Aus dem Universitätsklinikum Münster
Medizinische Klinik und Poliklinik B
– Direktor: Univ.-Prof. Dr. med. Dr. h.c. W. Domschke –

Role of the TL1A/DR3 pathway in NK cell effector functions

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Zusammenfassung

Bedeutung des TL1A/DR3-Signalwegs für die Effektorfunktionen Natürlicher Killerzellen

Stephanie Claudia Heidemann

Das vor kurzem identifizierte Zytokin der TNF-Superfamilie TL1A (TNFSF15) ist Ligand des Todesrezeptors DR3 (TNFRSF25), welcher nach Zellaktivierung in T-Zellen und Natürlichen Killerzellen (NK-Zellen) exprimiert wird. TL1A steigert die durch Interleukin (IL)-12 und IL-18 induzierte Produktion von IFN-γ in T-Zellen um das 10-fache oder mehr. Während ein Großteil der NK-Zellen infolge Stimulation mit den Zytokinen IL-12 und IL-18 DR3 exprimiert, führt die Kostimulation mit TL1A nur zu einer zweifachen Steigerung der IFN-γ-Produktion. Diese Studie hat sich deshalb mit der Fragestellung beschäftigt, ob der TL1A-DR3-Signalweg von Bedeutung ist für eine weitere durch IL-12 and IL-18 regulierte Effektorfunktion der NK-Zellen, die Lyse von Tumorzellen. Der Einfluß von TL1A auf die Zytotoxizität von mit IL-12 und IL-18 kostimulierten, aus mononukleären Zellen des peripheren Blutes (PBMC) isolierten NK-Zellen wurde in ^{51}Chromfreisetzungstests untersucht.

Es konnte kein zusätzlicher Einfluß von TL1A auf die durch IL-12 and IL-18 induzierte Zytotoxizität der NK-Zellen gegenüber der NK-sensitiven Tumorzelllinie K562 (CML) nachgewiesen werden. TL1A war jedoch in der Lage, die Zytotoxizität der NK-Zellen gegenüber der NK-resistenten Tumorzelllinie Daudi (Burkitt-Lymphom), welche nur durch Zytokin-aktivierte NK-Zellen lysiert wird, zu steigern. TL1A erhöhte die durch IL-12 und IL-18 induzierte Lyse der Daudi-Zellen durch PBMC um das zweifache und durch aus PBMC isolierten NK-Zellen um das 7-fache. TL1A steigerte ferner die durch IL-12 und IL-18 koaktivierte NK-Zell-vermittelte Lyse der Tumorzellinien WiDr and SW837, die von Adenokarzinomen des Kolon und Rektums abgeleitet sind, wenn auch in geringerem Ausmaß. Durch die hier nachgewiesene Fähigkeit, die Zytotoxizität von NK-Zellen gegenüber NK-resistenten Tumoren zu steigern, könnte dem TNF-Superfamilienmitglied TL1A in vivo eine Schlüsselrolle als Koaktivator der Zytotoxizität Natürlicher Killerzellen zukommen.

Mittels Durchflußzytometrie, ELISA bzw. H^3-Thymidin-Inkorporationsassays konnte die vorliegende Studie ferner belegen, daß aus einer Anzahl von getesteten Zytokinen und Zytokinkombinationen nur die Kombination von IL-12 und IL-18 zu einer signifikanten Induktion der DR3-Expression in NK-Zellen führt, daß TL1A einen synergistischen Effekt auf die Produktion von IFN-γ durch mit optimalen und suboptimalen Konzentrationen von IL-12 und IL-18 kostimulierten NK-Zellen hat und daß dieser Effekt überwiegend durch die Proliferation von NK-Zellen bedingt ist.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

Ab	antibody
ADCC	antibody-dependent-cellular-cytotoxicity
AP	activator protein
ASK	apoptosis signal-regulating kinase
ATCC	American type culture collection
BBS	borate buffered saline
BSA	bovine serum albumin
°C	degree celsius
CD	cluster of differentitation
Ci	curie
CML	chronic myelogenous leukemia
Cpm	counts per minute
Cr	Chromium
CSA	colon specific antigen
DcR3	decoy receptor 3 (TNFRSF6B)
DD	death domain
DNA	deoxyribonucleic acid
DR3	death domain receptor 3 (TNFRSF25)
E_{STD}	standard number of effector cells
E:T ratio	effector:target ration
EDTA	ethylenediamine tetraacetic acid
e.g.	exempli gratia (*Latin*; in *English*: for example)
ELISA	enzyme-linked immunosorbent assay
ER	experimental release
ERK	extracellular signal-regulated kinase
FAS/FasR	Fas receptor (TNFRSF6)
FasL	Fas ligand (TNFSF6)
Fig	Figure
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte colony-stimulating factor
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	human leucocyte antigen
h/hr	hour
hrs	hours
HUVEC	human umbilical vein endothelial cells
i.e.	id est (*Latin*; in *English*: this means)
IFN	interferon
Ig	immunoglobulin
IGIF	IFN-γ-inducing factor
IL	interleukin
JAK	Janus kinase
JNK	C-Jun N-terminal kinase
KIR	killer-cell immunoglobulin-like receptor

LIGHT	lymphotoxin-like inducible protein that competes with glycoprotein D for binding herpesvirus entry mediator on T cells (TNFSF14)
LPL	lamina propria lymphocytes
LU	lytic unit
mAb	monoclonal antibody
MACS	magnetic-activated cell sorting
MAPK	mitogen-activated protein kinase
MFI	mean fluorescence intensity
mg	milligram
MHC	major histocompatibility complex
MIC	MHC class I-related chain/molecule
min	minute(s)
MKK	mitogen-activated protein kinase (MAPK) kinase
ml	milliliter
MR	maximal release
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NCR	natural cytotoxicity receptor
ND	not determined
NF-κB	nuclear factor-kappa B
ng	nanogram
NK cell	natural killer cell
PAF	platelet-activating factor
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
pg	picogram
PMA	Phorbol 12-myristate 13-acetate
PSL	percent specific lysis
Rh	Rhesus factor
RIP	receptor interactive protein
RPMI	Rosewell Park Memorial Institue Medium
SD	standard deviation
SEM	standard error of the mean
SR	spontaneous release
T_{STD}	standard number of target cells
TAK	TGF-β activated kinase
TC	tri-color
TCR	T cell receptor
TH1	T helper cell type 1
TL1A	TNFSF15
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TNFRSF	tumor necrosis factor receptor superfamily

TNFSF	tumor necrosis factor superfamily
TNF/TNFR SFP	TNF/TNFR superfamilies of proteins
TRADD	TNFR1-associated death domain protein
TRAF	TNFR-associated factor
TRAIL	TNF-related apoptosis-inducing ligand (TNFSF10)
TRIS	trishydroxymethylaminomethane
TYK	tyrosine kinase
STAT	signal transducer and activator of transcription
ULBP	UL16-binding protein
v/v	by volume
w/v	weight by volume
µl	microliter

1 Introduction

1.1 Immunosurveillance

Immunosurveillance is mediated by the innate immunity, characterized by a rapid response upon host invasion by pathogens and transformed cells which does not require priming with specific antigens (76), and the adaptive immunity which provides antigen-specific recognition of pathogens and cancerous cells, an accelerated response upon re-exposure with the antigen, and long-term memory (84).

The innate immune system comprises the epithelial barriers, phagocytic cells (e.g. neutrophils and macrophages), the complement system, natural killer (NK) cells and cytokines which regulate the functions of the innate cells (76). Adaptive immunity is mediated by B and T cells (84).

1.2 Cellular effectors of immunosurveillance

1.2.1 Natural killer cells

Natural killer cells are white blood lymphocytes of the innate immune system, phenotypically characterized as CD56+ and CD3-, which play an important role in innate immune defense by eliminiating invading infectious pathogens and tumor cells through cytokine production and MHC-nonrestricted cytotoxicity.

NK cells, which comprise approximately 15% of all circulating lymphocytes, are also found in peripheral tissues, e.g. the liver, peritoneal cavity and placenta. While resting NK cells circulate in the blood, cytokine-activated NK cells are capable of extravasation and infiltration into most tissues invaded by pathogens and tumor cells (76).

NK cells can be divided into subsets based on their surface phenotype. It was hypothesized that phenotypically distinct NK cell population represent independent subsets with specialized NK cell effector functions. Recent studies however support the conclusion that the distinct phenotype of the subsets rather correlates with developmental stages or states of cell activation (68).

The pattern of cytokine production depends on the stage of NK cell differentiation. While immature NK cells produce T helper 2 cytokines (e.g. IL-4, IL-5 and IL-13, which regulate antibody-mediated responses), mature NK cells lose this ability, but acquire the ability to produce T helper 1 cytokines (i.e. pro-inflammatory cytokines, like IFN-γ, which are essential for the defense against intracellular pathogens) (68;76).

NK cells produce the immunomodulatory cytokines IFN-γ, TNF-α, GM-CSF which determine the early innate immune response and also regulate the delayed T cell response. When stimulated by products of infectious pathogens, antigen-presenting cells, e.g. macrophages and dendritic cells, induce NK cell production of IFN-γ via secretion of cytokines, e.g. interleukin (IL)-12, IL-15 and IL-18, for which NK cells constitutively express receptor complexes (12;22;72;87).

NK-cell secretion of IFN-γ plays an essential role in the immune defense against pathogens, and recent evidence further revealed the multiple antitumor effects of NK cell IFN-γ production. The proposed functions of IFN-γ include induction of antigen expression by up-regulation of MHC class I and II on many cell types, coordination of the interaction of leucocytes with the endothelium, regulation of cell proliferation and sensitivity to apoptosis, stimulation of the bactericidal activity of phagocytes and inhibition of angiogenesis (76).

NK cells discriminate between normal cells expressing an adequate amount of major histocompatibility complex (MHC) class I and cells with loss of or altered MHC class I expression as a consequence of tumor transformation or viral infection. Engagement of MHC class I-specific inhibitory receptors by classical or non-classical MHC class I molecules downregulates NK cell effector functions. Activating NK receptors include MHC class I-specific receptors, Non-MHC class I-specific receptors, e.g. natural cytotoxicity receptors (NKp30, NKp46, NKp44) and NKG2D which recognizes pathogen-associated and stress- and tumor-induced MHC class I-related ligands, and coreceptors (2B4 (CD24), CD16 (FcgammaRIII) etc.). Engagement of activating receptors by their ligands induces NK cell cytotoxicty, blastogenesis, cytokine production, and/ or migration. The FcgammaRIII (CD16) receptor on NK cells recognizes antigen-bound IgG antibodies and mediates antibody-dependent-cellular-

cytotoxicity (ADCC). A variety of co-stimulatory and adhesion molecules can also trigger cytotoxicity. NK cell effector function is the result of an integration of signals from inhibitory receptors and activating NK receptors and dependent upon the density and selection of MHC class I and MHC class I-related molecules expressed on target cells (17;22;41;54).

Cytolytic activity of NK cells is generally mediated through the Ca^{2+}-dependent granule exocytosis pathway involving perforin/granzyme release. The pore-forming protein perforin induces loss of osmotic stability and target cell necrosis (34) and permits the influx of granzymes which induce target cell apoptosis by activation of caspases and cleaving of Bcl2 familiy members (31;70). Perforin-dependent target cell membrane damage and necrosis are measured by the ^{51}Cr-release assay (92). It was recently demonstrated that tumor elimination mediated by NKG2D receptor-ligand interaction is perforin-dependent (30).
An alternative, Ca^{2+}-independent mechanism of NK cytotoxicity involves Fas/CD95 ligand-mediated apoptosis upon interaction of Fas/CD95 receptor-positive target cells with Fas ligand, expressed or induced on NK cells (89;93). A role of TRAIL and other TNF family ligands in apoptotic killing of tumor targets by NK cells has also been described (37;85).
Recent evidence suggests that the cytokines to which NK cells respond and the expression patterns of NKG2D ligands on tumor cells determine if NK cells exert their lytic function through perforin-, Fas ligand-, or TRAIL-dependent pathways (77).

1.2.2 T cells

T cells, which mediate adaptive immunity, are capable of generating an infinitely diverse repertoire of antigen-specific receptors. They can proliferate clonally very rapidly, and confer long-term protection after the initial immunization with an antigen with an accelerated response upon re-exposure (84).
T cells play an important role in tumor immunosurveillance (47;84). They can be divided in two sub-lineages based on the type of antigen-receptor expressed, i.e. αβ T cells and γδ T cells. Both subsets express surface CD3 and a rearranged T-cell

receptor (TCR) (84). αβ T cells express the α and β chains of the TCR, while γδ T cells express the γ and δ chain rearrangement (47). αβ T cells comprise the majority of T cells in the circulation and lymphoid tissues, and they include a subset called natural killer T (NKT) cells, which expresses several NK cell surface receptors (84). Most γδ T cells and a subset of NKT cells are unconventional T cells which, like NK cells, are not restricted to classical MHC class-I or MHC class II-bearing specific peptides (80).

The functional properties of γδ T cells are in general more characteristic of the innate immune system despite the expression of a rearranged TCR. A large number of γδ T cells are found in the human intestinal epithelium and the murine skin. γδ T cells mount a rapid immune response against transformed cells, while conventional T cells have a long activation period to permit clonal expansion (84). Like NK cells, some γδ T cells recognize the MHC class I-related proteins A and B (MICA/B) via the receptor NKG2D, but also via the T cell receptor (84). Unlike αβ T cells, in vitro activated γδ T cells were reported to mediate cytotoxicity against several tumor cell lines, including Daudi cells (47).

Although innate immune cells form an important first line of defense against cancerous cells, complete tumor rejection depends on the adaptive immune system, i.e. the majority of conventional T cells (84).

1.3 IL-12 and IL-18

IL-12 is primarily produced by antigen-presenting cells and plays an important role in Th1-biased, cell-mediated immune responses induced by intracellular pathogens, but it is also involved in the pathogenesis of chronic inflammatory disease (25;83;88). IL-12 powerfully stimulates NK and T cells. The effects of IL-12 on these cells include induction of IFN-γ production in the presence of accessory cells (14;40); enhancement of cytolytic activity (15); and stimulation of proliferation of activated cells (24).

IL-18 is an IL-1-related cytokine, produced by cells of the monocyte lineage, that was first described as an IFN-γ-inducing factor (IGIF) based on its ability to induce high level IFN-γ secretion by both NK cells and T cells (56;59). IL-18 also mediates other immunoregulatory functions shared with IL-12 including enhancement of NK lytic

activity and proliferation of activated T cells (59;91). IL-18-deficient mice show reduced production of IFN-γ, impaired NK cell activity, and a defective Th1 cell response, as do IL-12-deficient mice. In mice lacking both cytokines NK lytic activity and Th1 differentiation are further impaired which demonstrates the synergistic function of IL-12 and IL-18 in vivo (86).
NK cells, unlike T cells, constitutively express high affinity receptors for both IL-12 and IL-18. The synergistic effects of IL-12 and IL-18 could be the consequence of reciprocal up-regulation of receptors and of cooperative action at the transcriptional level resulting in optimal activation of IFN-γ transcription and other effector functions of T and NK cells (3;12;13;35;55;82).

IL-18, which enhances the expression of Fas ligand on NK cells (90), exerts its significant antitumor activity primarily via a Fas-dependent pathway (29). IL-12, however, augments cytotoxicity of NK-like cells by inducing expression of perforin (20), and IL-12-induced antitumor effects are predominantly mediated by the perforin pathway (29).
Recent evidence implies that NK cell recognition of tumor cells is a requirement for effective immunotherapy of cancer by cytokines (77). In murine models of NK cell-mediated tumor suppression, the perforin-dependent antitumor activity of IL-2 or IL-12 was initiated via the NKG2D receptor-ligand recognition pathway, and cytokines that stimulated perforin-mediated cytotoxicity were more effective against tumor metastases expressing NKG2D ligands. By contrast, the Fas-dependent IL-18-induced tumor rejection by NK cells did not involve the NKG2D receptor-ligand pathway but required tumor susceptibility to Fas ligand. Cotreatment with IL-2 and IL-18 stimulated both perforin- and Fas ligand-dependent NK cell lytic functions with potent synergistic tumor-suppressive effects (77). A similar action is conceivable for the combination of IL-12 and IL-18.

1.4 The TL1A/DR3 pathway

TL1A (TNFSF15), a new TNF-like molecule, was recently described as ligand of the TNF receptor (TNFR)-superfamily members DR3 (TNFRSF25) and TR6/DcR3 (52).
The TNF superfamily of proteins (TNF SFP) consists of 18 type 2 proteins that can have membrane-bound or soluble forms. Their receptors, which compose the TNFR superfamily of proteins, are type 1 transmembrane proteins characterized by cysteine-rich domains (46).
Ligand-receptor interaction of the TNF/TNFR superfamilies of proteins (TNF/TNFR SFP) activates signaling pathways involved in many biological processes, e.g. tissue homeostasis, cell death, development, organogenesis, and innate and adaptive immunity. Members of the TNF/TNFR SFP are expressed on cells of the immune system and play a key regulatory role in multiple functions of the immune response, including defense against microorganisms, inflammation, and autoimmunity (46).
The TNFR superfamily can be divided in two groups based on two principal classes of cytoplasmic adaptor proteins and distinct modes of signaling (46). The first group is characterized by a TRAF (TNFR-associated factor) binding domain. Ligation of the TRAF binding domain by TNFR-associated factors activates signal transduction pathways resulting in NF-κB activation and initiation of transcription. The second group contains a death domain (DD). Death domain-containing receptors were initially shown to induce apoptosis via the activation of caspases. Recent evidence has shown an involvement of DD-containing receptors in nonapoptotic immunomodulatory functions, e.g. T cell activation by Fas (52). DR3 belongs to this group of receptors. The members of the third group, which include DcR3/TR6, are called decoy receptors. They lack a cytoplasmic domain and were thought to inhibit signal transduction by competing with the signal-transducing receptor for the ligand.

DcR3 binds to FasL and LIGHT and inhibits FasL-induced apoptosis. Recent studies revealed its downregulating function in immune responses (52;96). Many human tumors overexpress DcR3, and DcR3 is believed to be released by tumor cells as soluble decoy receptor to escape the host immune response by neutralizing the cytotoxic effects of FasL, LIGHT and TL1A (96).

DR3 is a death-domain-containing receptor with highest homology to TNFR1 (52). Expression of DR3 is confined to lymphocytes and can be induced by activation (75). To date, TL1A is the only known ligand of DR3 (97). TL1A is a longer variant of VEGI, an endothelium-derived factor shown to inhibit endothelial cell growth and tumor progression (52). While resting T cells, B cells, NK cells, DC, and monocytes did not express TL1A mRNA, IL-1a, TNF or PMA induced transcript in human umbilical vein endothelial cells (HUVEC) (52). Like most TNF ligands, TL1A has a membrane-bound form, and ectopic expression of the complete cDNA results in the release of a bioactive, truncated form (52;71).

In anti-CD3, anti-CD28 stimulated T cells, TL1A enhances IL-2 responsiveness and secretion of the proinflammatory cytokines IFN-γ and GM-CSF (52). TL1A further has a synergistic effect on IL-12 and IL-18-induced IFN-γ production in human T cells and NK cells (64). There is evidence that TL1A is involved in the pathogenesis of inflammatory bowel disease (71), atherosclerosis and rheumatoid arthritis (97). TL1A has been reported to inhibit angiogenesis and to induce metalloproteinase and IL-8 gene expression (97).

In TF-1 cells, which express DR3 endogenously, TL1A binding activates NF-κB and MAPK (ERK, JNK, and p38) pathways by inducing the formation of a DR3 signaling complex involving TNFR1-associated death domain protein (TRADD), TNFR-associated factor-2 (TRAF2), and receptor interactive protein (RIP), but it does not induce apoptosis (95). The results suggested that DR3-induced activation of NF-κB was responsible for resistance to apoptosis in TF-1 cells. Moreover, interaction of TL1A with its receptor DR3 was recently shown to induce IL-8 gene expression by activating TRAF2 and NF-κB via the TAK1/ASK1-MKK4/MKK7-JNK2 kinase cascade (97).

1. 5 Aims of the study

Papadakis et al. recently reported that TL1A synergizes with IL-12/IL-18 to augment IFN-γ production in resting human peripheral blood T and NK cells. DR3 is not expressed on unstimulated T and NK cells, but it is induced on a small subset of T cells and on up to 70% of NK cells by IL-12 and IL-18. However, while engagement of DR3 by TL1A augments IL-12/IL-18 induced IFN-γ production 3-15 fold in T cell cultures, the increase in NK cell cultures is a modest two-fold. In T cells, TL1A enhances IFN-γ production by increasing both the number of IFN-γ producing cells and the amount of IFN-γ produced per cell, whereas in NK cells, only the number of IFN-γ producing cells is augmented in response to TL1A (64). The synergistic effect of TL1A on NK cell IFN-γ production therefore seems to be solely due to stimulation of NK cell proliferation and not to an enhanced IFN-γ production per cell. Given the induction of DR3 on most NK cells by IL-12/IL-18, this study investigated if TL1A also regulates another function of cytokine activated NK cells, cytotoxicity against tumor cells.

This study also analyzed if other cytokines known to activate NK cells induce DR3 expression, and it aimed at identifying concentrations of the combined DR3-inductive cytokines IL-12 and IL-18 which result in maximal effectiveness of the TL1A-DR3 pathway. A dose-kinetic analysis of the effect of IL-12/IL-18 on NK cell IFN-γ production, with and without co-stimulatory TL1A, was therefore performed. This study further investigated whether the synergistic effect of TL1A on IL-12/IL-18-induced IFN-γ production is mainly due to stimulation of NK cell proliferation, as suggested by previous data (64). To this end, NK cell DNA replication was measured in a H^3-Thymidine incorporation assay.

2 Materials

2.1 Peripheral blood cells

Blood for the isolation of peripheral blood mononuclear cells (PBMC) and natural killer (NK) cells was obtained from normal donors after informed consent in accordance with the Human Subjects policy of the Cedars-Sinai Medical Center Institutional Review Board.

2.2 Tumor cell lines

K562 ATCC CCL-243

The cell line K562 was derived from the pleural effusion of a patient with chronic myelogenous leukemia in terminal blast crisis (48;49). K562 are multipotential, hematopoietic malignant cells which are capable of spontaneously differentiating into progenitors of the erythrocytic, granulocytic and monocytic series (48). The strain purchased from ATCC most closely resembles the B population. The Philadelphia chromosome was not detected in 15 metaphases examined by ATCC. The line is Ebstein-Barr virus nuclear antigen-negative (1). K562 cells, which are highly sensitive to lysis by NK cells, are standard target cells for measurements of cytotoxicity in ^{51}Cr-release assays (23).

Daudi ATCC CCL-213

The Daudi cell line was derived from a Burkitt's lymphoma (39). Daudi is a well characterized B lymphoblast cell line which is frequently used in studies of mechanisms of leukemogenesis. The cells are negative for beta-2-microglobulin. The line carries the Epstein-Barr virus and is positive for Ebstein-Barr virus nuclear antigen, viral capsid antigen and surface immunoglobulin (1). While the line is resistant to lysis by unstimulated NK cells, it can be used for measurements of cytotoxicity of activated NK cells (23).

SW837 ATCC CCL-235

SW837 cells were established from a rectal adenocarcinoma and are highly resistant to lysis by unactivated NK cells (our data). The epithelial cells display a mutation in

the p53 gene (Cytosine → Thymine mutation in codon 248 (73)). The cells are negative for expression of colon specific antigen (CSAp) and colon antigen 3 and express the following antigens: HLA A23, A32, B15, B35; blood type O; Rh+ (1). SW837 are capable of synthesizing carcinoembryonic antigen (CEA) (44) and keratin (1).

WiDr ATCC CCL-218

The NK-resistant cell line (our data) was derived from a patient with a colorectal adenocarcinoma. The cells display a mutation of the p53 antigen (Guanine → Adenine mutation). WiDr express HLA A24, A32, B15, B18 and synthesize carcinoembryonic antigen (CEA), colon specific antigen (CSAp), transforming growth factor beta and keratin. The cells are negative for colon antigen 3 expression (1).

2.3 Cytokines

Ionomycin	Sigma-Aldrich Inc. (St.Louis, MO)
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich Inc. (St.Louis, MO)
Recombinant IFN-α	R&D Systems (Minneapolis, MN)
Recombinant IFN-β	R&D Systems (Minneapolis, MN)
Recombinant IFN-γ	R&D Systems (Minneapolis, MN)
Recombinant IL-2	R&D Systems (Minneapolis, MN)
Recombinant IL-12	Peprotech (Rocky Hills, NJ)
Recombinant IL-15	Peprotech (Rocky Hills, NJ)
Recombinant IL-18	R&D Systems (Minneapolis, MN)
Recombinant TL1A (aa72-251)	Human Genome Sciences, Inc. (Rockville, MD)
Recombinant TNF-α	Peprotech (Rocky Hills, NJ)

2.4 Antibodies

2.4.1 Flow cytometry

Anti-CD3 mAb, FITC-conjugated	Caltag (South San Francisco, CA)

(mouse IgG2a)	
Anti-CD3 mAb, PE-conjugated (mouse IgG2a)	Caltag (South San Francisco, CA)
Anti-CD14 mAb, FITC-conjugated (mouse IgG2a)	Caltag (South San Francisco, CA)
Anti-CD19 mAb, FITC-conjugated (mouse IgG1)	Caltag (South San Francisco, CA)
Anti-CD56 mAb, TC-conjugated (mouse IgG1)	Beckman Coulter (Fullerton, CA)
Anti-DR3 specific mAb (mouse IgG1; clone F05)	Human Genome Sciences, Inc., (Rockville, MD)
Anti-IFN-γ mAb, PE-conjugated	BD PharMingen (San Diego, CA)
Anti-mouse IgG1 mAb, PE-conjugated	Caltag (South San Francisco, CA)
Blocking antibodies mouse IgG1, IgG2a, IgG2b	Jackson ImmunoResearch (West Grove, PA)
Isotype mouse IgG1, TC-conjugated	Caltag (South San Francisco, CA)
Isotype mouse IgG1, unconjugated	Caltag (South San Francisco, CA)
Isotype mouse IgG1, PE-conjugated	BD PharMingen (San Diego, CA)
Isotype mouse IgG2a, FITC conjugated	Caltag (South San Francisco, CA)
Isotype mouse IgG2a, PE conjugated	Caltag (South San Francisco, CA)

2.4.2 ELISA

Anti-human IFN-γ mouse Ab	BD Pharmingen (San Diego, CA)
Secondary anti-human IFN-γ biotinylated mAb	BD Pharmingen (San Diego, CA)
Streptavidin-Alkaline phosphatase	Jackson Immuno Research (West Grove, PA)

2.5 Chemicals and buffers

2.5.1 Isolation of PMBC and NK cells

Heparin	Sigma Chemical Co. (St. Louis, MO)

HBSS-10x, w/o Ca^{2+} and Mg^{2+}	Mediatech Inc. (Herndon, VA)
Lymphocyte Separation Medium	Fisher Scientific (Tustin, CA)
Sodium Bicarbonate (7.5% sol.)	Mediatech Inc. (Herndon, VA)

2.5.2 Cell culture

Fetal bovine serum	Omega Scientific (Tarzana, CA)
Gentamicin Sulfate Solution	Omega Scientific (Tarzana, CA)
Minimum Essential Medium Eagle with Earle's salts and l-glutamine	Mediatech Inc. (Herndon, VA)
RPMI 1640 with 2 mM glutamine and 25 mM HEPES buffer	Mediatech Inc. (Herndon, VA)
Trypsin EDTA (0.25% Trypsin and 1mM EDTA-4Na)	Gibco Invitrogen Corp. (Grand Island, NY)

2.5.3 Flow cytometry

Bovine serum albumin	Sigma Chemical Co. (St. Louis, MO)
Brefeldin A	EMD (c/o Calbiochem; La Jolla, CA)
Paraformaldehyd	Sigma Chemical Co. (St. Louis, MO)
10x PBS, w/o Ca^{2+} and Mg^{2+}	Mediatech Inc. (Herndon, VA)
Saponin	Sigma Chemical Co. (St. Louis, MO)
Sodium Azide	Sigma Chemical Co. (St. Louis, MO)

2.5.4 ELISA

2-propanol	J.T. Baker (Phillipsburg, NJ)
β-Nicotinamide adenine dinucleotide phosphate ($NADP^+$)	Sigma Chemical Co. (St. Louis, MO)
Alcohol Dehydrogenase (ADH)	Sigma Chemical Co. (St. Louis, MO)
Diaphorase	Sigma Chemical Co. (St. Louis, MO)
Dimethyl Sulfoxide (DMSO)	Sigma Chemical Co. (St. Louis, MO)
H_3BO_4	Sigma Chemical Co. (St. Louis, MO)
Iodonitrotetrazolium chloride (INT)	Sigma Chemical Co. (St. Louis, MO)
KH_2PO_4	Fisher Scientific (Tustin, CA)
$MgCl_2$ $2H_2O$	Fisher Scientific (Tustin, CA)

NaCl	Fisher Scientific (Tustin, CA)
NaH_2PO_4 $2H_2O$	Fisher Scientific (Tustin, CA)
Na_2HPO_4	J.T. Baker (Phillipsburg, NJ)
NaN_2	Sigma Chemical Co. (St. Louis, MO)
NaOH	J.T. Baker (Phillipsburg, NJ)
PBS 10x, w/o Ca^{2+} & Mg^{2+}	Mediatech Inc. (Herndon, VA)
TRIS Base	Fisher Scientific (Tustin, CA)
Tris-HCl	J.T. Baker (Phillipsburg, NJ)
Tween 20	Fisher Scientific (Tustin, CA)

2.5.5 ^{51}Cr-release assay

Chromium-51 Radionuclide, 1mCi (37MBq), Specific Activity: 400-1200Ci (14.8-44.4TBq)/g, Sodium Chromate in Normal Saline (pH 8-10)	Perkin Elmer Life Sciences (Downers Grove, IL)
LKG Hi-Load (High safety scintillation Cocktail)	Perkin Elmer (Downers Grove, IL; formerly Wallac)
Triton X-100	Fisher Scientific (Tustin, CA)

2.5.6 H^3-Thymidine incorporation assay

BetaplateScint	Perkin Elmer (Downers Grove, IL; formerly Wallac)

2.6 Composition of prepared solutions

2.6.1 PBMC isolation

1x HBSS	10x HBSS, w/o Ca^{2+} and Mg^{2+}, sterile destilled water, pH 7.0 (pH equilibration with Sodium Bicarbonate (7.5% sol.))

2.6.2 NK cell Isolation

Buffer (PBS/0.5% BSA)	10x PBS w/o Ca^{2+} and Mg^{2+}, sterile destilled water, 0.5% (w/v) BSA, pH 7.2, degassed

2.6.3 Cell culture

Minimum Essential Medium Eagle with Earle's salts and l-glutamine	Minimum Essential Medium Eagle supplemented with 10% (v/v) fetal bovine serum and 50 µg/ml gentamycin
RPMI culture medium	RPMI 1640 with 2 mM glutamine and 25 mM HEPES buffer, supplemented with 10% (v/v) fetal bovine serum (heat inactivated) and 50 µg/ml gentamicin

2.6.4 Flow cytometry

Fixing solution (PBS/2% (w/v) Paraformaldehyd)	2% (w/v) Paraformaldehyd in 1x PBS w/o Ca^{2+} and Mg^{2+}, sterile-filtered
1x PBS	10x PBS w/o Ca^{2+} and Mg^{2+}, sterile destilled water, pH 7.2
Permeabilizing solution	0.5% (w/v) BSA, 0.02% (w/v) Sodium Azide, 0.1% (w/v) Saponin in 1x PBS w/o Ca^{2+} and Mg^{2+}, sterile-filtered
Staining solution (PBS/0.5% (w/v) BSA)	0.5% (w/v) BSA, 0.02% (w/v) Sodium Azide in 1x PBS w/o Ca^{2+} and Mg^{2+}, sterile-filtered

2.6.5 ELISA

Amplifier	1.0 mg Diaphorase, 10.0 mg INT (dissolve in 0.3 ml DMSO), 1.5 mg ADH, 0.6 ml 2-propanol in 10.0 ml Amplifier Buffer (0.025 M PO_4, pH 8.8)

Amplifier Buffer (10x)	17.8 g Na_2HPO_4 (0.25 M PO_4) in 500 ml destilled water, equilibrate pH by adding KH_2PO_4 (3.4 g KH_2PO_4 in 100 ml destilled water) to 500 ml of Na_2HPO_4 until pH reaches 8.8
Borate Buffered Saline (10x)	51.5 g H_3BO_4, 36.5 g NaCl in 500 ml destilled water, pH 8.5 (using NaOH)
PBS/Tween (10x)	2.89 g NaH_2PO_4 $2H_2O$, 11.94 g Na_2HPO_4, 87.67 g NaCl, 2.0 g NaN_2, 5.0 ml Tween 20 in 1000 ml destilled water
Tris/NaCl (10x)	60.5 g Tris Base, 87.67 g NaCl, 2.00 g NaN_2, 5.0 ml Tween 20, pH to 7.5 with 10 N HCl (approximately 37 ml)
Substrate	1.6 mg $NADP^+$ in 5.0 ml of Substrate Buffer (0.075 M Tris Base, pH 8.8)
Substrate Buffer (10x)	45.43 g Tris Base (0.75 M Tris Base), 1.52 g $MgCl_2$ $2H_2O$ in 500 ml destilled water, pH to 8.8 with 10 N HCl

2.7 Kits

2.7.1 NK cell Isolation

NK Cell Isolation Kit II - NK Cell Biotin-Antibody Cocktail: Cocktail of biotin-conjugated monoclonal antibodies against CD3, CD4, CD14, CD15, CD19, CD36, CD123 and Glycophorin A - Anti-Biotin Micro-Beads: Microbeads conjugated to a monoclonal antibiotin antinody (clone: Bio3-18E7:2; mouse IgG1)	Miltenyi Biotec Inc. (Auburn, CA)

2.7.2 Flow cytometry

Fix and Perm Cell Permeabilization Reagents - Fixation Medium (Reagent A) - Permeabilization Medium (Reagent B)	Caltag (South San Francisco, CA)

2.8 Laboratory equipment

Centrifuges	
- IEC Centra MP4R	Thermo Electron Cooperation (Waltham, MA; formerly International Equipment Company)
- IEC Centra HN	Thermo Electron Cooperation (Waltham, MA; formerly International Equipment Company)
- Sorvall TC 6	Thermo Electron Cooperation (Waltham, MA)
Emax plate reaeder	Molecular Devices (Menlo Park, CA)
Flow-cytometer	Becton Dickinson (Mountain View, CA)
Harvester 96®	Tomtec (Hamden, CT)
Heat Sealer	Packaging Aids Corporation (San Rafael, CA)
Incubator	Thermo Electron Cooperation (Waltham, MA; formerly Forma Scientific)
Microscopes	
- Olympus Microscope CH2	Olympus (San Diego, CA)
- Olympus Microscope CK2	Olympus (San Diego, CA)
Steril Card Hood, class II type A/B3	The Baker Company (Sanford, ME)
VarioMACS™ Separator	Miltenyi Biotec, Inc. (Auburn, CA)
Vortex Mixer	Barnstaed Thermolyne (Dubuque, IA)
Wallac 1450 Microbeta Liquid Scintillation	Perkin Elmer (Downers Counter Grove,

Counter	IL; formerly Wallac)

2.9 Other materials

2.9.1 Cell culture

12-well Culture Cluster, Flat bottom with lid	Corning Inc. Life Sciences (Acton, MA)
24-well Culture Cluster, Flat bottom with lid	Corning Inc. Life Sciences (Acton, MA)
96-well Culture Cluster, Flat bottom with lid	Corning Inc. Life Sciences (Acton, MA)
Cell Culture Flasks, 225 cm^2	Corning Inc. Life Sciences (Acton, MA)
MACS^{TM} Cell Separation Column LS	Miltenyi Biotec Inc. (Auburn, CA)
Pipet tips, filtered	VWR Scientific Products (Cerritos, CA)
Tissue Cultue Flasks, 250 ml	Greiner Bio-One North America Inc. (Monroe, NC)
Tissue Cultue Flasks, 50 ml	Greiner Bio-One North America Inc. (Monroe, NC)

2.9.2 ELISA

96-well plates, Flat bottom	Greiner Bio-One North America Inc. (Monroe, NC)

2.9.3 ^{51}Cr-release assay

96-well plates, V-bottom	Greiner Bio-One North America Inc. (Monroe, NC)
AeraSeal Sealing Film Soft Rubber Roller	Research Products International (Mount Prospects, IL)
Sealing Film, polypropylene	Research Products International (Mount Prospects, IL)
TempPlate RT Optically Clear Sealing Film	USA Scientific (Ocala, FL)

2.9.4 H^3-Thymidine incorporation assay

Bags for Wallac 1450 Microbeta Liquid	Perkin Elmer (Downers Grove, IL)

Scintillation Counter	
Filtermat A	Perkin Elmer (Downers Grove, IL)

2.10 Software

Elisa Master®	developed by R. Deem (Inflammatory Bowel Disease Center, Cedars-Sinai Medical Center, Los Angeles, CA)
CellQuest	Becton Dickinson (Mountain View, CA)
JMP IN 5.1 data analysis software	SAS Institute (Cary, NC)

3 Methods

3.1 Isolation and culture of PBMC, NK cells, and non-NK cells

3.1.1 Isolation of PBMC

Test principle

The density-gradient centrifugation technique with Ficoll-Hypaque is used to isolate peripheral blood mononuclear cells from whole blood by taking advantage of the different specific densities of blood cells. Hereby, anticoagulated blood is layered over Ficoll-Hypaque and centrifuged. Red blood cells and polymorphonuclear leucocytes have a higher specific density than Ficoll-Hypaque and therefore settle below the Ficoll-Hypaque layer. Mononuclear cells and platelets are less dense than Ficoll-Hypaque, therefore band over it, and can be collected from the plasma/Ficoll-Hypaque interface.

Procedure

60, 120 or 180 ml of peripheral blood were obtained from normal donors by venipuncture and treated with 1000 units of Heparin per ml of blood. PBMC were isolated on standard Ficoll-Hypaque density gradients. To this end, 15 ml of Lymphocyte Separation Medium were carefully overlayered with 30 ml of blood in 50 ml conical centrifuge tubes and centrifuged at 1000 x g for 20 minutes without brake at room temperature. The interface layer of each density gradient, consisting of mononuclear cells, was removed with a 10 ml glass pipet and placed into a 50 ml conical centrifuge tube for two subsequent cycles of washing of the cells. To this end, the cells were diluted with 40 ml of 1 x HBSS and centrifuged at 400 x g for 5 min. Next, the supernatant was decanted, the pellet resuspended and the cells of two 50 ml tubes were united in one 15 ml tube. Each 15 ml tube was filled with 14 ml of 1 x HBSS and centrifuged at 400 x g. The cell pellet was resuspended in RPMI 1640 containing 2 mM glutamine and 25 mM HEPES buffer, supplemented with 10% (v/v) fetal bovine serum and 50 μg/ml gentamicin.

PBMC were depleted of monocytes by adherence for two hours or overnight.

3.1.2 Isolation of NK cells and non-NK cells

Test principle

The magnetic-activated cell sorting (MACS) technology permits the enrichment or depletion of a particular cell type using magnetic cell labeling (53). For indirect magnetic cell labeling, cells are labeled with an unconjugated, biotinylated, or flourochrome-conjuagted primary monoclonal antibody directed against a characteristic surface marker of the cell type of interest (e.g. CD3 for T-cells, CD56 for NK cells). A cocktail of antibodies can also be employed to isolate or deplete a number of cells simultaneously. In a second step, cells are magnetically labeled with superparamagnetic MACS MicroBeads which are coupled to an appropriate secondary reagent (e.g. anti-immunoglobulin, anti-biotin, streptavidin, or anti-biotin, respectively). The cells are then passed over a magnetic separation column (MACS column), which is placed in the strong magnetic field of the MACS Separator. The magnetically labeled cells are retained in the column, while non-labeled cells pass through. After removal of the column from the magnetic field, the magnetically retained cells can be eluted from the column. The manufacturer states that the biodegradable, submicroscopic MicroBeads do not affect function, viability or light scattering properties of the magnetically labeled cells. Two magnetic cell separation strategies can be employed: positive and negative selection. Positive selection involves magnetic labeling of the cells of interest which are isolated by retention in a MACS column, followed by elution from the column after removal from the magnetic field. Negative selection depends on depletion of the non-target cells by magnetic labeling and subsequent retention in the MACS column, while the enriched unlabeled target cells pass through the column.

Procedure

In this study, NK cells were isolated from PBMC by negative selection (NK Cell Isolation Kit II, Miltenyi Biotec, Auburn, CA) and thus not exposed to potentially stimulatory mAb. Hereby, non-NK-cells were indirectly magnetically labeled with a cocktail of biotin-conjugated mononclonal antibodies, as primary labeling reagent, and anti-biotin monoclonal antibodies conjugated to MicroBeads, as secondary labeling reagent. The magnetically labeled non-NK cells were depleted by retention in

a MACS column in the magnetic field of a MACS Separator, while the unlabeled NK cells passed through the column.

Magnetic labeling

The NK cell isolation was performed according to the manufacturer's protocol, using from $10x10^7$ to $15x10^7$ PBMC obtained by density gradient centrifugation as described above. The suspension of PBMC was centrifuged at 400 x g for 5 min and the supernatant was removed completely. The cell pellet was then resupended in 40 µl of buffer per 10^7 total cells and 10 µl of Biotin-Antibody Cocktail per 10^7 total cells were added. After mixing the suspension was incubated for 10 min at 4-8 ºC. Then the suspension was diluted with 30 µl of buffer per 10^7 total cells and 20 µl of Anti-Biotin MicroBeads were added per 10^7 total cells. The well mixed suspension was incubated for 15 min at 4-8 ºC. The cells were washed with buffer by adding 10-20 x labeling volume and centrifuged at 400 x g for 5 min. The supernatant was pipetted off completely and up to 10^8 cells were resuspended in 500 µl buffer.

Magnetic separation

The MACS Separation column LS placed in the magnetic field of the VarioMACS™ Separator was rinsed with 3 ml of buffer. The cell suspension was then applied onto the column. The cells were allowed to pass through the column and the effluent was collected as the fraction of unlabeled cells representing the enriched NK cell fraction. Subsequently, the column was washed by adding 3 ml of buffer three times allowing the column reservoir to empty before applying the next amount of buffer. The entire effluent was collected representing the enriched NK cells.

For some experiments, the cells retained in the column were eluted and tested as the non-NK fraction. The cell fractions suspended in NK cell isolation buffer were centrifuged and resuspended in RPMI 1640 containing 2 mM glutamine and 25 mM HEPES buffer, supplemented with 10% fetal bovine serum (heat inactivated) and gentamycin (50 µg/ml).

3.1.3 Culture conditions

PBMC, purified NK cells and the non-NK fraction were cultured at 0.5 x 10^6 cells/ml in RPMI 1640 containing 2 mM glutamine and 25 mM HEPES buffer, supplemented with 10% (v/v) fetal bovine serum (heat inactivated) and gentamycin (50 μg/ml) under conditions stated in the figures. Supernatants were frozen for IFN-γ analysis by ELISA.

3.1.4 Stimulation with cytokines

Recombinant TL1A (aa 72-251) was used at 50 ng/ml. Recombinant IL-12 and IL-18 were used as indicated in the Figure legends. The cytokines tested for the induction of DR3 expression on NK cells were used at concentrations as follows: recombinant IFN-α, IFN-β, IFN-γ, and IL-2, 200 U/ml; recombinant IL-15, 50 ng/ml and TNF-α, 10 ng/ml; and Phorbol 12-myristate 13-acetate (PMA) and ionomycin, 10 ng/ml and 0.2 μg/ml, respectively.

3.2 Culture of tumor cell lines

The cell lines K562, Daudi and SW837 were all grown in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (heat inactivated) and gentamycin (50 μg/ml). WiDr cells were cultured in Minimum Essential Medium Eagle with Earle's salts and l-glutamine, with 10% (v/v) fetal bovine serum and gentamycin. The cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2. New cultures were started at 0.5-1x10^5 cells/ml. The leukemic suspension cell lines were split to a density of 0.2x10^6 daily or every two days and thus used in the log phase of growth for cytotoxicity assays or DR3 induction, respectively.

The adherent epithelial cell lines were used when reaching approximately 70% confluency. They were subcultured 2 times per week at a ratio of 1:3 to 1:5 and harvested by using Trypsin EDTA (0.25% Trypsin and 1mM EDTA-4Na) as follows: The medium was removed from the plates and the adherent cells were washed with PBS w/o Ca^{2+}& Mg^{2+} in order to completely remove traces of serum which contains substrates of trypsin as well as trypsin inhibitor. After adding Trypsin EDTA the plates were incubated at 37 ºC and periodically observed under an inverted

microscope until the cells had detached from the bottom of the plates. To stop lysis new medium was added to the plates. The cells were collected and centrifuged. The cell pellet was resuspended in new medium and the cells were dispensed in new flasks.

3.3 Flow cytometric analysis

Test principle

Flow cytometry is a technology that permits the simultaneous analysis of multiple physical and biological characteristics of individual cells in a suspension, based on light scattering and fluorescent properties (9;51). To this purpose, cells are transported to a laser intercept in a fluid stream. When the cells are illuminated by the laser beam, they scatter light and cell constituents labeled with fluorescent molecules emit flourescence. Scattered and fluorescent light is collected by lenses. Beam splitters and filters direct the resulting light signals to detectors which transform the light signals into electronic signals. The data on the light scattering and fluorescent properties of each particle or event is processed in a computer and can be analysed to provide information about subpopulations within a sample. Sorting decisions can further be initiated by the electronics system.

The technique of hydrodynamic focusing is used to ensure optimal illumination of the cells by the laser beam. Hereby, the sample is injected into a stream of sheath fluid within the flow chamber. The flow of sheath fluid accelerates the cells and precisely aligns them in front of the beam of laser light.

Light scattering depends on a cell's physical properties, namely its size, membrane and internal complexity. Forward-scattered light (FCS), which is proportional to cell-surface area or size, is diffracted light, detected just off the axis of the incident laser beam in the forward direction. Side-scattered light (SSC), which depends on cell granularity (or internal complexity), is a measurement of refracted and reflected light, collected in approximately 90 degrees to the laser beam. Correlated measurements of FSC and SSC permit the differentiation of major leucocyte subpopulations (neutrophils, monocytes, lymphocytes).

For immunophenotyping, cell constituents, e.g. individual antigenic surface markers of a cell, are specifically labeled with monoclonal antibodies conjugated to fluorescent dyes (fluorochromes), which are then used to identify cells carrying the constituent (positive cells).

A flourochrome is a compound which absorbs light energy over a characteristic spectrum of wavelengths. Due to the absorption of light, an electron in the flourochrome is raised to a higher energy level. When the excited electron decays to its ground state, it emits the excess energy as a photon of light. This transition of energy defines fluorescence.

As cells pass through the laser beam, each cell carrying a fluorochrome label produces a fluorescent signal. The amount of flourescent signal detected is directly proportional to the number of flourochrome-conjugated antibodies bound on the cell and consequently to the number of copies of the labeled constituent present in the cell. Multiple cell constituents or antigens can be detected simultaneously by using antibodies conjugated to different fluorochromes. Several flourochromes can be used in combination if the wavelength of the laser excites the different fluorochromes and if their peak emission wavelengths are far enough apart, so that each fluorescent signal can be detected by a separate detector. Modern flow cytometers are equipped with multiple lasers with different wavelengths, so that even more fluorochromes can be used simultaneously.

To subtract auto-fluorescence of cells and unspecific binding of the specific antibody directed against the cell constituent in question, a sample of cells of each test condition is labeled with an isotype control antibody, i.e. an unspecific immunoglobulin of the isotype of the specific antibody, and the control is then analyzed together with the test samples. A threshold value of fluorescence intensity attributed to specifically bound flourochrome-conjugated antibody is defined, which is used to demarcate positive from negative cells.

The following flourochromes were used in this study:

Flourescein iso-thiocyanate (FITC), peak emission wavelength at 530 nm

Phycoerythrin (PE), peak emission wavelength at 570 nm

Tri-Color (TC), peak emission wavelength at 670 nm

The staining pattern, combined with FCS and SSC data, allows to identify a particular cell type or subpopulation and to count its relative percentage. If the employed antibodies are used in excess, it is further possible to compare the mean fluorescence intensities of different cell populations and to determine which population expresses a greater per cell quantity of the labeled cell constituent.

3.3.1 Evaluation of NK cell purity

The purity of the NK cell fraction isolated by magnetic-activated cell sorting (cf. 2.2.1.2) was determined by flow cytometry. To determine the percentage of NK cells, T cells, B cells and monocytes in the enriched NK cell fraction, aliquots were directly stained for the NK cell marker CD56, the T cell marker CD3 and for the B cell marker CD19 or the monocyte marker CD14, respectively. To this end, $1.5x10^6$ cells suspended in medium were washed by centrifuging at 400 x g for 5 min, resuspending in 1 ml of staining solution (cf. Materials) and subsequent centrifuging. Next, the supernatant was removed and the cell pellet was resuspended in 300 µl of staining solution (PBS/0.5% (w/v) BSA). The cell suspension was then subdivided in three 100-µl aliquots of $0.5x10^6$ cells each. Two aliquots were incubated on ice for 30 min with 2.5 µl each of anti-CD3 PE-conjugated mAb, of anti-CD56 TC-conjugated mAb and of either anti-CD19 FITC-conjugated mAb or anti-CD14 FITC-conjugated mAb, respectively. The third aliquot incubated with 5 µl each of mouse IgG1-TC-conjugated and of mouse IgG2a-PE conjugated served as isotype control. The aliquots were washed afterwards with staining solution and the cells were fixed in 300 µl of PBS/1% (w/v) Paraformaldehyd by adding 150 µl of staining solution (PBS/0.5% (w/v) BSA) to the cell pellets, vortexing and diluting with 150 µl of fixing solution (PBS/2% (w/v) Paraformaldehyd). Expression of CD56, CD3 and CD19 or CD14, respectively, on the enriched NK cell fraction was analyzed by flow cytometry. Based on forward and side scatter the gate was set to include lymphocytes and monocytes and to exclude cellular debris and aggregates. $5x10^4$ cells were acquired. FITC was acquired on the FL-1 fluorescence detector, PE on FL-2 and TC on FL-3 using log amplification. Percentage non-specific staining by isotype control Ab was substracted from percentage specific staining of the test condition.

The flow cytometric analysis revealed a yield of a population of greater than 95% CD56+, CD3- NK cells in all experiments.

3.3.2 Analysis of DR3 surface expression

Test principle

To study surface NK cell DR3 expression, NK cells were indirectly labeled with an anti-DR3 specific monoclonal antibody or an isotype control antibody, respectively, as primary labeling reagent, and an anti-mouse antibody conjugated to phycoerythrin, as secondary labeling reagent. After blocking with mouse IgG to prevent unspecific binding, the cells were further stained for CD3 and CD 56 to evaluate DR3 surface expression of NK cells (CD3-, CD56+), T cells (CD3+, CD56-) and NKT cells (CD3+, CD56+) by flow cytometric analysis.

Procedure

$0.5x10^6$ PBMC per staining condition were washed with staining solution (PBS/0.5% (w/v) BSA). After centrifuging for 5 min at 400xg the cell pellet was resuspended in 100 µl of staining solution per $0.5x10^6$ PBMC. Next, the cell suspension was subdivided in 100 µl-aliquots of $0.5x10^6$ PBMC per stainining condition. To prevent unspecific binding of the subsequently used antibodies the aliquots were blocked with 1 µl goat IgG each for 20 min on ice (without subsequent washing). Next, the aliquots were incubated with 2 mg anti-DR3 specific monoclonal antibody or 10 µl isotype control antibody (mouse IgG1) on ice for 30 min. The aliquots were then washed with staining solution to remove excess unbound antibodies and incubated with 2.5 µl anti-mouse IgG1 secondary antibody conjugated to phycoerythrin for 30 min on ice. After washing with PBS/0.5% (w/v) BSA and blocking with 1 µl of mouse IgG for 20 min on ice, all aliquots were stained with 2.5 µl CD3-FITC and CD56-TC for 20 min on ice, fixed in PBS/1% Paraformaldehyd and analyzed by flow cytometry. DR3 expression was analyzed on NK cells by gating on lymphocytes based on forward and side scatter and further gating on CD3-CD56+ cells. FITC was acquired on the FL-1 fluorescence detector, PE on FL-2 and TC on FL-3 using log amplification. $5x10^4$ cells were acquired and both the percentage of NK cells expressing DR3 and the mean

fluorescence intensity of DR3-positive cells were obtained. Percentage non-specific staining by isotype control Ab was subtracted from percentage specific staining of the test condition.

3.3.3 Analysis of intracellular IFN-γ production

Test principle

For analysis of intracellular IFN-γ production, cytokine-activated PBMC were incubated with Brefeldin A for the last 4 hrs of the incubation time. Brefeldin A is a lactone antibiotic produced by fungal organisms, which inhibits protein secretion of cells by interfering with anterograde protein transport from the endoplasmatic reticulum (ER) to the Golgi apparatus (38;57). Proteins thus accumulate inside the ER and can be detected intracellularly by fluorochrome-conjugated monoclonal antibodies. To this end, PMBC were surface-stained with flourochrome-conjugated anti-CD3 and anti-CD56 antibodies for the phenotyping of NK cells, T cells and NKT cells first. Fixation of the cell structure and the surface staining with formaldehyde followed. The cell membrane was then permeabilized with Permeabilization Medium (Reagent B, Caltag (South San Francisco, CA)). After the cells had been blocked with mouse IgG to prevent unspecific binding, intracellularly accumulated IFN-γ was stained with flourochrome-conjugated anti-IFN-γ antibody, which could enter the cells through the created pores in the cell membrane. Subsequently, phenotyping of the cells and assessment of intracellular IFN-γ production were performed by flow cytometry.

Procedure

PBMC were cultured with IL-12 and IL-18 with or without TL1A for 24, 48 and 72 hrs. Brefeldin A (10 µg/ml) was added to the cells for the last 4 hrs of the incubation time. Next, $0.5x10^6$ PBMC per staining condition were washed with PBS/0.5% (w/v) BSA. The cell pellet was resuspended in 100 µl of PBS/0.5% (w/v) BSA per $0.5x10^6$ PBMC and the cell suspension was subdivided in 100 µl-aliquots of $0.5x10^6$ PBMC per stainining condition. All aliquots were stained with 2.5 µl CD3-FITC and CD56-TC for 20 min on ice. After washing with PBS/0.5% (w/v) BSA and centrifuging, the supernatant was removed and the cell pellets were resuspendend by vortexing

carefully. 100 µl of Fixation Medium (Reagent A) were added to each aliquot. Next, the aliquots were incubated at room temperature for 10 min and washed with PBS/0.5% (w/v) BSA. After centrifuging and removing the supernatant the cell pellets were resuspendend by vortexing carefully and 100 µl of Permeabilization Medium (Reagent B) and 1 µl of mouse IgG were added to the aliquots. The aliquots were blocked on ice for 10 min and either 10 µl of anti-IFN-γ PE or of isotype control antibody (mouse IgG1-PE) were added. After incubating for 20 min on ice the aliquots were washed with permeabilizing solution. The cells were fixed in 300 µl of PBS/1% (w/v) Paraformaldehyd by adding 150 µl of permeabilizing solution to the cell pellets, vortexing and diluting with 150 µl of fixing solution. Intracellular IFN-γ production was analyzed on NK cells by flow cytometry by gating on lymphocytes based on forward and side scatter and further gating on CD3-CD56+ cells. $5x10^4$ cells were acquired and both the percentage and the mean fluorescence intensity of IFN-γ-producing NK cells were obtained. Percentage non-specific staining by isotype control Ab was subtracted from percentage specific staining of the test condition.

3.4 Detection of IFN-γ by ELISA

Test principle

The Enzyme-Linked Immunosorbent Assay (ELISA) is used to detect and quantitate antibodies or antigens in unknown samples (16).

The ELISA can be employed for qualitative or quantitative measurements. The qualitative ELISA, which examines the presence of a substance in an unknown sample, yields negative or positive results. The quantitative ELISA requires that a purified standard of the substance to be measured is available. In the quantitative ELISA, the optical density of the unknown sample is interpolated into a standard curve, composed of a serial dilution of the target antigen.

Depending on the measured substance different techniques of ELISA are employed. The quantitative sandwich ELISA used in this study is a frequently applied technique for antigen measurement. Hereby, capture antibody is bound to a solid phase, usually the bottom of a microtiter plate well. The antigen-containing unknown samples and

the standards which consist of a serial dilution of the antigen are then applied to the plate. The antigen is allowed to complex with the bound capture antibody. Unbound antigen is then removed by washing the plate, and a detection antibody is allowed to bind to the antigen, thereby completing the sandwich. Next, an enzyme-conjugated secondary antibody, which binds the Fc region of the detection antibody, is added. The wells are washed again, and a substrate is applied to the wells. The substrate is converted by the enzyme which catalyzes the formation of a colored product. Color development during an incubation period is directly proportional to the amount of enzyme-conjugated secondary antibody, which is in proportion to the amount of target substance detected. In the quantitative ELISA, the optical density is monitored spectophotometrically and related to the concentration of the antigen by calibration to the standard curve, composed of a serial dilution of the target antigen. The sandwich ELISA can be used if capture and detection antibody recognize separate epitopes on the antigen, so that they do not hinder each other's binding.

Procedure

IFN-γ was quantitated in culture supernatants by amplified sandwich ELISA. To this end, 96 well microtiter plates were coated overnight in a humidified box at 4ºC with 50 µl/well of monoclonal mouse anti-IFN-γ Ab at 5.0 µg/ml in 1xBorate Buffered Saline (BBS). After washing three times with 200 µl/well 1xPBS/Tween the wells were blocked with 150 µl/well PBS/0.5% (w/v) BSA for 30 min at room temperature. The use of non-human serum protein (bovine serum albumin) prevents non-specific binding of proteins antigens in the culture supernatants by anti-IFN-γ Ab. The blocking solution was then removed (no washing). 50 µl/well of standards, consisting of a serial dilution of recombinant human IFN-γ at 0.2, 0.4, 0.6, 1.2, 1.6, 2.0 and 2.4 ng/ml, and 50 µl/well of the samples of unknown IFN-γ concentration, both diluted in PBS/0.5% (w/v) BSA, were applied to the wells in duplicate for each condition. The plates were incubated for 24-72 hrs in a humidified box at 4ºC. Sample and standard IFN-γ was detected by adding (no emptying or washing) 50 µl/well of secondary anti-IFN-γ biotinylated mAb at 0.5 µg/ml in 0.5% (w/v) BSA/PBS and incubating for 2 hrs at room temperature in a humidified box. After washing four times with 200 µl/well

1xPBS/Tween, the biotinylated mAb was labeled by 100 µl/well of streptavidin-alkaline phosphatase, diluted 1 to 1000, for 30 min at room temperature in a humidified box. The plates were washed two times with 200 µl/well of 1xPBS/Tween followed by four phosphate-free washes with 200 µl/well of 1xTris/NaCl. 50 µl/well of substrate (0.2 mM NADPH) were added for 30 min, and the NADH signal was amplified using 2-propanol (3%) with iodonitrotetrazoliun violet (1 mM), alcohol dehydrogenase (75 µg/ml) and diaphorase (50 µg/ml). Color development occurred within 15 min at 20ºC. Plates were read at 490 nm on an Emax plate reader. Sample concentration was calculated by interpolating the optical density of the samples into a standard curve consisting of a serial dilution of IFN-γ, generated with the software *Elisa Master*®, developed by R. Deem (Inflammatory Bowel Disease Center, Cedars-Sinai Medical Center, Los Angeles, CA).

3.5 ^{51}Cr-release assay

Test Prinicple

The evaluation of NK cell cytotoxicity is dependent upon the labeling of target cells with a substance that is released upon disruption of the target cell plasma membrane as a result of lysis mediated by effector cells. Although several non-radioactive, mostly flow-cytometric techniques are employed to measure cytotoxicity, the ^{51}Chromium-release assay is considered the gold standard cytotoxicity measure (23;36). The ^{51}Cr-release assay primarily measures perforin-mediated osmotic lysis (23). Hereby, target cells are incubated with the radioactive ^{51}Chromium and then mixed with effector cells. After an incubation period of effector and target cells the amount of ^{51}Chromium released from the target cells into the supernatant is directly proportional to the number of target cells killed.

Peripheral blood mononuclear cells, which can be easily obtained by venipuncture, are commonly used as effector cells. The cell line K562 is the standard target for measurements of cytotoxicity. The NK-sensitive K562 can be used to assess the cytolytic acivity mediated by freshly isolated, unstimulated NK cells, while the Daudi cell line, which is resistant to lysis by unstimulated NK cells, is frequently used to evaluate cytolytic acivity of activated NK cells. The cell lines are maintained under

optimal culture conditions in the log phase of growth to ensure reproducible assay results.

In this study, the standard 4-hr incubation period of effector cells and ^{51}Chromium-labeled target cells was reduced to 2 hrs to shorten the considerable length of the assay procedure. Initial experiments had demonstrated that the maximal level of cytotoxicity mediated by NK cells during a 2-hr incubation period was not significantly different from the maximal level of NK cell cytotoxicity mediated during a 4-hr incubation period (Fig. 4.4).

^{51}Chromium released into supernatants by lysed target cells is determined in triplicate for all experimental conditions in a beta counter, and mean cpm is calculated.

Cytotoxicity is quantified by the percentage of target cells killed (percent specific lysis (PSL)). The following formula is used to calculate percent specific lysis:

PSL = [(ER - SR)/(MR - SR)] x 100

ER denotes the experimental release mean cpm, SR is the spontaneous release release mean cpm, and MR is the maximal release mean cpm.

Maximal release of ^{51}Chromium results from lysis of all target cells. Spontaneous release of ^{51}Chromium refers to lysis of target cells that is not mediated by effector cells. It is an indicator of the well-being of the target cells and should not exceed 20 % of maximal lysis.

The determination of PSL is used to assess the validity of the ^{51}Chromium-release assay. A valid cytotoxicity assay is characterized by an increasing dose-response relationship between PSL and increasing E:T ratios (Fig. 3.1).

Lytic unit (LU) is the widely preferred and used standard measure of cytotoxicity, as it permits an assessment of cytotoxic activity which is independent of any particular E:T ratio. It is defined as the number of effector cells that is required to lyse a predetermined percentage, commonly 30%, of a specified standard number of target cells (T_{STD}). T_{STD} was equal to 10^4 target cells in this study. LU_{30} was calculated by multiplying the E:T ratio which results in 30% lysis of 10^4 targets by the number of targets in the assay ($T_{STD}=10^4$). Usually, results are presented as the number of LU_{30} contained in a predetermined number of effector cells (E_{STD}), in general 10^7 effector cells (Fig. 3.1). Increasing values of the measure $LU_{30}/10^7$ effector cells represent an

increase of NK cell cytotoxic activity. The measure $LU_{30}/10^7$ effector cells summarizes the data and facilitates the comparison of test conditions in the NK assay (23).

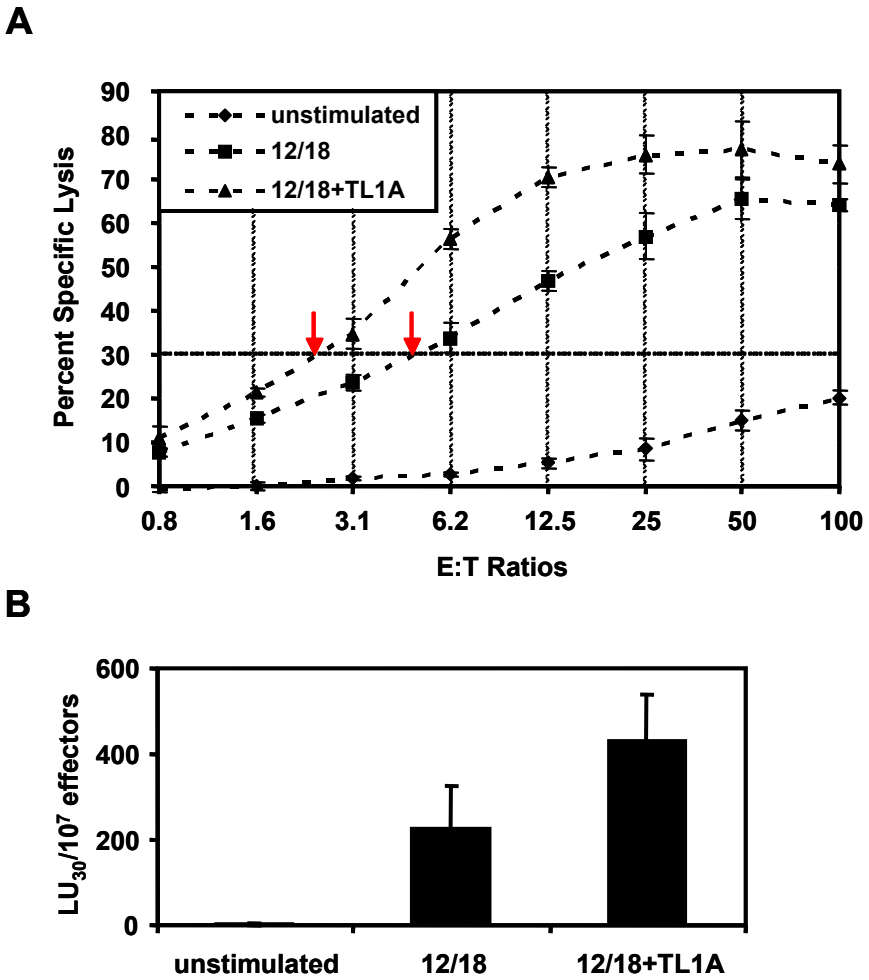

Figure 3.1: Results of a representative 2-hr ^{51}Cr-release assay with PBMC. *A,* Dose-response relationship between Percent Specific Lysis and E:T ratios. PBMC were cultured for 96 hrs with medium (*Control*) or with IL-12 (40 pg/ml) and IL-18 (54 ng/ml) without or with TL1A (50 ng/ml), and their cytotoxicity was tested against Daudi cells in a 2-hr ^{51}Cr-release assay. *B,* LU_{30} of a treatment condition was calculated by multiplying the E:T ratio which resulted in 30% lysis of 10^4 targets by the number of targets in the assay ($T_{STD}=10^4$). Results are presented as the number of LU_{30} contained in a predetermined number of effector cells (E_{STD}), in general 10^7 effector cells ($(LU)_{30}/10^7$ effectors).

In summary, lytic units were calculated as:

$LU_{30}/10^7$ effector cells = $E_{STD}/(E{:}T_{30})$ x (T_{STD})

in which E_{STD} is 10^7 effector cells (abbreviated: effectors), $E{:}T_{30}$ is the E:T ratio at which 30% of the target cells are killed and T_{STD} is 10^4 target cells.

Procedure

Cytolytic activity was determined by 2-h ^{51}Cr-release assays. K562, Daudi, SW837 and WiDr were used as target cells. NK cells, non-NK cells, and PBMC were used as effector cells. Target cell pellets (3x 10^6 cells) were labeled with 150 µCi of ^{51}Cr at 37°C for 1 h with gentle mixing every 15 min. The target cells were washed twice with Hanks' Buffered Salt Solution and adjusted to the concentration of 0.1 x 10^6 cells/ml in RPMI 1640 medium (supplemented as described above). 1 x 10^4 target cells/well were incubated in 200 µl RPMI 1640 medium with effector cells at 8 different effector/target (E:T) ratios, ranging from 100:1 to 0.8:1 for PBMC and the non-NK cell fraction and from 10:1 to 0.08:1 for purified NK cells.

To plate effector and target cells at serial E:T ratios effector cells were added to triplicate wells of a 96-well V-bottom plate and diluted to appropriate concentrations first. To this end, the effector cell concentration was adjusted to 20 x 10^6 cells/ml for PBL and non-NK cells and to 2.0 x 10^6 cells/ml for purified NK cells. A 100-µl aliquot of RPMI 1640 medium (specified above) was dispensed to each testing well of a 96-well V bottom plate. Next, 100-µl aliquots of the effector cell suspension were applied to row "A" wells in triplicate for each condition of treatment. Serial twofold dilutions were performed by mixing the contents of row "A" wells, removing 100-µl aliquots of the cell suspension from these wells and adding them to row "B" wells with a multi-channel pipettor. The contents of row "B" wells were mixed with the multichannel pipettor and the dilution procedure was continued down the plate to row "H". A 100-µl aliquot of the cell suspension was discarded from the final row "H". As a result of this, each well across the row contained 100-µl aliquots of effector cell suspension at concentrations ranging from 10 x 10^6 cells/ml for PBL and non-NK cells or 1.0 x 10^6 cells/ml for purified NK cells in row "A" to 0.07 x 10^6 cells/ml for PBL and non-NK cells or 0.007 x 10^6 cells/ml for purified NK cells in row "H". Next, 100 µl of the ^{51}Cr-labeled target cell suspension, adjusted to 0.1 x 10^6 cells/ml, were added to each well. E:T ratios ranging from 100:1 to 0.8:1 for PBMC and the non-NK cell fraction and from 10:1 to 0.08:1 for purified NK cells resulted. Triplicate wells containing target cells alone (1 x 10^4 target cells/200 µl RPMI 1640 medium/well) were set up to determine spontaneous release (SR) by adding 100-µl aliquots of RPMI

1640 medium and of the target cell suspension. To measure maximum target ^{51}Cr-release (MR) 100-µl aliquots of 1% Triton X-100, diluted with medium, and of the target cell suspension were added to triplicate wells (Fig. 3.2).

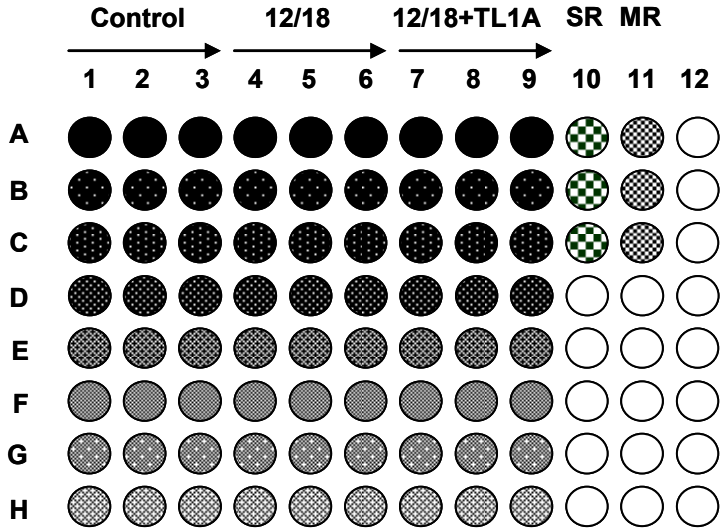

Figure 3.2: A plating scheme for the cytotoxicity assay with PBMC. A 100-µl aliquot of medium was dispensed to each testing well (wells "1" to "9" in rows "A" to "H") of a 96-well V bottom plate. Effector cell suspension (100 µl/well) was applied to row "A" wells in triplicate for each condition of treatment. Serial twofold dilutions were performed by mixing the contents of row "A" wells, removing 100-µl aliquots of the cell suspension from these wells and adding them to row "B" wells. This dilution procedure was continued down the plate to row "H". A 100-µl aliquot of the cell suspension was discarded from the final row "H". 100 µl of the ^{51}Cr-labeled target cell suspension were added to each well, resulting in a total volume of 200 µl per well. *SR*, Spontaneous release; *MR*, Maximal release.

Assay plates were centrifuged briefly at 200 x g to and incubated at 37°C in an atmosphere of 5% CO_2 in air for 2-3 h. Next, the supernatants were harvested by transfering 80 µl of supernatant from each well to the counting tray containing 80 µl of scintillation fluid/well with a multichannel pipettor. ^{51}Cr released into supernatants by lysed target cells was measured in a Wallac 1450 Microbeta Liquid Scintillation Counter, and mean cpm calculated. The results are reported as lytic units $(LU)_{30}/10^7$ effectors. Percent specific lysis and lytic units were calculated using a computer program developed by R. Deem (Cedars-Sinai Medical Center, Los Angeles, CA).

3.6 H^3-Thymidine incorporation assay

Test principle

The H^3-Thymidine Incorporation Assay is used to determine proliferation of lymphocytes following activation by cytokines or antigens. Cells undergoing proliferation display an increased rate of protein and DNA synthesis. The increase in DNA synthesis can be assessed by adding H^3-Thymidine, a radioisotope-labeled DNA precursor, to the culture medium. The amount of DNA taken up by the dividing cells is directly proportional to the level of cellular proliferation. The amount of H^3-Thymidine incorporated into cellular DNA is measured in a beta counter.

Procedure

Purified NK cells were cultured in a 96-well plate at 0.2 x 10^6 cells/ml for 72 hrs with the cytokines IL-12 and IL-18 combined at 5-fold serial dilutions ranging from 108 ng/ml to 6 ng/ml for IL-18 and from 2000 pg/ml to 8 pg/ml for IL-12, without and with TL1A. Tritiated thymidine was diluted with RPMI 1640, supplemented as described above, to a concentration of 40 µCi/ml. After removing 100 µl-aliquots of supernatant from each treatment condition for measurement of IFN-γ, 100 µl-aliquots of tritiated thymidine were added to the wells resulting in a final concentration of 20 µCi/ml of tritiated thymidine. The plate was incubated for further 24 hrs and then frozen at -20°C. The effect of TL1A on NK cell proliferation at titrated concentration ratios of IL-12/IL-18 was subsequently determined with liquid scintillation counting. After the plates had been thawed, the cells of each treatment condition were harvested on a filter mat according to the instructions of the Harvester 96®. Hereby, excess tritiated thymidine that had not been incorporated into cellular DNA is removed. The filer mat was then dried in an oven for 10 min and sealed in a plastic bag containing 8 ml of BetaPlateScint. The filter mat was completely immersed in the BetaPlateScint with the help of a rubber roller. H^3-Thymidine incorporation into the cells of each treatment condition was measured in a Wallac 1450 Microbeta Liquid Scintillation Counter. The results were reported in counts per minute (cpm).

3.7 Statistical analysis

The paired t-test was performed using JMP IN 5.1 data analysis software to determine the significance of the difference in cytotoxicity of IL-12/IL-18 treated PBMC without and with TL1A.

4 Results

4.1 Regulation of DR3 expression on NK cells by cytokines

DR3 is the receptor for TL1A, the only DR3-ligand of several tested by Migone et al (52). DR3 is not expressed on non-activated resting NK cells (64). In a previous study, Papadakis et al. showed that DR3 could be induced on up to 70% of NK cells by maximally effective concentrations of the combined cytokines IL-12 and IL-18, while IL-12 or IL-18 alone upregulated DR3 on approximately 20% of NK cells (64).

Culture Condition	% DR3-positive NK cells
Control	0.7
IFN-α	0.6
IFN-β	0.2
IFN-α+IFN-β	1.0
IFN-α+IFN-β+IL-12	2.3
PMA+Ionomycin	0.2
IL-15	2.4
IL-2	2.6
IL-2+IL-15	0.1
IL-2+IL-12	0
IFN-γ	5.6
TNF-α	1.1
IL-18 (54 ng/ml) + IL-12 (1000 pg/ml)	63.9

Table 4.1: Regulation of DR3 expression on NK cells by cytokines. PBMC were incubated with medium *(Control)* or with cytokines alone or in combination as indicated for 48 hrs or with PMA+Ionomycin for 18 hrs, stained indirectly for DR3 and analyzed by flow cytometry. Percentage of DR3 specific staining in the NK cell population (CD56+CD3-cells) is shown for each culture condition. Percentage specific staining by isotype control Ab was subtracted from percentage specific staining of the test condition.

Other cytokines known to activate NK cells (12) might also induce DR3 expression. We, therefore, tested several of these stimuli for DR3 up-regulation. To this end, freshly isolated PBMC were cultured for 24 and 48 hrs with the cytokines specified in Table 4.1 or for 18 hrs with PMA + ionomycin and DR3 expression was analyzed on

the NK cell subset, as described in Methods. Only IL-12/IL-18, of a panel of cytokines and cytokine combinations tested, was capable of significant induction of DR3 (Table 4.1).

4.2 Dose-kinetic analysis of the effect of IL-12/IL-18 on NK cell IFN-γ production and proliferation and effect of co-stimulatory TL1A

4.2.1 NK cell IFN-γ production at titrated concentrations of IL-12/IL-18 and effect of co-stimulatory TL1A

To identify concentrations of the DR3-inductive cytokines IL-12 and IL-18 which result in maximal effectiveness of the TL1A-DR3 pathway, NK cell IFN-γ production at titrated concentrations of IL-12 and IL-18 was determined and the effect of TL1A was studied in each condition.

To this end, purified NK cells were cultured in a 96-well plate at 0.2 x 10^6 cells/ml for 72 hrs with the cytokines IL-12 and IL-18 combined at 5-fold serial dilutions ranging from 108 ng/ml to 6 ng/ml for IL-18 and from 2000 pg/ml to 8 pg/ml for IL-12, without or with TL1A. Culture supernatants were collected for IFN-γ analysis (Fig. 4.1A). The fold increase in IFN-γ production induced by TL1A was calculated (Fig. 4.1B).

Fig. 4.1A shows that the combined cytokines IL-12/IL-18 dose-dependently augment IFN-γ production. A maximum was reached when IL-12 was used at 1000 pg/ml, while using IL-18 at 108 ng/ml, without and with TL1A. Remarkably however, the TL1A-induced fold increase in IFN-γ production was comparable at the various concentration ratios of the two cytokines. The TL1A-induced fold increase ranged from 2.0 to 3.1, with an average of 2.6; hereby, very low concentration ranges of IL-12 and IL-18 (combinations of IL-18 with 8 pg/ml of IL-12; combinations of 40 and 200 pg/ml of IL-12 with 6 ng/ml of IL-18), which deviated significantly from the average, were not taken into account. The synergistic effect of TL1A on IFN-γ production at both sub-optimal and optimal concentrations of IL-12 and IL-18 suggested an efficient upregulation of DR3 on a large proportion of NK cells even at

sub-optimal concentrations of IL-12/IL-18, when total IFN-γ production was markedly reduced.

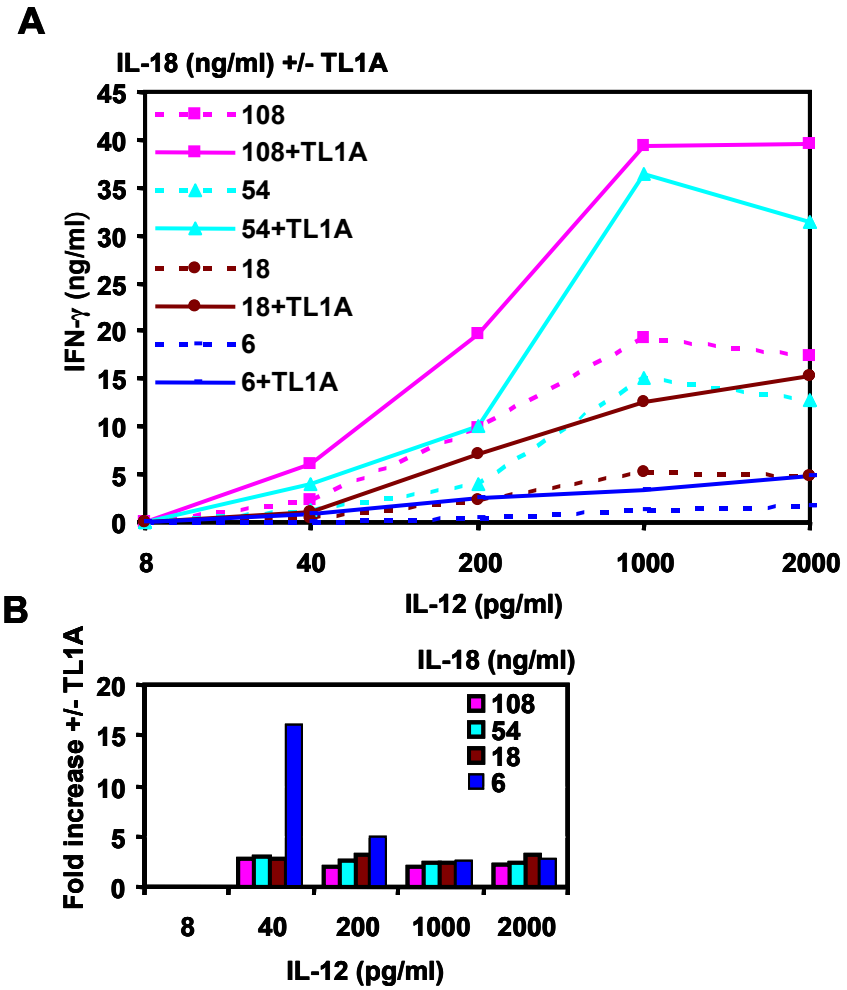

Figure 4.1: NK cell IFN-γ production at titrated concentrations of IL-12/IL-18 and the effect of co-stimulatory TL1A. *A,* Purified NK cells were cultured at 0.2 x 10^6 cells/ml for 72 hrs with the cytokines IL-12 and IL-18 combined at 5-fold serial dilutions ranging from 108 ng/ml to 6 ng/ml for IL-18 and from 2000 pg/ml to 8 pg/ml for IL-12, without and with co-stimulatory TL1A. The culture supernatants were collected for IFN-γ analysis. The amount of IFN-γ accumulating in the culture supernatant over time, as determined by ELISA, is shown. *B,* The fold increase in IFN-γ production induced by TL1A was calculated.

4.2.2 NK cell proliferation at titrated concentrations of IL-12/IL-18 and effect of co-stimulatory TL1A

Papadakis et al. had shown by flow cytometry that while in T cells, TL1A enhanced IFN-γ production by increasing both the number of IFN-γ producing cells and the amount of IFN-γ produced per cell (increase in mean fluorescence intensity), in NK cells, only the number of IFN-γ producing cells was significantly augmented in

response to TL1A (64). These findings suggested that the synergistic effect of TL1A on IL-12/IL-18-induced-IFN-γ production of NK cells was mainly due to NK cell proliferation. A 24-h H^3 Thymidine incorporation assay was performed to study if this observation corresponded to an induction of DNA replication by TL1A and if the extent of proliferation varied at different concentrations of IL-12 and IL-18.

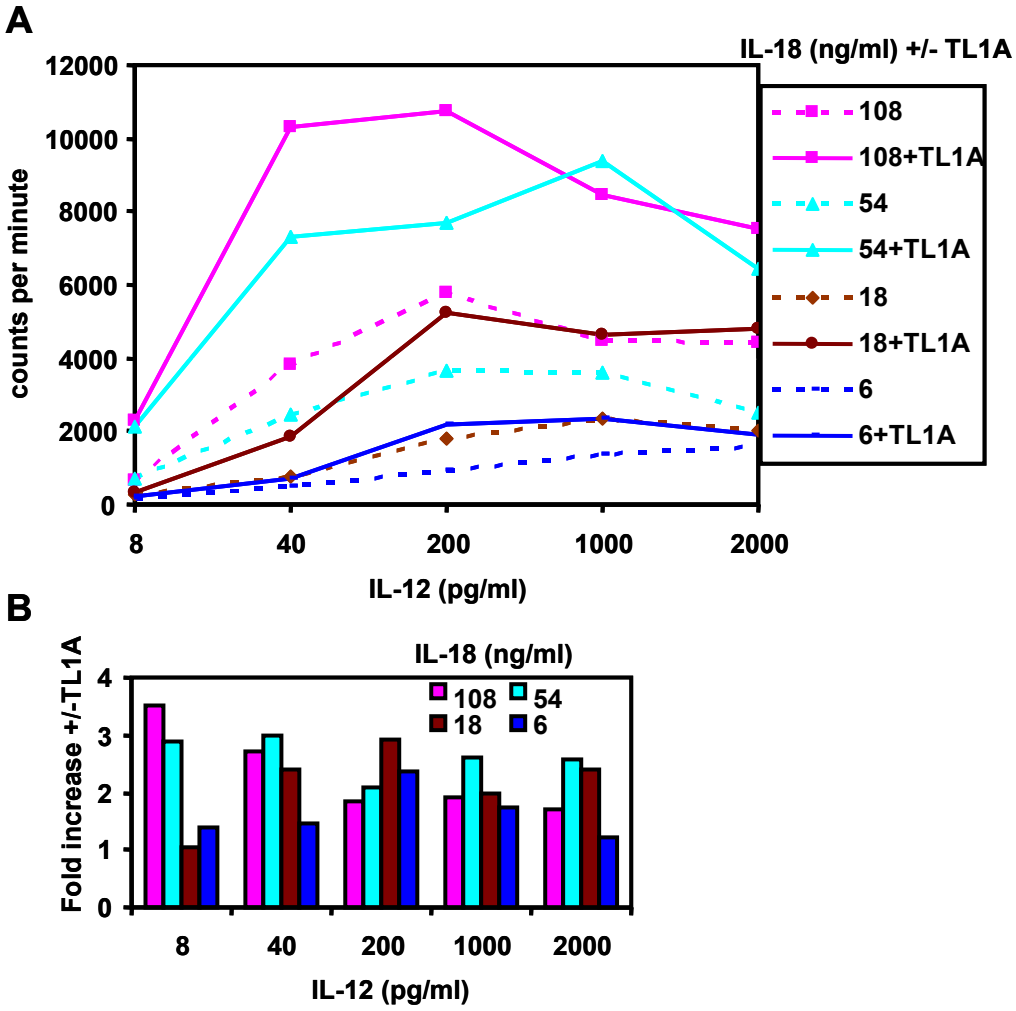

Figure 4.2: DNA replication of NK cells at titrated concentrations of IL-12 and IL-18 and effect of co-stimulatory TL1A. *A,* Purified NK cells were cultured for 72 hrs with the cytokines IL-12 and IL-18 combined at 5-fold serial dilutions, ranging from 108 ng/ml to 6 ng/ml for IL-18 and from 2000 pg/ml to 8 pg/ml for IL-12, without and with TL1A. After removing the culture supernatant from each treatment condition for measurement of IFN-γ (cf. 4.2.1), tritiated thymidine was added to the wells and NK cells were incubated for further 24 hrs. The extent of cell proliferation in each test condition was then determined by liquid scintillation counting. The results are represented in counts per minute. *B,* The fold increase in proliferation induced by TL1A was calculated.

To this end, purified NK cells were cultured for 72 hrs with the cytokines IL-12 and IL-18 combined at 5-fold serial dilutions without and with TL1A, as described above

(cf. 4.2.1). After removing 100 µl-aliquots of culture supernatant from each treatment condition for measurement of IFN-γ, tritiated thymidine was added to the wells and NK cells were incubated for further 24 hrs. The extent of DNA replication at the titrated concentrations of IL-12/IL-18 was then determined by liquid scintillation counting.

The counts revealed induction of NK cell DNA replication by IL-12/IL-18, peaking at concentrations of 200 or 1000 pg/ml of IL-12 and 54 or 108 ng/ml of IL-18, respectively (Fig. 4.2A). Proliferation was further enhanced by co-stimulatory TL1A, with TL1A inducing an average fold increase of 2.2 (Fig. 4.2B).

4.3 Effect of TL1A on IL-12/IL-18-induced NK cell cytotoxicity against NK-sensitive K562 target cells

Papadakis et al. demonstrated previously that TL1A augments IL-12/IL-18-induced IFN-γ production in NK cells by about two-fold, largely due to NK proliferation (64). Given the dramatic induction by IL-12/IL-18 of DR3 on NK cells, this study analyzed if TL1A affected another NK effector function, cytotoxicity, as well as IFN-γ production. A late time-point (72 hrs) was chosen, since TL1A requires 72 hrs for maximal augmentation of IFN-γ production of NK and T cells co-stimulated with IL-12 and IL-18. The reason for this finding and the prolonged kinetics of other TNF-family members for IFN-γ augmentation is unknown (64;71).

PBMC and purified NK cells were cultured for 72 hrs with maximally effective concentrations of IL-12 and IL-18 (PBMC*:* 1 ng/ml and 54 ng/ml, respectively; NK cells*:* 400 pg/ml and 135 ng/ml, respectively) without and with TL1A (50 ng/ml). PBMC were also incubated with medium only (Control). Since most purified NK cells in culture without stimulation died, a control condition of NK cells cultured with medium only was not performed. Their cytolytic activity was then tested against the standard NK-sensitive target cell line K562 in a 2-h ^{51}Cr-release assay. An incubation time of 72 hrs was chosen as DR3 expression on NK cells reaches its peak at 48 hrs after combined stimulation with IL-12/IL-18 (unpublished data) and optimal IFN-γ

production in response to IL-12/IL-18 and TL1A occurs at 64-72 hrs (64). The culture supernatants were collected to measure the IFN-γ production in each condition.

While the TL1A/DR3 pathway was functional as evidenced by enhanced IFN-γ production in response to TL1A (Fig. 4.3, right panels: 2.1-fold increase in PBMC and 2.4-fold increase for NK cells), there was no significant difference in cytolytic activity with TL1A (Fig. 4.3, left panels).

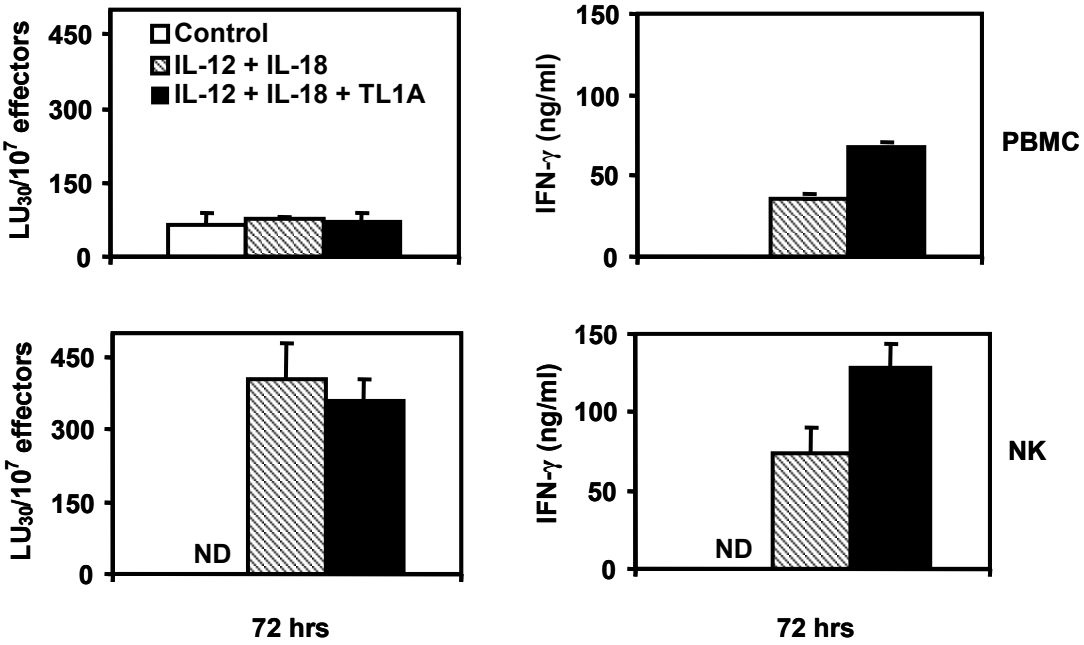

Figure 4.3: Effect of TL1A on IL-12/IL-18-induced NK cell cytotoxicity against NK-sensitive K562 target cells. PBMC and purified NK cells ($0.5x10^6$/ml; different donors) were cultured for 72 hrs with maximally effective concentrations of IL-12 and IL-18 (PBMC: 1 ng/ml and 54 ng/ml, respectively; NK cells: 400 pg/ml and 135 ng/ml, respectively) without or with TL1A (50 ng/ml). PBMC were also cultured with medium only (*Control).* Cytotoxicity was tested against the NK-sensitive cell line K562 in ^{51}Cr-release assays and expressed in $LU_{30}/10^7$ (left panels). Culture supernatants were collected at 72 hrs and analyzed for IFN-γ content by ELISA (right panels). For NK cells, the results are representative of two similar experiments. *LU_{30},* the number of effector cells required to lyse 30% of a standard number of target cells, here 10^4 target cells *Bars,* SD. *ND,* not determined, since most purified NK cells in culture without stimulation died.

To investigate if a 2-h incubation period of ^{51}Cr-labeled target cells with NK cells resulted in the same level of percent specific lysis as a 4-h incubation period, a 4-h ^{51}Cr-release assay was performed under the same experimental conditions as described above. A comparable level of cytotoxicity was reached in 2-h and 4-h ^{51}Cr-release assays (Fig. 4.4A). While TL1A did not significantly enhance cytotoxicity in

either assay, it potentiated IL-12/IL-18 induced IFN-γ production of NK cells, as determined by analysis of IFN-γ contents in culture supernatants (Fig. 4.4B).

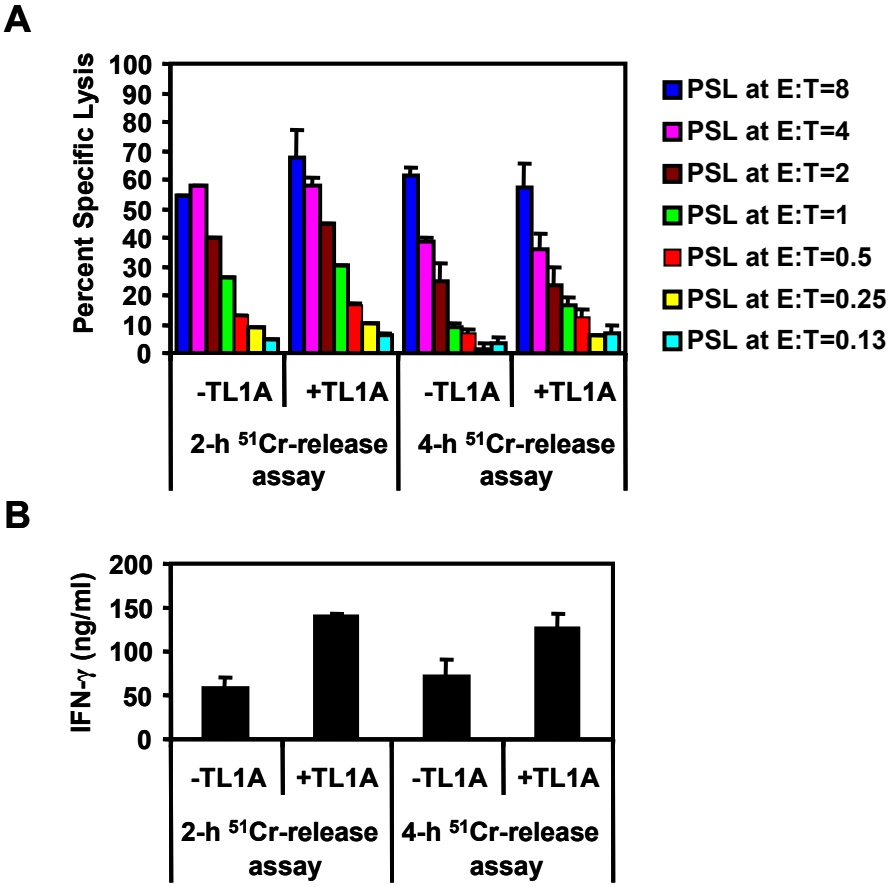

Figure 4.4: Comparison of percent specific lysis in a 2-h and a 4-h ^{51}Cr-release assay. *A,* Purified NK cells ($0.5x10^6$/ml; different donors) were cultured for 72 hrs with IL-12 and IL-18 (400 pg/ml and 135 ng/ml, respectively) without or with TL1A (50 ng/ml). Cytotoxicity was tested against the NK-sensitive cell line K562. Percent Specific Lysis (PSL) is shown for the indicated Effector:Target (E:T) ratios in a 2-h and a 4-h ^{51}Cr-release assay. *B,* Culture supernatants were collected at 72 hrs and analyzed for IFN-γ content by ELISA. *PSL*, Percent Specific Lysis. *Bars,* SD.

While the spontaneous release was markedly reduced in the 2-h ^{51}Cr-release assay, the maximal release of ^{51}Chromium was comparable in the 2-h and the 4-h ^{51}Cr-release assay (Table 4.2).

	2-h ^{51}Cr-release assay	4-h ^{51}Cr-release assay
Spontaneous release (mean cpm)	**246**	**573**
Maximal release (mean cpm)	**3901**	**3911**

Table 4.2: Spontaneous and maximal release of ^{51}Chromium in a 2-h and a 4-h ^{51}Cr-release assay. Purified NK cells ($0.5x10^6$/ml; different donors) were cultured for 72 hrs with IL-12 and IL-18 (400 pg/ml and 135 ng/ml, respectively) without or with TL1A (50 ng/ml). Cytotoxicity was tested against the NK-sensitive cell line K562. ^{51}Chromium release into supernatants following NK-mediated lysis of targets (cf. Fig.4.4), spontaneous release of ^{51}Chromium by target cells, and maximal release of ^{51}Chromium following osmotic lysis of targets were determined in triplicate for all experimental conditions in a beta counter, and mean counts per minute (cpm) were calculated.

4.4 Time-kinetic analysis of the effect of TL1A on IL-12/IL-18-induced NK cell cytotoxicity against K562 tumor cells

This study further investigated if TL1A had an effect on IL-12/IL-18 induced cytotoxicity of NK cells against K562 at altered time courses. The effect of TL1A on NK cell cytotoxicity might set in before or after 72 hrs of co-stimulation with IL-12/IL-18 and TL1A. Also the efficacy of recombinant TL1A might wane over time. To avoid a loss of function of TL1A before DR3 had been induced in NK cells by IL-12/IL-18, TL1A was added to the test conditions only after a 48-h incubation period with IL-12/IL-18, when NK cell expression of DR3 had reached its maximum (64).

The effect of TL1A on IL-12/IL-18 induced cytotoxicity of NK cells against the standard K562 target cell line was studied under the same conditions as previously reported (IL-12 at 1000 pg/ml, IL-18 at 54 ng/ml, without and with TL1A).

The results, summed up in the following table (Table 4.3), reveal that TL1A did not enhance IL-12/IL-18 induced cytotoxicity of NK cells and PBL at altered time courses. The TL1A/DR3 pathway was functional in all test conditions, as evidenced by TL1A enhancement of IL-12/IL-18-induced IFN-γ production or proliferation. The potentiating effect of TL1A on IL-12/IL-18-induced NK cell IFN-γ production was not evident when culturing NK cells with IL-12/IL-18, without and with TL1A, for 24 hrs or when co-stimulating with TL1A for the last 3 or 6 hrs of a 51-h or 54-h

incubation period with IL-12/IL-18. However, TL1A was shown to increase the proliferation of NK cells under these conditions. The identical donor was used and a simultaneous experimental set-up was performed in the experiments where NK cells were co-stimulated with TL1A for the last 3 or 6 hrs of a 51-h or 54-h incubation period with IL-12/IL-18.

Cells	Incubation Period		Effect of TL1A on cytotoxicity	Effect of TL1A on IFN-γ production	Effect of TL1A on proliferation
	IL-12/IL-18	TL1A			
NK	24 hrs	24 hrs	%	%	1.6 fold increase
NK (same donor)	51 hrs 54 hrs	Last 3 hrs Last 6 hrs	% %	% %	ND 1.4 fold increase
NK	69 hrs	Last 21 hrs	%	1.3 fold increase	ND
PBMC (same donor)	96 hrs 120 hrs	96 hrs 120 hrs	% %	1.9 fold increase 2.9 fold increase	ND ND

Table 4.3: Time-kinetic analysis of the effect of TL1A on NK cell cytotoxicity against K562 tumor cells at maximal concentrations of IL-12/IL-18. PBMC or purified NK cells ($0.5x10^6$/ml) were cultured with IL-12 and IL-18 (1000 pg/ml and 54 ng/ml, respectively) without or with TL1A (50 ng/ml) for the specified incubation times. Cytotoxicity was tested against the NK-sensitive cell line K562 in 2-h ^{51}Cr-release assays. Culture supernatants were collected at each time point and analyzed for IFN-γ content by ELISA. A 24-h H^3 Thymidine incorporation assay was performed at 24 hrs of stimulation with IL-12/IL-18 +/-TL1A and when co-stimulating with TL1A for the last 6 hrs of a 54-h incubation period with IL-12/IL-18. *ND*, not determined.

4.5 Effect of low level stimulation with IL-12/IL-18 on NK cell DR3 expression and IFN-γ production

The used maximal concentrations of IL-12 and IL-18, while strongly inducing DR3, might maximize NK cell cytotoxicity (Fig. 4.3, left panels), and thus obscure an effect of TL1A on NK cell cytotoxicity.

Therefore, it was determined whether a lower concentration of IL-12, with maintained IL-18, would effectively induce DR3 expression on NK cells, and perhaps not maximally stimulate IFN-γ production and cytotoxicity.

The following two experiments analyzed the effect of a reduced concentration of IL-12 (40 pg/ml) with maintained IL-18 (54 ng/ml) on DR3 up-regulation and intracellular IFN-γ production of NK cells.

4.5.1 NK cell DR3 expression at low level stimulation with IL-12 and IL-18

First, the effects of maximal and reduced concentrations of IL-12 on DR3 expression were compared by flow cytometry.

PBMC were cultured with medium (Control) or a maximal dose of IL-18 (54 ng/ml), while using the co-stimulatory IL-12 at either 40 pg/ml (potentially optimal stimulation) or at 1000 pg/ml, as used in the previous cytotoxicity experiment (maximal stimulation). At 24 and 48 hrs, PBMC were stained with CD3-FITC, CD-56 tricolor and DR3-PE, and DR3 expression was analyzed in the NK cell subset by flow cytometry, as described in Methods.

After 48 hrs of incubation, when DR3 expression reached a peak in all test conditions, a significant induction of DR3 was maintained at low level stimulation with IL-12/IL-18 (IL-12 reduced to 40 pg/ml, maintained IL-18). The number of DR3-positive NK cells at low level stimulation still mounted to 2/3 of the number at maximal stimulation (40% vs. 64% at 48 hrs, Fig. 4.5A). A further analysis of the mean fluorescence intensity (MFI) of DR3-positive NK cells showed that the amount of DR3 expressed on a per cell basis had decreased only marginally at low level stimulation with IL-12/IL-18: At 48 hrs, the MFI of DR3-expressing cells was 30 at minimal stimulation, compared to a MFI of 40 at maximal stimulation (Fig. 4.5B).

The effect of TL1A on IL-12/IL-18-induced NK cell DR3 expression cannot be assessed in this experimental set-up. Data (not shown) revealed that DR3 expression of IL-12/IL-18-activated NK cells was markedly reduced with TL1A, compared to without TL1A. This finding could imply that TL1A and the anti-DR3 mAb compete for the same epitope of the DR3 receptor or that bound TL1A masks the epitope which binds anti-DR3.

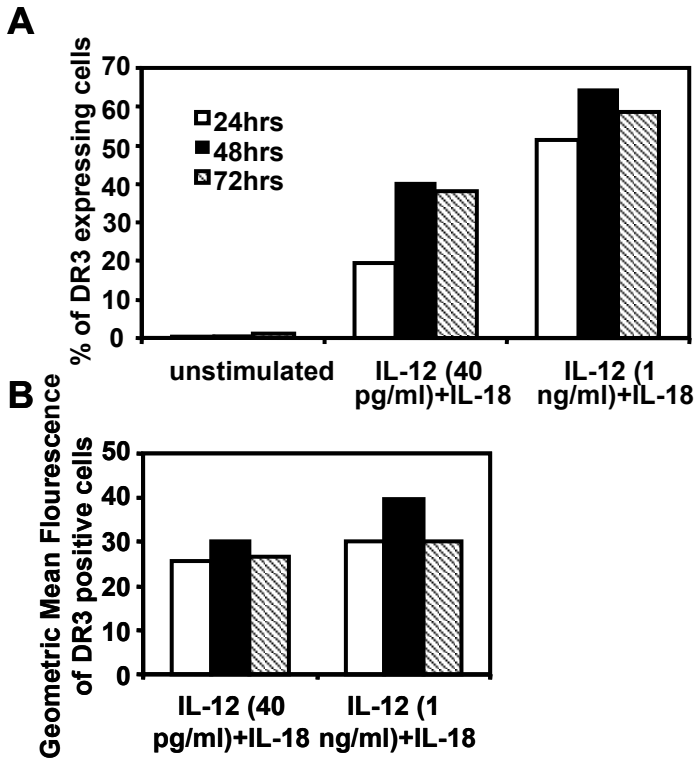

Figure 4.5: NK cell DR3 expression at low level stimulation with IL-12/IL-18. PBMC were cultured with medium (*Control*) or a maximal dose of IL-18 (54 ng/ml), while using the co-stimulatory IL-12 at either 40 pg/ml or at 1000 pg/ml. At 24 and 48 hrs, PBMC were stained with CD3-FITC, CD-56-TC and DR3-PE, and DR3 expression was analyzed in the NK cell subset by flow cytometry. *A,* The percentage of DR3-expressing NK cells is shown. *B,* Mean Fluorescence Intensity of DR3 expressing NK cells. The data is representative of two experiments with similar results. Percentage non-specific staining by isotype control Ab was subtracted from percentage specific staining of the test condition.

4.5.2 Intracellular IFN-γ production of NK cells at low level stimulation with IL-12 and IL-18 and effect of co-stimulatory TL1A

Next, the effect of a reduced concentration of IL-12 (with maintained IL-18) on intracellular IFN-γ production of NK cells was studied by flow cytometry.

PBMC were cultured with medium (Control) or IL-18 (54 ng/ml) and IL-12 at either 40 pg/ml or at 1000 pg/ml without and with TL1A (50 ng/ml) for 24 and 48 hrs. Brefeldin A was added to each treatment condition for the last 4 hrs of the incubation time. Cells were stained with CD3-FITC and CD56-tricolor, fixed and permeabilized as described in Methods. Fixed and permeabilized cells were stained with anti-IFN-γ-PE or an isotype control mAb. Intracellular IFN-γ production was analyzed in the NK cell subset by flow cytometry.

A marked decrease in the percentage of IFN-γ producing NK cells was observed at a reduced dose of IL-12 with maintained IL-18 (Fig. 4.6A). Figure 4.6A further shows that TL1A modestly increases the percentage of IFN-γ producing NK cells at maximal stimulation with IL-12/IL-18 (at 48 hrs: increase from 56% to 61%). Remarkably however, the TL1A-induced increase in IFN-γ producing NK cells is more pronounced at reduced level stimulation with IL-12/IL-18 (at 48 hrs: increase from 7% to 16%).

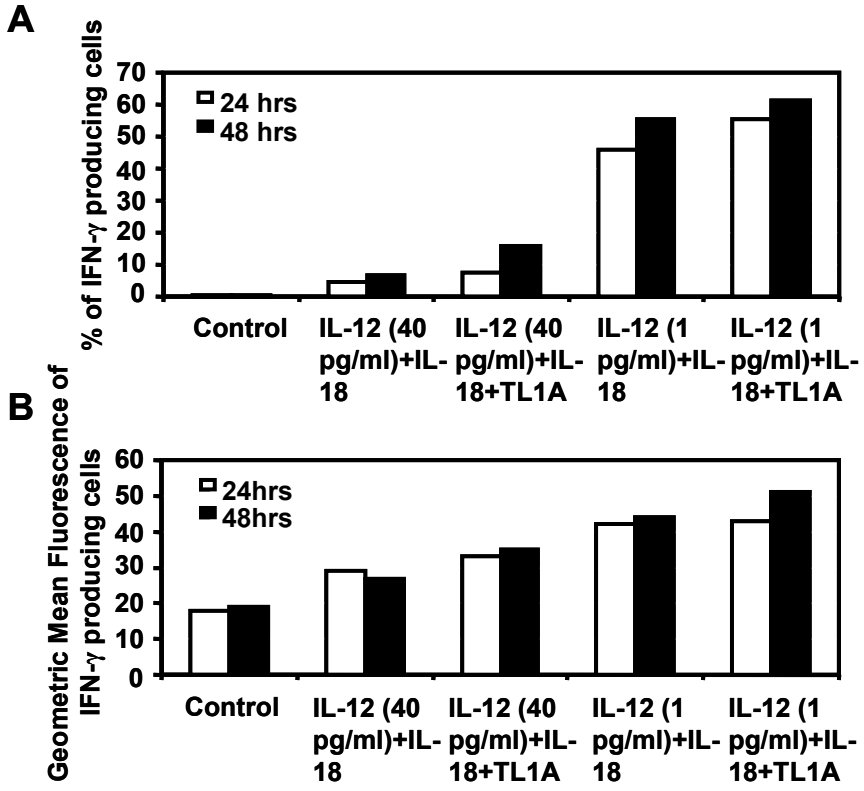

Figure 4.6: Intracellular IFN-γ production of NK cells at low level stimulation with IL-12/IL-18 without and with co-stimulatory TL1A. PBMC were cultured with medium (*Control*) or IL-18 (54 ng/ml) and IL-12 at either 40 pg/ml or at 1000 pg/ml without and with TL1A (50 ng/ml) for 24 and 48 hrs. Brefeldin A was added to each treatment condition for the last 4 hrs of the incubation time. Cells were stained with CD3-FITC and CD56-TC, fixed and permeabilized as described in Methods. Fixed and permeabilized cells were stained with anti-IFN-γ-PE or an isotype control mAb. Intracellular IFN-γ production was analyzed in the NK cell subset by flow cytometry. *A,* The percentage of IFN-γ producing NK cells is shown. *B,* Mean Fluorescence Intensity of IFN-γ producing NK cells. Percentage non-specific staining by isotype control Ab was subtracted from percentage specific staining of the test condition.

The mean fluorescence intensity (MFI) of IFN-γ producing NK cells is slightly lower at low level stimulation with IL-12/18 (at 48 hrs: 27 (without TL1A) and 35 (with TL1A)), compared to the MFI at maximal stimulation (at 48 hrs: 44 (without TL1A) and 51 (with TL1A)) (Fig. 4.6B).

4.6 Effect of TL1A on NK cell cytotoxicity against NK-sensitive K562 target cells at low level stimulation with IL-12 and IL-18

The preceding experiments identified the combination of 54 ng/ml of IL-18 and 40 pg/ml of IL-12 as a concentration ratio inducing DR3 effectively, while stimulating IFN-γ production only moderately. This concentration might fail to maximize NK cytotoxicity, so that a potential synergistic effect of TL1A on NK cytotoxicity might be revealed.

This concentration ratio of IL-12/IL-18 was tested in cytotoxicity experiments (Fig. 4.7, left panels). PBMC and purified NK cells of the same donor were cultured for 72 and 96 hrs with IL-12 at 40 pg/ml and IL-18 at 54 ng/ml with and without TL1A (50 ng/ml), and their cytolytic activity was tested against the K562 cell line in a 2-h ^{51}Cr-release assay. The culture supernatants were collected to measure the IFN-γ production in each condition.

At the reduced concentration of IL-12, cytotoxicity was not decreased, and TL1A still did not significantly enhance IL-12/IL-18-induced cytolytic activity of PBMC and NK cells. The effect of TL1A on IFN-γ production was unimpaired: TL1A induced a marked increase in IFN-γ production by PBMC and NK cells (PBMC 72 hrs: 3.9 fold/96 hrs: 4.6 fold; NK cells 72 hrs: 1.5 fold/96 hrs: 1.5 fold), which demonstrates the functionality of the TL1A/DR3 pathway (Fig. 4.7, right panels).

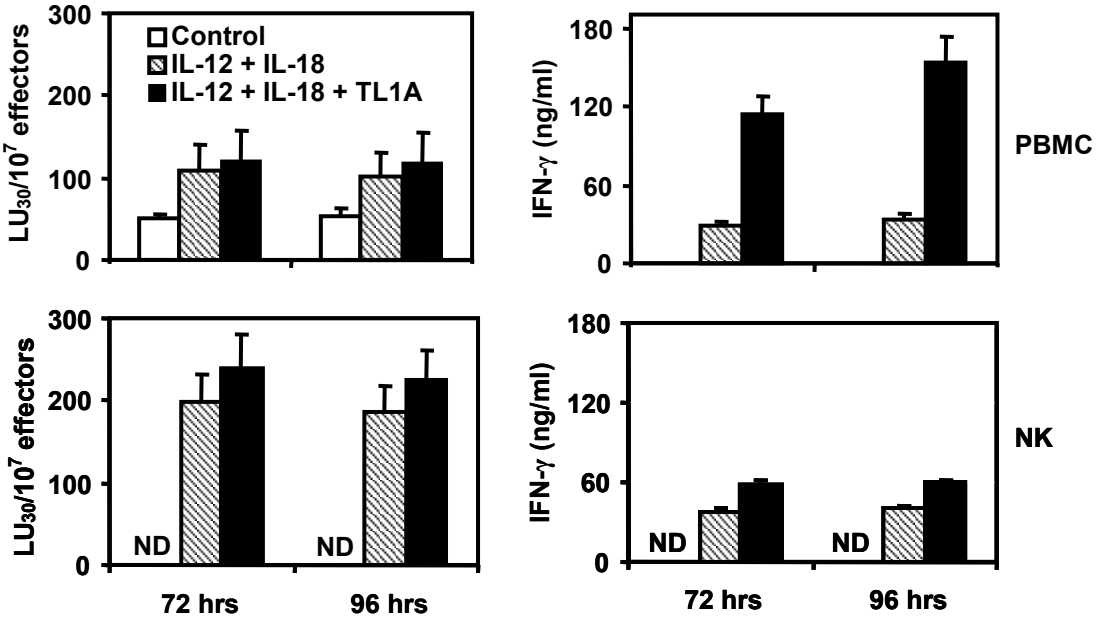

Figure 4.7: Effect of TL1A on the cytotoxicity of PBMC and NK cells against NK-sensitive K562 target cells at low level stimulation with IL-12 and IL-18. PBMC and purified NK cells of the same donor ($0.5x10^6$/ml) were cultured for 72 and 96 hrs with medium *(Control)* or a maximal dose of IL-18 (54 ng/ml) and a lower dose of IL-12 (40 pg/ml) without or with TL1A (50 ng/ml), and their cytotoxicity was tested against the NK-sensitive cell line K562 in a 2-h ^{51}Cr-release assay (upper and lower left panel). Culture supernatants were collected at 72 hrs and analyzed for IFN-γ content by ELISA (right panels). For PBMC, the results are representative of 3 experiments. *LU_{30}*, the number of effector cells required to lyse 30% of a standard number of target cells, here 10^4 target cells. *Bars,* SD. *ND,* not determined.

4.7 Time-kinetic analysis of the effect of TL1A on NK cell cytotoxicity against K562 tumor cells at low level stimulation with IL-12/IL-18

TL1A might enhance NK cell-mediated tumor lysis at low level stimulation with IL-12/IL-18 over a shorter or a more prolonged time-course.

Therefore, the effect of TL1A on NK cytotoxicity was examined in the same conditions for 120 hrs and 144 hrs. PBMC and purified NK cells of one donor or PBMC of another donor were cultured for 120 hrs or for 144 hrs, respectively, with medium (Control) or IL-12 at a low concentration (40 pg/ml) and IL-18 at a maximal dose (54 ng/ml) with and without TL1A (50 ng/ml). Their cytolytic activity was tested

against the K562 cell line in a 2-h ^{51}Cr-release assay. The culture supernatants were collected to measure the IFN-γ production in each condition.

No significant difference in NK cell cytotoxicity against K562 targets was detected with and without TL1A at 120 or 144 hrs (Fig. 4.8A), while TL1A had a synergistic effect on IL-12/IL-18-induced IFN-γ production (Fig. 4.8B).

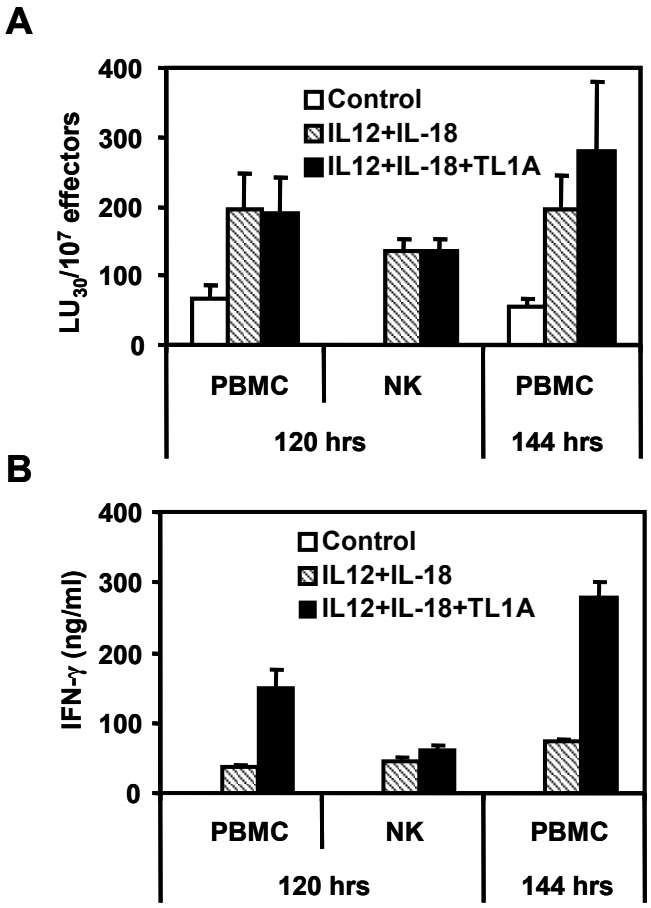

Figure 4.8: Time-kinetic analysis of the effect of TL1A on NK cell cytotoxicity against K562 tumor cells at low level stimulation with IL-12/IL-18. PBMC and purified NK cells of one donor or PBMC of another donor were cultured for 120 hrs or for 144 hrs, respectively, with medium (*Control*) or IL-12 at a low concentration (40 pg/ml) and IL-18 at a maximal dose (54 ng/ml) with and without TL1A (50 ng/ml). Their cytolytic activity was tested against the K562 cell line in ^{51}Cr-release assays and expressed in lytic units. Culture supernatants were collected to measure the IFN-γ production in each condition. LU_{30}, the number of effector cells required to lyse 30% of a standard number of target cells, here 10^4 target cells. *Bars,* SD. *ND,* not determined.

TL1A could not be shown to enhance NK cell cytotoxicity against K562 at a reduced concentration of IL-12 (with maintained IL-18) at a shorter time course, at 48 or 72

hrs, either (Table 4.4). Replicate experiments further confirmed these findings (Table 4.4).

Cells	Incubation period	Effect of TL1A on cytotoxicity	Effect of TL1A on IFN-γ production
PBMC (same donor)	48 hrs 72 hrs	% %	ND 2.7 fold increase
PBMC	96 hrs	%	1.5 fold increase
PBMC (same donor)	72 hrs 96 hrs 120 hrs 144 hrs	% % % %	4.7 fold increase 4.2 fold increase 4 fold increase 3.2 fold increase
PBMC (same donor)	72 hrs 96 hrs 120 hrs	% % %	3.6 fold increase 2.9 fold increase 2.8 fold increase
PBMC NK (same donor)	120 hrs 120 hrs	% %	4.1 fold increase 1.7 fold increase

Table 4.4: Time-kinetic analysis of the effect of TL1A on NK cell cytotoxicity against K562 tumor cells at low level stimulation with IL-12/IL-18. PBMC or purified NK cells ($0.5x10^6$/ml) were cultured with medium or a maximal dose of IL-18 (54 ng/ml) and a lower dose of IL-12 (40 pg/ml) without or with TL1A (50 ng/ml) for the indicated incubation periods, and their cytotoxicity was tested against the NK-sensitive cell line K562 in a 2 hour ^{51}Cr-release assay. Culture supernatants were collected at 72 hrs and analyzed for IFN-γ content by ELISA. *ND,* not determined.

4.8 Effect of TL1A on IL-12/IL-18-induced cytotoxicity of PBMC tested against cell lines lysed only by activated NK cells

The NK-sensitive cell line K562 (chronic myelogenous leukemia) is the standard target for measurements of cytotoxicity, and it can be used to assess the cytolytic acivity mediated by freshly isolated, unstimulated NK cells. The Daudi cell line (Ebstein-Barr virus-associated Burkitt's lymphoma), which is resistant to lysis by fresh NK cells, is used for assays of cytotoxicity mediated by activated NK cells (23). This study investigated whether TL1A had an effect on NK cell lytic activity against the NK-resistant target cell lines Daudi, SW837 (rectal adenocarcinoma) and WiDr (colorectal adenocarcinoma) (Fig. 4.9).

To this end, PBMC were cultured with IL-12 at a reduced concentration (40 pg/ml) and IL-18 (54 ng/ml) with and without TL1A (50 ng/ml) for 72 and 96 hrs, and their cytolytic activity was tested against the NK-resistant cell lines in 2-h ^{51}Cr-release assays. The culture supernatants were collected to measure the IFN-γ production in each condition. The results were compared to the cytotoxicity of PBMC cultured under the same experimental conditions against NK-sensitive K562 cells (Fig. 4.9, upper right panel).

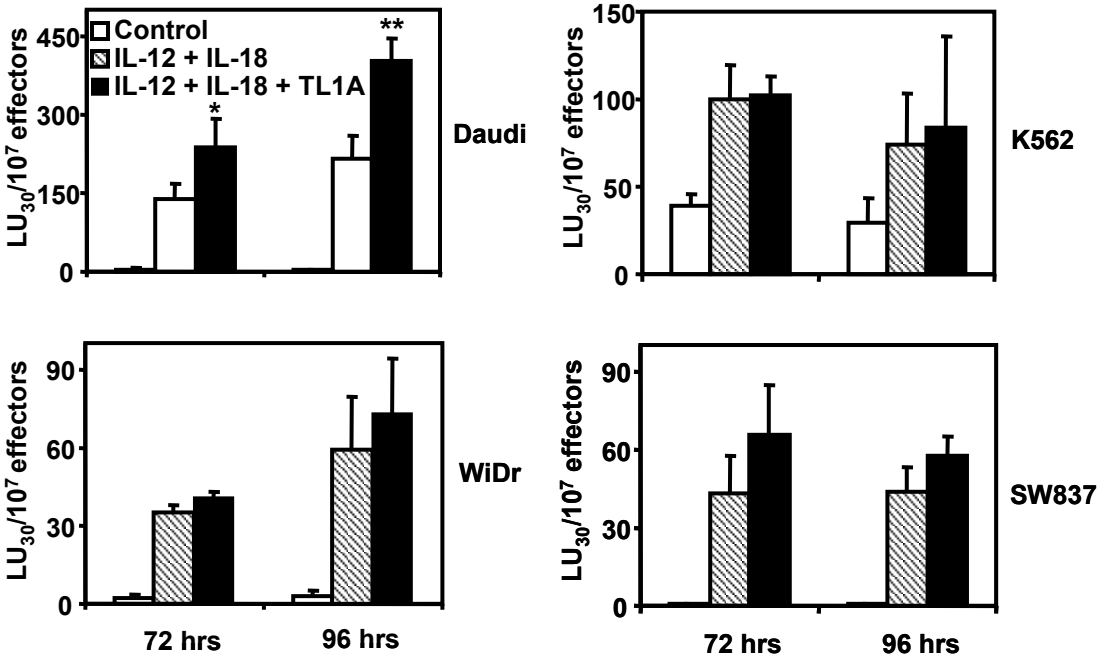

Figure 4.9: Effect of TL1A on IL-12/IL-18 induced cytotoxicity of PBMC tested against cell lines lysed only by activated NK cells. PBMC were cultured and tested for their cytotoxicity against the NK-resistant target cells Daudi (n=7), WiDr (n=3) and SW837 (n=3) in ^{51}Cr-release assays (left upper panel and lower panels) and compared to the cytotoxicity toward K562 cells (right upper panel). The results are shown in $LU_{30}/10^7$ effector cells. Cytotoxicity of IL-12/IL-18 and TL1A treated NK cells toward Daudi cells was significantly higher than cytotoxicity induced by IL-12/IL-18 alone (* $p < 0.0004$ at 72 hrs and ** $p < 0.009$ at 96 hrs, paired t-test). Cytotoxicity of TL1A stimulated NK cells against WiDr and SW837 cells approached significance (WiDr cells: $p < 0.39$ at 72 hrs, $p < 0.18$ at 96 hrs; SW837 cells: $p < 0.11$ at 72 hrs, $p < 0.05$ at 96 hrs, paired t-test). LU_{30}, the number of effector cells required to lyse 30% of a standard number of target cells, here 10^4 target cells. *Bars,* SEM.

As represented in Fig. 4.9 (upper left panel) as percent increase in LU_{30} of seven independent experiments, TL1A had the most profound effect on PBMC tested against Daudi target cells enhancing cytotoxicity 2-fold at 96 hrs of incubation. The effect of TL1A on IL-12/IL-18-induced cytotoxicity of PBMC against the NK-resistant

epithelial cell lines WiDr and SW837 showed a similar trend (Fig. 4.9, lower panels). However, the enhancement of cytotoxicity by TL1A was less pronounced, compared to the Daudi cell line, and the difference in LU_{30} of PBMC cultured with and without TL1A was not statistically significant as determined by paired t-test.

4.9 Effect of TL1A on IL-12/IL-18-induced cytotoxicity of purified NK cells and cytotoxic T-cells towards NK-resistant cell lines

Because T lymphocytes present in PBMC (a subset of human γδ T lymphocytes) are capable of lysing Daudi target cells (15), it was necessary to clarify whether the fore-mentioned observations with PBMC resulted from NK or non-NK cell cytotoxicity. NK cells, purified by magnetic cell separation, were tested on Daudi targets, compared to the non-NK cell fraction.

To this end, NK cells, the non-NK cell fraction and PBMC of the same donor were incubated with IL-12 (40 pg/ml) and IL-18 (54 ng/ml) with and without TL1A (50 ng/ml) for 72 and 96 hrs. The cytolytic activity of PBMC, NK cells and non-NK cells was tested in 2-h ^{51}Cr-release assays against Daudi. The culture supernatants were collected to measure the IFN-γ production in each condition.

While the non-NK population, which included CD56+ T cells, displayed some IL12/IL18 induced cytotoxicity against Daudi cells, which was enhanced by TL1A (Fig. 4.10, lower left panel), TL1A had the most significant effect on the lytic activity of purified NK cells (Fig. 4.10, middle left panel). TL1A augmented IL-12/IL-18-induced cytotoxicity by purified NK cells against Daudi by 7-fold compared to a 2-fold increase in NK cell IFN-γ production (Fig. 4.10, middle right panel). In non-NK cells (Fig. 4.10, bottom panels), consisting mostly of T cells, TL1A increased IFN-γ production by 7-fold, but cytotoxicity by only 2-fold. These results imply that the enhancing effect of TL1A on the cytotoxicity of PBMC against the Daudi cell line is mediated mainly by augmenting the cytotoxicity of IL-12/IL-18 activated NK cells.

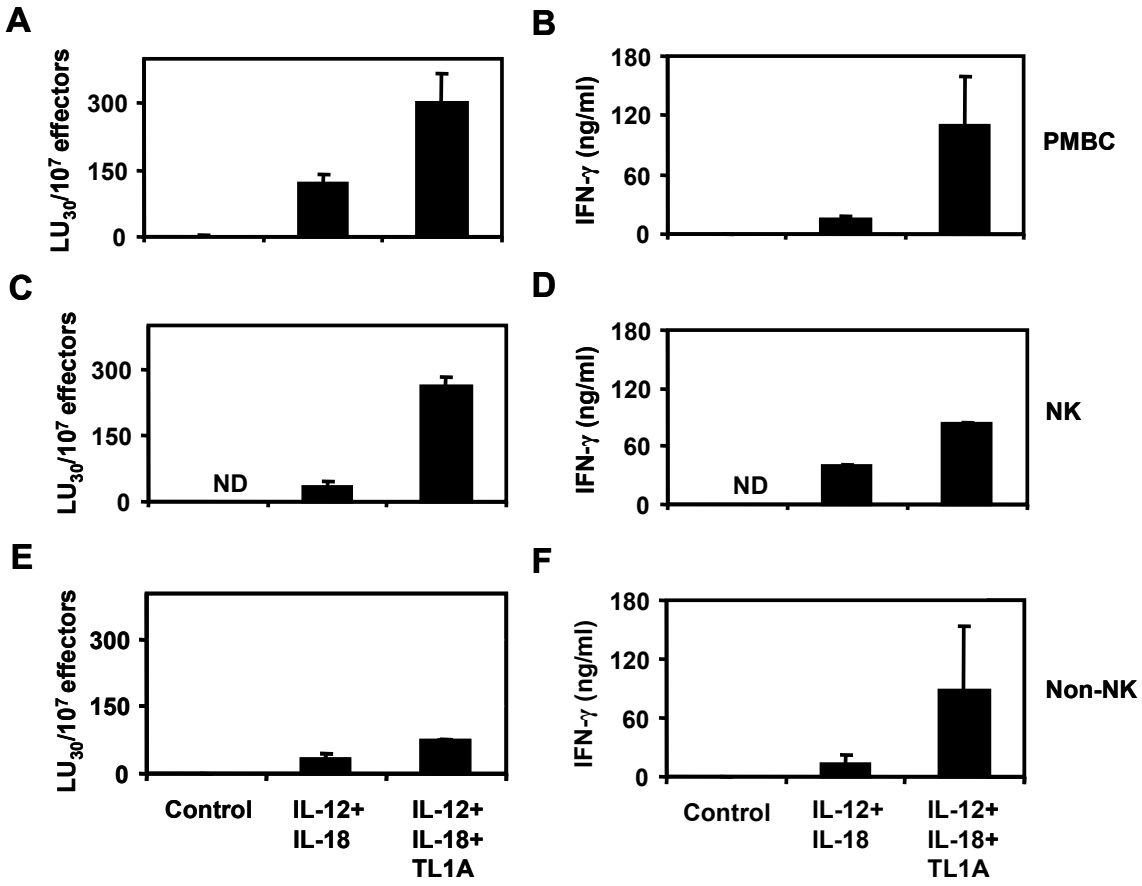

Figure 4.10: Effect of TL1A on IL-12/IL-18 induced cytotoxicity of purified NK cells and cytotoxic T-cells towards Daudi. NK (CD56+, CD3-) cells were purified from PBMC by negative selection on a MACS column. The cells retained on the column were eluted and designated as the non-NK fraction. NK cells, the non-NK cell fraction and unseparated PBMC from the same donor were incubated with medium *(Control)* or IL-18 at 54 ng/ml and IL-12 at 40 pg/ml without or with TL1A (50 ng/ml) for 72 and 96 hrs, and their cytolytic activity was tested against Daudi in a 2-h ^{51}Cr-release assay. The results for 96 hrs are shown in $LU_{30}/10^7$ effector cells. *LU_{30}*, the number of effector cells required to lyse 30% of a standard number of target cells, here 10^4 target cells. *Bars,* SEM. *ND,* not determined. Since most purified NK cells in culture without stimulation died, no control condition of unstimulated NK cells cultured for 3 and 4 days could be done. IFN-γ production was measured in culture supernatants by ELISA. Mean values and SEM of two experiments are shown. *ND,* not determined.

TL1A was shown to enhance IL-12/IL-18-induced cytotoxicity of PMBC towards Daudi cells by two-fold, while it potentiated the lytic activity of purified NK cells by 7-fold. The effect of TL1A on IL-12/IL-18-induced NK cell cytotoxicity towards the resistant epithelial cell lines WiDr and SW837 might therefore be more pronounced, when purified NK cells are used as effectors of the ^{51}Cr-release assay instead of unseparated PBMC.

The cytotoxicity of NK cells and non-NK cells, purified by magnetic cell separation, was therefore tested on WiDr and SW837. To this end, NK cells and the non-NK cell fraction of the same donor were incubated with IL-12 (40 pg/ml) and IL-18 (54 ng/ml) with and without TL1A (50 ng/ml) for 72 and 96 hrs. The cytolytic activity of NK cells and non-NK cells was tested in 2-h ^{51}Cr-release assays against WiDr and SW837. The culture supernatants were collected to measure the IFN-γ production in each condition.

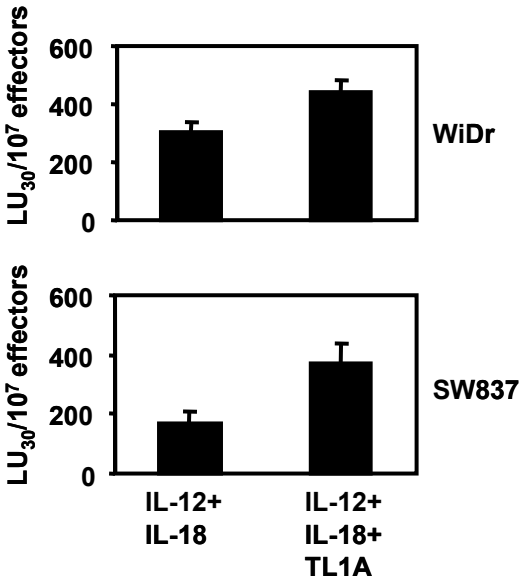

Figure 4.11: Effect of TL1A on IL-12/IL-18 induced cytotoxicity of purified NK cells towards WiDr and SW837. NK (CD56+, CD3-) cells were purified from PBMC by negative selection on a MACS column. NK cells were incubated with IL-18 at 54 ng/ml and IL-12 at 40 pg/ml without or with TL1A (50 ng/ml) for 72 and 96 hrs, and their cytolytic activity was tested against WiDr and SW837 in a 2 hour ^{51}Cr-release assay. The results for 96 hrs are shown in $LU_{30}/10^7$ effector cells. LU_{30}, the number of effector cells required to lyse 30% of a standard number of target cells, here 10^4 target cells. Since most purified NK cells in culture without stimulation died, no control condition of unstimulated NK cells cultured for 3 and 4 days could be done. The results are representative of two similar experiments. *Bars,* SD.

As reported in Fig. 4.11, TL1A increased IL-12/IL-18-induced cytotoxicity of purified NK cells towards WiDr by 1.4-fold and towards SW837 by 2.2-fold.
Non-NK cells, consisting mainly of T cells, were incapable of mediating lytic activity against WiDr and SW837 after stimulation with IL-12/IL-18 without and with TL1A (data not shown).

TL1A enhancement of IL-12/IL-18-induced NK cytotoxicity against NK-resistant colorectal adenocarcinoma-derived epithelial cells was less powerful, compared to the NK-resistant B cell line Daudi. However, the results further confirmed a synergistic effect of TL1A on IL-12/IL-18-induced NK cytotoxicity against NK-resistant tumor cells.

5 Discussion

5.1 Summary of the results

The TNF-like protein TL1A was identified as ligand of DR3 and DcR3 when Migone et al. tested a panel of TNF-superfamily receptors for binding affinity to TL1A in 2002 (52), and it is the only known ligand of DR3 to date (98). This study showed that only the combination of IL-12 and IL-18, of a panel of cytokines and cytokine combinations tested, is capable of significant induction of DR3 on NK cells (Table 4.1). Combined stimulation with maximally effective concentrations of IL-12 and IL-18 powerfully induces DR3 expression on up to 70% of NK cells (64). Papadakis et al. further showed that IL-12 or IL-18 alone are also capable of moderate induction of DR3 on approximately 20% of NK cells (64). The effects of TL1A on NK cell functions are thus dependent on IL-12 and IL-18. TL1A might therefore contribute to the regulation of NK cell immune responses by IL-12 and IL-18, for example in inflammation (71). This conclusion is supported by recent evidence revealing a role of TL1A in the pathogenesis of chronic inflammatory conditions, e.g. inflammatory bowel disease, rheumatoid arthritis and atherosclerosis (71;98). Moreover, pro-inflammatory cytokines, including IL-12 and IL-18, are produced by antigen-producing cells upon stimulation with antigen in host defense against pathogens (12;22;72;88), and a substantial infiltration of tumors by inflammatory cells has also been reported (75). TL1A could therefore function in vivo as a co-activator of NK cell effector functions in host defense both against pathogens and transformed cells.

This study further demonstrated that TL1A synergizes with both sub-optimal and optimal concentrations of IL-12 and IL-18 to augment NK cell IFN-γ production (Fig 4.1). This finding implies that both sub-optimal and optimal concentrations of IL-12 and IL-18 induce a functional level of DR3 expression on a large proportion of NK cells, which was confirmed in a subsequent experiment analyzing DR 3 expression on NK cells at maximal and reduced concentrations of IL-12/IL-18 (Fig. 4.5). These results are in line with previous findings of Prehn et al., who examined the effect of TL1A on IFN-γ production of peripheral blood lymphocytes (PBL) and lamina

propria lymphocytes (LPL), pre-treated with PHA and co-stimulated with high-dose IL-12 and IL-18 at low or high concentration. Their results implied that TL1A synergizes with IL-12 and both low or high dose IL-18 to augment IFN-γ production of PBL and LPL (71).
This study further confirmed the results of Papadakis et al., who showed by flow cytometry that only the number of IFN-γ producing cells was augmented in response to TL1A, while the amount of IFN-γ produced per cell was not increased (64). This observation implied that the synergistic effect of TL1A on IL-12/IL-18-induced IFN-γ production is mainly due to stimulation of NK cell proliferation. This study provided further evidence for this conclusion by demonstrating that TL1A induced an average 2.2-fold increase of IL-12/IL-18-induced NK cell DNA replication (Fig. 4.2), which was correlated to an average 2.6-fold increase of IL-12/IL-18-induced NK cell IFN-γ production by TL1A (Fig. 4.1).

This study further revealed that TL1A, while not affecting lysis of NK-sensitive CML-derived K562 cells, strongly stimulates IL-12/IL-18-induced NK cell cytotoxicity against NK-resistant Daudi cells (Burkitt's lymphoma), lysis that is completely dependent on NK activation (Fig. 4.9 and Fig. 4.10). Lysis of the colorectal adenocarcinoma-derived epithelial cell lines WiDr and SW837 is similarly, but less powerfully, stimulated (Fig. 4.9 and 4.11).
Although only a few target cell lines (K562, Daudi, WiDr, and SW837) were tested for susceptibility to lysis by PBMC or purified NK cells activated by IL-12/IL-18 without and with TL1A, the results suggested that TL1A preferentially potentiates lytic activity of NK cells against NK-resistant targets (Daudi, WiDr and SW837), while not increasing the already substantial cytolytic activity against NK-sensitive targets (K562). Further studies of the effect of TL1A on NK cytotoxicity against additional targets will be needed to determine the generality of this action of TL1A.

Interestingly, TL1A resulted in a 2-fold increase in cytotoxicity of Non-NK cells, largely T cells, and thus, the effect of TL1A on cytotoxicity is not limited to NK cells.
The data presented here indicated that TL1A has a greater impact on NK cell-mediated cytotoxicity against Daudi cells than on NK cell IFN-γ production, as

evidenced by a 7 fold enhancement of cytotoxicity versus a 2 fold increase in IFN-γ production. For Non-NK cells, consisting of a large majority of T cells, a 7 fold increase in IFN-γ production, but only a 2 fold increase in lysis following stimulation by TL1A was demonstrated. These results suggest that TL1A stimulates proliferation and effector IFN-γ and lytic function in both NK and T cells, but for NK cells potentiating the cytolytic activity of activated NK cells predominates, while for T cells, stimulation of IFN-γ production and proliferation of a small subset is the main effect (64;65).

5.2 Mechanisms potentially involved in TL1A selective enhancement of IL-12/IL-18-induced NK cytotoxicity towards NK-resistant target cells

This study did not investigate the mechanisms by which TL1A selectively enhances NK cytotoxicity against NK-resistant tumor targets. Since augmentation of cytotoxicity against NK-resistant targets by TL1A is dependent on IL-12/IL-18, TL1A could contribute to IL-12/IL-18-induced modulations of NK lytic mechanisms and/or NK-target cell recognition and NK activation. TL1A might enhance IL-12/IL-18-induced modulations of NK cell-target interactions which confer NK cell susceptibility of previously resistant targets. To date, determinants of target cell susceptibility to NK cell-mediated lysis and mechanisms of target resistance, respectively, have not been fully elucidated.

5.2.1 General mechanisms of tumor resistance to NK cell-mediated lysis

General mechanisms of tumor resistance to immune surveillance applicable to NK cell-mediated lysis have been formulated (19;32). They either affect NK-target cell recognition and NK cell activation or the mechanisms of NK cell killing (necrotic and apoptotic pathways).

One possibility of tumor resistance to NK cytotoxicity might be downregulated expression of NK activating receptors (e.g. natural cytotoxicity receptors (NCRs), NKG2D, 2B4 (CD24)). Soluble ligands for activating receptors can impair NK-target cell recognition. Certain tumors release soluble forms of MHC class I-related ligand

A (MICA) or ULBP, ligands of the NK cell activating receptor NKG2D, by proteolytic cleaving, which in turn impairs the expression of functional NKG2D receptor and enables cancer cells to escape NKG2D-mediated cytolytic activity of NK cells (28;74;79). It was also reported that sustained localized expression of MICA on tumor cells can elicit NKG2D downregulation and impair NK cell-mediated cytotoxicity (60).

Absence or reduced expression of NK cell activating receptor ligands (e.g. MHC class-I related molecules (MIC) A and B, ULBP) and upregulation of MHC class I molecules at the target cell surface, which activate MHC-specific inhibitory receptors, might also interfere with tumor recognition by NK cells (19;32). Tumor cells can display apoptosis resistance involving several distinct molecular mechanisms (31). Mechanisms of resistance towards the perforin/granzyme pathway of NK cell-mediated lysis can involve overexpression of the serine protease inhibitor PI-9/SPI-6 which inhibits granzyme B and is expressed in a variety of human tumors (11;50), or impaired binding of perforin to the target cell membrane (42)

5.2.2 Surface phenotype of the NK-sensitive tumor target K562 and the NK-resistant tumor cell lines Daudi, SW837 and WiDr

Phenotypic differences of Daudi and K562 target cells might explain their distinct levels of susceptibility to lysis by nonactivated NK cells. However, both Daudi and K562 are MHC class I-deficient (10). K562 cells express the MHC class I-related molecules ULBP1 and ULBP2 (18), which are ligands to the NK cell activating receptor NKG2D, while Daudi cells only express ULBP1 (67). WiDr cells express the MHC class I-related chain A and ULBP 2 and 3 (67); it is not known to date if SW837 cells also express MHC class I-related molecules. Both Daudi and K562 are Fas receptor-negative, which is reflected by the fact that both are resistant to Fas-mediated apoptotic killing by freshly isolated NK cells (94). NK cell cytotoxicity against a large variety of solid tumor lines including colon carcinoma is, however, mediated by apoptotic killing only or by both pathways (93). A susceptibility to apoptotic killing might therefore complement the low susceptibility of WiDr and SW837 to perforin-dependent cytotoxicity of activated NK cells (cf. Fig. 4.9 and 4.11).

It was recently reported that the secreted member of the TNF receptor superfamily Decoy receptor 3 (DcR3)/TR6/M68 is overexpressed in many human primary tumors, including Epstein-Barr-Virus-associated lyphomas (58) and adenocarcinoma of the colon and rectum, as well as the colon adenocarcinoma cell lines SW480 and SW1116 (7). Know ligands of DcR3 are Fas ligand, LIGHT and TL1A. Binding to DcR3 blocks the effector functions of these three ligands. Tumor cell release of DcR3, which neutralizes the cytotoxic and immunoregulatory effects of Fas ligand, LIGHT and TL1A, is therefore regarded as a mechanism of tumor escape to host immune response. Recent studies analyzed the multiple effects of DcR3 on many cells (97), and surprisingly, Wu et al. demonstrated that DcR3 sensitizes cells to TRAIL-induced apoptosis (97). Since TL1A was further able to induce apoptosis in transient systems where DR3 and/or the signaling molecules were overexpressed (52;96), the TL1A/DcR3 pathway might contribute to NK cell-mediated lysis of DcR3 overexpressing tumor cells. It has not been analyzed to date if K562, Daudi, WiDr, and SW837 express DcR3. If the NK-resistant targets Daudi (Ebstein-Barr virus-associated Burkitt's lymphoma), WiDr (colorectal adenocarcinoma) and SW837 (rectal adenocarcinoma) overexpress DcR3, while K562 (chronic myelogenous leukemia) does not express DcR3, a selective effect of membrane-bound TL1A on NK cytotoxicity against on Daudi, WiDr and SW837 would be conceivable.

5.2.3 Tumor resistance towards the perforin/granzyme pathway

The following findings suggest that mechanisms involved in the perforin/granzyme pathway might determine target cell susceptibility to lysis by NK cells.

Lehmann et al. showed that the lack of NK-susceptibility of the tumor target ML-2 is caused by a resistance of these cells to cytotoxic effector molecules of nonactivated NK cells (42). Perforin from nonactivated NK cells was shown to bind to the cell membrane of the NK-sensitive tumor K562, but it did not bind to the surface of the NK-resistant target ML-2, derived from a patient with AML. In this context, it was demonstrated that activation of NK cells with IL-12 and IL-2 confers NK-susceptibility of previously resistant targets by enhancing perforin binding to the target cell membrane (43).

It was furthermore reported that failure of IFN-γ to induce membrane expression of platelet-activating factor (PAF) is a mechanism of resistance of Daudi cells and other NK-resistant targets to perforin-mediated lysis by NK cells (18). Activation by interaction with the tumor targets K562 and Daudi leads to naive NK cell release of choline phosphate-containing lysolipids including platelet-activating factor (PAF) in addition to perforin. PAF can simultaneously bind to its specific membrane receptor and to perforin and contributes to perforin-induced membrane damage in the NK-sensitive target K562, but does not have an agonistic effect on the lysis of Daudi cells. It was shown that IFN-γ secreted from NK cells activated by target cell contact induces PAF-receptor expression on perforin-sensitive K562 cells, but not on perforin-resistant Daudi cells. Transfection of the PAF-receptor cDNA into MHC class I and Fas-receptor negative Daudi cells restored susceptibility to perforin-induced lysis by naive NK cells.

Moreover, it was possible to demonstrate that the immunological synapse formed between effector and target cell, which provides a microenvironment for the release of cytotoxic granules (6;27), displays distinct organization modes during NK cell interaction with resistant and susceptible targets (80;95).

In conclusion, it is conceivable that stimulation of NK cells by cytokines such as IL-12 and IL-2 or IL-18 preceding target cell contact might elaborate the perforin-mediated granule exocytosis pathway, for example by inducing the release by NK cells of additional cytokines and molecules like platelet-activating factor (PAF) that enhance binding of perforin and subsequent target lysis.

5.2.4 Effects of cytokines on NK cell recognition of tumor targets and triggering of NK cells

In order to explain the susceptibility of NK-resistant cells to lysis by activated NK cells Moretta et al. postulated that culture of NK cells with cytokines might induce the de novo expression of activating receptors that enable NK cells to recognize additional ligands on the surface of tumor targets (54). Previously NK-resistant target cells could subsequently provide an activating signal to NK cells which is capable of overriding the previously dominant inhibition signal. The expression of the natural cytotoxicity receptor NKp44, for example, is induced on NK cells upon activation

with IL-2 (54). However, the results of Pende et al. suggest that this activating receptor is not predominantly involved in NK-cell mediated killing of Daudi cells. Pende et al. demonstrated that mAB-mediated masking of NKG2D abrogated the cytotoxicity of IL-2-activated NK cells against Daudi cells. This finding suggests that NK cell killing of Daudi cells is dependent on signals generated by the NKG2D receptor on NK cells, which interacts with ULBP1, a MHC class I-related molecule, expressed on Daudi cells (66;67). To date, it has not been shown whether NK cytotoxocity against K562, which express NKG2D receptor ligands ULBP 1 and 2 (18), is NCR and NKG2D-dependent or only NKG2D-dependent. More recently, however, there has been evidence that NKG2D might function as a coreceptor rather than a primary receptor during NK cell activation (4).

NKG2D expression and ULBP binding to NK cells were further shown to be upregulated in response to IL-12 stimulation of NK cells (82). Contact of unactivated NK cells, and potentially γδ T cells, with MHC class I-negative Daudi cells might be insufficient to induce an activating signal for NK cell- and/or γδ T cell-mediated lysis of Daudi cells. As a consequence of upregulated NKG2D expression and ULPB binding, cytokine-activated NK cells and γδ T cells might then respond to such signals and lysis of Daudi cells could be triggered.

Ortaldo et al. furthermore demonstrated that costimulation of the murine activating NK cell receptor Ly49D with IL-12 and IL-18 is capable of over-riding signals from the inhibitory Ly49G2 receptor for IFN-γ production in vivo and in vitro (63). Like the human killer-cell immunoglobulin-like receptors (KIR), murine Ly49 receptors have binding specificity for MHC class I alleles and in general display a great functional homology to the human KIR (41). Ortaldo et al. showed that IL-12 and Ly49D crosslinking resulted in elevated sustained biochemical activation of STAT4 and IFN-γ mRNA (63). Since IL-12 and IL-18 synergistic induction of IFN-γ mRNA is the result of a STAT4-dependent increase in AP-1 binding to the IFN-γ promoter (55), Ortaldo et al. proposed that this signaling pathway could also be involved in their finding (63). Moreover, Ortaldo et al. were recently able to extend their evidence for synergistic action of NK cell receptors and cytokines to additional NK cell activating receptors which use distinct signal transduction pathways (62). In murine

NK cells, the DAP12-associated receptor Ly49H, the TCRζ-associated receptor NKRp1, and the DAP10- and DAP12-associated receptor NKG2D were shown to synergize with IL-12 or IL-18 in the induction of IFN-γ, both in the absence and presence of signals from the inhibitory Ly49G2 receptor. Interestingly, co-regulation of inhibiting and activating receptors by cytokines was also found in T cells and NKT cells (62). Ortaldi et al. showed that the synergistic response of IL-12 and the NK activating receptors Ly49D and NKG2D required both the p38 MAP kinase and the ERK-dependent signal transduction pathways, which converge to enhance expression of the *Ifng* gene (62).

These results show that NK receptors cooperate with cytokines mediating NK cell effector functions and that co-stimulation of NK receptors with cytokines can reverse otherwise dominant inhibitory signals. A similar mechanism might also explain IL-12/IL-18-induced NK cell lysis of NK-resistant tumor cells.

5.2.5 Signal transduction pathways regulated by IL-12, IL-18 and TL1A

Even with all of these findings it is not clear which mechanisms may account for the enhancing effect of TL1A on IL-12/IL-18-induced NK cell cytotoxicity against NK-resistant targets.

TL1A has been shown to activate NF-κB and MAPK (JNK, p38 and ERK1) in the human erythroleukemic cell line TF-1 (96). Moreover, interaction of TL1A with its receptor DR3 was recently reported to induce IL-8 gene expression by activating TRAF2 and NF-κB via the TAK1/ASK1-MKK4/MKK7-JNK2 kinase cascade (98).

Binding of IL-18 to its receptor (IL-1 receptor-related protein (IL-1Rrp)) induces activation of IL-1-receptor-associated kinase (IRAK-1 and IRAK-4), TNFR-associated factor-6 (TRAF6), NF-κB and JNK (which in turn activates AP-1) in NK and Th1 cells (3;83). Experiments analyzing the signal transduction of IL-18 in the NK cell line 92 suggest that mitogen-activated protein kinases (MAPKs) p42erk-2, p44erk-1, p38 and STAT3 might also play a role in IL-18 responses (35).

Binding of IL-12 to its specific receptor complex leads to activation of the Janus kinases TYK2 and JAK2, resulting in phosphorylation and activation of signal transducer and activator of transcription (STAT) 4 and other STATs (12;99). STAT4 plays a pivotal role in all biological responses induced by IL-12, including IFN-γ

production (12). IL-12 also activates p38 mitogen-activated protein kinase (MAPK) in NK cells and T cells (99;100).

The synergistic effects of IL-12 and IL-18 could be the consequence of reciprocal upregulation of receptors and of cooperative action at the transcriptional level resulting in optimal activation of IFN-γ transcription and other effector functions of T and NK cells (3;55).

By cooperating with IL-12 and IL-18 in the activation of these signaling pathways TL1A could contribute to tumor recognition and activation of NK cells and/or NK cell lytic mechanisms and thereby maximize NK cytotoxicity against NK-resistant target cells.

5.2.6 Potential role of TL1A in activation-induced cell death of NK cells

Alternatively, TL1A might enhance IL-12/IL-18-induced NK cytotoxicity by promoting NK cell survival and recycling for continous killing of target cells. After lysing a susceptible target a NK cell can prepare to kill again, become inactivated or undergo activation-induced cell death (45). Contact with susceptible targets usually induces sustained inactivaton of NK cells and reduces the ability to mediate lysis of additional target cells (2;69), due to depletion of effector molecules and/or postreceptor desensitization (8;26). Although cytokines can enhance NK cell responsiveness and recovery of lytic activity, IL-2 and IL-12-activated NK cells are particularly susceptible to activation-induced cell death upon contact with susceptible targets and FcR-specific ligands (5;21;33;61). TL1A could inhibit activation-induced cell death of NK cells, as DR3-induced NF-κB activation was shown to be responsible for resistance to apoptosis in TF-1 cells (96). It remains unclear why this would selectively affect NK cell-mediated lysis of NK-resistant targets.

5.3 Conclusion

This study demonstrated that TL1A selectively enhances IL-12/IL-18-induced NK cell cytotoxicity against Daudi cells and other NK-resistant tumor targets, susceptible to lysis only by activated NK cells. TL1A thereby markedly potentiates the lytic activity of activated NK cells. The striking induction of DR3 on NK cells by the activating cytokines IL-12 and IL-18 would make possible a powerful enhancement of NK cytotoxicity in inflammatory sites where TL1A is expressed.

The signaling pathways involved in TL1A enhancement of IL-12/IL-18-induced cytotoxicity and the mechanisms underlying its preferential effect on NK cell-mediated lysis of resistant targets deserve further attention, and may elucidate a possible role for TL1A in tumor surveillance and therapy.

Specifically, the following points should be addressed. The panel of target cells should be extended to determine the generality of the demonstrated effect of TL1A on NK cytotoxicity. Hereby, it would be interesting to assess if TL1A-activated NK cells acquire the ability to kill selectively certain types of targets (e.g. B cell lines). Tumor targets could also be evaluated for DcR3 expression.

The surface phenotype of NK cells co-stimulated by IL-12/IL-18 and TL1A could be analyzed, since TL1A might affect surface expression and function of NK cell triggering receptors (CD16, NCR, NKG2D, CD244) as well as receptors induced upon NK cell activation (CD69, CD70, Nkp44).

It would further be warranted to analyze the effect of TL1A stimulation on the perforin/granzyme pathway, in particular the perforin granule content, as well as the other NK cell lytic mechanisms, i.e. the Fas ligand- and TRAIL-dependent pathways.

Finally, the hypothesis that TL1A may protect NK cells from activation-induced cell death could be assessed.

6 Abstract

TL1A (TNFSF15) is a recently identified cytokine belonging to the TNF super-family, a ligand for death domain receptor 3 (TNFRSF25 or DR3), which is induced by activation on T cells and natural killer (NK) cells. TL1A augments IFN-γ production by IL-12/IL-18 responsive human T cells by 10-fold or more. Although IL-12/IL-18 induces DR3 expression on most NK cells, addition of TL1A increases IFN-γ production by only 2-fold. This study, therefore, investigated whether the TL1A-DR3 pathway is also implicated in another IL-12/IL-18-regulated NK cell function: lysis of tumor cells. The effect of TL1A on IL-12/IL-18-induced NK cell cytotoxicity was tested in ^{51}Cr-release assays.

While TL1A had no additional effect on IL-12/IL-18 induced cytotoxicity against an NK-susceptible tumor (K562), TL1A promoted cytotoxicity against NK-resistant targets (Daudi), susceptible to lysis only by activated NK cells. Thus, with IL-12/IL-18 activation, TL1A enhanced lysis of Daudi by both PBMC (2-fold) and purified NK cells (7-fold). TL1A also increased lysis of the colorectal adenocarcinoma epithelial derived lines (WiDr and SW837) by IL-12/IL-18 activated cells, but to a lesser degree. TL1A markedly increased cytotoxicity of IL-12/IL-18 activated NK cells against targets cells dependent on NK activation for lysis, and could function in vivo as a key co-activator of NK cytotoxicity.

This study further established by flow cytometry, ELISA or H^3-Thymidine incorporation assay, respectively, that only combined IL-12/IL-18, of a panel of cytokines and cytokine combinations tested, was capable of significant induction of DR3 on NK cells, that TL1A synergized with both optimal and sub-optimal concentrations of IL-12 and IL-18 to enhance NK cell IFN γ production, and that this effect was mainly due to NK cell proliferation.

7 References

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