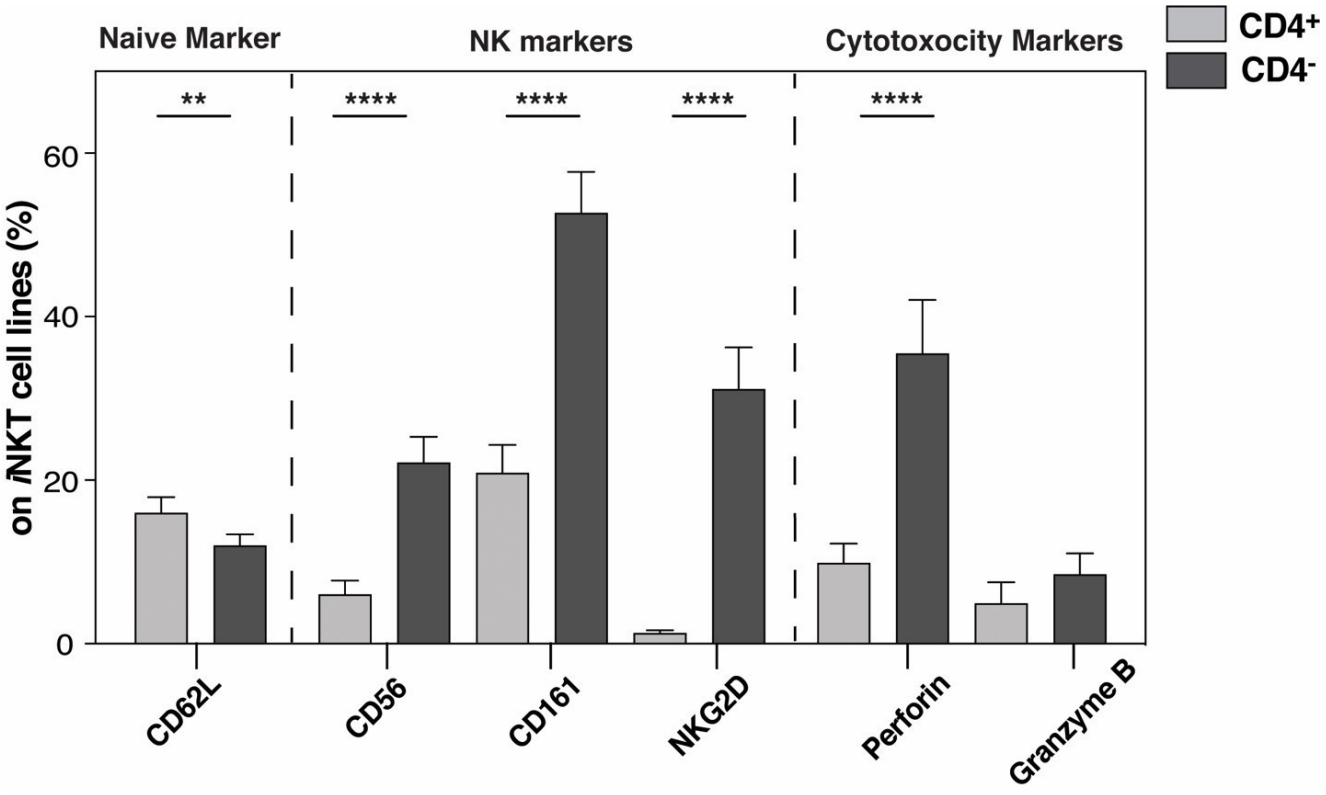


Figure 11. Phenotype of human iNKT cell lines.

iNKT cells were expanded from PBMCs as described in figure 9. On day 13, the iNKT cells were identified as $CD3^+iV\alpha24^+$ (6B11) cells. The figure shows the frequency of positive cells for each indicated marker on $CD4^+$ (right, light grey) or $CD4^-$ (left, dark grey) human iNKT cell lines. The figure shows pooled data from three independent experiments (n = 15).

We next analysed the expression of CD253/TRAIL, CD261/DR4, and CD262/DR5 on the expanded iNKT cell lines (**Figure 12**). As we did for the primary iNKT cells, we first analysed the expression of these markers based on their cellular location. No difference was observed for the surface or intracellular CD253/TRAIL expression on iNKT cell lines (**Figure 12A**). However, the expression of CD261/DR4 and CD262/DR5 was primarily localized to the intracellular compartment of the iNKT cell lines (**Figure 12B, C**).

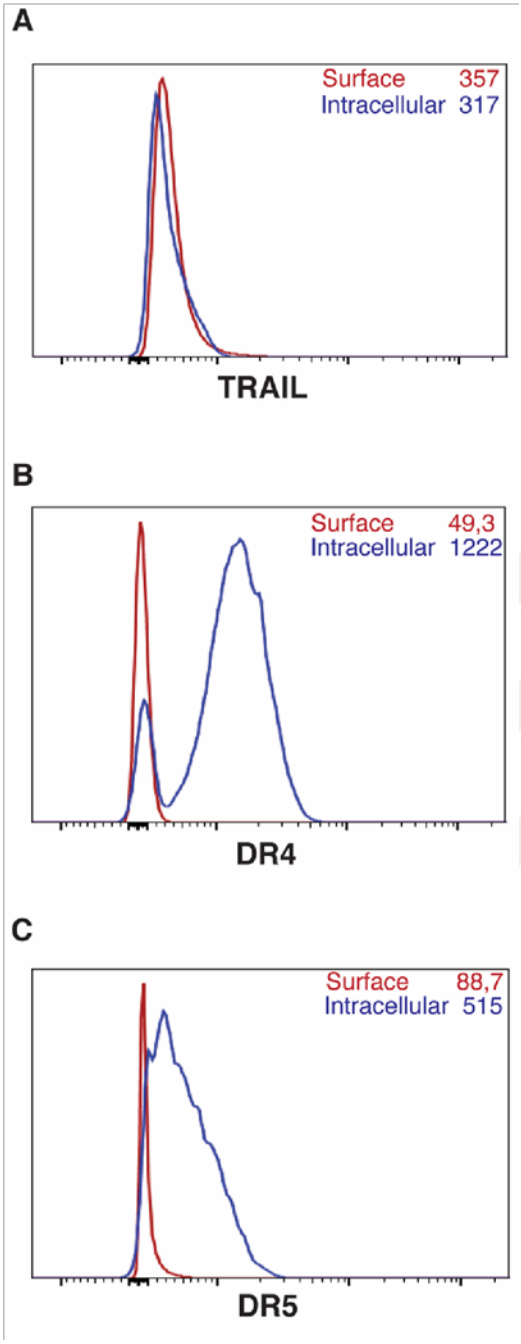


Figure 12. Surface and intracellular expression of CD253/TRAIL, CD261/DR4, and CD262/DR5 in NKT cell lines.

NKT cells lines were expanded from PBMCs of 5 healthy donors as described in figure 9. iNKT cell lines, identified as $CD3^+$ $iV24^+$ (6B11) cells, were analysed for **(A)** CD253/TRAIL, **(B)** CD261/DR4, and **(C)** CD262/ DR5 surface and intracellular expression. For each marker, a representative histogram is shown on the left and summary data are given on the right. In the histograms, the surface expression is depicted in red and the intracellular expression in blue. In the bar graphs, the intracellular expression is shown with dark grey bars and the surface expression with light grey bars. Representative data from one of two independent experiments are shown (n = 5).

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We also analysed the expression of death receptors (CD261/DR4, CD262/DR5, and CD95/FAS) and death ligands (CD253/TRAIL and CD178/FASL) on $CD4^+$ and $CD4^-$ primary iNKT cells and iNKT cell lines in parallel. For this, total expression of each marker was assessed with the same antibody-conjugate in a combined surface-intracellular staining. No difference was observed for CD261/DR4 (**Figure 13A**) and CD262/DR5 (**Figure 13B**) between $CD4^+$ and $CD4^-$ primary

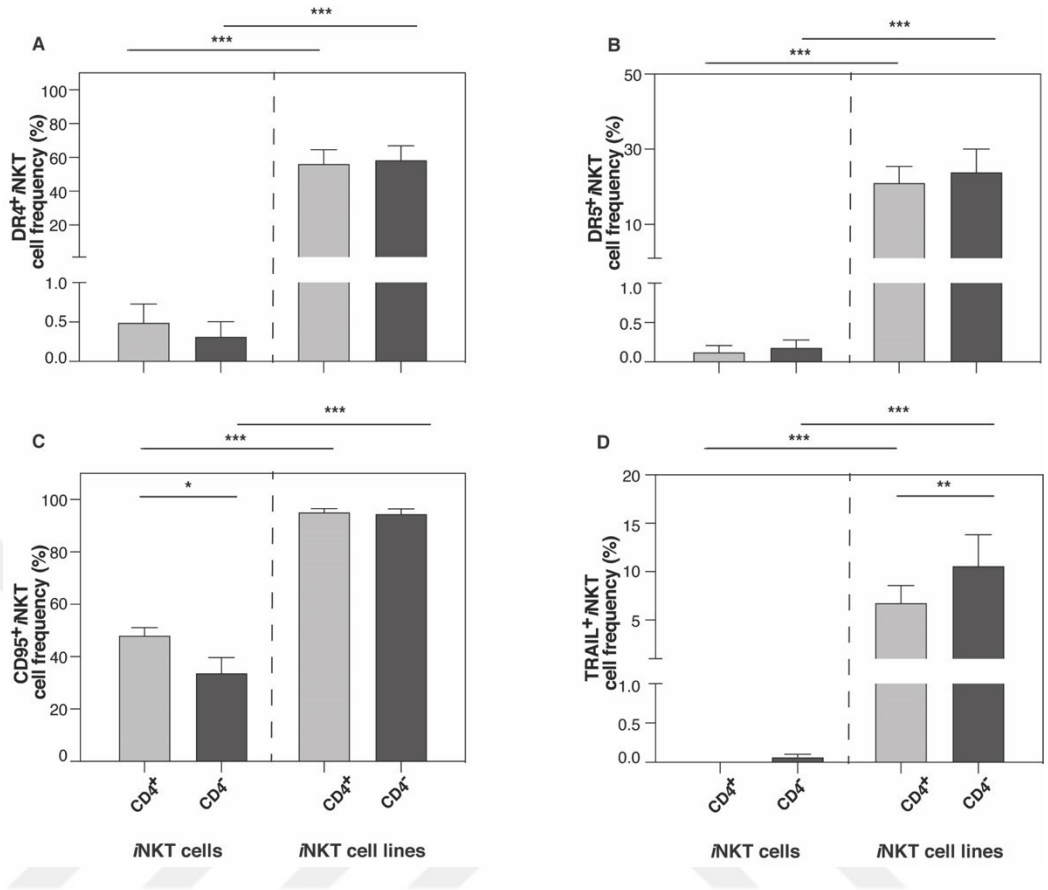


Figure 13. Frequency of CD261/DR4, CD262/DR5, CD95/FAS, and CD253/TRAIL on primary and expanded iNKT cells.

PBMCs were isolated from healthy donors and prepared individually for the flow cytometric analysis. iNKT cell lines were expanded from the PBMCs of healthy donors as described in figure 9. iNKT cells were identified as Live $CD14^-$ $CD20^-CD3^+iV\alpha24^+$ (6B11) within the PBMCs and as $CD3^+$ $iV\alpha24^+$ (6B11) cells within the iNKT cell lines. The samples were stained with the same antibody-fluorophore conjugate for both surface and intracellular compartments. Summary data for the percentages of **(A)** CD261/DR4, **(B)** CD262/DR5, **(C)** CD95/FAS, and **(D)** CD253/TRAIL on primary iNKT cells (left) and iNKT cell lines (right) that were $CD4^+$ (light grey) or $CD4^-$ (dark grey). The figures show pooled data of two independent experiment with six samples each (n = 12).

iNKT cells or iNKT cell lines. In primary samples, fewer $CD4^-$ than $CD4^+$ iNKT cells expressed CD95 (**Figure 13C**). Independent of the CD4 expression, more cells expressed the death receptors (CD261/DR4, CD262/DR5, and CD95/FAS) in iNKT cell lines than in primary iNKT cells (**Figure 13A-C**). CD253/TRAIL expression was also elevated in the iNKT cell lines, and this was more prominent for the $CD4^-$ than the $CD4^+$ iNKT cells (**Figure 13 D**). Similar to the primary iNKT cells, we could not detect CD178 expression on the iNKT cell lines (data not shown).

We analysed next whether αGalCer or PMA/ionomycin stimulation of the iNKT cell lines modulates the expression of these markers. We could not confirm the enrichment of TRAIL$^+$ CD4$^-$ iNKT cell population in this setting at the basal level (**Figure 14 A**), in contrast to **Figure 13 D**. All iNKT cells upregulated TRAIL expression after αGalCer stimulation and this modulation was stronger in the CD4$^+$ than CD4$^-$ subsets (**Figure 14 A**). CD261/DR4 expression remained unchanged after stimulation, however, a lower DR4$^+$ frequency was detected on CD4$^-$ iNKT cells than on CD4$^+$ iNKT cells regardless of the condition (**Figure 14 B**). As such, fewer CD4$^-$ than CD4$^+$ iNKT cells expressed CD262/DR5 in all conditions, which was more evident after the stimulation due to the elevated DR5 expression on CD4$^+$ iNKT cells (**Figure 14 C**).

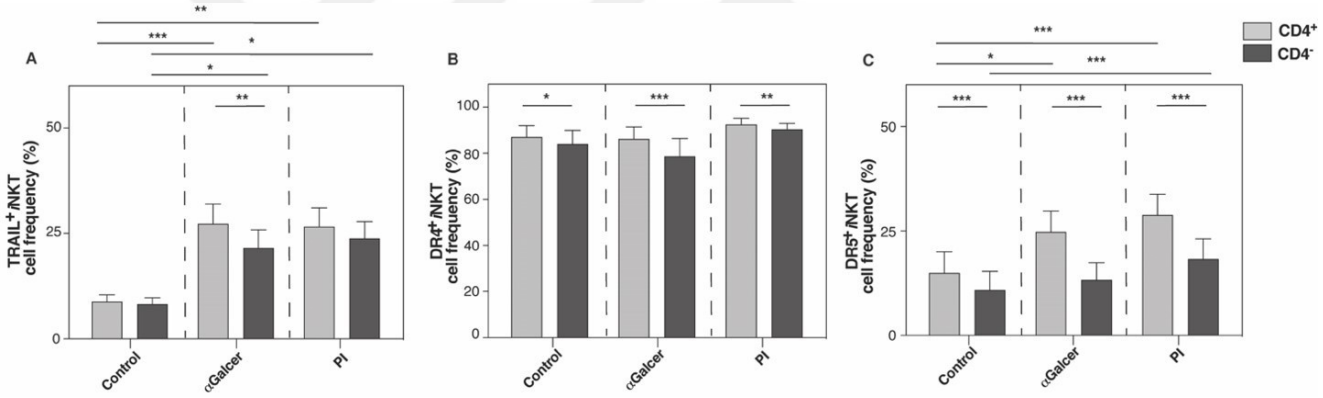


Figure 14. Expression of CD253/TRAIL, CD261/DR4, and CD262/DR5 on CD4$^+$ and CD4$^-$ iNKT cell lines after stimulation.

iNKT cell lines were expanded from PBMCs and restimulated with 100 ng/ml αGalCer or with 25ng /ml PMA and 1µg/ml ionomycin (PI) on day 13 for 4 hours. After 4 hours, iNKT cells (CD3$^+$ Vα24$^+$ cells or CD3$^+$ 6B11$^+$ cells) were analysed for the expression of **(A)** CD253/TRAIL, **(B)** CD261/DR4, and **(C)** CD262/DR5 in both CD4$^+$ and CD4$^-$ iNKT cell subsets. Each figure shows pooled data from three independent experiments (n = 12).

We next determined whether the stimulation would alter the cellular distribution of CD261/DR4, i.e. if a translocation to the surface could be observed. The stimulation reduced the frequency of iNKT cell that expressed CD261/DR4 only intracellular (**Figure 15**). This reduction was compensated with a minor increase of the frequency of iNKT cells expressing only surface CD261/DR4 expressing (**Figure 15**). However, the stimulation increased the frequency of iNKT cells without detectable DR4 (**Figure 15**).

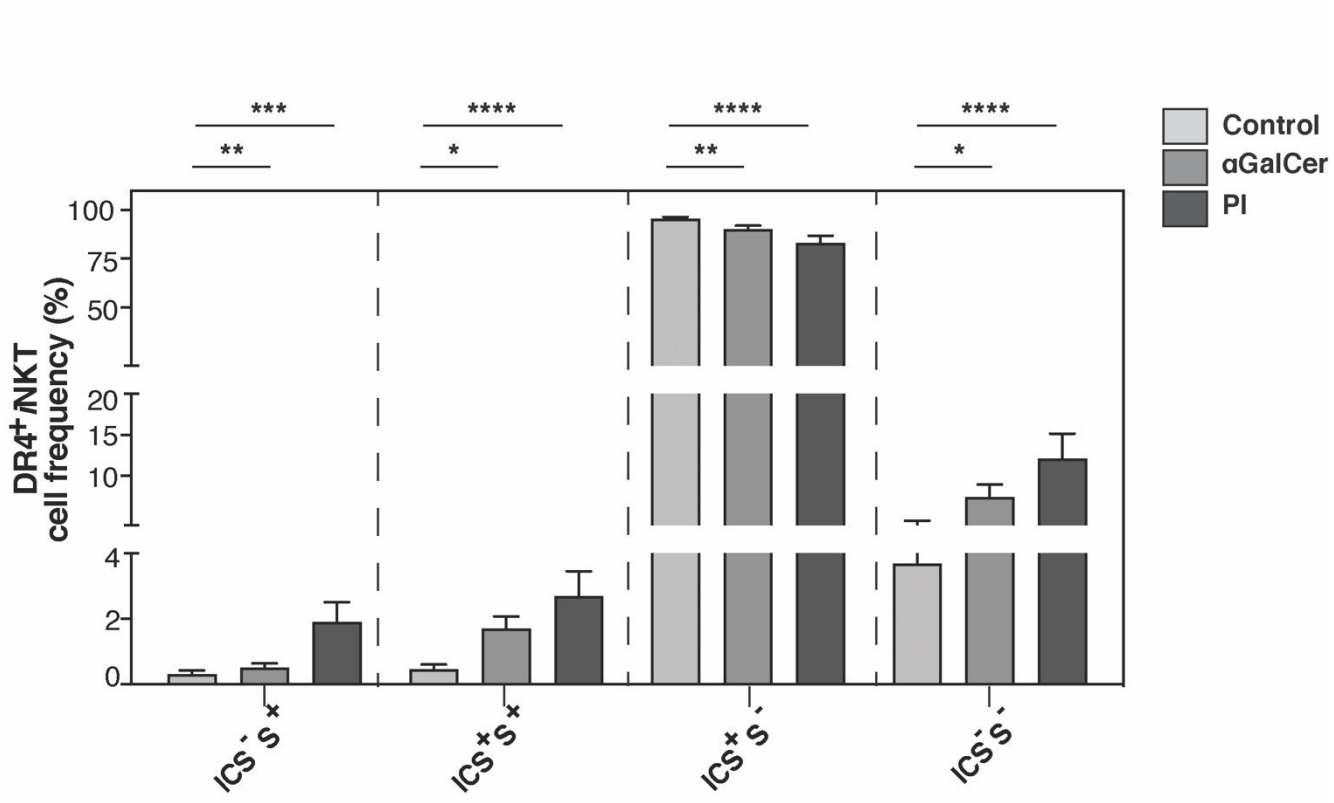


Figure 15. Subcellular distribution of DR4 expression after stimulation of iNKT cell lines.

iNKT cells were expanded from PBMCs and restimulated with 100 ng/ml αGalCer or PMA (25ng/ml) and ionomycin (1μg/ml) (PI) on day 13. After 6 hours, iNKT cells ($CD3^+$ $Vα24^+$ or $CD3^+$ $6B11^+$ cells) were analysed for the expression of CD261/DR4 on the surface (S) (surface stain with αDR4-PE-Cy7 antibodies) or intracellular (ICS) (intracellular stain with αDR4-APC antibodies) and sub-grouped based on CD261/DR4 expression as ICS^-S^+, ICS^+S^+, ICS^+S^-, and ICS^-S^-. The frequency of each subgroup is shown for control (light grey), αGalCer re-stimulated (grey), and PMA/ionomycin stimulated (dark grey) cells. After 2 hours of stimulation, brefeldin A (0.5 μl/ml) and monensin (0.33 μl/ml) were added to the cultures. The panel shows the summary of pooled data from four independent experiments (n = 14).

Finally, we checked the cytokine production of the iNKT cell lines after stimulation. Both $CD4^+$ and $CD4^-$ iNKT cells can produce Th_1 cytokines, like IFNɣ and TNF, but the Th_2 cytokine IL-4 is produced mainly by $CD4^+$ iNKT cells [16,17,76]. We confirmed that antigen specific stimulation with αGalCer induces comparable levels of IFNɣ and TNF production by $CD4^+$ and $CD4^-$ iNKT cells (**Figure 16 A, B, left**). A larger frequency of IL4 production was observed in $CD4^+$ than $CD4^-$ iNKT cells (**Figure 16 C**).

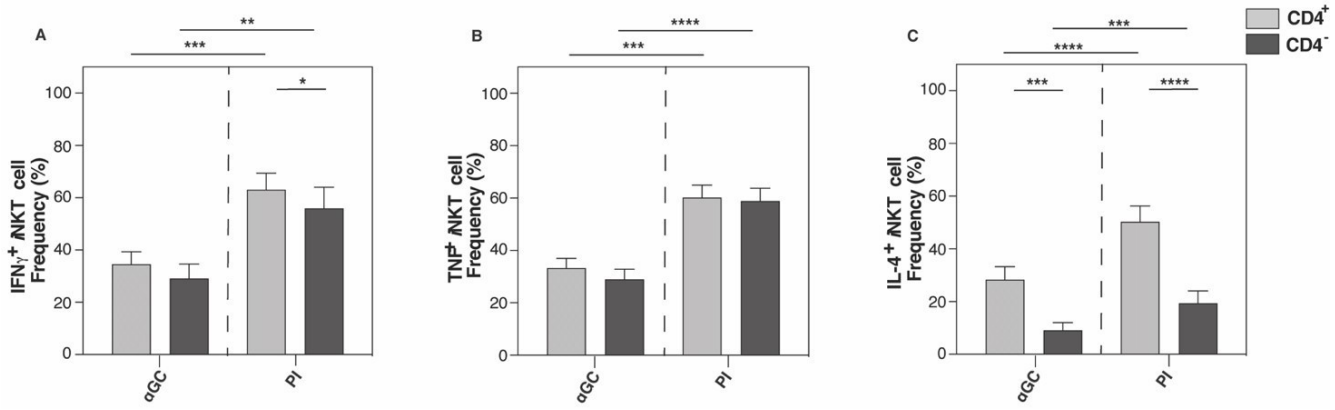


Figure 16. IFNγ, TNF, and IL-4 production by $CD4^+$ and $CD4^-$ iNKT cell lines after stimulation.

iNKT cells were expanded from PBMCs and restimulated with 100 ng/ml soluble αGalCer or PMA (25ng/ml) and ionomycin (1µg/ml) (PI) on day 13. After 4 hours, iNKT cells ($CD3^+$ $Vα24^+$ cells or $CD3^+$ $6B11^+$ cells) were analysed for the expression of **(A)** IFNγ, **(B)** TNF, and **(C)** IL-4 in both $CD4^+$ and $CD4^-$ iNKT cells. Each figure shows the pooled data from three independent experiments (n = 12).

4.4 Impact of chronic TRAIL signalling on iNKT cell lines

We wanted to mimic the tumour microenvironment of a $TRAIL^+$ solid tumour and investigate how iNKT cells behave under continuous TRAIL exposure. To this end, we supplemented the culture media of the expanding iNKT cells with 10 ng/ml rhTRAIL every other day for 5 days starting from day 8 of the expansion (**Figure 17 A**). The total iNKT cell frequency (**Figure 17 B**, black bars on the left) and the total fold expansion (**Figure 17 C**, black bars on the left) did not change by being exposed to rhTRAIL chronically. Furthermore, we checked how rhTRAIL treatment impacts the relative frequency and fold expansion of $CD4^+$ and $CD4^-$ subsets. There was no difference in the relative frequency of each subset due to chronic rhTRAIL treatment (**Figure 17 B**, right), however the expansion of $CD4^-$ iNKT cells was slightly reduced (**Figure 17 C**, right).

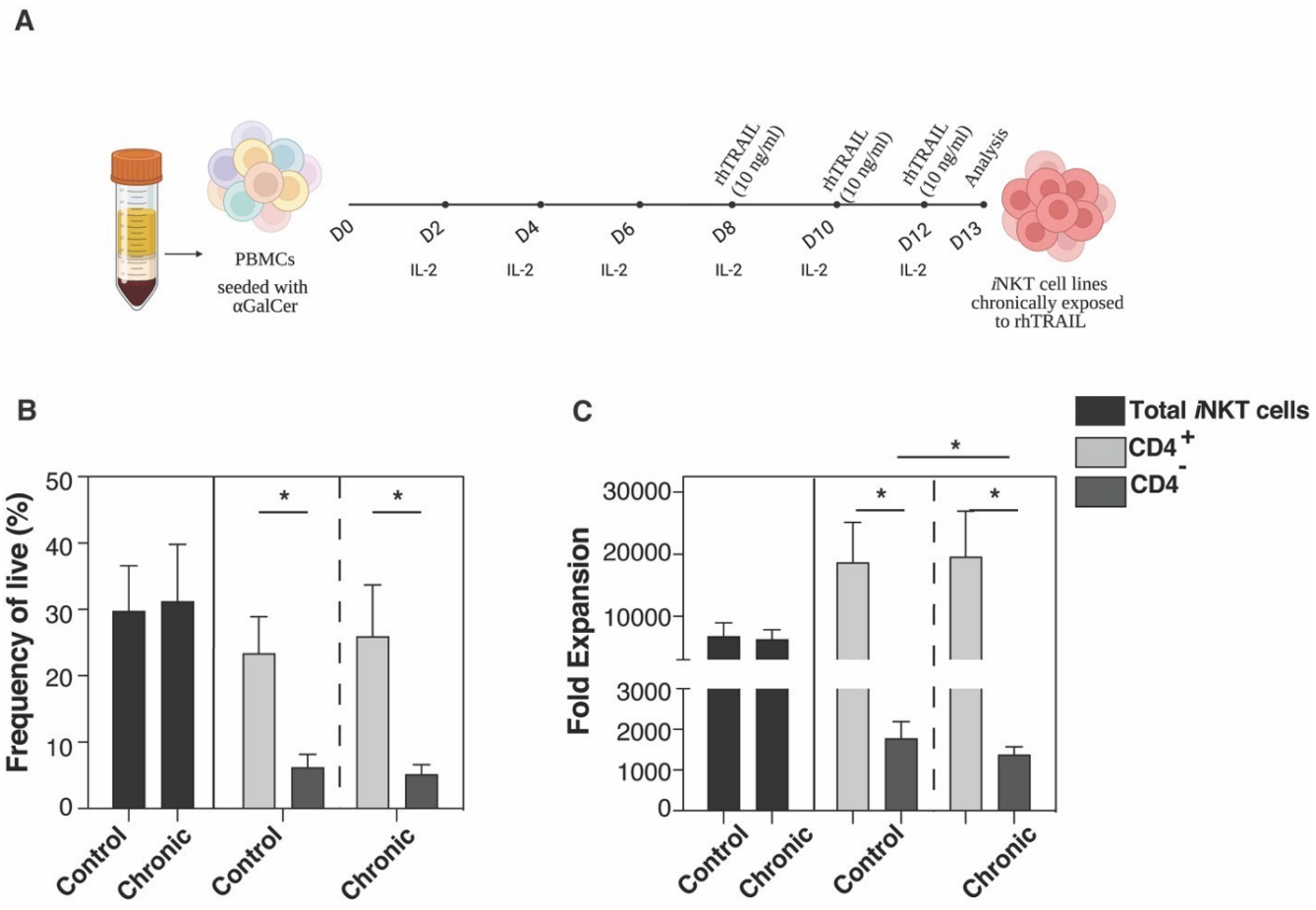


Figure 17. Chronic exposure to soluble rhTRAIL alters the expansion of human CD4⁻ iNKT cells in vitro.

(A) iNKT cell lines were expanded from healthy donor PBMCs as described in figure 9 and treated with soluble 10 ng/ml rhTRAIL starting from day 8 and every other day thereafter. On day 13, the cells were analysed by flow cytometry. **(B)** The frequency and **(C)** the fold expansion of generated $CD4^+$ and $CD4^-$ iNKT cells in the presence (chronic) or absence (control) rhTRAIL is shown. In each figure, the total frequency of live and total fold expansion is shown on the left side (black bars). On the right side, the frequency of $CD4^+$ (light grey bars) and $CD4^-$ (dark grey bars) iNKT cells is shown. The fold expansion was determined as follows: (total number of live cells recovered on day 13 * percentage of $CD3^+$ i$V\alpha24^+$ cells on day 13) / (total number of PMBCs seeded on day 0 * percentage of $CD3^+$ $V\alpha24^+$ cells in the starting population on day 0). The graphs show pooled data of two independent experiments with three donors each (n = 6).

Next, we analysed the expression of death receptors and CD253/TRAIL on iNKT cell lines following chronic exposure to rhTRAIL. No difference was observed in the expression of CD261/DR4, CD262/DR5, CD95/FAS, or CD253/TRAIL between $CD4^+$ and $CD4^-$ iNKT cell lines in the control samples (**Figure 18**). The frequency of $CD261/DR4^+$, $CD262/DR5^+$, $CD95/FAS^+$, and $CD253/TRAIL^+$ cells remained unchanged in each subset after being chronically exposed to rhTRAIL (**Figure 18**). However, a slightly higher frequency of $DR5^+$ iNKT cells was detected on $CD4^+$ then $CD4^-$ iNKT cells following the chronic rhTRAIL exposure (**Figure 18 B**).

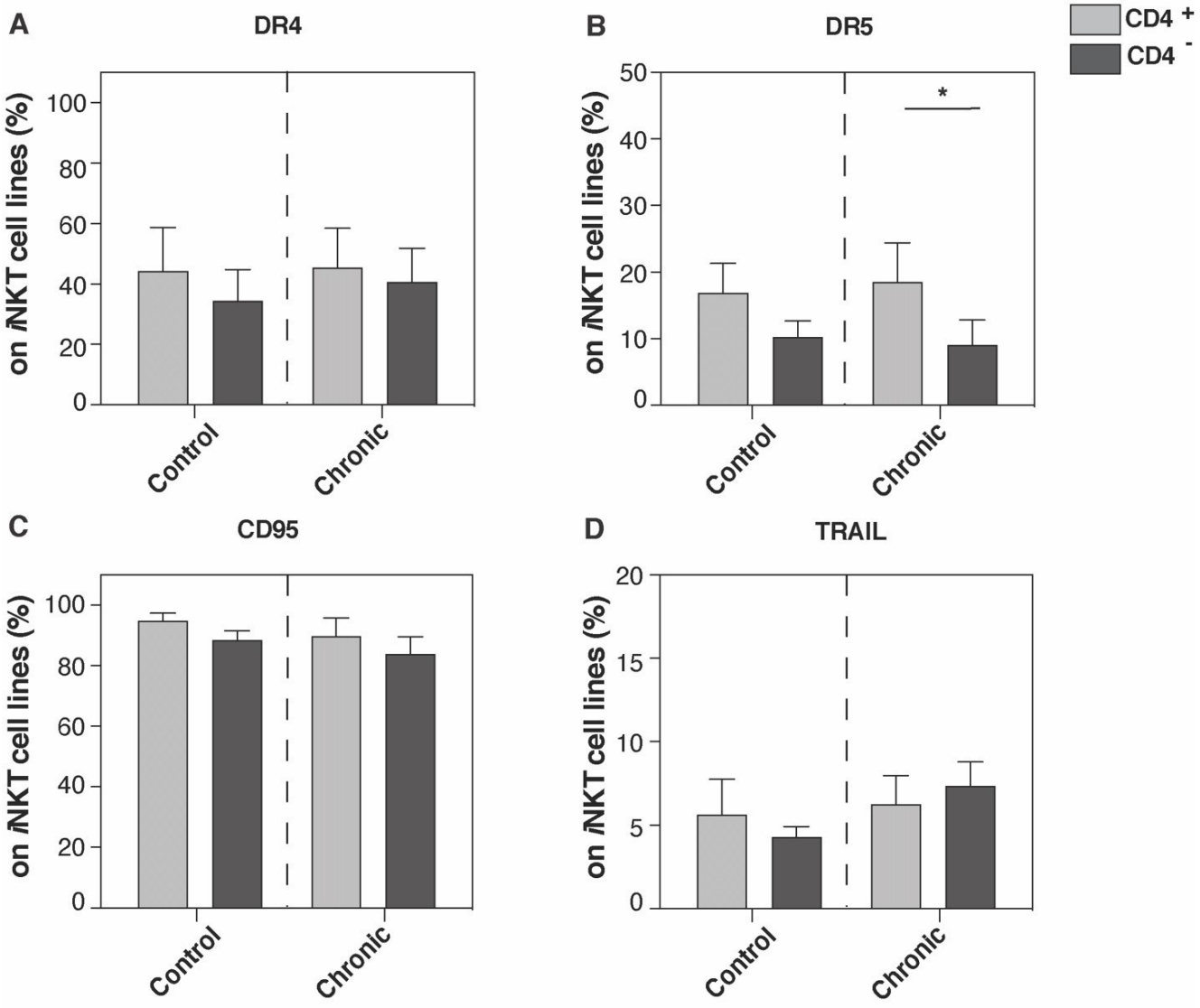


Figure 18. Chronic rhTRAIL treatment favoured DR5 expression on CD4⁺ iNKT cell lines in vitro.

iNKT cell lines were expanded from healthy donor PBMCs as described in figure 9 and treated with soluble 10 ng/ml rhTRAIL starting from day 8 and thereafter. On day 13, iNKT cells ($CD3^+$ $V\alpha24^+$ cells) were analysed for the expression of **(A)** CD261/DR4, **(B)** CD262/DR5, **(C)** CD95/FAS, and **(D)** CD253/TRAIL on $CD4^+$ and $CD4^-$ iNKT cell subsets in the presence (chronic) or absence (control) of rhTRAIL. Each graph shows pooled data of two independent experiments (n = 6).

The NK cell markers, CD56/NCAM, CD161/KLRB1, and CD314/NKG2D are preferentially expressed on $CD4^-$ iNKT cells [16,18] , which we confirmed here (**Figure 19**, left half of each plot). Chronic rhTRAIL treatment did not alter the frequency of these markers on the iNKT cell lines (**Figure 19**).

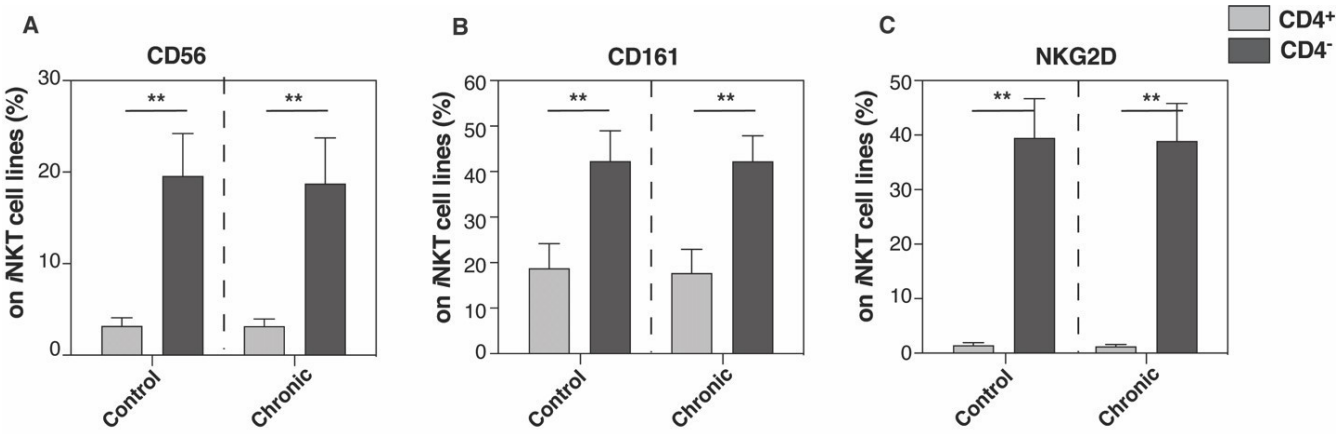


Figure 19. The frequency of NK markers on iNKT cell lines was unchanged by chronic rhTRAIL exposure.

iNKT cell lines were expanded from healthy donor PBMCs as described in figure 9 and treated with soluble 10 ng/ml rhTRAIL starting from day 8 thereafter. On day 13, iNKT cells ($CD3^+$ $V\alpha24^+$ cells) were analysed for the expression of **(A)** CD56, **(B)** CD161, and **(C)** NKG2D on $CD4^+$ and $CD4^-$ iNKT cell subsets in the presence (chronic) or absence (control) of rhTRAIL. Each graph shows pooled data of three independent experiments (n = 10).

We next determined whether chronic rhTRAIL exposure affects the expression of the cytotoxicity markers, perforin and granzyme B, on iNKT cell lines. As expected [16–18], $CD4^-$ iNKT cell lines had a higher frequency of perforin at the basal level (**Figure 20 A**, left). Following chronic rhTRAIL exposure, we noted a decrease in the frequency of $perforin^+$ $CD4^-$ iNKT cells (**Figure 20 A**). In contrast, chronic rhTRAIL treatment did not alter the frequency of granzyme B expressing iNKT cells (**Figure 20 B**).

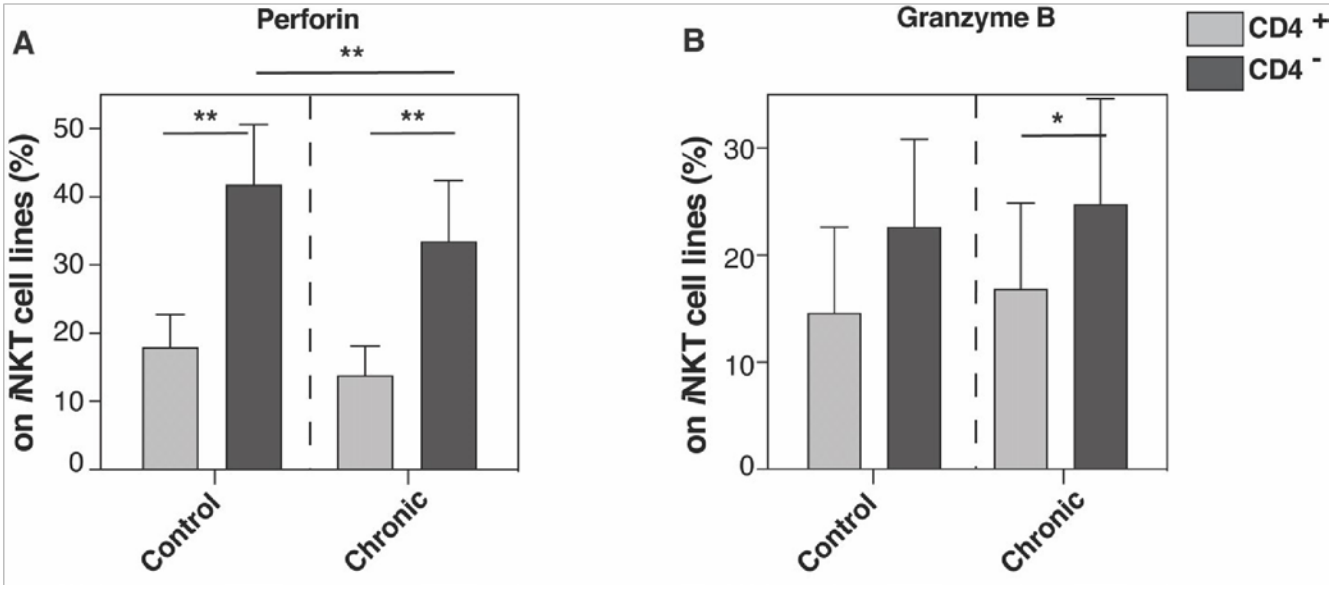


Figure 20. Chronic TRAIL exposure during the expansion period decreased the frequency of $perforin^+CD4^-$ iNKT cells in the generated cell lines.

iNKT cell lines were expanded from healthy donor PBMCs as described in figure 9 and treated with soluble 10 ng/ml rhTRAIL starting from day 8 and thereafter as described in Figure 17 A. On day 13, iNKT cells ($CD3^+$ $iV\alpha24^+$ cells) were

analysed for the expression of **(A)** perforin and **(B)** granzyme B in $CD4^+$ and $CD4^-$ iNKT cell subsets in the presence (chronic) or absence (control) of rhTRAIL. Each graph shows pooled data of three independent experiments (n = 10).

Finally, we evaluated the expression of these markers and the cytokine production in iNKT cell lines in chronically rhTRAIL treated samples after antigen specific stimulation. In both $CD4^+$ and $CD4^-$ iNKT cell lines, CD261/DR4 and CD253/TRAIL expressing iNKT cell frequency is elevated in iNKT cells lines generated under chronic rhTRAIL exposure compared to control group (**Figure 21 A and C**). Except a higher CD261/DR4 expression in $CD4^+$iNKT cells, no difference was observed between $CD4^{+/-}$ iNKT cell lines for the expression of CD262/DR5 and CD253/TRAIL in the chronically rhTRAIL treated group (**Figure 21 B and C)**. In addition to this, for all markers, the chronic rhTRAIL treatment did not alter their expression patterns in both $CD4^{+/-}$ iNKT cell subsets after antigenspecific stimulation (**Figure 21 A, B, and C**). Further, we checked whether being exposed to chronic rhTRAIL treatment changes the cytokine production of iNKT cell subsets after antigen specific stimulation. $CD4^+$ and $CD4^-$ iNKT cells did not alter their cytokine profile after being exposed to chronic rhTRAIL (**Figure 21 D, E, and F).**

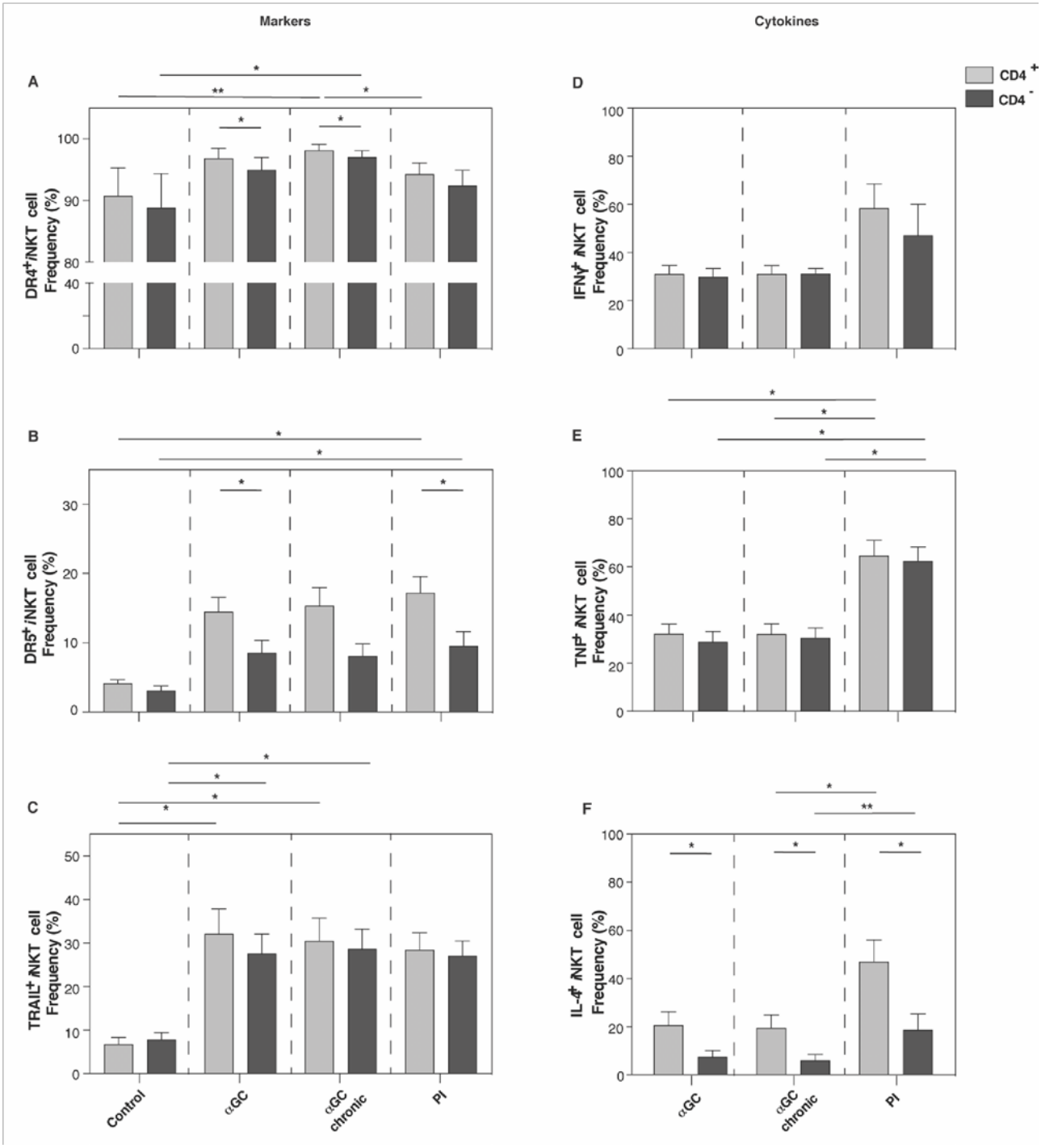


Figure 21. Chronic rhTRAIL treatment has minor impact on DR4 expression but not on the cytokine production of iNKT cell lines after stimulation.

iNKT cell lines were expanded from healthy donor PBMCs as described in figure 9 and treated with soluble 10 ng/ml rhTRAIL starting from day 8 and every other day after as described in Figure 17 A. On day 13, cell lines were stimulated for 4h either with 100 ng/ml αGalCer or with PMA (25 ng/ml) and ionomycin (1 µg/ml) (PI) in the presence of Golgi plug (0.5 µl/ml) and Golgi stop (0.33µl /ml). iNKT cells ($CD3^+V\alpha24^+$ cells) were analysed for the expression of **(A)** CD261/DR4, **(B)** CD262/DR5, **(C)** CD253/TRAIL and for the production of cytokines **(D)** IFNγ, **(E)** TNF, and **(F)** IL-4 in both $CD4^+$ and $CD4^-$ iNKT cell subsets. Each figure shows the pooled data of two independent experiments (n = 6).

4.5 Impact of acute rhTRAIL signalling on iNKT cell lines

Next, we aimed to mimic the first entry of iNKT cells into a TRAIL$^+$ solid tumour microenvironment. To this end, iNKT cell lines were exposed to 10 ng/ml rhTRAIL for the last 16 hours of the expansion period (**Figure 22 A**). Acute rhTRAIL treatment had no impact on the total frequency or the fold expansion of iNKT cell lines (**Figure 22 B and C**, left, black bars). Similar, the frequency and the calculated fold expansion of CD4$^+$ and CD4$^-$ iNKT cell lines was unaffected by the acute rhTRAIL exposure (**Figure 22 B and C**, right, light bars).

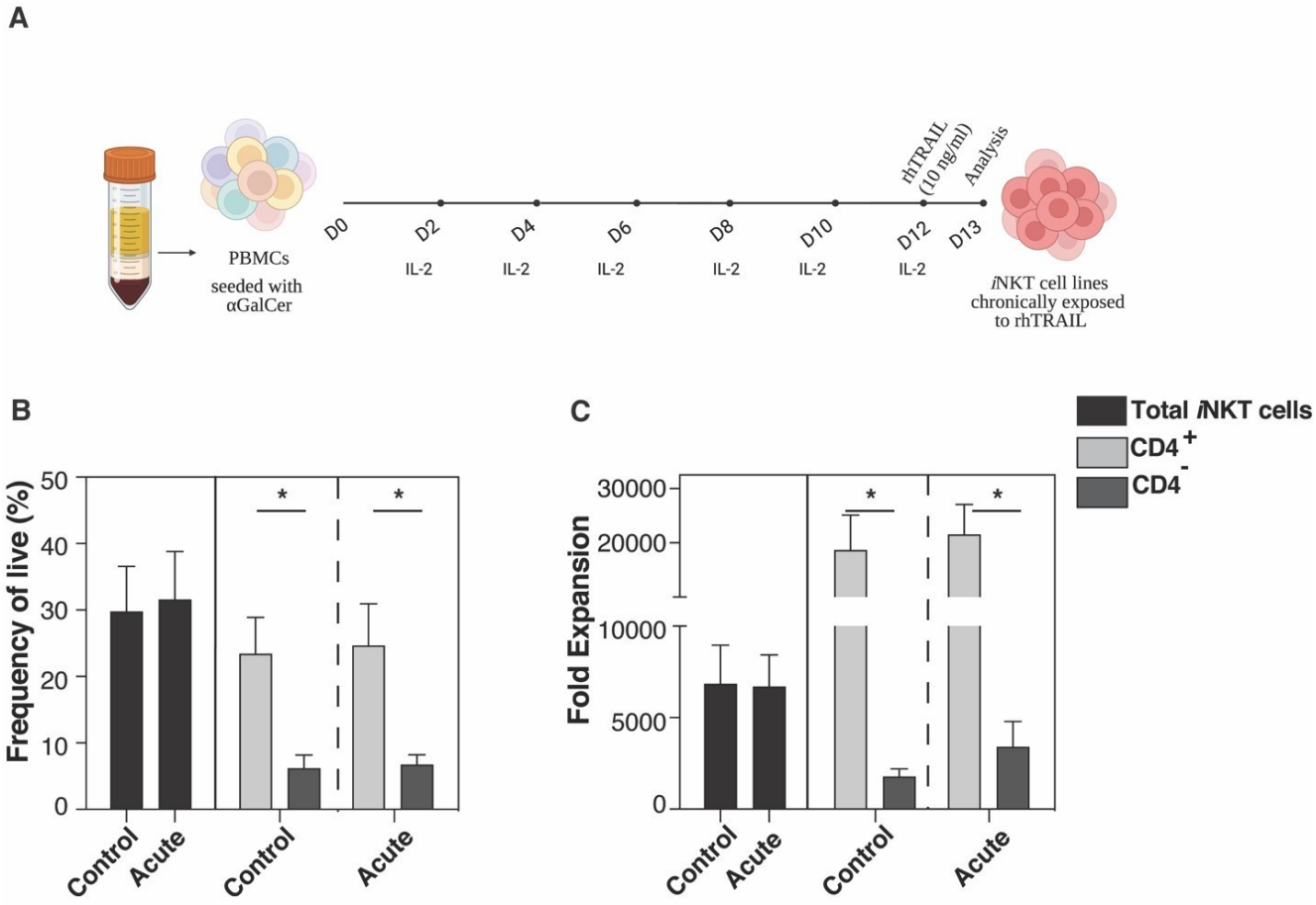


Figure 22. Acute exposure to soluble rhTRAIL does not alter the expansion of human CD4$^+$ and CD4$^-$ subsets of iNKT cell lines in vitro.

iNKT cells were expanded from PBMCs as described in figure 9 and were **(A)** treated with 10 ng/ml soluble rhTRAIL for the last 16 hours of the expansion period. On day 13, the cells were analysed by flow cytometry. **(B)** The frequency and **(C)** the fold expansion of CD4$^+$ and CD4$^-$ iNKT cells in the generated cell lines cultured in the presence of rhTRAIL (10 ng/ml, Acute) and alone (Control). In each figure, the total frequency of live and total fold expansion is shown on the left side (black bars). On the right side, CD4$^+$ (light grey bars) and CD4$^-$ (dark grey bars) specific frequency of live and fold expansion data is shown. The fold expansion was determined as follows: (total number of live cells recovered on day 13 * percentage of CD3$^+$ Vα24$^+$ cells on day 13) / (total number of seeded PBMCs * percentage of CD3$^+$ Vα24$^+$ cells in the starting population on day 0). The figures show the pooled data of two independent experiments with three donors each (n = 6).

In regard to the phenotype, no change was observed for the frequency of CD261/DR4, CD262/DR5, CD95/FAS, or CD253/TRAIL on $CD4^+$ and $CD4^-$ iNKT cell lines after acute exposure to rhTRAIL (**Figure 23**).

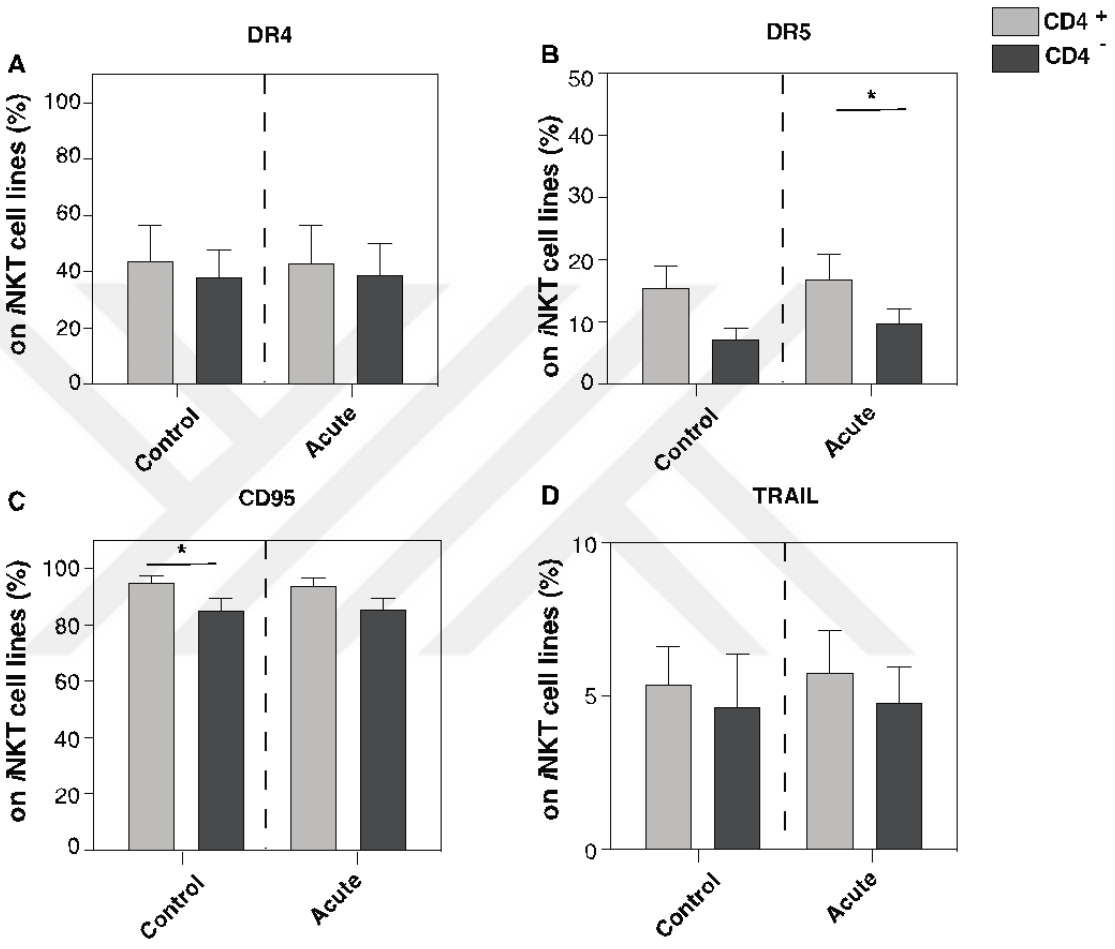


Figure 23. Acute rhTRAIL treatment has no impact on the expression of CD261/DR4, CD262/DR5, CD95/FAS, and CD253/TRAIL on iNKT cell lines in vitro.

iNKT cell lines were expanded from healthy donor PBMCs and on day 12 treated with 10 ng/ml soluble rhTRAIL for the last 16 hours of the expansion period. On day 13, iNKT cells ($CD3^+$ $V\alpha24^+$ cells) were analysed for the expression of **(A)** CD261/DR4, **(B)** CD262/DR5, **(C)** CD95/FAS, and **(D)** CD253/TRAIL in $CD4^+$ and $CD4^-$ iNKT cell subsets. Each figure shows the pooled data of two independent experiments (n = 6).

Next, we analysed the expression of NK cell markers on $CD4^{+/-}$ iNKT cell subsets after acute rhTRAIL treatment. The expression of CD56/NCAM, CD161/KLRB1, and CD314/NKG2D remained high on $CD4^-$ iNKT cells and no changes were observed due to the acute rhTRAIL treatment (**Figure 24 A-C**).

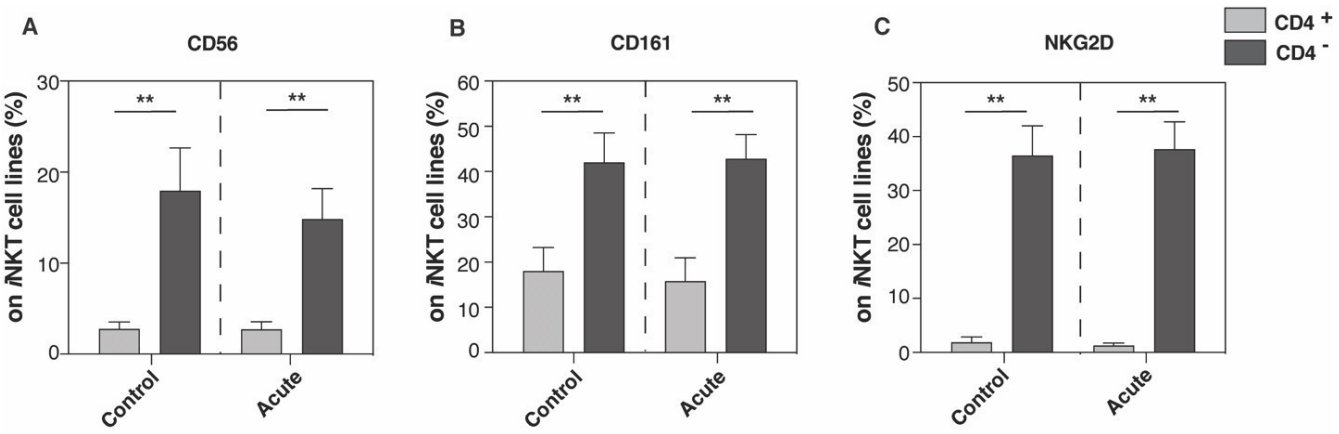


Figure 24. Acute rhTRAIL exposure did not influence the expression of CD56, CD161, and CD314/NKG2D on iNKT cell lines.

iNKT cell lines were expanded from healthy donor PBMCs and treated with soluble 10 ng/ml rhTRAIL for the last 16 hours of the expansion period. On day 13, iNKT cells ($CD3^+$ $V\alpha24^+$ cells) were analysed for the expression of **(A)** CD56, **(B)** CD161, **(C)** CD314/NKG2D in $CD4^+$ and $CD4^-$ iNKT cell subsets. Each figure shows the pooled data of three independent experiments (n = 10).

The last two phenotypic markers analysed were perforin and granzyme B. Acute rhTRAIL exposure decreased the frequency of perforin$^+$ $CD4^+$ iNKT cells (**Figure 25 A**). No change was detected in $CD4^-$ iNKT cells for perforin production or on $CD4^+$ and $CD4^-$ iNKT cells for granzyme B production (**Figure 25 A and B**).

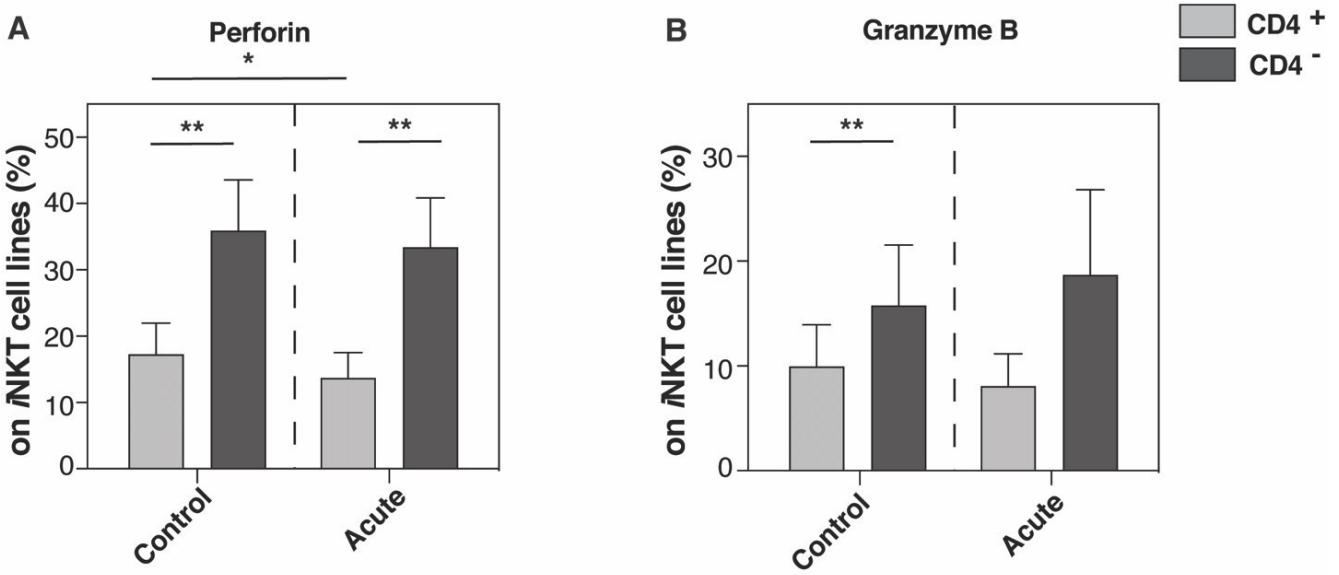


Figure 25. Acute rhTRAIL exposure decreases the frequency of perforin$^+$ iNKT cells within the $CD4^+$ iNKT cells.

iNKT cell lines was expanded from healthy donor PBMCs and treated with soluble 10 ng/ml rhTRAIL for the last 16 hours of the expansion period. On day 13, iNKT cells ($CD3^+$ $V\alpha24^+$ cells) were analysed for the expression of **(A)** perforin and **(B)** granzyme B in $CD4^+$ and $CD4^-$ iNKT cell subsets. Each figure shows the pooled data of three independent experiments (n = 10).

Finally, we evaluated the impact of acute rhTRAIL treatment on the expression of these markers and the cytokine production in iNKT cell lines after antigen specific stimulation. The frequency of cells expressing CD261/DR4, CD262/DR5, or CD253/TRAIL on iNKT cell lines was unchanged by the acute rhTRAIL exposure (**Figure 26 A, B, C**). Similarly, the expression of the cytokines IFNg, IL-4, and TNF was unaffected by the acute rhTRAIL treatment (**Figure 26 D, E, F**).

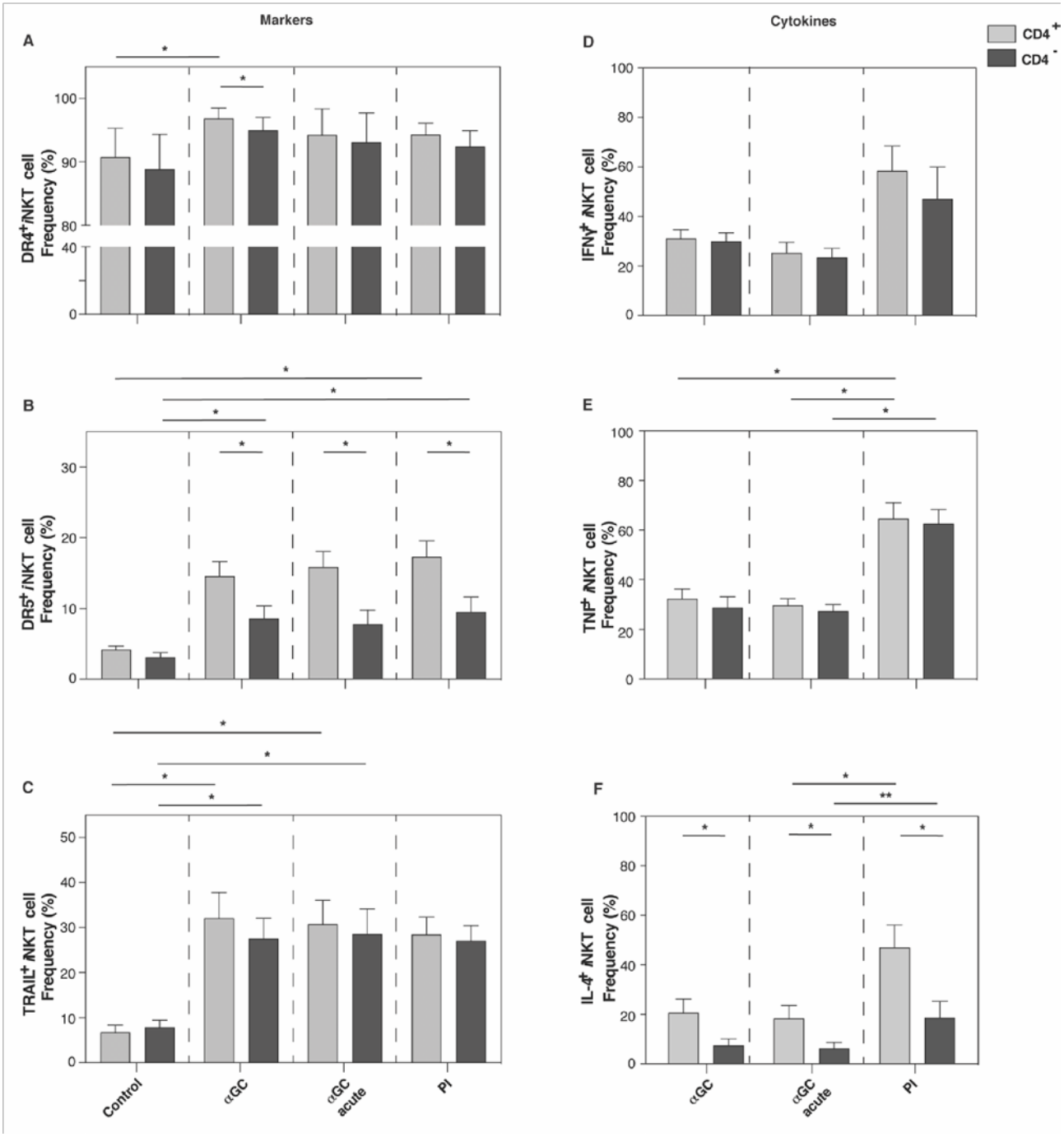


Figure 26. Acute rhTRAIL exposure does not influence the expression of CD261/DR4, CD262/DR5, CD253/TRAIL or the cytokine production of iNKT cells lines.

iNKT cell lines was expanded from healthy donor PBMCs as described in figure 9. Expanded cell lines were treated with soluble 10 ng/ml rhTRAIL for the last 16 hours of the expansion period as described in figure 22 A. On day 13, cell lines were restimulated for 4 hours with 100ng/ml αGalCer or PMA (25ng/ml) and ionomycin (1µg/ml) (PI) in the presence of Golgi Plug (0,5µl/ml) and Golgi Stop (1µl/3ml). iNKT cells ($CD3^+V\alpha24^+$ cells) were analysed for the expression of **(A)** CD261/DR4, **(B)** CD262/DR5, **(C)** CD253/TRAIL and for the production of the cytokines **(D)** IFNγ, **(E)** TNF, and **(F)** IL-4 in both $CD4^+$ and $CD4^-$ iNKT cell subsets. Each figure demonstrates the pooled data of two independent experiments (n = 6)

5. DISCUSSION

In this thesis, we investigated for the first time the expression of the death ligand CD253/TRAIL and its receptors, CD261/DR4 and CD262/DR5, on primary iNKT cells ex vivo and on expanded iNKT cell lines. We also correlated the expression of these markers with the phenotypic and functional profile of the generated iNKT cell lines.

We show that human primary iNKT cells express no CD253/TRAIL (**Figure 3, Figure 7 A**) but weakly CD262/DR5 (**Figure 5, Figure 7A**). Our initial results suggested no CD261/DR4 expression on primary iNKT cells (**Figure 4**), however, with increased sample size, we detected a small fraction of $DR4^+$ human iNKT cells ex vivo (**Figure 7 A**). We further showed that human primary iNKT cells express CD95/FAS, the receptor of CD178/FASL (**Figure 6 C**). Although the expression of CD95 on human primary iNKT cells was shown before, the frequency of $CD95^+$ iNKT cells greatly differs between these studies, with values from 10% to 30% [15,17,18]. In our case, around 40% human primary iNKT cells were $CD95^+$ (**Figure 7 A**). Unlike CD95/FAS, no CD178/FASL expression was detected on human iNKT cells ex vivo (**Figure 7 A**).

In accordance with published data [16], we show that $CD4^+$ and DN cells constitute the main two populations among primary iNKT cells (**Figure 7 C**) and that primary iNKT cells express NK cell markers such as CD56 (neural cell adhesion marker) and CD161 (lectin like receptor 1) (**Figure 7 B**).

iNKT cells can be activated TCR-dependent via specific antigens and TCR-independent via cytokines like IL-12 and IL-18. Both antigen-specific and cytokine-driven activation upregulated CD253/TRAIL and CD262/DR5 expression on human primary iNKT cells (**Figure 8 A and C**). CD253/TRAIL expression was observed after 24 hours, whereas both stimulations upregulated CD262/DR5 expression as early as 4 hours (**Figure 8**).

Primary iNKT cells are rare in the PBMCs of healthy donors [9,10], making a phenotypic and functional analysis of primary iNKT cells ex vivo difficult. Therefore, we expanded primary iNKT cells in vitro to generate iNKT cell lines (**Figure 9**) after adopting published protocols [76–78] . This allowed an approximately 300-fold expansion of the iNKT cell (**Figure 9 D**), constituting about half of all remaining cells at the end of the culture (**Figure 9 C**). As expected [16], the $CD4^+$ iNKT cell preferentially expanded (**Figure 10 A**). Furthermore, less $CD4^+$ iNKT cell lines expressed the NK cell markers CD56/NCAM, CD161/KLRB1, and CD314/NKG2D, as well as the cytotoxicity marker perforin (**Figure 11**).

In contrast to primary iNKT cells, the iNKT cell lines expressed CD253/TRAIL and mainly on surface (**Figure 12 A**). Furthermore, the iNKT cells lines expressed CD261/DR4 and CD262/DR5, which was mainly intracellular. (**Figure 12 B and C).** Little information has been published on the subcellular distribution of these death receptors. Cytoplasmic CD262/DR5 and nuclear CD261/DR4 expression was reported in $CD16^+$, $CD56^+$, and T cells in psoriatic skin lesion [79]. In some cancer cell lines, nuclear CD262/DR5 prevented the maturation of miRNA let-7 through its interaction with the microprocessor complex Drosha/DGCR8, which was suggested to promotes tumour malignancy [80]. Furthermore, in placental trophoblast cells, the nuclear localization of CD262/DR5 correlated with resistance to TRAIL-induced apoptosis, whereas the surface expression, following TNF stimulation, sensitized these cells to TRAIL [81]. In our study, CD262/DR4 was the main receptor for CD253/TRAIL expressed by iNKT cells and its expression was mainly intracellular (**Figure 15**) and unaffected by restimulation (**Figure 13 A, 14 B**).

We then evaluated how chronic and acute rhTRAIL exposure modulates the phenotype, function, and proliferation of iNKT cell lines. Neither chronic nor acute TRAIL exposure had an adverse impact on the survival or the expansion of iNKT cell lines (**Figure 17 B and C, Figure 22 B and C**) or altered their expression of CD261/DR4, CD262/DR5, CD253/TRAIL, CD95/FAS, granzyme B, or the the NK cell markers CD56/NCAM, CD161/KLRB1, CD314/NKG2D (**Figure 18, 19, 20 B, 23, 24 and 25 B**). We only observed minor changes for the expansion $CD4^-$ iNKT cells during chronic rhTRAIL exposure (**Figure 17 C**) and the frequency of $perforin^+$ $CD4^-$ and $perforin^+$ $CD4^+$ iNKT cells after chronic (**Figure 20 A**) and acute (**Figure 25 A**) rhTRAIL exposure. Furthermore, we show that the expression of CD261/DR4, CD262/DR5, and CD253/TRAIL was not altered on iNKT cell lines after a second antigenic stimulation following both chronic (**Figure 21**) or acute (**Figure 26**) rhTRAIL exposure. Similar, the cytokine profile of the iNKT cell lines after restimulation was unaltered by either the chronic (**Figure 21**) or acute (**Figure 26**) rhTRAIL exposure.

6. CONCLUSION

In this thesis, the expression and the potential role of the death ligand CD253/TRAIL and its death receptors CD261/DR4 and CD262/DR5 was studied on human primary iNKT cells and expanded iNKT cell lines. Our data show that iNKT cells lines are not sensitive to

long- or short-term TRAIL-induced signalling and their phenotype and function remained unchanged.

These data greatly expand our understanding of TRAIL-DR4/DR5 signalling in human iNKT cells. Importantly, the preserved function of human iNKT cells lines after long- and short-term TRAIL exposure, suggests that iNKT cells would be unaffected by $TRAIL^+$ tumours and are, therefore, an attractive therapeutic candidate to fight such tumours.

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8. APPENDIX

DOKUZ EYLÜL ÜNİVERSİTESİ
GİRİŞİMSEL OLMAYAN ARAŞTIRMALAR ETİK KURULU

Konu: Karar hk.
Sayı : 193

01.03.2018

Sayın Ph.D.Gerhard Wingender,

Kurulumuz tarafından 01.03.2018 tarih ve 3801-GOA protokol numaralı 2018/06-27 karar numarası ile görüşülen **"TRAIL Aracılığı ile Gerçekleşen Sinyallerin İnsan İNKT Hücrelerinin Fonksiyonlarına ve Proliferasyonuna Etkisi (The role of TRAIL mediated signals on the proliferation and function of human İNKT cells)"** konulu araştırmanıza ilişkin Kurulumuz kararı ekte sunulmuştur.

Bilgilerinizi ve gereğini rica ederim.

Prof.Dr.Banu ÖNVURAL
Başkan

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Thesis Subject: Glioblastoma Multiform (gbm), Yetişkinlerde Görülen Malignant Beyin Tümörleri Arasında En Yaygın Olarak Rastlanan Beyin Tümörü Şeklidir.
Anemi, Hem Hematolojik Kökenli Hem De Solid Tümöre Sahip Kanser Hastalarında, Hastalığın Seyri Boyunca Herhangi Bir Evresinde Görülen Ve Hastalığın Seyrini Etkileyen Bağımsız Bir Prognostik Faktördür. Rekombinant Insan Eritropoeitini (rhuepo) Klinik Olarak Kronik Böbrek Hastalığı Ile Antiviral Hıv Terapisi Ilişki Anemide Kullanıldığı Gibi Kemoterapinin Tetiklediği Aneminin (cıa) Gözlendiği Kanser Hastalarında Da Kullanılmaktadır. Bu Çalışmamızda, U-87 Malignant Glioma (u-87 Mg) Hücre Hattında Öncelikle, Hangi Eritropoetin (epo) Reseptörlerinin Bulunduğunun Belirlenmesi Ve Sonrasında Bu Epo Reseptörleri Aracılığı Ile Proliferasyon, Migrasyon, Invazyon Ve Angiogenesiz Gibi Hangi Biyolojik Olayların Düzenlendiğinin Belirlenmesi Amaçlamıştır.
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Thesis Subject: Malignant Melanoma Is The Deadliest Type Of Skin Cancers, Seen With Low Frequency. Lack Of Diagnostic And Prognostic Markers Are Important Obstacles During Early Detection And Selection Of Appropriate Adjuvant Therapy For Malignant Melanoma Patients.ımmunotherapy Trials Targeting Malignant Melanoma Patients Have Been Developing In Larger Extents In The Last 2 Decades, Considering Melanoma Is The Most Immugenic Cancer Type Among All Cancers. Based On These Facts, We Decided To Search For Prognostic Biomarkers Which Will Guide Clinicians To Define Appropriate Malignant Melanoma Patients To Apply Autologous Vaccination To As A Post Surgical Adjuvant Therapy. We Were Able To Obtain 28 Primary Cell Cultures Of Mm Patients And Their Gene Expression Profiles In Luminex Platform. Our Findings

Generated Time: 04 September 2023 10:52:49

Propose That Autologous Vaccination Will Be Beneficial For Malignant Melanoma Patients Having Higher Ct Gene Expression For Increasing Overall Survival.
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(Yurt İçi) (Still continues) .

UIDB - HORİZON 2020, BURSİYER, HORIZON 2020 DISCOVER, Enforcing Institution: İZMİR BİYOTIP VE GENOM MERKEZİ, Sponsor Institution: AVRUPA BİRLİĞİ KOMİSYONU (EUROMED) (Yurt Dışı) , 01 January 2018, 01 December 2022.

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INDÜKLENEN HÜCRE ÖLÜMÜNE SÜLFORAFAN'IN ETKİSİ, Enforcing Institution: KURULUŞ GÜNCELLENMESİ GEREKİYOR, Sponsor Institution: , 15 April 2015, 15 April 2017.

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Scholarships and Fundings

Project Info BIDEB Supports

Number of Panelist/Observer/Reporter Jobs
121C257, Targeted induction of NKTFH cells in vivo to improve vaccination protocols, Burslu, Yürürlükte, BİDEB - Eğitim Burs Ve Etkinlik Destekleri Grubu, BİDEB-2247A / Deneyimli Araştırmacı, BİDEB, Project Participation/Leave Dates: 9/1/22 - Devam ediyor, Project Start/End Dates: 9/1/22 - 9/1/25.
214S171, Santral Sinir Sistemi Hücrelerinde Inflamazom Aktivasyonuna ve
Inflamazom Aktivasyonu Ile Indüklenen Hücre Ölümüne Sülforafan'ın Etkisi,
Burslu, Sonuçlandı, SBAG - Sağlık Bilimleri Araştırma Destek Grubu, 1001 Araştırma, ARDEB, Project Participation/Leave Dates:

Number of Observer/Consultant Jobs

Number of Reporter Jobs
4/15/15 - Devam ediyor, Project Start/End Dates: 4/15/15 - 4/15/17.
110O167, Sünede Faydalı Genlerin Belirlenmesi, Burslu, Sonuçlandı, TOVAG - Tarım, Ormancılık Ve Veterinerlik Araştırma Destek Grubu, 1001 - Araştırma, ARDEB, Project Participation/Leave Dates: 4/1/13 - Devam ediyor, Project Start/End Dates: 11/1/10 - 11/1/13.

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ARDEB/BİDEB 0	TEYDEB 0	Total 0

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PLOS ONE

RESEARCH ARTICLE

The Ca^{2+} concentration impacts the cytokine production of mouse and human lymphoid cells and the polarization of human macrophages *in vitro*

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Abstract

Various aspects of the *in vitro* culture conditions can impact the functional response of immune cells. For example, it was shown that a Ca^{2+} concentration of at least 1.5 mM during *in vitro* stimulation is needed for optimal cytokine production by conventional αβ T cells. Here we extend these findings by showing that also unconventional T cells (invariant Natural Killer T cells, mucosal-associated invariant T cells, γδ T cells), as well as B cells, show an increased cytokine response following *in vitro* stimulation in the presence of elevated Ca^{2+} concentrations. This effect appeared more pronounced with mouse than with human lymphoid cells and did not influence their survival. A similarly increased cytokine response due to elevated Ca^{2+} levels was observed with primary human monocytes. In contrast, primary human monocyte-derived macrophages, either unpolarized (M0) or polarized into M1 or M2 macrophages, displayed increased cell death in the presence of elevated Ca^{2+} concentrations. Furthermore, elevated Ca^{2+} concentrations promoted phenotypic M1 differentiation by increasing M1 markers on M1 and M2 macrophages and decreasing M2 markers on M2 macrophages. However, the cytokine production of macrophages, again in contrast to the lymphoid cells, was unaltered by the Ca^{2+} concentration. In summary, our data demonstrate that the Ca^{2+} concentration during *in vitro* cultures is an important variable to be considered for functional experiments and that elevated Ca^{2+} levels can boost cytokine production by both mouse and human lymphoid cells.

Introduction

Various cell media have been developed for *in vitro* cell cultures to optimize the growth and survival of particular cell types. For example, the RPMI1640 media is frequently used for *in vitro* cultures of mouse and human lymphocytes [1–3]. However, it was suggested that the Ca^{2+} concentration of RPMI1640 (0.49 mM) is actually suboptimal for the *in vitro* stimulation of conventional mouse [4] and human [5] αβ T cells, as measured by cytokine production, and that a 1 mM $CaCl_2$ supplement is required to obtain the maximal cytokine response. Whether the function of unconventional T cells or of other lymphoid and myeloid cells similarly is impacted by the Ca^{2+} concentration *in vitro* is currently unknown. Unconventional T cells differ from examples of unconventional T cells are invariant Natural Killer T (*i*NKT) cells and mucosalassociated invariant T (MAIT) cells, which both express an αβTCR, and γδ T cells, which express a γδTCR. Both *i*NKT and MAIT cells express a highly conserved invariant TCR αchain, which recognizes glycolipids or riboflavin derivates in the context of the nonpolymorphic MHC class I homologs CD1d or MR1, respectively [6–9]. γδ T cells are largely MHCunrestricted and although the antigen for many γδ T cells is not known, some respond to phosphorylated isoprenoid metabolites or lipids [10, 11]. These unconventional T cells develop as memory T cells and can provide a first line of defence during immune responses [12]. B cells are the second main adaptive lymphoid cell type and are characterized by the expression of a BCR [13]. As an example of myeloid cells, we choose here macrophages, which are phagocytic and antigen-presenting effector cells of the innate immune system [14]. Depending on the way of stimulation, macrophages can differentiate into several functionally distinct subsets, often referred to as classically activated M1 or alternatively activated M2 macrophages [14–16]. To determine the impact of the Ca^{2+} concentration on lymphoid and myeloid cells besides conventional αβ T cells, we here compared their immune response *in vitro* in the presence of normal RPMI1640 medium ($RPMI^{norm}$) and RPMI1640 medium supplemented with 1 mM Ca^{2+} ($RPMI^{suppl}$). Our data indicated that elevated Ca^{2+} concentrations during PMA/ionomycin stimulation *in vitro* increased the cytokine production by both mice and human lymphoid cells for most cytokines tested. Furthermore, the polarization of human macrophages shifted towards an M1 phenotype in the high-Ca^{2+} environment. Consequently, the Ca^{2+} concentration during *in vitro* cultures is an important variable to be considered for functional experiments.

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Abbreviations: αGalCer, αgalactosylceramide; FCS, fetal calf serum; ICCS, intracellular cytokine staining; *i*NKT, invariant Natural Killer T; MAIT, mucosalassociated invariant T; MFI, mean fluorescent intensity; PBMCs, peripheral blood mononucleated cells; ROS, reactive oxygen species; RT, room temperature.

Results

Cytokine production of mouse unconventional T cells and of B cells is augmented by increased Ca^{2+} concentrations *in vitro*

Following activation, *i*NKT cells are able to produce a wide range of cytokines, including T_h1 cytokines, like IFNγ and TNF; T_h2 cytokines, like IL-4 and IL-13, the T_h17 cytokine IL-17A, as well as IL-10 [17–19]. When splenic mouse *i*NKT cells (**S1 Fig**) were stimulated *in vitro* with PMA and ionomycin, the increased Ca^{2+} concentration had no detrimental effect on *i*NKT cell survival (**S2A Fig**). However, we observed a marked and significant increase in the production of all cytokines tested (IFNγ, IL-2, IL-4, IL-10, IL-13, IL-17A) by *i*NKT cells when stimulated in media supplemented with calcium (**Fig 1**). Similar to *i*NKT cells, γδ T cells can produce a wide range of cytokines following stimulation [20]. No detrimental effect of the Ca^{2+} supplementation on γδ T cell survival was observed (**S2B Fig**). However, we noticed a significant increase in the production of IFNγ, IL-2, and IL-4 by γδ T cells stimulated in elevated Ca^{2}

+ levels (Fig 2A–2C). In contrast, the changes for IL-10 and IL-17A remained non-significant (S3A and S3B Fig). We also analysed the impact of Ca^{2+} levels on B cells. The survival of stimulated B cells was not impaired by the Ca^{2+} concentration *in vitro* (S2C Fig). However, an increase in the production of IL-2 and IL-10 was observed (Fig 2D and 2E), while IFNγ (S3C Fig) remained unaffected. Therefore, the PMA/ionomycin stimulation of mouse lymphoid

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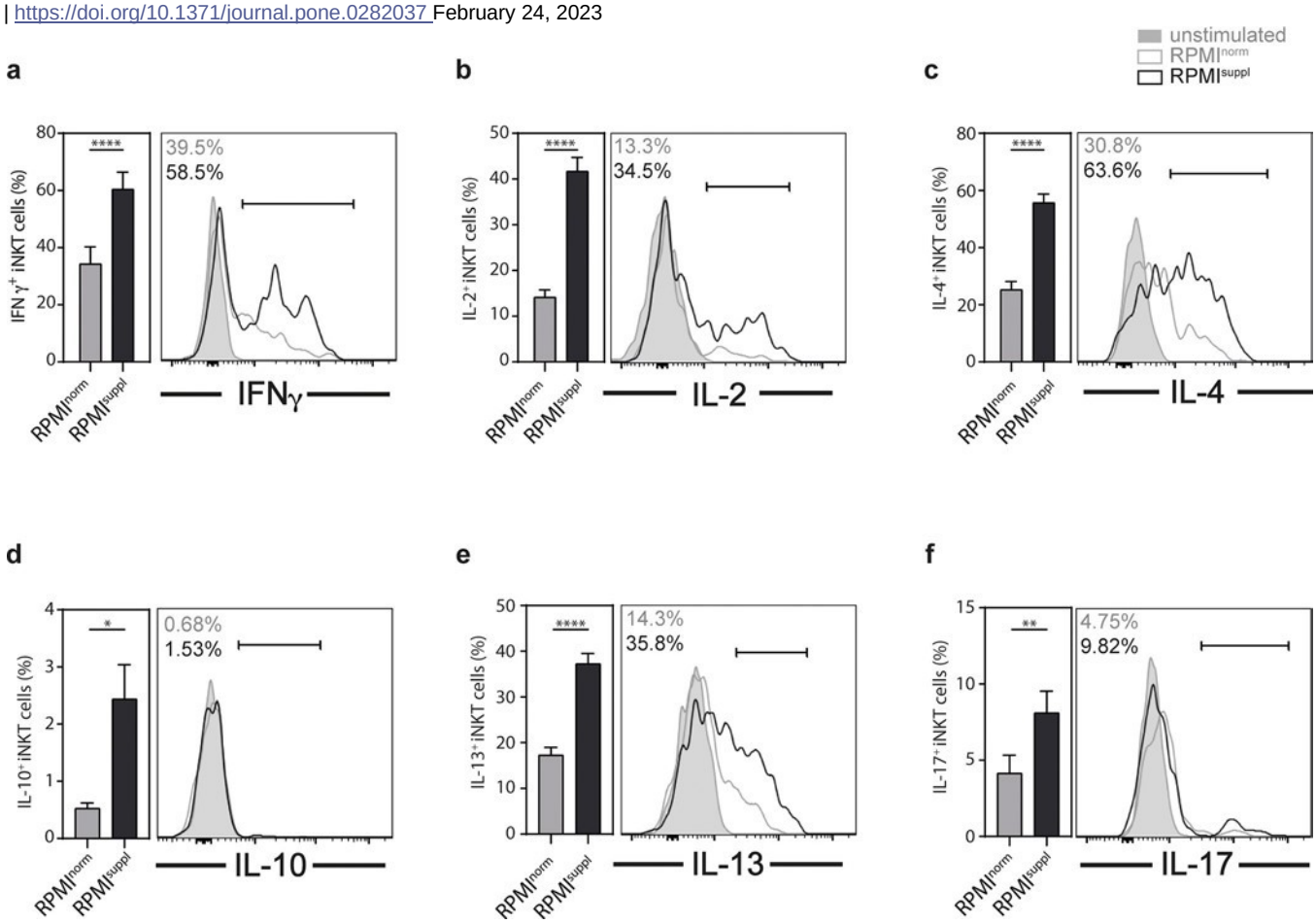


Fig 1. Ca^{2+} supplementation *in vitro* increases the cytokines production of mouse *i*NKT cells. Splenocytes from C57BL/6 mice were stimulated 4 h with 50 ng/ml PMA and 1 μg/ml ionomycin in either normal RPMI1640 medium ($RPMI^{norm}$) or RPMI1640 medium supplemented with 1 mM Ca^{2+} ($RPMI^{suppl}$). The production of **(a)** IFNγ, **(b)** IL-2, **(c)** IL-4, **(d)** IL-10, **(e)** IL-13, and **(f)** IL-17A by *i*NKT cells (live $CD8\alpha^-$ $CD19/CD45R^-$ $CD44^+$ $TCR\beta/CD3\varepsilon^+$ CD1d/ PBS57-tetramer$^+$ cells) was analysed by intracellular cytokine staining (ICCS). Summary graphs (left panels) and representative data (right panels) from gated *i*NKT cells are shown, respectively. Data were pooled from three independent experiments with three mice per group per experiment (n = 9).

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cells in complete RPMI medium supplemented with 1.0 mM Ca^{2+} improves the detection of numerous cytokines, without impacting the survival of the cells.

Cytokine production of human unconventional T cells and of B cells is augmented by increased Ca^{2+} concentrations *in vitro*

Having established that increased Ca^{2+} concentrations during *in vitro* stimulation can augment the cytokine production of mouse lymphoid cells, we next tested its effect on human lymphoid cells. For primary human *i*NKT cells, the elevated Ca^{2+} levels only increased the production of TNF slightly (Fig 3A), without effects on the other cytokines tested (IL-2, IFNγ, IL4, IL-17A) or on the expression of the activation marker CD69 (S4 Fig). For primary human

Vδ2$^+$ T cells, some (TNF, IFNγ) (Fig 3B and 3c) but not all (IL-2, IL-4) were boosted by the Ca^{2+} supplementation, which did also not influence CD69 expression (S5A–S5C Fig). For primary human MAIT cells, the elevated Ca^{2+} levels increased the production of all cytokines tested (IL-2, TNF, IFNγ) (Fig 3D–3F), without changes to the expression of CD69 (S5D Fig). A similar effect of the Ca^{2+} concentrations was noted for primary human B cells (IL-2, TNF, CD69) (Fig 4, S5E Fig). For some immune cells, the low frequency in the peripheral blood

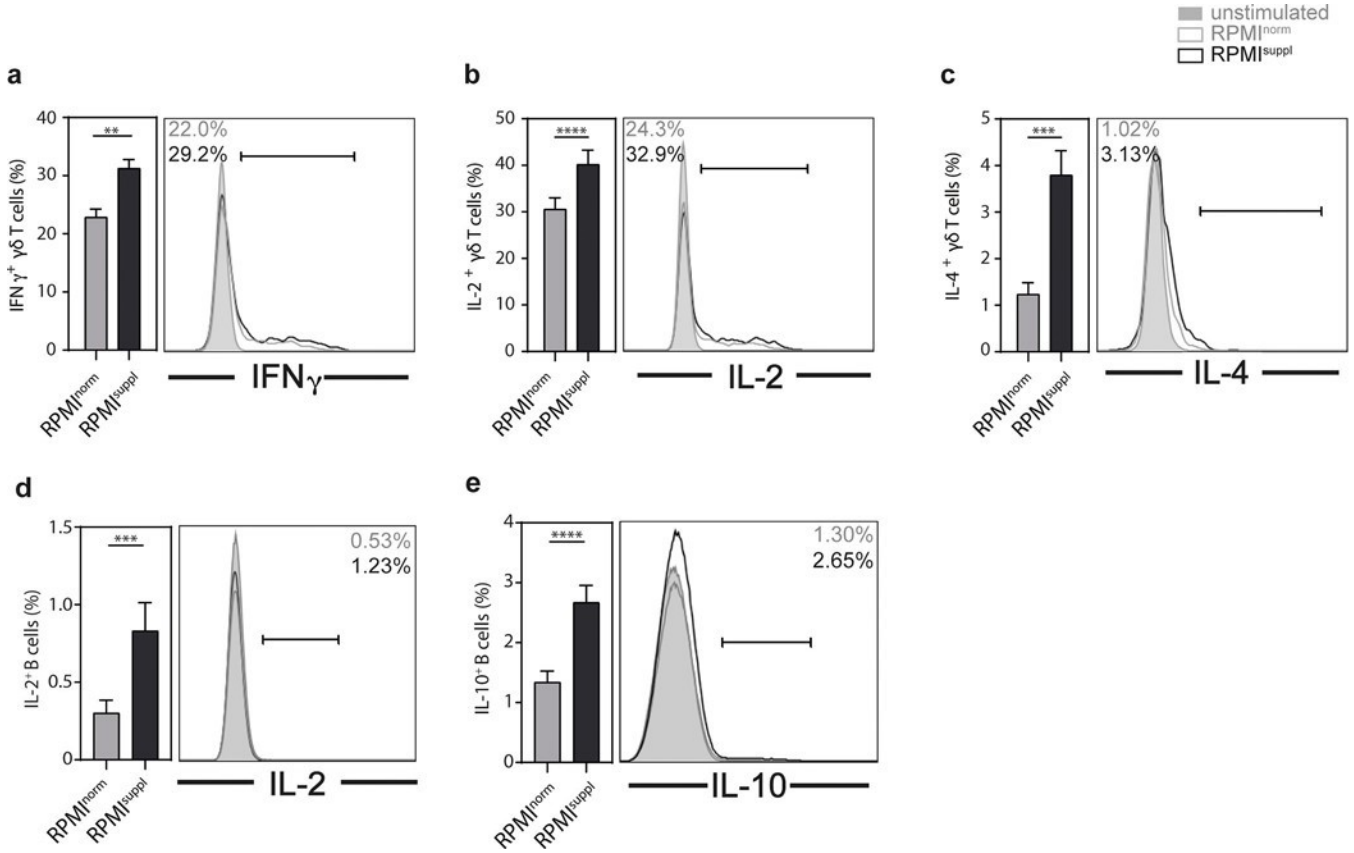


Fig 2. Ca^{2+} supplementation *in vitro* increases the production of some cytokines by mouse γδ T and B cells. Splenocytes from C57BL/6 mice were stimulated 4 h with 50 ng/ml PMA and 1 μg/ml ionomycin in either normal RPMI1640 medium ($RPMI^{norm}$) or RPMI1640 medium supplemented with 1 mM Ca^{2+} ($RPMI^{suppl}$). The production of **(a)** IFNγ, **(b)** IL-2, and **(c)** IL-4 by γδ T cells (live CD19/CD45R$^-$ CD4$^-$ CD8α$^-$ CD3ε$^+$ γδTCR$^+$ cells) and the production of **(d)** IL-2 and **(e)** IL-10 by B cells (live CD3ε$^-$ CD4$^-$ CD8α$^-$ CD19/CD45R$^+$ cells) was analysed by ICCS. Summary graphs (left panels) and representative data (right panels) from gated γδ T cells and B cells are shown, respectively. Data were pooled from three independent experiments with three mice per group per experiment (n = 9).

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makes their analysis directly *ex vivo* difficult, which is why protocols were established to expand them *in vitro*. We, therefore, also tested the impact of elevated Ca^{2+} concentrations on *in vitro* expanded *i*NKT and Vδ2$^+$ T cells. For expanded human *i*NKT cells, the elevated Ca^{2+} levels only increased the production of TNF slightly (Fig 5B), without effects on the other cytokines tested (IL-2, IFNγ, IL-4, IL-17A) (S6A–S6D Fig) and decreased CD69 expression (Fig 5A). For expanded human Vδ2$^+$ T cells, some (IFNγ, GM-CSF) (Fig 5D and 5E) but not all (CD69, TNF, IL-4) (S6E–S6G Fig) markers were boosted by the Ca^{2+} supplementation, whereas the production of IL-2 surprisingly decreased (Fig 5C). For all human lymphoid cell populations tested, the Ca^{2+} supplementation *in vitro* did not impair the cell survival (S7A–S7F Fig). Therefore, similar to mouse lymphoid cells, the detection of cytokines in primary

human lymphoid cells can be improved by increasing the Ca^{2+} levels during the *in vitro* stimulation.

Increased Ca^{2+} during *in vitro* stimulation of macrophages increases cell death and induces a shift toward M1 polarization

Given the clear ability of increased *in vitro* Ca^{2+} concentrations to augment the cytokine production of stimulated mouse and human lymphoid cells, we tested next the impact on myeloid cells. To this end, we initially measured the impact of elevated Ca^{2+} levels on primary human

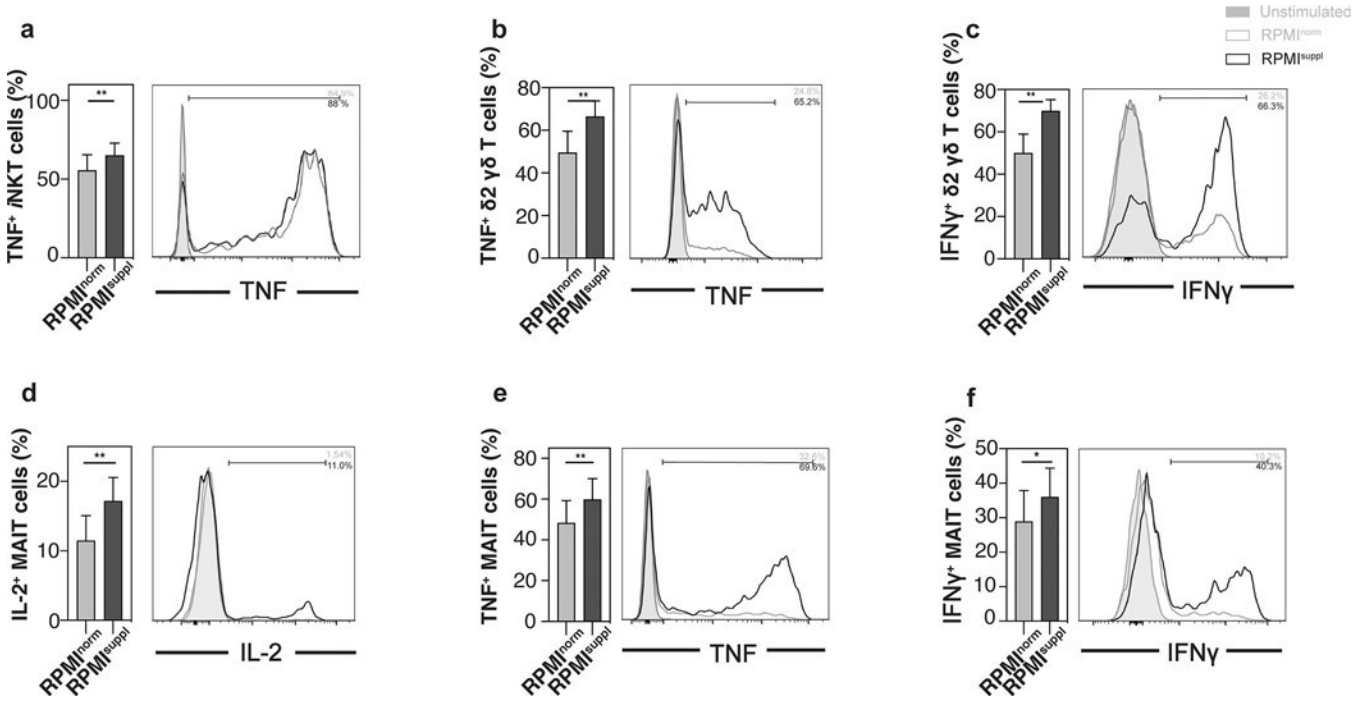


Fig 3. Ca^{2+} supplementation *in vitro* modulates cytokine production by primary human *i*NKT cells, Vδ2$^+$ T cells, and MAIT cells. PBMCs were isolated from the residual leukocyte units of healthy donors. PBMCs were stimulated for 4 h with 25 ng/ml PMA and 1 μg/ml ionomycin in either normal RPMI1640 medium ($RPMI^{norm}$) or RPMI1640 medium supplemented with 1 mM Ca^{2+} ($RPMI^{suppl}$). **(a)** Human Vα24*i* NKT cells (live $CD14^-$ $CD20^-$ $CD3^+$ $6B11^+$ cells) were analysed for the expression of TNF. **(b, c)** Human Vδ2$^+$ T cells (live $CD14^-$ $CD20^-$ $CD3^+$ γδTCR^{low} or Vδ2$^+$ cells) were analysed for the expression of (b) TNF and (c) IFNγ. **(d-f)** Human MAIT cells (live $CD14^-$ $CD20^-$ $CD3^+$ Vα7.2$^+$ $CD161^+$ cells) were analysed for the production of (d) IL-2, (e) TNF, and (f) IFNγ. Summary graphs (left panels) and representative data (right panels) from gated *i*NKT cells, Vδ2$^+$ T cells, and MAIT cells are shown, respectively. Data were pooled from three (*i*NKT cells, Vδ2$^+$ T cells; n = 9) and four independent (MAIT cells; n = 12) experiments with three samples each.

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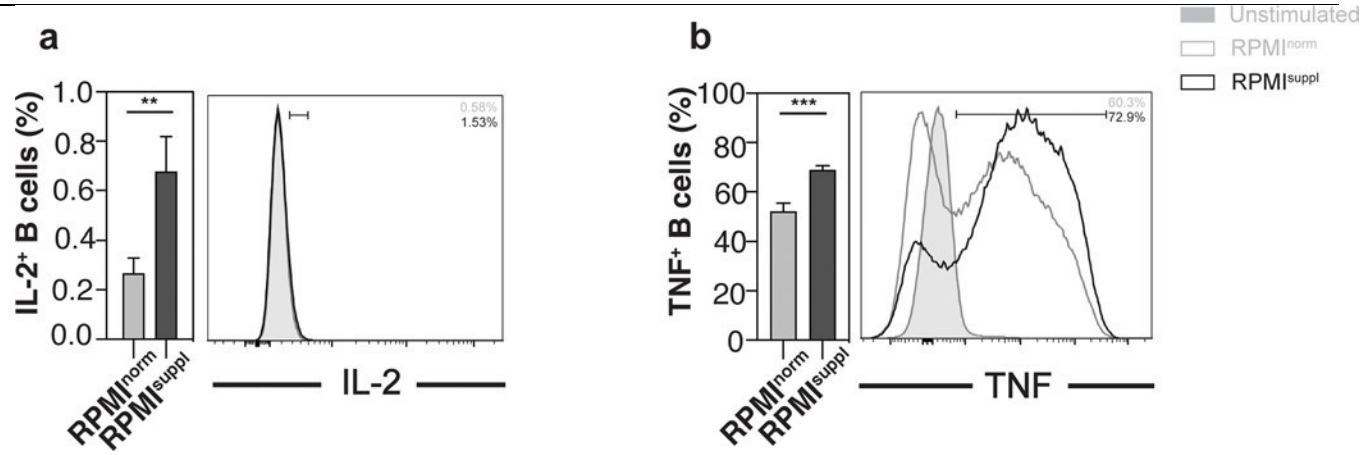


Fig 4. Ca^{2+} supplementation *in vitro* increases the cytokine production of primary human B cells. PBMCs were isolated from residual leukocyte units of healthy donors. PBMCs were stimulated for 4 h with 25 ng/ml PMA and 1 μg/ml ionomycin in either normal RPMI1640 medium ($RPMI^{norm}$) or RPMI1640 medium supplemented with 1 mM Ca^{2+} ($RPMI^{suppl}$). Human B cells ($CD14^-$ $CD3^-$ $CD20^+$ cells) were analysed for the production of **(a)** IL-2 and **(b)** TNF. Summary graphs (left panels) and representative data (right panels) from gated B cells are shown, respectively. Data were pooled from four independent experiments with three samples each (n = 12).

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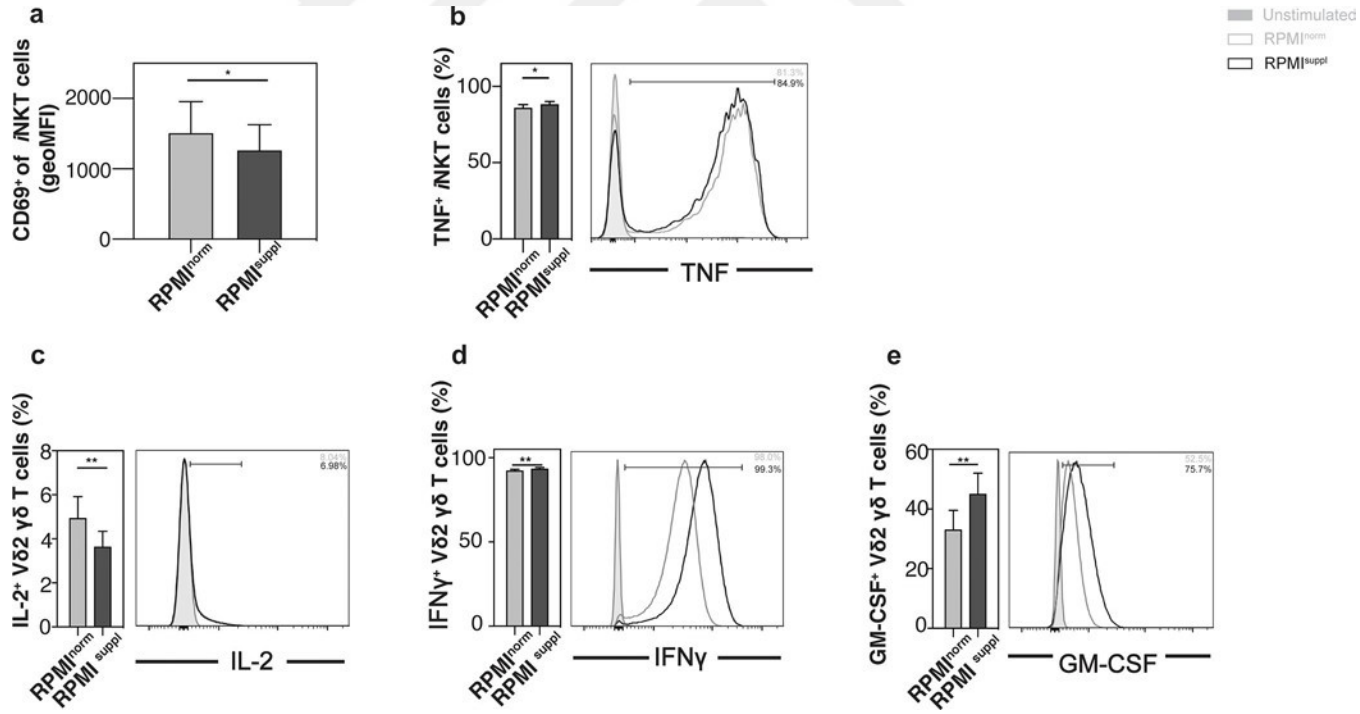


Fig 5. Ca^{2+} supplementation *in vitro* modulates cytokine production by expanded human *i*NKT cells and $V\delta2^+$ T cells. (a, b) *i*NKT cells were expanded *ex vivo* in the presence of αGalCer. The expanded cells were stimulated for 4 h with 25 ng/ml PMA and 1 μg/ml ionomycin in either normal RPMI1640 medium ($RPMI^{norm}$) or RPMI1640 medium supplemented with 1 mM Ca^{2+} ($RPMI^{suppl}$). Vα24*i* NKT cells (live $CD14^-$ $CD20^-$ $CD3^+$ $6B11^+$ cells) were analysed for the expression of (a) CD69 and the production of (b) TNF. **(c-e)** $V\delta2^+$ T cells were expanded *in vitro* in the presence of Zoledronic acid. Human $V\delta2^+$ T cells (live $CD14^-$ $CD20^-$ $CD3^+$ $V\delta2^+$ cells) were analysed for the production of (c) IL-2, (d) IFNγ, and (e) GM-CSF. Summary graphs (left panels) and representative data (right panels) from gated *i*NKT cells and $V\delta2^+$ T cells are shown, respectively. Data were pooled from four (*i*NKT cells; n = 12) and three ($V\delta2^+$ T cells, n = 9) independent experiments with three samples each. https://doi.org/10.1371/journal.pone.0282037.g005

PBMC monocytes and noticed that some (TNF, IFNγ; Fig 6) but not all (IL-2; S5F Fig) cytokines were boosted by the Ca^{2+} supplementation, without impact on cell survival (S7G Fig). These data suggested that the cytokine detection by primary myeloid cells could benefit from *in vitro* Ca^{2+} supplementation as well. Blood monocytes have the ability to

differentiate into functionally distinct macrophages subsets and, therefore, we tested next the impact of the Ca^{2+} concentration on human monocyte-derived macrophages. Primary human monocyte-derived macrophages (M0 macrophages) were polarized into M1 with LPS and IFNγ, into M2a with IL-4 stimulation, or into M2c with IL-10 with or without Ca^{2+} supplementation. Surprisingly, and in contrast to the findings with human lymphoid cells (S[1] Fig), the polarization of M0 macrophages in elevated Ca^{2+} concentrations increased cell death, regardless of the subtype they were polarized into (Fig 7A–7c). Similar results were seen when M0 macrophages were cultured alone in Ca^{2+} supplemented medium (S8 Fig). When M0 macrophages were cultured alone or when they were polarized into M1 macrophages, higher Ca^{2+} levels increased the expression of the M1 markers HLA-DRα and CD86 (S9A Fig). In contrast, when M0 macrophages were cultured alone or when they were polarized into M2a macrophages, Ca^{2+} supplementation decreased the expression of the M2a markers CD200R and CD206 (S9B Fig). The Ca^{2+} levels did not influence the expression of CD163 on M2c polarized macrophages (S9C Fig). Importantly, when the expression of the M1 markers HLA-DRα and CD86 was analysed on M2a and M2c macrophages, Ca^{2+} supplementation increased the expression of HLA-DRα on M2a macrophages (Fig 7D) and of CD86 on both M2 macrophages subsets (Fig 7E and

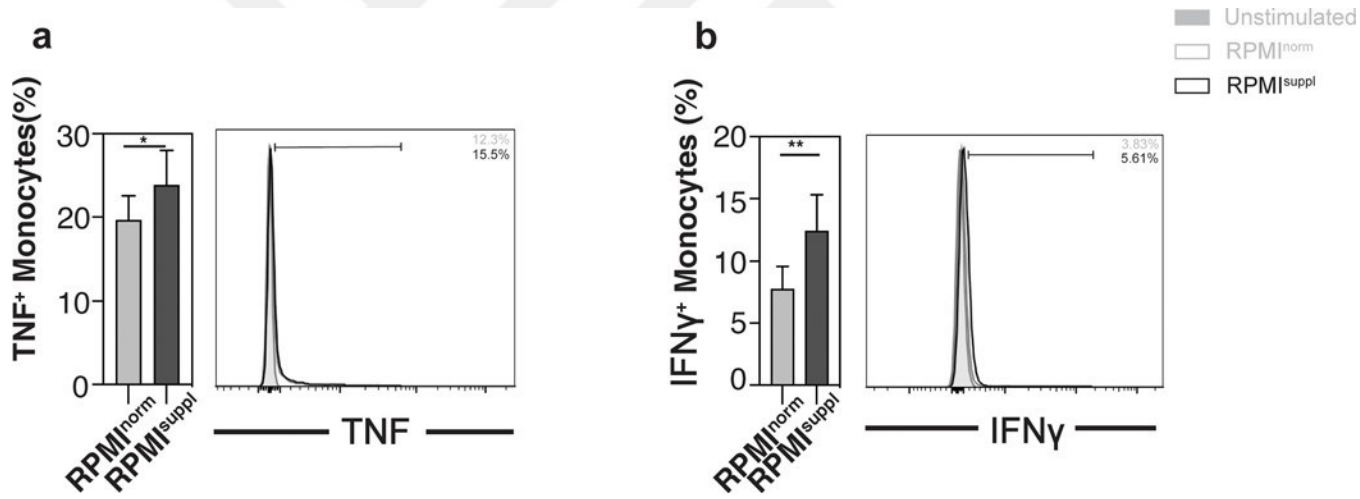


Fig 6. Ca^{2+} supplementation *in vitro* increases the cytokine production of primary monocytes. PBMCs were isolated from residual leukocyte units of healthy donors. PBMCs were stimulated for 4 h with 25 ng/ml PMA and 1 μg/ml ionomycin in either normal RPMI1640 medium ($RPMI^{norm}$) or RPMI1640 medium supplemented with 1 mM Ca^{2+} ($RPMI^{suppl}$). Human monocytes ($CD3^-$ $CD20^-$ $CD14^+$ cells) were analysed for production of **(a)** TNF and **(b)** IFNγ. Summary graphs (left panels) and representative data (right panels) from gated monocytes are shown, respectively. Data were pooled from four independent experiments with three samples each (n = 12).

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polarization of human monocyte-derived macrophages *in vitro*. However, these phenotypic changes appeared not to translate into functional changes, as the production of TNF and CXCL10 by M1 macrophages (Fig 7G and 7H) and the production of TGFβ and IL-4 by M2a and M2c macrophages (Fig 7I and 7J) was not influenced by the Ca^{2+} supplementation. These data suggest that for myeloid cells the impact of an increased Ca^{2+} concentration on the cytokine production depends on the activation status of the cells and needs to be tested cell type specifically.

[1] F). These data indicate that increased Ca^{2+} concentrations support phenotypic M1

Discussion

Here we demonstrate that supplementing the RPMI1640 medium with 1 mM Ca^{2+} can increase the cytokine production by both mice and human lymphoid cells during PMA/ionomycin stimulation *in vitro* without impacting the survival of the cells. This effect appeared stronger for primary lymphoid cells than *in vitro* expanded cells. Primary human monocytes responded similarly with an augmented cytokine production to the elevated Ca^{2+} concentrations. However, for human monocyte-derived macrophages a distinct effect was observed: elevated Ca^{2+} concentrations *in vitro* led to increased cell death and promoted phenotypic M1 polarization without impacting cytokine production.

Previous work on conventional mouse [4] and human [21] αβ T cells showed that the 0.49 mM Ca^{2+} in RPMI1640 are suboptimal to drive cytokine production during *in vitro* stimulation and a minimal concentration of 1.5 mM was suggested. The basal level of intracellular Ca^{2+} in T cells is approx. 100 nM and can increases to 1 μM following stimulation [22]. Therefore, it is not self-evident how an increase of the extracellular Ca^{2+} concentration above 0.49 mM can boost cytokine production by conventional αβ T cells [4, 21]. Irrespective of the mechanism, this observation is important to optimize the analysis of conventional αβ T cells *in vitro*. Here, we confirmed that unconventional αβ T cells, γδ T cells, and B cells from both mouse and human also produce more cytokines after *in vitro* stimulation in the presence of elevated Ca^{2+} levels. The Ca^{2+} concentration of the extracellular fluid *in vivo* in humans was measured to be 2.2–2.7 mM [23]. This indicates that the Ca^{2+} supplementation brings the

Ca^{2+}

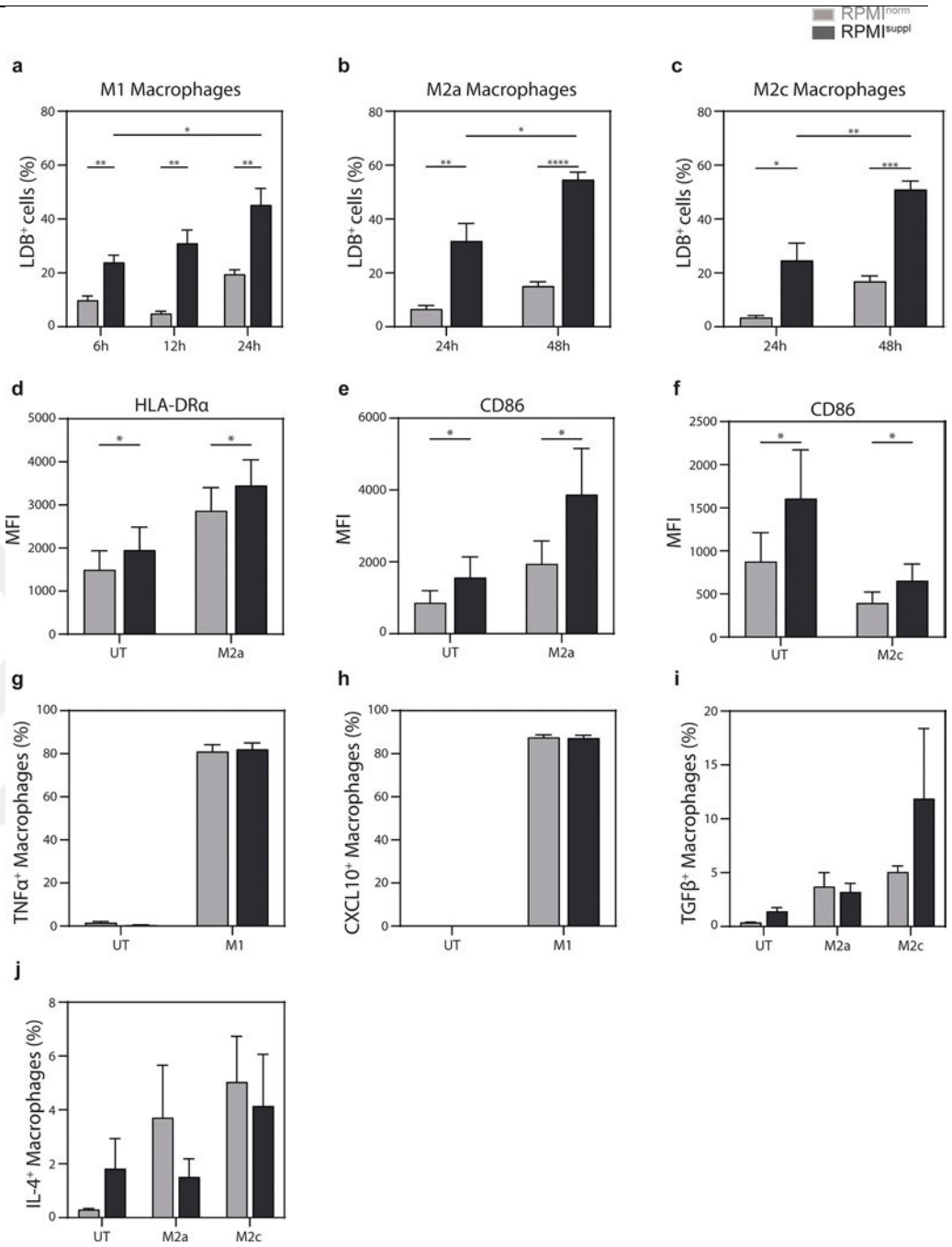


Fig 7. Ca^{2+} supplementation *in vitro* increases cell death in polarized human macrophages and favours an M1 phenotype. Primary human monocyte-derived macrophages were cultured untreated (UT) or polarized into M1 (100 ng/ ml LPS, 20 ng/ml IFNγ), M2a (20 ng/ml IL-4), or M2c (20 ng/ml IL-10) macrophages for the indicated hours in either normal RPMI1640 medium ($RPMI^{norm}$) or RPMI1640 medium supplemented with 1 mM Ca^{2+} ($RPMI^{suppl}$) as indicated. **(a-c)** The bar graphs show the relative percentages of cells positive for LIVE/DEAD Fixable Blue Dead Cell Stain (LDB^+ cells), indicating dead cells, for (a) M1, (b) M2a, and (c) M2c macrophage. **(d-f)** The expression (mean fluorescent intensity, MFI) of (d) HLA-DRα (UT; M2a, 24 hours) and (e, f) CD86 ((e): UT; M2a, 24 hours; (f): UT; M2c, 24 hours) on live macrophages is shown. The data shown are means ± SEM of biological replicates of six donors, pooled from two independent experiments with similar results. **(g-j)** The production of (g) TNF and (h) CXCL10 by M1 macrophages (6 hours) and of (i) TGFβ, and (j) IL4 by M2a (48 hours) or M2c (48 hours) macrophages was analysed ICCS. The data shown are means ± SEM of biological replicates of five (TGFβ, IL-4) or six donors (CXCL10, TNF), pooled from two independent experiments with similar results.

https://doi.org/10.1371/journal.pone.0282037.g007 concentration of the $RPMI^{suppl}$ medium (approx. 1.8 mM) close to the physiological conditions *in vivo*.

For primary mouse lymphoid cells, the elevated Ca^{2+} concentration increased the production of 6 out of 6 cytokines tested for *i*NKT cells (Fig 1), 3 out of 5 for γδ T cells (Fig 2A–2C; S3A and S3B Fig), and 2 out of 3 for B cells (Fig 2D and 2E; S3C Fig), without a decrease of the production of any of the cytokines tested. Interestingly, the boosting Ca^{2+} effect appeared weaker for primary human lymphoid cells: the elevated Ca^{2+} concentration increased the production of 1 out of 5 cytokines tested for *i*NKT cells (Fig 3A; S4B–S4E Fig), 2 out of 4 for γδ T cells (Fig 3B and 3C; S5A–S5C Fig), 3 out of 3 for MAIT cells (Fig 3D–3F), and 2 out of 2 for B cells (Fig 4), again without any decreases of any of the cytokines tested. The reason for this species difference is unclear at this point. The cytokine response of the *in vitro* expanded cell lines under increased Ca^{2+} concentrations was comparable to the primary cells: both primary and expanded *i*NKT cells showed increased production for 1 (TNF) out of 5 cytokines tested (Figs 3A and 5B; S6A–S6D Fig). Primary γδ T cells showed increased production for 2 (IFNγ, TNF) out of 4 (Fig 3B and 3C; S5B and S5C Fig) and expanded γδ T cells showed increased production for 2 (GM-CSF, IFNγ) out of 5 (Fig 5D and 5E; S6F and S6G Fig) cytokines tested. Surprisingly, expanded γδ T cells showed a decrease of IL-2 with increased Ca^{2+} (Fig 5C), which is the only instance in which we noticed a decrease in cytokine production.

Similar to the lymphoid cells, primary human monocytes increased the production of 2 out of 3 cytokines tested (Fig 6; S5F Fig) when stimulated in the presence of elevated Ca^{2+} levels (Fig 6; S5F Fig). However, the data we obtained with human primary monocytederived macrophages were in contrast to the ones from lymphoid cells and primary human monocytes. Most importantly, we noticed a clear increase in the frequency of macrophages that died when incubated for more than six hours in the presence of elevated Ca^{2+} levels (Fig 7A–7C; S8 Fig). Calcium-induced ER-stress [24, 25] and mitochondrial changes [26] can trigger ROS- (reactive oxygen species) production in macrophages, which can lead to cell death [24, 25, 27–29]. This might explain why macrophages are more sensitive to the Ca^{2+} concentration of the medium. Furthermore, we observed a shift towards an M1 phenotype for both M0 macrophages (S9 Fig) as well as M1 and M2 macrophages (Fig 7D–7F) in the presence of elevated Ca^{2+} levels. However, these phenotypic changes appeared not to translate into functional changes (Fig 7G–7J). Incidentally, ROS-induced NFκB/MAPK activation in macrophages [30, 31] supports M1 polarization [32–34]. However, given the large amount of macrophage cell death we observed, we cannot exclude the possibility that the apparent M1 shift is the result of M1 macrophages potentially being less sensitive to this Ca^{2+}-induced cell death. We are aware of only two other studies on the impact of Ca^{2+} on the cytokine production of macrophages. Both showed that calcium influx can impair LPS-induced IL-12 production of mouse macrophages [35, 36] without affecting the production of TNF or IL-6 [36]. Therefore, it is unclear at this stage whether elevated Ca^{2+} concentration *in vitro* can influence the cytokine production of macrophages.

In summary, our data demonstrate that the Ca^{2+} concentration during *in vitro* cultures is an important variable to be considered for functional experiments and that supplementing the media with 1 mM Ca^{2+} can boost the cytokine production by both mice and human lymphoid cells.

Material and methods

Human samples

Residual leukocyte units from healthy donors were provided by Dokuz Eylul University Blood Bank (Izmir, Turkey) after obtaining informed written consent from all donors. The ethical approval for the study was obtained from the 'Noninvasive Research Ethics Committee' of the Dokuz Eylul University (approval number: 2018/06-27/3801-GOA). All protocols performed were in accordance with the relevant guidelines and regulations for human samples.

Mice

All mice were housed in the vivarium of the Izmir Biomedicine and Genome Center (IBG, Izmir, Turkey) in accordance with the respective institutional animal care committee guidelines. C57BL/6 and BALB/c mice were originally purchased from the Jackson Laboratories (Bar Harbor, ME, USA). All mouse experiments were performed with prior approval by the institutional ethic committee ('Ethical Committee on Animal Experimentation' of the Izmir Biomedicine and Genome Center, approval number: 19/2016) in accordance with national laws and policies. All the methods were carried out in accordance with the approved guidelines and regulations and following 3R (replacement, reduction, refinement) procedures. These aspects of this study are reported in accordance with the ARRIVE guidelines [37]. Mice were sacrificed by cervical dislocation and as all mouse experiments were performed *ex vivo*, no anaesthesia was required.

Reagents, monoclonal antibodies, and flow cytometry

α-galactosylceramide (αGalCer) was obtained from Avanti Polar Lipids (Birmingham, AL, USA). Monoclonal antibodies were purchased from either BioLegend (San Diego, CA, USA), eBiosciences (San Diego, CA, USA), BD Biosciences (Franklin Lane, NJ, USA), or R&D Systems (Minneapolis, MN, USA). The list of antibodies against the mouse and human antigens used in this study, with clone name, vendor, and the conjugated fluorochrome, is given in **S1 Table**. Anti-mouse CD16/32 (2.4G2) antibody (Tonbo Biosciences, San Diego, CA, USA) or Human TruStain FcX (BioLegend) was used to block Fc receptors according to the manufacturers' recommendations. Unconjugated mouse and rat IgG antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Dead cells were labelled with Zombie UV Dead Cell Staining kit (BioLegend) or with LIVE/DEAD Fixable Blue Dead Cell Stain kit (ThermoFisher Scientific, Waltham, MA, USA). Flow cytometry of fluorochromeconjugated antigen-loaded CD1d tetramers were performed as described [38]. Cells were analysed with LSR-Fortessa (BD Biosciences), and data were processed with CellQuest Pro (BD Biosciences) or FlowJo (BD Biosciences) software. Graphs derived from digital data are displayed using a 'bi-exponential display'. Cell were gated as follows: (a) mouse: Vα14*i* NKT cells (live $CD8\alpha^-$ $CD19/CD45R^-$ $CD44^+$ $TCR\beta/CD3\varepsilon^+$ CD1d/PBS57-tetramer$^+$ cells), γδ T cells (live $CD19/\ CD45R^-$ $CD4^-$ $CD8\alpha^-$ $CD3\varepsilon^+$ $\gamma\delta TCR^+$ cells), and B cells (live $CD3\varepsilon^-$ $CD4^-$ $CD8\alpha^-$ $CD19/\ CD45R^+$ cells); (b) human: Vα24*i* NKT cells (live $CD14^-$ $CD20^-$ $CD3^+$ $6B11^+$), $\delta2^+$ T cells *ex vivo* (live $CD14^-$ $CD20^-$ $CD3^{high}$ $\gamma\delta TCR^{low}$ cells, or live $CD14^-$ $CD20^-$ $CD3^+$ $V\delta2^+$ cells) and *in vitro* (live $CD14^-$ $CD20^-$ $CD3^+$ $V\delta2^+$ cells), MAIT cells (live $CD14^-$ $CD20^-$ $CD3^+$ $V\alpha7.2^+$ $CD161^+$ cells), B cells (live $CD3^-$ $CD14^-$ $CD20^+$ cells), and macrophages (live $CD68^+$ cells). Representative data are shown in **S1 Fig**.

Cell preparation

Single-cell suspensions from mouse spleens were prepared as described [39]. In brief, the spleens were filtered through a 70 μm cell strainer (BD Biosciences) and red blood cells and dead cells were eliminated through Lymphoprep (StemCell Technologies, Vancouver, Canada) density gradient centrifugation (300 *g*, 10 min, RT). Human PMBCs were obtained from the residual leukocyte units of healthy donors via density gradient centrifugation (300 *g*, 30 min, RT) with Ficoll-Paque Plus (GE Healthcare, Chicago IL, USA). To obtain monocytes, a second density gradient centrifugation with 46% iso-osmotic Percoll (GE Healthcare) was performed (400 *g*, 10 min, RT) [40].

In vitro expansion of human *i*NKT cells

Human *i*NKT cells were expanded from resting PBMCs of healthy donors as described before [41]. Briefly, freshly isolated PBMCs (1 x 10^6 cell/ml, 5 ml/well) were treated with 100 ng/ml αGalCer (KRN7000, Avanti Polar Lipids) and cultured for 13 days. 20 IU/ml human recombinant IL-2 (Proleukin, Novartis, Basel, Switzerland) was added to the cultures every other day starting from day 2. From day 6 onwards, the concentration of IL-2 was increased to 40 IU/ml. At the end of the expansion, an aliquot of each sample was collected and analysed for *i*NKT cell expansion by flow cytometry. Expansion was done in RPMI 1640 (Gibco, Waltham, MA, USA, or Lonza, Basel, Switzerland) supplemented with 5% (v/v) Human AB serum (Sigma-
Aldrich, St. Louis, MO, USA), 1% (v/v) Penicillin/Streptomycin, 1 mM sodium pyruvate (Lonza), 1% (v/v) non-essential amino acids (Cegrogen Biotech, Stadtallendorf, Germany), 15 mM HEPES buffer (Sigma-Aldrich), and 55 μM 2-mercaptoethanol (AppliChem, Darmstadt, Germany).

In vitro expansion of human Vδ2$^+$ T cells

Human Vδ2$^+$ T cells were expanded from PBMCs of healthy donors similar to published protocols [41–43]. Briefly, freshly isolated PBMCs (1 x 10^6 cell/ml, 5 ml/well) were cultured with 5 μM Zoledronic acid (Zometa, Novartis) in the presence of 100 IU/ml human recombinant IL-2 (Proleukin, Novartis) for 13 days. IL-2 was replenished every other day and from day 6 onwards the concentration was increased to 200 IU/ml. The cultures were performed in RPMI1640 (Gibco or Lonza) supplemented with 5% (v/v) Human AB serum (Sigma-Aldrich), 1% (v/v) Penicillin/Streptomycin (Gibco), 1 mM sodium pyruvate (Lonza), 1% (v/v) nonessential amino acids (Cegrogen), 15 mM HEPES buffer (Sigma-Aldrich), and 55 μM 2-mercaptoethanol (AppliChem).

Human macrophage generation

Purified monocytes were cultured in RPMI1640 (Gibco or Lonza) medium containing 5% FCS (Corning, NY, USA), 1% Penicillin/Streptomycin (Gibco), and 10 ng/ml human recombinant M-CSF (Peprotech, London, UK) for their differentiation into macrophages. 3 x 10^6 cells/ well were seeded in low-attachment 6-well plates (Corning) and incubated for 7 days at 5% CO_2 and 37˚C. Macrophages were collected and cultured in 24-well cell culture plates as 5 x 10^5 macrophages/well for 24 hours in RPMI1640 medium containing 5% FCS, 1% Penicillin/ Streptomycin. On the following day, the cell culture media was replaced with fresh media and macrophages were stimulated with the relevant polarization factors as described below. The cells were verified to be over 90% CD68$^+$ by flow cytometry.

In vitro stimulation

Splenocytes were stimulated *in vitro* with 50 ng/ml PMA and 1 μg/ml ionomycin (both SigmaAldrich) for 4 h at 37˚C in the presence of both Brefeldin A (GolgiPlug) and Monensin (GolgiStop, both BD Biosciences). As GolgiPlug and GolgiStop were used together, half the amount recommended by the manufacturer where used, as suggested previously [44]. Cells were stimulated in complete^{mouse} RPMI medium (RPMI 1640 supplemented with 10% (v/v) FCS (Corning), 1% (v/v) Pen-Strep-Glutamine (10.000 U/ml penicillin, 10.000 μg/ml streptomycin, 29.2 mg/ml L-glutamine (Gibco)) and 50 μM 2-mercaptoethanol (AppliChem), containing 0.42 mM Ca^{2+}). For human studies, freshly isolated PBMCs or expanded cell populations were stimulated *in vitro* with 25 ng/ml PMA and 1 μg/ml PMA for 4 h at 37˚C in the presence of Brefeldin A or monensin. Cells were stimulated in complete^{human} RPMI medium (RPMI 1640 supplemented with 10% (v/v) FCS, 1% (v/v) Penicillin/Streptomycin, 1 mM sodium pyruvate (Lonza), 1% (v/v) non-essential amino acids (Cegrogen), 15 mM HEPES buffer (SigmaAldrich), and 55 μM 2-mercaptoethanol (AppliChem). Considering the Ca^{2+} found in the FCS (3.9 mM) [21] the supplemented RPMI medium contained approx. 0.8 mM Ca^{2+} ($RPMI^{norm}$). To obtain the $RPMI^{suppl}$ medium containing elevated Ca^{2+} concentrations, 1 mM $CaCl_2$ (Sigma-Aldrich) was added to the $RPMI^{norm}$ medium (i.e. approx. 1.8 mM Ca^{2+}).

Human macrophage polarization

Macrophages were stimulated (i) for M1 polarization with 100 ng/ml LPS (InvivoGen, San Diego, CA, USA) and 20 ng/ml IFNγ (R&D Systems) for 6h, 12h, or 24h; (ii) for M2a with 20 ng/ml IL-4 (R&D Systems) for 24h or 48h; or (iii) for M2c with 20 ng/ml IL-10 (R&D Systems) for 24h or 48h. The particular incubation times for the experiments are specified in the figures.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Normal distribution was tested using D'Agastino–Pearson Test. Statistical comparisons were drawn using either a twotailed paired Student's t-test (normally distributed data) or Wilcoxon matched-pairs signed rank test (not normally distributed data) (Excel, Microsoft Corporation; GraphPad Prism, GraphPad Software). p-values <0.05 were considered significant and are indicated with (*p < 0.05, **p < 0.01, ***p < 0.001, ****p <0.0001. Each experiment was repeated at least twice, and background values were subtracted. Graphs were generated with GraphPad Prism (GraphPad Software).

Supporting information

S1 Fig. Gating strategy to identify leukocytes and macrophage purity. (A, B) Exemplary dot plots illustrating the gating strategy employed to identify the indicted (A) mouse or (B) human lymphoid cells. **(C)** Purity of the differentiated macrophages: human PBMC-derived monocytes were incubated for 7 days in RPMI1640 medium containing 10 ng/ml M-CSF for macrophage differentiation and the percentage of $CD68^+$ macrophages was determined by intracellular staining. Left: representative flow cytometry data (blue = undifferentiated; red = differentiated); Right: Summary data (n = 3; un-diff = undifferentiated; diff = differentiated). **(D-F)** Evaluation of macrophage polarization. Primary human monocytederived macrophages were left unstimulated (UT) or stimulated for 12 h (D) with

100 ng/ mL LPS and 20 ng/mL IFN-γ for M1 polarization (M1), (E) with 20 ng/mL IL-4 for M2a polarization (M2a), or (F) with 20 ng/ mL IL-10 for M2c polarization (M2c). Expression of indicated surface markers was analyzed by flow cytometry. The bar graphs indicate mean fluorescent intensity (MFI). The biological replicates of 6 independent donors pooled from 2 independent experiments are shown. (PDF)

S2 Fig. Stimulation of murine *i*NKT cells, γδ T cells, or B cells in $RPMI^{suppl}$ has no negative impact on cell viability. Splenocytes from C57BL/6 mice were stimulated 4 h with 50 ng/ml PMA and 1 μg/ml ionomycin in either normal RPMI1640 medium ($RPMI^{norm}$) or RPMI1640 medium supplemented with 1 mM Ca^{2+} ($RPMI^{suppl}$). **(a)** *i*NKT cells; **(b)** $V\delta2^+$ T cells; and **(c)** B cells were stained and analysed by flow cytometry. The bar graphs show the relative percentages of cells positive for LIVE/DEAD Fixable Blue Dead Cell Stain, indicating dead cells. Data were pooled from three independent experiments with three mice per group per experiment (n = 9). (TIF)

S3 Fig. Ca^{2+} supplementation *in vitro* has no impact on the production of some cytokines by mouse γδ T and B cells. (a-c) Splenocytes from C57BL/6 mice were stimulated 4 h with 50 ng/ml PMA and 1 μg/ml ionomycin in either normal RPMI1640 medium ($RPMI^{norm}$) or RPMI1640 medium supplemented with 1 mM Ca^{2+} ($RPMI^{suppl}$). The production of **(a)** IL-10 and **(b)** IL-17A by γδ T cells (live $CD19/CD45R^-$ $CD4^-$ $CD8\alpha^-$ $CD3\varepsilon^+$ $\gamma\delta TCR^+$ cells) and the production of **(c)** IFNγ by B cells (live $CD3\varepsilon^-$ $CD4^-$ $CD8\alpha^-$ $CD19/CD45R^+$ cells) was analysed by ICCS. Summary graphs (left panels) and representative data (right panels) from gated γδ T cells and B cells are shown, respectively. Data were pooled from three independent experiments with three mice per group per experiment (n = 9). (TIF)

S4 Fig. Ca^{2+} supplementation *in vitro* has no impact on the expression of CD69 and the production IL-2, IFNγ, IL-4, and IL-17 by primary human *i*NKT cells. PBMCs were isolated from the residual leukocyte units of healthy donors. PBMCs were stimulated for 4 h with 25 ng/ml PMA and 1 μg/ml ionomycin in either normal RPMI1640 medium ($RPMI^{norm}$) or RPMI1640 medium supplemented with 1 mM Ca^{2+} ($RPMI^{suppl}$). Human Vα24*i* NKT cells (live $CD14^-$ $CD20^-$ $CD3^+$ $6B11^+$ cells) were analysed for the expression of the activation marker **(a)** CD69 and the production of the cytokines **(b)** IL-2, **(c)** IFNγ, **(d)** IL-4, and **(e)** IL-17. Summary graphs (left panels) and representative data (right panels) from gated *i*NKT cells are shown, respectively. Data were pooled from three independent experiments with three samples each (n = 9). (JPG)

S5 Fig. Ca^{2+} supplementation *in vitro* has no impact on expression of CD69 on human primary $V\gamma2^+$ T cells, MAIT cells, and B cells, and on the production of the IL-2 by human monocytes and IL-4 by $V\gamma2^+$ T cells. PBMCs were isolated from residual leukocyte units of healthy donors and were stimulated for 4 h with 25 ng/ml PMA and 1 μg/ml ionomycin in either normal RPMI1640 medium ($RPMI^{norm}$) or RPMI1640 medium supplemented with 1 mM Ca^{2+} ($RPMI^{suppl}$). Human $V\delta2^+$ T cells (live $CD14^-$ $CD20^-$ $CD3^+$ $\gamma\delta TCR^{low}$ or $V\gamma2^+$ cells) were analysed for the expression of **(a)** CD69 and the production of **(b)** IL-2 and **(c)** IL-4. **(d)** Human MAIT cells (live $CD14^-$ $CD20^-$ $CD3^+$ $V\alpha7.2^+$ $CD161^+$ cells) were analysed for the expression of CD69. **(e)** Human B cells ($CD14^-$ $CD3^-$ $CD20^+$ cells) were analysed for the expression of CD69. **(f)** Human monocytes ($CD3^-$ $CD20^-$ $CD14^+$ cells) were analysed for the production of IL-2. Summary graphs (left panels) and representative data (right panels) are shown for the cytokine data. Data were pooled from three ($V\delta2^+$ T cells; n = 9) and four

(MAIT cells, B cells, monocytes; n = 12) independent experiments with three samples each. (JPG)

S6 Fig. Ca^{2+} supplementation *in vitro* has no impact on the expression of CD69 by expanded Vγ2$^+$ T cells and on the production of some cytokines by expanded human *i*NKT and Vγ2$^+$ T cells. *i*NKT cells were expanded *ex vivo* in the presence of αGalCer. Vγ2$^+$ T cells were expanded *in vitro* in the presence of Zoledronic acid. The expanded cells were stimulated for 4 h with 25 ng/ml PMA and 1 μg/ml ionomycin in either normal RPMI1640 medium ($RPMI^{norm}$) or RPMI1640 medium supplemented with 1 mM Ca^{2+} ($RPMI^{suppl}$). Vα24*i* NKT cells (live $CD14^-$ $CD20^-$ $CD3^+$ $6B11^+$ cells) were analysed for the production of (a) IL-2, (b) IFNγ, (c) IL-4, and (d) IL-17 were measured by ICCS. Human Vδ2$^+$ T cells (live $CD14^-$ $CD20CD3^+$ Vγ2$^+$ cells) were analysed for the expression of (e) CD69 and the production of (f) TNF, (g) IL4. Summary graphs (left panels) and representative data (right panels) from gated *i*NKT cells and Vγ2$^+$ T cells are shown, respectively. Data were pooled from four and three independent experiments with three samples each for *i*NKT cells (n = 12) and Vγ2$^+$ T cells (n = 9), respectively. (JPG)

S7 Fig. Stimulation of human lymphoid cells in $RPMI^{suppl}$ has no negative effect on cell viability. PBMCs were isolated from residual leukocyte units of healthy donors and were stimulated either directly (a-d) or after in vitro expansion of indicated cells (e, f). The cells were stimulated for 4 h with 25 ng/ml PMA and 1 μg/ml ionomycin in either normal RPMI1640 medium ($RPMI^{norm}$) or RPMI1640 medium supplemented with 1 mM Ca^{2+} ($RPMI^{suppl}$). Primary (a) *i*NKT cells, (b) Vδ2$^+$ T cells, (c) B cells; and (d) MAIT cells, (e) monocytes, or *in vitro* expanded (f) *i*NKT cells and (g) Vδ2$^+$ T cells were stained and analysed by flow cytometry. The bar graphs show the relative percentages of cells positive for LIVE/DEAD Fixable Blue Dead Cell Stain, indicating dead cells. Data were pooled from three (a-b, g) or four (c—f) independent experiment with three samples each (n = 9–12). (JPG)

S8 Fig. Ca^{2+} supplementation *in vitro* increases cell death of M0 macrophages. Primary human monocyte-derived macrophages were cultured for 6, 12, 24, or 48 hours in either normal RPMI1640 medium ($RPMI^{norm}$) or RPMI1640 medium supplemented with 1 mM Ca^{2+} ($RPMI^{suppl}$). The bar graphs show the relative percentages of cells positive for LIVE/DEAD Fixable Blue Dead Cell Stain (LDB^+ cells), indicating dead cells. Data shown are means ± SEM of biological replicates of six donors, pooled from two independent experiments with similar results. (JPG)

S9 Fig. Ca^{2+} supplementation *in vitro* affects the expression of macrophage polarization markers. Primary human monocyte-derived macrophages were cultured untreated (MØ) or polarized into M1 (100ng/ml LPS, 20 ng/ml IFNγ, 12 hours), M2a (20 ng/ml IL-4, 24 hours), or M2c (20ng/ml IL-10, 24 hours) macrophages in either normal RPMI1640 medium (RPMI-^{norm}) or RPMI1640 medium supplemented with 1 mM Ca^{2+} (RPMI^{suppl}). The surface expression (mean fluorescent intensity, MFI) of indicated **(a)** M1 markers (HLA-DR, CD86, CD64), **(b)** M2a markers (CD200R, CD206), and an **(c)** M2c marker (CD163) are shown. The data shown are means ± SEM of biological replicates of six donors, pooled from two independent experiments with similar results. (JPG)

S1 Table. Details on the antibodies used in this study.
(PDF)

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cancers

Review

The Role of TRAIL/DRs in the Modulation of Immune Cells and Responses

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Abstract: Expression of TRAIL (tumor necrosis factor–related apoptosis–inducing ligand) by immune cells can lead to the induction of apoptosis in tumor cells. However, it becomes increasingly clear that the interaction of TRAIL and its death receptors (DRs) can also directly impact immune cells and influence immune responses. Here, we review what is known about the role of TRAIL/DRs in immune cells and immune responses in general and in the tumor microenvironment in particular. **Keywords:** apoptosis; immune regulation; tumor; myeloid cells; lymphoid cells

1. Introduction

The 'Tumor Necrosis Factor-Related Apoptosis Inducing Ligand' (TRAIL, CD253, TNFSF10) is a member of the 'Tumor Necrosis Factor Superfamily' (TNFSF), along with other closely related ligands, like TNF and CD178 (FasL, CD95L) [1–4]. Although, TRAIL is a type II transmembrane protein, protease cleavage at the membrane can generate a soluble version. In humans, five receptors for TRAIL are known. DR4 (CD261, TRAIL-R1, TNFRSF10A) and DR5 (CD262, TRAIL-R2, TNFRSF10B) contain a functional intracellular death domain (DD) required for signaling. Depending on the particular signaling pathway utilized, these signals can lead to three di↵erent outcomes [1,2,4]. On the one hand, cell death can be induced either via caspase 8- and caspase 3-dependent apoptosis or by necroptosis in a caspase-independent manner. On the other hand, DR4/DR5 signals can support survival, cell migration, and proliferation. Details on the respective signaling pathways have been reviewed previously [2,5–7]. The other three receptors described in humans lack a functional death domain and are, therefore, considered decoy receptors. These include the membrane-bound DcR1 (CD263, TRAIL-R3, TNFRSF10C) and DcR2 (CD264, TRAIL-R4, TNFRSF10D), and the soluble osteoprotegerin (OPG, TRAIL-R5, TNFRSF11B), which also can bind RANKL (TNFSF11) [8]. In contrast to humans, mice only express three receptors, DR5, DcR1 (Tnfrsf23), and DcR2 (Tnfrsf22) [1,4].

Early work indicated that particular tumor cells are highly susceptible towards TRAIL-induced apoptosis, with little harm to healthy cells [1–4]. Due to its great potential as anti-tumor drug, much work on TRAIL and its receptors has been and still is devoted on its development for anti-tumor therapy [1,4,9,10]. In addition to the cancer cells and their surrounding stroma, the tumor microenvironment contains innate and adaptive

immune cells that can recognize and destroy tumors. TRAIL expression by immune cells is one major mean by which immune cells can induce apoptosis of tumor cells. However, it becomes increasingly clear that TRAIL/DRs interaction can also directly

*Cancers***2019**, *11*, 1469; doi:10.3390/cancers11101469 www.mdpi.com/journal/cancers impact the function of immune cells in many ways. In this review, we will first outline what role TRAIL and its receptors can play in immune cells in general (Section 2) and will discuss some open questions (Section 3). Then, we will outline how these and other findings are relevant for the anti-tumor responses (Section 4).

2. Expression and Function of TRAIL/DRs in Immune Cells

2.1. Myeloid Cells

2.1.1. Neutrophils

Human blood-derived neutrophils constitutively express mRNA for TRAIL [11–13]. However, how much of the TRAIL can be found on the cell surface, if any at all, seems to depend on the donor [11–13]. It was suggested that TRAIL is rather pre-stored intracellular in granules [14–16] and that some of this pre-stored TRAIL is in the cleaved soluble form which could facilitate secretion [14,16]. Indeed, upon activation, human neutrophils upregulate TRAIL and secrete functional soluble TRAIL. This was observed most potently with IFN↵ and IFN [14,15,17,18], but also, to a lesser extent [13], with IFN [11,13,15]. Several other pro-inflammatory stimuli, like fMLP, IL-8, Hsp96 [15]; IL-17 [19]; the CD184-ligand SDF1 [20]; and the TLR2-ligand Pam3C [14]; were shown to boost the TRAIL-release by human neutrophils. In contrast, no impact on the TRAIL expression of human neutrophils was reported for IL-1, G-CSF, GM-CSF, TGF , and the TLR3-ligand Poly I:C [11,13,14,17]. Conflicting data were reported for TNF and the TLR4-ligand LPS, with some reports finding either a boost of TRAIL on neutrophils [14,15,18], no e↵ect [13,17], or even a down-regulation of TRAIL [11]. In mice, TRAIL is not expressed by neutrophils [21,22], but can be upregulated following IFN stimulation [23]. Interestingly, stimulation of mouse neutrophils with IL-6 and G-CSF induced a tumor-promoting N2 phenotype, characterized by TRAIL down-regulation [24,25], but TRAIL expression could be rescued by IFN or TNF [26].

Most studies reported expression of DR5 [11,12,17,20] and DcR1 [11,12,20,27–29] on human neutrophils, and only two reports show DR4 and DcR2 expression [20,29]. Importantly, the expression of DR4/DR5 on neutrophils tended to be lower than the expression of the decoy receptors DcR1/DcR2 [11,12,17,28,29]. Activation of human neutrophils with TNF or SDF1 [11,20] or of mouse neutrophils with IFN [23] increased the expression of DR4/DR5. In contrast, TNF stimulation [11] or ER stress [30] down-regulated DcR1/DcR2 on human neutrophils. In mice, the expression of DR5 was observed on neutrophils [28,31–33], but DcR1 and DcR2 were not detected in the one study that tested their expression [34].

In line with the expression of death receptors, most [20,23,28,29] but not all [17] studies indicated that freshly isolated neutrophils are not sensitive towards TRAIL-induced apoptosis. However, the neutrophils became sensitive following activation [11,20,23,29] or after aging [12,20]. Interestingly, upon such aging, senescent neutrophils upregulated CD184/CXCR4 [35] and became receptive to SDF1, which increased TRAIL-sensitivity, via upregulation of DR4 and DR5 [20]. This aided the migration of the senescent neutrophils to the bone marrow for apoptotic removal in the mouse model [20].

Functionally, most reports are in line with the interpretation that TRAIL/DR-activity is involved in the apoptotic removal of activated, stressed, or aged neutrophils in vivo. As neutrophils are major drivers of inflammation [36–38], their elimination usually limits the inflammation and the resulting tissue damage and promotes the resolution of the inflammation. Therefore, TRAIL-deficient mice displayed reduced neutrophil apoptosis, leading to increased neutrophil numbers and inflammation. This was noted during TLR-ligands induced sepsis [39], in the bleomycin model of lung fibrosis [21], and following *Streptococcus pneumoniae* infection of the CNS [31]. Consequently, the administration of soluble TRAIL or agonistic ↵DR5-antibodies increased neutrophil apoptosis, leading to ameliorated inflammation following *Streptococcus pneumoniae* infection [31] and during sepsis induced by bacteria [32,34] or TLR-ligands [39]. It was also observed in other systems that blocking of neutrophil apoptosis augments inflammation and tissue damage [40]. However, the TRAIL-sensitivity of activated neutrophils was not seen in all models, as, for examples, following *S. pneumoniae* infection of the lung neutrophil-apoptosis was una↵ected by the absence of TRAIL [41].

Additionally, the TRAIL produced by activated human neutrophils themselves could mediate cytotoxicity of TRAIL-sensitive tumors [13,14,17,42,43]. However, blood-derived human neutrophils of some tumor patients (squamous cell carcinoma [44]; B cell chronic lymphocytic leukemia [45]) expressed less TRAIL than healthy donors and IFN↲-therapy in vivo enhanced TRAIL expression on neutrophils of chronic myeloid leukemia (CML) patients [18]. Besides tumor cytotoxicity, neutrophil-derived TRAIL was also shown to be involved in the resolution of inflammations by targeting macrophages. Neutrophil-derived TRAIL could induce apoptosis of alveolar and lung macrophages in *S. pneumoniae* infected mice [41]. This apoptosis of *S. pneumoniae*-infected macrophages supported the bacterial clearance in the airways and limited the inflammation and the ensuing tissue damage [41].

2.1.2. Monocytes and Macrophages

Freshly purified human blood monocytes have a basal level of intracellular TRAIL expression [11,46]. Conflicting data were reported for the surface expression of TRAIL on human blood monocytes; with two studies reporting no TRAIL-expression [46,47] and two studies showing expression [11,48]. In vitro generated human monocyte-derived macrophages express high levels of TRAIL intracellularly, but low levels on the surface [48]. TRAIL expression can be rapidly induced in human monocytes via IFN↲ [47,49–52] and IFN [53]. Furthermore, secretion of soluble TRAIL after IFN↲ stimulation has been reported for human monocytes [17]. In addition, installation of the IFN↲-inducer BCG into the bladder of bladder cancer patients led to an increase in TRAIL expression on tumor macrophages [54]. For the treatment of human blood monocytes with LPS, increased TRAIL surface expression was detected after 24 h incubation [48], but not after 12 h [47]. Apart from that, PEDF, an anti-angiogenic and anti-inflammatory agent widely used in clinical trials for cancer treatment [55], induces TRAIL production by human monocyte-derived macrophages [56]. In contrast, exposure of human $CD14^+$ monocytes to C-reactive protein (CRP) led to a down-regulation of TRAIL [57]. In mice, approx. 25% of peritoneal macrophages express TRAIL [58]. TLR2 (lipoteichoic acid), TLR3 (poly I:C) and TLR4 (LPS) ligands, but not a TLR9 ligand (CpG), induced TRAIL expression in murine macrophages [59]. Moreover, murine bonemarrow derived macrophages (BMDMs) treated with PEDF in vitro or tumor infiltrating macrophages from PEDF-treated mice upregulated TRAIL expression [56]. Chemically induced ER stress also led to upregulation of soluble TRAIL in a murine monocytic cell line and in primary mouse peritoneal macrophages [60].

Human peripheral monocytes and monocyte-derived macrophages express functional DR4 and DR5, with DR5 being expressed usually higher than DR4 [28,47]. In contrast, tissue resident macrophages have very low DR5 expression [28]. Although one study demonstrated surface expression of DcR1 in human peripheral monocytes [47], other studies reported that decoy receptors are barely expressed by monocytes [28,52]. Macrophages tend to express higher level of DcR1 compared to monocytes [28]. However, macrophages are heterogeneous with several subpopulations displaying di↲erent functional activities. Macrophages can di↲erentiate into two main types in the presence of certain polarization factors when recruited into peripheral tissues [61]. Pro-inflammatory signals, like LPS and IFN treatment, polarize macrophages towards M1 or classically activated macrophages, that are cytotoxic, pro-inflammatory, and are potent in fighting tumors and pathogens. In contrast, anti-inflammatory cytokines, like IL-4, IL-10, and IL-13, induce M2 or alternatively activated macrophage, that play a role in immune suppression, tumor promotion, angiogenesis, and tissue remodeling [61]. Death receptors are di↲erentially expressed on M1 and M2 type macrophages. M2-like tumor associated macrophages (TAMs) and M2 polarized THP-1 macrophages express more DR5 than M1 macrophages [28,62,63]. Consistently, agents that support M2 polarization enhance DR4 and DR5 expression on human monocytes and monocyte-derived macrophages [28]. Furthermore, inflammatory mediators such as IFN [47] and IFN↲ [52] can downregulate surface expression of DR5 on human blood monocytes. In contrast, one study reported that LPS augments DR4 expression on human monocytes [64]. Furthermore, M1 macrophages in the synovium of rheumatoid arthritis patients expressed higher levels of DR5 than M2 macrophages [65]. In mice, monocytes and macrophages express DR5 constitutively [28,62,65–68] and its expression can be augmented by the DNA-binder trabectedin [62].

Peripheral human monocytes are sensitive to TRAIL-induced apoptosis with 50% reduction in their survival 72 h after TRAIL treatment in vitro [28]. Furthermore, pre-treatment with the anti-inflammatory cytokines IL-4 or IL-10 seemed to sensitize monocytes towards TRAIL-mediated cell death [28,69]. TRAIL also induced apoptosis in the human macrophage cell line U937 [70] and in murine peritoneal macrophages [71]. M2 macrophages are more sensitive to TRAIL-induced apoptosis than M1 macrophages due to their higher DR5 surface expression [28] and due to the increased O-glycosylation of DR5, which augments receptor oligomerization [63]. Sensitivity of monocytes and macrophages to TRAIL-mediated apoptosis was also

reported in disease models. In a mouse tumor model, TRAIL treatment in vivo was shown to suppress tumor growth by the elimination of monocytes and M2-like TAMs in the tumor [28,62]. In the atherosclerosis ApoE $^{/}$ mouse model, TRAIL treatment in vivo selectively induced apoptosis in vascular infiltrating inflammatory macrophages [66,67]. This may be due to the upregulation of DR5 on vascular macrophages by ER stress in advanced stages of atherogenesis [72]. In general, activated macrophages are sensitive to TRAIL-induced apoptosis and this is crucial for the regulation of homeostasis [58,71]. Besides inducing apoptosis, TRAILmediated signals can make monocytes and macrophages also anti-tumorigenic [17,47,50,52,53,73].

Apart from apoptotic e↵ects, TRAIL/DR interaction can also impact monocytes and macrophages in other ways. For instance, TRAIL-treatment can enhance monocytic maturation of primary human CD34^{+} hematopoietic stem cells without inducing any cytotoxicity [74]. Furthermore, TRAIL-mediated DR4-triggering could induce migration of THP-1 monocytes and of LPS-activated primary human monocytes [64]. Moreover, treatment with soluble TRAIL induced the expression of pro-inflammatory cytokines in human monocytederived macrophages and in mouse peritoneal macrophages [75]. Similarly, in tumor-challenged nude mice injected with soluble TRAIL, TAMs were reported to exhibit increased expression of proinflammatory cytokines [75]. In contrast to these pro-inflammatory e↵ects of TRAIL, one study reported an antiinflammatory role. In a mice model of colitis-associated colorectal cancer (CAC), early treatment of TRAIL in vivo protected the mice by reducing the inflammation, probably by decreasing the levels of infiltrating macrophages and pro-inflammatory cytokines and increasing the percentage of M2 macrophages [76]. Additionally, after stimulation with LPS and TRAIL in vitro, the murine RAW264.7 macrophage cell line displayed a reduction in iNOS, IL-6, and TNF expression compared to LPS alone [76].

2.1.3. Dendritic Cells

Human dendritic cells (DCs), either isolated from blood or monocyte-derived, expressed no surface TRAIL in most [47,49,77–80] but not all [46,81] studies. However, TRAIL could be detected intracellular [46,77–80] and in the culture supernatant [79]. Similar to neutrophils, the activation of DCs by IFN↵ [49,77,82–88] or IFN [77,81,85] increases the levels of membrane-bound and soluble TRAIL. Known inducers of IFN↵/IFN , like TLRligands [77,84,88–90] and virus particles [85,88,91–93], also induce TRAIL expression/secretion by human DCs. Conflicting results obtained with LPS could be due to the timing, as TRAIL induction was not observed after 12 h [49,81] but after 1-2 days [82,90] of incubation. Disagreement also exists about the impact of IFN on the TRAIL expression on human DCs [49,85]. Interestingly, it was suggested that CD40-ligation [77] or the action of regulatory T cells (Tregs) [94] could inhibit the upregulation of TRAIL on stimulated human DCs. Similar to humans, in mice, TRAIL was found to be upregulated on mouse DCs by IFN↵ and IFN directly [86] or indirectly [94–96]. Furthermore, IL-15 was suggested to be an inducer of TRAIL expression on mouse DCs [97,98].

With the exception of one report showing a weak staining for surface DR5 [90], most studies did not detect surface expression of DR4/DR5 [46,90,99,100] or DcR1/DcR2 [90,100] on human DCs.
However, DR4 and DR5 could be detected by mRNA [82,99] and intracellular staining [46]. The surface expression of DR4 and DR5 was upregulated after 2 days of stimulation with LPS in vitro [82]. However, in another study the incubation with LPS for 20 h resulted in a di↵erent outcome, with DR4 being unchanged and DR5 being down-regulated [90]. In the mouse system, DR5 surface expression was reported for bone-marrow derived DCs [101].

Surprisingly, little is known about the sensitivity of DCs towards TRAIL-induced apoptosis. Two studies, one on human monocyte-derived DCs [90] and one on mouse bone-marrow derived DCs [101], suggest that stimulated DCs are less sensitive towards TRAIL-induced apoptosis than immature DCs.

In contrast, many studies demonstrated that human DCs can exert TRAIL-dependent cytotoxic activity against various tumors cells. This was reported for unstimulated DCs [46,102], for IFN↵/ stimulated monocytederived DCs [49,77,81–83,87,92,103,104], for pDCs [78,79], and for DCs stimulated with TLR-ligands [77,84,88] or virus particles [85,88,91,93,105]. Similar observations were made in the mouse system [86,95,96,98]. Interestingly, two studies suggest that TRAIL/DR interaction can also have a direct impact on the TRAIL^{+} DCs in an apoptosis-independent manner, although, they conflict in their implication. For human monocyte-derived DCs stimulated with LPS, TRAIL acted like a co-stimulatory molecule, as blocking TRAIL reduced the upregulation of activation markers and the production of cytokines by the DCs [90]. In contrast, in a mouse study, the engagement of DR5 on mouse DCs impaired the antigen-presenting functions of the DCs, leading to reduced priming of CD4^{+} and CD8^{+} T cells [106].

2.1.4. Other Myeloid Cells

Basophils and Mast Cells Primary human blood-derived basophils expressed DR4 and DR5 in most but not all donors [107,108] and were resistant towards TRAIL-induced apoptosis [107]. In contrast, neoplastic basophils from chronic myeloid leukemia (CML) patients were TRAIL sensitive, despite lower expression of DR5 [107]. Human cord bloodderived mast cells (CBMCs) were reported to express only DR5 on the cell surface, although mRNA for DR4, DcR1, and DcR2 was detected in most of the donors [109]. These CBMCs were also sensitive towards TRAILinduced apoptosis [109,110]. In contrast, primary human lung mast cells were reported to be negative for surface DR4 or DR5 expression [108].

Eosinophils

Human blood-derived eosinophils did not express surface TRAIL [111], but eosinophils upregulated TRAIL during inflammatory responses [111–113]. Eosinophils can express all four death receptors (DR4, DR5, DcR1, and DcR2), although the expression levels reported varied [29,113,114]. Interestingly, during inflammation, human eosinophils down-regulated the expression of the pro-apoptoticreceptors(DR4, DR5)andupregulatedtheexpressionofthedecoyreceptors(DcR1, DcR2). This was observed in asthmatic patients [113,114] and in patients with parasitic infections [114] or with Churg-Strauss syndrome, a disease characterized by eosinophilia [115]. Importantly, treatment with soluble TRAIL did not induce apoptosis in human eosinophils, but rather increased cell survival [29,113], although variability between donors was noted [29]. TRAIL was also shown to promote lung eosinophilia in an indirect fashion. Human and mouse bronchial epithelial cells incubated with soluble TRAIL produced CCL20 [116,117]. CCL20 caused the influx of IL-5producing $CD4^+$ T cells into the lung in mice [116] and IL-5 is an essential chemoattractant for eosinophils [118,119]. In line with the role of TRAIL in promoting eosinophilia and acute inflammation are animal studies on allergic asthma [116], rhinoviral infection [120], and eosinophilic esophagitis (EoE) [121]. However, one study probing the role of TRAIL late during the allergic asthma inflammation, suggested a protective role for TRAIL [122]. Furthermore, data on mouse models of chronic airway inflammation yielded conflicting results [123,124]. These findings could suggest that the sensitivity of eosinophils towards TRAIL-induced apoptosis might di↵er during early and late stages of the inflammation.

Myeloid Derived Suppressor Cells

Inhibitory myeloid derived suppressor cells (MDSCs), either derived from monocytes or granulocytes, are prevalent in the tumor microenvironment [125]. Their recruitment/induction can be promoted in mice by chemokines that tumor cells produce following treatment with soluble TRAIL [126]. Human and mouse MDSCs express DR5 [127] and, curiously, the tumor environment could cause an upregulation of DR5 on mouse MDSCs in vivo [127]. It was suggested that the increase of DR5 on MDSCs is a consequence of ER stress [62,127], which also led to a down-regulation of DcR1 and DcR2 on human MDSCs [127]. Consequently, human and mouse MDSCs are sensitive towards TRAIL-induced apoptosis [127], which was utilized already therapeutically to remove MDSCs in vivo in preclinical [62,127] and clinical settings [30]. However, another study found little DR5 expression on tumor-associated mouse MDSCs [28], suggesting that the TRAIL-sensitivity of MDSCs could vary depending on the tumor studied.

2.2. Lymphoid Cells

2.2.1. Conventional Natural Killer Cells and Innate Lymphoid Cells 1

Freshly purified Natural Killer (NK) cells ($CD3^{neg}$ $CD56^{+}$) from human blood, either peripheral blood or umbilical cord blood, did in most reports not express membrane TRAIL [128–130]. Although one study suggested that TRAIL could be detected by intracellular staining [130], another one could not even detect expression of TRAIL mRNA in blood-derived NK cells [131], possibly reflecting donor di↵erences. In those reports were a subset of blood-derived NK cells stained for surface TRAIL, it was found exclusively on the $CD56^{bright}$ population [128,132] and large inter-individual variability was noted [132]. In contrast to the blood, most [128,133,134] but not all [135] studies demonstrated surface TRAIL expression on human liver $CD56^{bright}$ NK cells. A similar expression profile was seen in mice, where only a subset of NK cells in the liver, but not in blood, spleen, or lung, constitutively expressed TRAIL [136–138]. In recent years it became clear, however, that these $TRAIL^{+}$ $CD56^{bright}$ cells are most likely not conventional NK (cNK) cells, but rather ILC1s, a related subset of innate lymphoid cells (ILCs) [139–141]. Indeed, TRAIL expression by ILC1s but not resting cNK cells seems to be a generic feature of these cells in humans [133] and in mice [142–150]. Interestingly, the expression of surface TRAIL on mouse cNK/ILC1s, but not on other cells, was dependent on CD335 (NKp46, Ncr1) [151–155] as in cells lacking CD335, TRAIL remained intracellular [151,153]. The distinction between cNK cells ($CD11b^{+}$ CD49a $CD49b^{+}$ CD186 Eomes TRAIL) and ILC1s (CD11b $CD49a^{+}$ CD49b $CD186^{+}$ Eomes $TRAIL^{+}$) is further complicated by the recent finding that mouse cNK cells can, under the influence of TGF , convert into ILC1-like cells ($CD49a^{+}$ $CD49b^{+}$ $TRAIL^{+}$) [147,156,157].

This is, therefore, another example of the plasticity of innate lymphoid cells [141,158].

Nonetheless, both cNK cells and ILC1s can upregulate the expression of TRAIL following stimulation. This was seen with IL-2 [128,129,135,146,151,159–161], IL-15 [151,159–162], IFN↵/ [50,51,131,163–166], and IFN [136,137,167–170]. Upregulation of TRAIL on cNK/ILC1 was also seen in patients treated with IFN↵ in vivo within 4–6 h [131,164,165,171–173], which negatively correlated with viral titers [131,172]. Given the prominent role of IFNs in anti-viral immune responses, it is not surprising that TRAIL upregulation was also seen following viral infections or stimulation with purified TLR3 or TLR9 ligands [51,148,166–169,174–177].

Little information is available on the expression of DRs on cNK/ILC1s. Whereas, resting human bloodderived NK cells were reported to express low levels of surface DcR2 [27], after in vitro activation only expression of DR5 and DcR2 was seen [160]. However, in vitro stimulated human NK cells were not sensitive towards TRAIL-induced apoptosis [160].

NK cells are prototypic cytotoxic cells, utilizing either soluble factors, like TNF, or granzymes and perforin stored in cytotoxic granules, or the death receptors CD178 (FasL, CD95L) and TRAIL to induce apoptosis in target cells [178,179]. The involvement of $TRAIL^{+}$ cNK/ILC1s for anti-tumor response has been reviewed previously [179,180]. Importantly, cNK/ILC1s can also regulate and limit adaptive immune responses [158,181,182] and TRAIL-mediated cytotoxicity is one of several mechanisms to achieve this. In particular, activated but not resting T cells upregulate death receptors (see Section 2.2.2) and become sensitive towards apoptosis induced by $TRAIL^{+}$ cNK/ILC1s. This was observed for $CD4^{+}$ [177,183] and $CD8^{+}$ [176] T cells and appears particularly important in the case of chronic virus infections, like MCMV in mice [177] and hepatitits B virus (HBV) [176]. By this TRAIL/DR-dependent removal of activated, antigen-specific T cells during the chronic MCMV infection, the cNK/ILC1s were able to restrain the T cell responses in mice and to limit tissue damage and the risk for autoimmunity [177]. Another TRAIL/DR-dependent means to limit T cell responses by mouse cNK/ILC1s was the induction of apoptosis in immature but not mature DC in vivo [101]. Interestingly, engaging DR5 on mouse

DCs by TRAIL$^+$ cNK/ILC1s was also reported to impair antigen-presenting functions of the DCs in an apoptosis-independent manner [106].

2.2.2. Conventional ↲ T cells

Resting human and mouse T cells do not express TRAIL, but can upregulate it following TCR-mediated activation [58,160,184–191], although, in some reports, TCR-stimulation alone was not su cient [59,192,193]. In general, this upregulation of TRAIL was stronger on CD4$^+$ T cells than on CD8$^+$ T cells [50,185–187]. Furthermore, in humans, a high degree of variability of TRAIL-upregulation on T cells between di↲erent donors was observed. In line with this, the degree of TRAIL-upregulation on mouse T cells was influenced by the genetic background [191]. In most studies, IFN↲/IFN cytokines alone, similar to NK cells, could induce TRAIL expression on human T cells [50,51,185–187] and they could boost TCR-induced TRAIL expression [186,187,192,194].

Expression of all four TRAIL death receptors on naïve human [27,92,183] or mouse [32] T cells appears absent, with one study reporting DcR2 expression on human blood-derived CD8$^+$ T cells but not CD4$^+$ T cells [27]. Where changes following TCR-triggering were reported, the reports suggest an upregulation of all death receptors, however, at varying degrees [92,160,183,184,190,195]. Interestingly, TCR-mediated stimulation of human T cell lines led to a down-regulation of DR4 and DR5, without changes in DcR1 or DcR2 [184], suggesting that the regulation of the death receptors might di↲er between primary and secondary responses.

In line with the expression of death receptors, naïve or freshly stimulated human T cells were not sensitive towards TRAIL-induced apoptosis [160,184,196,197]. However, following repeated or chronic stimulation, T cells can become sensitive towards TRAIL-induced apoptosis. One important example for this is the elimination of antigen-specific T cells during viral infections by TRAIL$^+$ cNK/ILC1s, as outlined above (see Section 2.2.1), and by TRAIL$^+$ pDCs [92]. In a similar fashion, mouse Tregs upregulatedmembraneboundTRAILafterTCRmediatedstimulationandinducedapoptosisine↲ector T cells in vitro and in vivo [190]. Another example is the TRAIL-sensitivity of CD8$^+$ T cells that have been primed without CD4$^+$ T cell help [198,199], mediated by CD27/CD70 interaction [200], although, common -chain cytokines, like IL-2, IL-7, or IL15, could substitute for CD4$^+$ T cell help [199,201–203]. 'Helpless' CD8$^+$ T cells expand normally during the primary response, but upon secondary stimulation undergo TRAIL-mediated 'activation-induced cell death' (AICD) [198,204]. Such 'helpless' CD8$^+$ T cells could also release soluble TRAIL and induce fratricide [198] and their TRAIL-expression was implicated in their capability to transfer tolerance (infectious tolerance) [205,206]. Such 'helpless' CD8$^+$ T cells were also detected in HIV patients with low peripheral CD4$^+$ T cell numbers [207]. Interestingly, TRAILinduced apoptosis also influenced, at least in mice, the balance of Th1- versus Th2-T cells. After TCR-driven in vitro stimulation, mouse Th2 cells expressed TRAIL, but were resistant towards TRAILdriven apoptosis [191]. In contrast, Th1 cells did not express TRAIL, but were sensitive towards TRAIL-induced apoptosis [191]. Due to this di↲erential sensitivity, TRAIL/DR-engagement could impair Th1 and favor Th2 responses in vitro [191] and in vivo [59,195,208].

Importantly, TRAIL/DR expression on T cells serves also several non-apoptotic functions. The proliferation of human [196,197,209,210] and mouse [195,209,211–215] conventional, naïve T cells following TCRmediated stimulation was impaired by concomitant TRAIL/DR-engagement. This inhibition was particularly strong with suboptimal TCR-stimulation [197,209] and both DR4 and DR5 were implicated [196,197,211,212]. Such DRtriggered signaling impaired proximal TCR-signals, $Ca2^+$ influx, cell cycle progression, and subsequent cytokine production [196,197,209,212,213]. In contrast to naïve T cells, the proliferation of activated mouse CD4$^+$ CD25$^+$ Tregs was enhanced by TRAIL/DR-engagement in vitro and in vivo [195,216,217].

2.2.3. Innate-like T cells

Invariant Natural Killer T cells

Expression of functional TRAIL on human [218] and mouse [137,219,220] invariant Natural Killer T (*i*NKT) cells could be induced by antigenic stimulation, although IFN was suggested to be involved as well [137]. *i*NKT cells were discovered and studied extensively due to their anti-tumor activity [221,222]. Besides

granzyme/perforin- [223,224] and CD178- [225,226] mediated cytotoxicity, TRAIL-dependent anti-tumor responses have been described for human [218,225,227] and mouse [219,228,229] *i*NKT cells.

T cells

Expression of membrane bound and soluble TRAIL by human T cells could be induced by TCR-triggering together with IL-2 [230–234] and/or by NKG2D-engagement [235,236]. TRAIL expression on blood-derived IL17-producing V 9V 2 T cells was also noted during bacterial meningitis [237]. Furthermore, T cell-agonists could augment serum levels of soluble TRAIL in vivo and this correlated positively with the clinical response in prostate cancer [230] but not in breast cancer [234] patients. Interestingly, the role of TRAIL in the cytotoxicity of the T cells appears to be influenced by the mean by which the target cell is recognized. When the target cells were recognized via NKG2D-engagement, then the cytotoxicity of the T cells was largely dependent on TRAIL [232,235,236]. In contrast, when the target recognition was TCR-driven, then TRAIL played a minor role and the cytotoxicity was largely dependent on perforin [231,233].

2.2.4. B cells

TRAIL expression on resting B cells was not observed in humans [53,238] but in mice on about $^{1}/_{5th}$ of splenic B cells [189]. Upregulation of TRAIL on human B cells was seen with IFN↲ and IFN↲-inducers, like TLR9 ligands [51], but not following stimulation via the BCR, PHA/IL-2, or IFN [51,53]. Furthermore, naïve B cells expressed DR4 [27,239,240] and DR5 [32,239–241] and their expression levels increased upon stimulation [239,240,242]. Consequently, germinal center (GC) or memory B cells expressed higher levels of DR4 and DR5 than naïve cells [239,240]. However, conflicting data were reported for the expression of DcR1 and DcR2 on B cells [27,239,240].

Whereas naïve B cells are insensitive towards TRAIL-induced apoptosis, they develop sensitivity following activation [239,243]. In line with this, human $CD5^{+}$ B cells [240] and human or mouse plasma cells [243] were reported to be particular sensitive towards TRAIL-induced apoptosis. The lack of protective CD40 ligation on plasma cells was suggested to be the reason for this sensitivity [243]. However, the impact of CD40 appears also dependent on the stage of B cell development or the type of stimulation. On the one hand, CD40 ligation concomitant to IFN↲-mediated stimulation could boost TRAIL upregulation on naïve human B cells [51]. On the other hand, it was suggested that CD40 ligation could protect B cells from TRAIL-induced apoptosis early after BCR-mediated activation [240,244] but not 3–5 days later [239]. The connection between CD40 and TRAIL is complicated by two additional aspects. First, the hetero-oligomerization of CD40 and DR5 could dampen the activation of primary human B cells [242]. Second, CD40-ligation on B cells can induce expression of CD25 (IL2R↲) [245] and IL-2 signaling was reported to cause down-regulation of TRAIL on B cells [246].

Importantly, the TRAIL/DR-interaction could influence the isotype of the antibodies produced by activated B cells. Data from in vivo experiments that either blocked TRAIL/DR-interaction [211,247] or triggered DRs with soluble TRAIL [247] indicated that TRAIL/DR-interaction impairs the production of IgG1 and, to a lesser extent, IgG2a antibodies.

3. Common Themes and Open Questions on the Role of TRAIL/DRs in Immune Cells

3.1. Regulation of TRAIL-Sensitivity

The sensitivity of immune cells towards TRAIL-induced apoptosis is regulated on several levels.

3.1.1. TRAIL Expression

As outlined above, the expression levels of TRAIL on immune cells can be influenced by intrinsic signals, like ER stress or senescence, as well as many extrinsic signals, like cytokines.

3.1.2. Membrane-Bound vs. Soluble TRAIL

Functional TRAIL can either be membrane-bound or soluble, after cleavage from the surface [1,5]. However, thebioactivityofmembrane-boundandsolubleTRAILdi↲erssignificantly. First, thecytotoxic potential of soluble TRAIL was suggested to be 100–1000-fold lower than that of membrane-bound TRAIL [248]. Second, soluble TRAIL, in contrast to the membrane-bound version, was not able to impair the activation of conventional T cells [197] or to promote the proliferation of Tregs [195]. Mechanistically, it was reported that soluble TRAIL could trigger only DR4, whereas membrane-bound

TRAIL could trigger both DR4 and DR5 [249]. Given these distinctions, it is particular important to keep in mind that not all of the TRAIL-bioactivity found in cell culture supernatants or in body fluids stems solely from soluble TRAIL (see Section 3.2).

3.1.3. Expression Levels of the Death Receptors

Similar to TRAIL and as outlined in detail above, the expression levels of the death receptors on immune cells can be influenced by many intrinsic and extrinsic signals. The relative expression of these death receptors is relevant for two reasons. First, it was suggested that the a nity of DR4/DR5 towards TRAIL is higher than that of DcR1/DcR2 [250,251]. Second, it appears that the ratio of functional (DR4, DR5) vs. decoy receptors (DcR1, DcR2) can dictate the sensitivity towards TRAIL. Immune cells could become TRAIL-sensitive by upregulation of DR4/DR5 and/or by the down-regulation of DcR1/DcR2 [11,28,30]. In contrast, an inverse ratio predicts TRAIL-resistance [28,47,90,127]. Although, DR4 and DR5 appear on a first glance redundant, slight functional di↵erences likely allow to fine-tune the response towards TRAIL. Such di↵erences include, for example, the higher a nity of DR5 than DR4 for TRAIL [250,251], the inability of DR5 to bind soluble TRAIL [249], and the ability of DR5 to form hetero-oligomers [242,250]. However, other di↵erences likely remain to be discovered.

3.1.4. Receptor Interactions

Besides the absolute expression levels of death receptors, their activity could also be influenced by the formation of hetero-oligomers. This has been suggested for DR5 and CD40, which reduced CD40-signaling [242], and for DR5 and DcR2, which reduced DR5-signaling [250].

3.1.5. Signaling Pathways

Finally, the outcome of DR-triggering is regulated on the level of the intracellular signaling pathways, which can lead to apoptosis, necroptosis, or increased survival and proliferation. The regulation of these pro- and anti-apoptotic signaling pathways are still poorly understood and are discussed in detail elsewhere [2,5–7]. However, it is likely that these pathways contain several potential new drug targets to regulate the resolution phase of immune responses.

3.2. TRAIL on Exosomes

Most studies that investigated the TRAIL-activity in cell culture supernatants assumed that the activity is due to soluble TRAIL released from the cells in the culture. However, functional TRAIL could also be detected in exosomes released from various cells, including human T cells [252–254], human neutrophils [16], human placental explants [255], mouse bone-marrow-derived DC [256], and from various tumors [257–260]. Furthermore, TRAIL$^+$ exosomes have been detected in sera of tumor patients [257] and in the synovial fluid of arthritis patients [261]. The TRAIL in these exosomes was the full-length, membrane-bound version and not the shorter version of soluble TRAIL [253,257]. Given that the bioactivity of soluble and membrane bound TRAIL are very di↵erent (see Section 3.1), it is important to know if the TRAIL-activity in the culture supernatant is due to soluble or exosome-bound TRAIL. However, few studies measured the molecular weight of the TRAIL recovered from the cell culture supernatants. Even transwell-studies could be misleading, as exosomes seem to move freely across pore sizes of 1 µm and can transmit 15–33% of the biological activity across 0.4 µm pores [262,263]. As most cells release exosome [264,265], a reasonable working hypothesis appears to be that all TRAIL$^+$ immune cells are able to release TRAIL$^+$ exosomes. How this, compared to soluble TRAIL, influences immune responses needs to be addressed in future studies.

3.3. TRAIL's Role in the Resolution of Immune Responses

Looking at the findings outlined so far in a broad sense, it appears that one of the main roles of TRAIL/DRs in the immune system is during the resolution phase of immune responses. By removing senescent, chronically activated, or stressed immune cells at sites of inflammation, TRAIL/DRs regulate innate and adaptive immune responses by terminating the response and by limiting thereby tissue damage and the risk of autoimmunity.

3.3.1. Removing E↵ector Cells

Activated or senescent neutrophils become sensitive towards TRAIL-induced apoptosis [11,12,20,23,29]. As neutrophils are major drivers of inflammation [36–38], their TRAIL-dependent removal supported the resolution of the inflammation [21,23,31,32,34,39,41]. Furthermore, activated T cells are sensitive towards TRAIL-induced apoptosis [176,177,183,190]. Other immune e↵ector cells known to be sensitive towards TRAILinduced apoptosis are sub-optimal activated, 'helpless' $CD8^+$ T cells upon secondary stimulation [198,204,207]; terminally di↵erentiated cells, like T cell blasts [210,266]; plasma cells [243]; MDSCs [30,62,127]; and hematopoietic cancers (e.g., [100,188,238]). Activated immune cells greatly increase the synthesis of proteins, which can stress the endoplasmic reticulum (ER), leading to an 'unfolded protein response' (UPR) [267–269]. Similar, pathogens and chronic cell activation can cause ER stress [267,269,270]. Indeed, it was shown that ER stress increases the TRAIL-sensitivity of macrophages [271] and MDSCs [30,127]. Furthermore, *Streptococcus pneumoniae* infected alveolar macrophages were susceptible towards TRAIL-induced apoptosis [41]. However, beyond these two examples, the link between ER stress and TRAIL-sensitivity is not yet established. The two exceptions in the pattern of TRAIL-induced removal of e↵ector cells, seem to be immature DCs and eosinophils. First, mouse cNK/ILC1s could induce apoptosis in immature but not mature DC in vivo in a TRAIL/DR-dependent manner [101]. Second, the survival and functions of eosinophils were reported to be augmented by TRAIL/DRs [116,120,121]. However, two studies that investigate the role of TRAIL either late during an allergic asthma inflammation [122] or during a chronic airway inflammation [123], suggested that TRAIL now induces apoptosis of eosinophils. These reports might indicate that the impact of TRAIL on eosinophil di↵ers during early and late stages of the inflammation.

3.3.2. Impairing E↵ector Cells

Besides their direct apoptotic removal of e↵ector cells, TRAIL/DR-activity can also impair the expansion/function of e↵ector cells. Either directly, by impairing the activation and proliferation of pathogenic T cells, or indirectly, by augmenting the proliferation of inhibitory Tregs (see Section 2.2.2).

3.3.3. Limiting Tissue Damage

In line with the idea that the activity of TRAIL/DRs limits ongoing immune response and supports the transition into the resolution phase, is the fact that TRAIL-deficiency or TRAIL/DR-blockage exacerbates, whereas the injection of functional TRAIL ameliorates pathogen burden. This has been noted for *Streptococcus pneumoniae* infection of the CNS [31] or the lung [41], for systemic *Listeria monocytogenes* [33] or MCMV [177] infection, and for influenza vaccination [272] or infection [273]. At first, it might appear counterintuitive to curtail anti-pathogenic immune responses. However, this inhibition is likely aimed at limiting tissue damage. Without an e cient resolution in the absence of TRAIL/DRs, immune responses continue and could become damaging to the host tissue, which eventually could lead to autoimmunity. Indeed, augmented tissue damage and signs of autoimmunity in the absence of TRAIL were observed, for example, following influenza [22], MCMV [177], rhinovirus [120], *Listeria monocytogenes* [33], and *Streptococcus pneumoniae* [31] infections and during sepsis induced by bacteria [32,34] or TLR-ligands [39]. This probably also contributes to the increased susceptibility of TRAIL-deficient mice towards experimental autoimmune diseases, as reported for collageninduced arthritis (CIA) [274], diabetes [67,274,275], and experimental autoimmune encephalomyelitis (EAE) [195,215].

3.3.4. Avoiding Autoimmunity

The idea that TRAIL/DR-activity limits tissue damage induced by unrestrained immune responses is also supported by the observation that TRAIL/DR-blockage exacerbates, whereas the injection of biologically active TRAIL ameliorates autoimmune diseases. This has been observed for colitis [214], collagen-induced arthritis (CIA) [211,276,277], diabetes [275,278], experimental autoimmune encephalomyelitis (EAE) [215,217,279–281], experimental autoimmune thyroiditis (EAT) [208,216], and systemic lupus erythematosus (SLE) [247].

4. TRAIL/DRs in the Tumor Microenvironment

4.1. Anti-Tumor Cytotoxicity of TRAIL$^+$ Immune Cells

Many immune cells express TRAIL constitutively or following activation and thereby can be cytotoxic to TRAIL-sensitive tumor cells in vitro and in vivo. This has been reported for neutrophils [13,14,17,42,43], monocytes/macrophages [17,47,52,73], DCs [46,49,77–79,81–83,86,87,91,98, 102–104], pDCs

[84,85,88,91,93,95,96,105], cNK/ILC1s [134,136,137,163,228,282], *i*NKT cells [218,219, 225,227,229], T cells [231,235], and conventional T cells [186,194,283–286].

4.2. TRAIL Susceptibility of Tumors and Immune-Surveillance

Malignant transformation of cells often leads to sensitivity towards TRAIL-induced apoptosis in a cellautonomous manner [1,2]. As many activated immune cells express TRAIL, the selective pressure of the antitumor immune response forces the evolution of the tumor. This is best illustrated by TRAIL-deficient mice, which are more susceptible towards endogenous tumors developing either spontaneously [287] or induced by the chemical carcinogen methylcholanthrene (MCA) [228]. Furthermore, tumors in TRAIL $^{/}$ mice developed metastases more frequently [137,288]. Interestingly, this TRAIL-dependence of metastases was more prominent for some organs, like liver, than others, like lung [137,288], indicating organ di↵erences of immunesurveillance mechanisms. The greater sensitivity towards MCA-induced tumors could also be mimicked by repeated injection of a blocking anti-TRAIL-antibody [289]. Additionally, tumors developing in TRAIL $^{/}$ mice retained TRAIL-sensitivity, whereas tumors developing in TRAIL-proficient animals acquired TRAILresistance [289]. These reports demonstrate that TRAIL is an important mechanism, besides CD178(FasL,CD95L)andperforin, inthetumorimmune-surveillance. cNK/ILC1s[136,137,228,282,289] and *i*NKT cells [290–292] have been suggested to be major players in this tumor immune-surveillance. The presence of TRAIL^{+} ILC1s in the liver [139–141] might also explain some of the organ specificity of TRAIL-mediated immunesurveillance [137,288].

4.3. Tumor Mechanisms to Evade TRAIL-Mediated Cytotoxicity

The observation that tumors developing in TRAIL $^{/}$ but not in wild-type mice retained TRAIL-sensitivity [289] indicates that tumor cells can develop mechanisms to avoid TRAIL-mediated killing [1,2]. The tumor can achieve this by means that are not unique to TRAIL/DRs, like the upregulation of antiapoptotic molecules [293] or an interference with general aspects of the death-receptor signaling, e.g., by mutating the death domain (DD) [294], by inactivating caspase-8 [295,296], or by overexpressing c-FLIP [297,298]. Several other tumor cell-intrinsic strategies were reported that limit the apoptotic-signals induced by DR4 or DR5 themselves. First, changes in glycosylation can alter the sensitivity of DR4/DR5 [299–302]. Second, the surface expression of DR4/DR5 can be reduced either by epigenetic changes in the respective genes [303,304], by autophagic removal [305], or by relocation to the nucleus [306]. Third, the tumor can upregulate the decoy receptor DcR1 and DcR2 [1,2,62], which reduces the TRAIL-binding to DR4 and DR5. Consequently, expression of DcR1/DcR2 correlated with poor prognosis for patients with breast cancer [307], prostate carcinoma [308], or acute myeloid leukemia (AML) [309,310]. In contrast, conflicting data have been reported for the correlation between DR4/DR5 expression and patient survival for renal cell carcinoma [311] and hepatocellular carcinoma (HCC) [304]. Furthermore, some single-nucleotide polymorphisms (SNPs) in TRAIL of HCC patients are correlated with overall patient survival [312]. In summary, these reports indicate that TRAIL/DRs are an important player in the anti-tumor response.

4.4. TRAIL/DR-Related Immune-Tumor Cross-Talk in the Tumor Microenvironment

The TRAIL-DR interaction does not only impact the tumor directly, but can also be utilized by the tumor to shape the tumor microenvironment. Treatment of TRAIL-resistant tumor cells with soluble TRAIL or agonistic ↵DR5-antibodies could promote tumor survival/proliferation [306,313], invasion and metastases [314–317], and cytokine production [126,316,318–321]. Such cytokines could induce chemotaxis and recruitment of various myeloid cells [126,318–320]. Many mechanisms have been described inside the tumor microenvironment that benefit the tumor [2,322–324]. In regard to TRAIL/DRs, several points can be mentioned. It was noted that neutrophils [18,44,45] and DCs [98,102] from the tumor itself or from blood of tumor patients express less TRAIL than cells from control tissue/donors. This reduction might be due to the proteolytic cleavage of TRAIL from the surface, as it was shown for CD178 [325–327]. Or the reduction could be a consequence of stimulation, as it was reported, for example, that mouse neutrophils stimulated with IL6 and G-CSF loose TRAIL expression and their anti-tumor properties [24,25]. Furthermore, DR5-triggering of mouse DCs by TRAIL^{+} cNK/ILC1s reduced their cross-presentation and -priming capacity for tumor-antigens, leading to reduced anti-tumor T cell responses [106]. Finally, expression of DcR1/DcR2 by human stromal cells in the tumor microenvironment could reduce the ligation of DR4/DR5 on tumor cells [328]. All of these mechanisms would reduce the anti-tumor activity of the immune system directly, benefiting the tumor.

Moreover, some other TRAIL/DR-dependent mechanisms promote tumor growth indirectly. TRAIL promoted the recruitment and polarization of immune-suppressive M2-like cells [126] and the proliferation of Tregs [190,195,216,217]. Furthermore, it is known that the tumor microenvironment triggers persistent ER stress in infiltrating immune cells and promotes immunosuppressive responses [329]. In regard to TRAIL/DRs, ER stress of myeloid cells has been linked to pro-inflammatory [271] but also anti-tumorigenic activities [30,127]. This latter point illustrates that not all impacts of TRAIL/DRs would benefit TRAIL-resistant tumors. In line with the role of TRAIL/DRs in the elimination of stressed or senescent immune cells, some TRAIL/DR-actions could also suppress tumor growth. For example, the tumor environment induced upregulation of DR5 on mouse MDSCs in vivo for some [127] but not all [28] tumors. Furthermore, mouse and human MDSCs [127] and mouse TAMs [28,63] were shown to be sensitive to TRAIL-induced apoptosis. Consequently, treatment of head and neck cancer patients with agonistic ↵DR5-antibodies [30] or of tumor-bearing mice with soluble TRAIL [28,62] was able to limit tumor growth to some extent. Additionally, the endothelial cells of tumor-associated blood vessels of some [330] but not all [28] tumor-bearing mice upregulated DR5 and were TRAIL-sensitive, which led to blood vessels collapse and inhibited tumor growth [330]. An overview of the anti- and pro-tumorigenic activities of TRAIL/DRs in the tumor microenvironment is given in Figure 1.

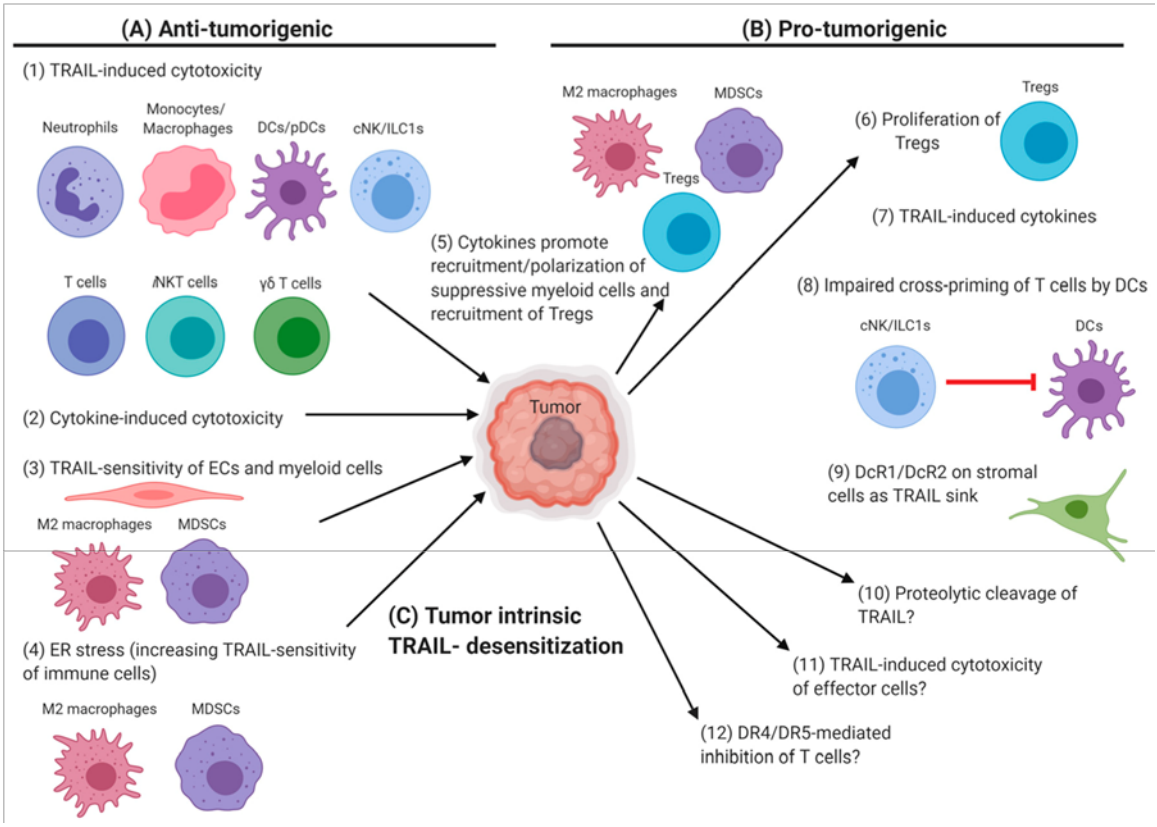


Figure 1. Anti- and pro-tumorigenic activities of TRAIL/DRs in the tumor micro-environment: (**A**) Antitumorigenic activities: (1) Several immune cells have been reported to be cytotoxic against tumor cells in an at least partially TRAIL-dependent manner. (2) Besides TRAIL killing the tumor directly, TRAIL-induced cytokines, like TNF, can be cytotoxic for the tumor as well. (3) Endothelial cells (ECs) in the tumor can become TRAILsensitive, potentially leading to blood vessels collapse, causing a disruption of the blood supply for the tumor. Similar, pro-tumorigenic M2 macrophages and MDSCs are known to be sensitive towards TRAIL-induced apoptosis. (4) Although, ER stress inside of the tumor is usually associated with pro-tumorigenic e↵ects [329] it can also have an anti-tumorigenic impact. This is mainly due to the increased TRAIL-sensitivity of antiinflammatory M2 macrophages and MDSCs. (**B**) Pro-tumorigenic activities: (5) TRAIL/DR-induced cytokines produced by the tumor can promote the recruitment and polarization of M2 macrophages and MDSCs, and can promote the recruitment of Tregs. (6) For Tregs it was shown that TRAIL-induced triggering of DR4/DR5 on Tregs can promote their proliferation. (7) TRAIL-induced cytokines, either produced by the tumor or immune cells, can promote tumor survival and proliferation directly or indirectly, e.g., by supporting neovascularization. (8) Triggering of DR4/DR5 on DCs by TRAIL$^+$ cNK/ILC1s could suppress the cross-priming of tumor-specific T cells by the DCs. (9) Expression of DcR1 and DcR2 on tumor stroma cells can act as sink for TRAIL, reducing its availability to induce tumor cytotoxicity. (10–12) For several other mechanisms, an impact of TRAIL/DRs has not been demonstrated for the tumor microenvironment yet, but their role could be hypothesized based on published data in other contexts.

(10) The tumor might promote the proteolytic cleavage of membrane TRAIL from immune cells. (11) TRAIL, either derived from immune cells or from the tumor itself, might promote cytotoxicity of TRAIL-sensitive immune e↵ector cells. (12) TRAIL-mediated triggering of DR4/DR5 on recently activated T cells might inhibit their proliferation. (**C**) To support survival, the tumor can evolve several cell-intrinsic mechanisms to reduce its sensitivity towards TRAIL-induced cytotoxicity. These include the upregulation of anti-apoptotic molecules, the inactivation of signaling molecules, the upregulation of DcR1/DcR2, and changes in DR4/DR5 localization and glycosylation. Alternatively, the tumor can re-purpose the DR4/DR5-signals, for example, to support tumor survival and proliferation, to induce cytokine production by the tumor, and to promote tumor invasion and metastases.

5. Conclusions

APC	antigen presenting cell
AICD	activation-induced cell death
AML	acute myeloid leukemia
BCR	B cell receptor
BM-DCs	bone marrow-derived dendritic cells
BMDMs	bone-marrow derived macrophages
CAC	colitis-associated colorectal cancer
CBMCs	cord blood-derived mast cells
CCL	C-C motif chemokine ligand
CD	cluster of di↵erentiation
CIA	collagen-induced arthritis
CML	chronic myeloid leukemia
cNK	conventional NK
CNS	central nervous system
CRP	C-reactive protein
CXCR	C-X-C chemokine receptor
DC	dendritic cell
DcR	decoy receptor
DD	death domain
DR	death receptor
DTx	diphtheria toxin
EAE	experimental autoimmune encephalomyelitis
EAT	experimental autoimmune thyroiditis
EoE	eosinophilic esophagitis
Eomes	eomesodermin
ER	endoplasmic reticulum

fMLP	N-Formylmethionyl-leucyl-phenylalanine
GC	germinal center
G-CSF	granulocyte-colony stimulating factor

GM-CSF granulocyte-macrophage colony-stimulating factor The original observation that TRAIL preferentially induces apoptosis in tumor cells, while sparing healthy cells, initiated intense research on the development of TRAIL/DR-based anti-cancer therapies. Besides the tumor, innate and adaptive immune cells are a major constituent of the tumor microenvironment, where they can use TRAIL to fight the tumor by inducing apoptosis of tumor cells. However, it becomes increasingly clear that TRAIL/DRs interaction can also directly impact the function of immune cells in many ways. As outlined in this review, many relevant aspects are yet unclear. Therefore, a better understanding of how TRAIL/DRs influence both tumor and immune cells and their interaction within the tumor microenvironment will be essential for the development of successful TRAIL/DR-based anti-cancer therapies.

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Abbreviations

HBV	hepatitis B virus
HCC	hepatocellular carcinoma
Hsp	heat shock protein
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cell
LPS	lipopolysaccharide
MCA	methylcholanthrene
MCMV	murine cytomegalovirus
MDSCs	myeloid derived suppressor cells
mRNA	messenger RNA
MSC	mesenchymal stem cell
*i*NKT	invariant Natural Killer T
NK	Natural Killer
OPG	osteoprotegerin
Pam3C	tri-palmitoyl-S-glyceryl cysteine
pDC	plasmacytoid dendritic cell
PEDF	pigment epithelium derived factor
PHA	phytohemagglutinin
Poly I:C	polyinosinic:polycytidylic acid
RANKL	Receptor activator of nuclear factor kappa-B ligand
RNA	ribonucleic acid
SDF1	stromal cell-derived factor 1
SLE	systemic lupus erythematosus
TAMs	tumor associated macrophages
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper type
TLR	toll like receptor
TNF	tumor necrosis factor
TNFRSF	TNF receptor superfamily
TRAIL	tumor necrosis factor–related apoptosis–inducing ligand
Treg	regulatory T cell
UPR	unfolded protein response.

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