Study on NK cells cytotoxicity toward endothelial cells

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Cytotoxicité des cellules tueuses naturelles vis-à-vis des cellules endothéliales organospécifiques : vers une immunothérapie tumorale

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Cytotoxicité des cellules tueuses naturelles vis à vis des cellules endothéliales organospécifiques : vers une immunothérapie tumorale

Résumé : Plusieurs mécanismes peuvent réduire l'angiogenèse tumorale d'où les stratégies visant à bloquer les cellules endothéliales (CE). Les cellules tueuses naturelles (NK) (natural killer cells) stimulées, s'arment pour l'élimination des cellules « dangereuses ». Notre hypothèse est qu'en conditions pathologiques (tumeur), les CE, acteurs de l'angiogenèse tumorale seraient reconnues comme telles et candidates à l'attaque par les NK. Les interactions entre les NK et les CE sont abordées à l'aide de CE humaines *in vitro,* quant aux mécanismes moléculaires de l'adhésion des NK en conditions statiques et conditions de flux. Ceci montre que les NK activées par l'IL-2 reconnaissent et adhérent aux CE selon leur origine tissulaire. Ce mécanisme est indépendant des sélectines mais dépend soit des intégrines, soit des co-récepteurs similaires aux lectines de type C. La cytotoxicité des NK vis-à-vis des CE s'exerce par la voie perforine-granzyme. En outre, stimulées par l'IL-2, les NK induisent la translocation de Bid et libération du cytochrome C dans les CE cibles lesquelles expriment les récepteurs de "mort", voie alternative d'apoptose. Ce modèle *in vitro* est validé avec des NK du sang humain. A visée *in vivo*, les expériences réalisées avec des CE murines et des NK de la rate de souris indiquent que l'efficacité des NK activées par l'IL-2 est directement reliée à leur adhésion, laquelle dépend de l'origine tissulaire des CE. Nous démontrons que l'IL-12 (interleukine connue pour inhiber l'angiogenèse tumorale) active les NK en synergie avec l'IL-2. Les NK reconnaissant et tuant les CE *in vitro* suggère l'hypothèse *qu'in vivo* elles inhibent l'angiogenèse tumorale.

Mots clés : angiogenèse, cellules endothéliales, cellules tueuses naturelles, cytotoxicité.

Study on NK cells cytotoxicity toward endothelial cells

Résumé en anglais: There are several mechanisms by which tumor angiogenesis may be reduced. They are addressed to tumor endothelial cell (EC) to block their activation and proliferation. Among the natural protection mechanisms, natural killer (NK) cells play an important role. Stimulated NK cells may eliminate potentially "dangerous" cells. Hence, we hypothesized that ECs involved in tumor angiogenesis may be recognized as "dangerous" cells and killed by NK cells. First, the adhesive interactions between NK cells and ECs were investigated. Using human *in vitro* cell lines, molecular mechanisms of NK cells adhesion to ECs under static and flow in vitro conditions were examined, showing that IL-2 activated NK cells were able to adhere to ECs. The molecular mechanism was shown not to be selectin - dependent but integrin and/or C type lectin like - dependent. Subsequently, cytotoxicity of NK cells toward ECs was analyzed. Its molecular mechanism was mainly perforin-granzyme dependent. NK cells were shown to induce in target ECs Bid translocation and cytochrome c releasing. To validate *in vitro* cellular model most of these experiments were repeated with freshly isolated human blood NK cells. The *in vivo* approach necessitated that adhesion and cytotoxicity were performed using murine ECs lines and freshly isolated NK cells from murine spleen. It was found that IL-12 synergizes with IL-2 to stimulate NK cells killing activity toward ECs. The demonstration that, under specific conditions stimulated NK cells are able to kill ECs *in vitro* allows hypothesizing that *in vivo* NK cells may participate in tumor angiogenesis inhibition.

Key words: angiogenesis, endothelial cells, natural killer cells, natural cytotoxicity.

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LIST OF ABBREVIATIONS

Ab	Antibody
ACE	Angiotensin converting enzyme
ADCC	Antibody dependent cell cytotoxicity
AIF	Apoptosis inducing factor
Apaf-1	Apoptotic protease activating factor
APC	Allophycocyanin
ATCC	American type culture collection
BCR	B cell receptor
Bid	*B*H3 domain *I*nteracting *D*eath agonist
bp	Base pair
BSA	Bovine serum albumin
CAD	Caspase-activated DNase
CCR	Chemokine receptor
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CLR	C-lectin related
CRTAM	Class I-restricted T cell-associated molecule
CS1	Cell surface glycoprotein subset 1
CSFs	Colony stimulating factors
CTLR	C-type lectin-like receptors
CX3CR	CX3C chemokine receptor
CXCR	CXC chemokine receptor
DAPI	4',6-diamidino-2-phenylindole
DD	Death domain
DIOC6(3)	3, 3'-dihexyloxacarbocyanine iodide
DNA	Deoxyribonucleic acid
DUS	Doppler ultrasonography
E/T ratio	Effector to target ratio
ECs	Endothelial cells
EDTA	Ethylene-diamine-tetra-acetic acid
EGTA	Ethyleneglycol-bis (β-amonoethyl ether)-tetraacetic acid
FACS	Fluorescence-activated cell sorting
FADD	Fas-associated protein with death domain
FasL	Fas lignad
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FL	Fluorescence
FTC	Fluorescein
FVB/N	Friend Virus B NIH Jackson
GlyCAM-1	Glycosylation dependent cell adhesion molecule 1
GM-CSF	Granulocyte macrophage colony stimulating factors
gp	Glycoprotein
HBrMEC	Human brain microvascular endothelial cells
HCl	Hydrochloric acid
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
HeuN	Human autologous lung carcinoma cell line
HEVs	High endothelial venules
HLA	Human leukocyte antigen
HLMEC	Human lung microvascular endothelial cells
HMLNEC	Human mesenteric lymph node endothelial cells
HPLNEC.B3	Human peripheral lymph node endothelial cells
HSkMEC.2	Human skin microvascular endothelial cells
HUVEC	Human umbilical vein endothelial cells
ICAM	Intramolecular cell adhesion molecules
IFN	Interferon
IL	Interleukin
ILT-2	Immunoglobulin-like transcript 2
IU	Infectious units

K562	Human chronic myelogenous leukemia cells
KIR-L	Inhibitory members of the killer Ig-like receptor family
KIR-S	Activating members of the killer Ig-like receptor family
KLRG-1	Killer cell lectin-like receptor G1
Lag3	Lymphocyte-activation gene 3
LAIR	Leukocyte-associated immunoglobulin-like receptor-1
LAK	Lymphokine-activated killer
LFA	Lymphocyte function-associated antigen
LLT1	Lectin-like transcript-1
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
mBMMEC FVB	Bone marrow microvascular endothelial cells from FVB mouse
mBrMEC FVB	Brain microvascular endothelial cells isolated from FVB mouse
MCF7	Breast carcinoma cell line
MCMV	Murine cytomegalovirus
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
mNK	Murine purified natural killer cell
mPLNMEC FVB	Peripheral lymph nodes microvascular endothelial cells isolated from FVB mouse
mPPMEC FVB	Peyer's patches microvascular endothelial cells isolated from FVB mouse
MPR	Mannose 6-phosphate receptor
mRNA	Messenger ribonucleic acid
MUC1	Mucin 1
NAA	Non-essential amino acids
N-CAM	Neural cell adhesion molecule
Ncl2	Nectin-like protein 2
NCR	Natural cytotoxicity receptors
NK	Natural killer
NKL	Natural killer cell line
NKp	Human purified natural killer cell
NKR	NK-cell receptor
NTBA	NK-T-B antigen
OSECs	Organ-specific endothelial cell lines
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PECAM-1	Platelet endothelial cell adhesion molecule
PE-Cy5	Phycoerythrin-cyanin 5 tandem
PI	Propidium iodide
PKH26GL	Red fluorescent cell linker kit for general cell membrane labeling
PKH67GL	Green fluorescent cell linker kit for general cell membrane labeling
PSGL	P-selectin glycoprotein ligand
RANTES	Regulated on activation, normal T-cell expressed and secreted
rh	Recombinant human
rIL-2/12/15	Recombinant interleukin 2/12/15
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SFV	Semliki Forest virus
SFV-LacZ	Recombinant SFV expressing influenza Escherichia coli β-galactosidase
SFV-mIL-12	Recombinant SFV expressing murine IL-12
SIGLEC	Sialic acid binding Ig-like lectins
Tactile	T cell-activated increased late expression
TCR	T cell receptor
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAF	TNF receptor associated factor

TRAIL	TNF-related apoptosis-inducing ligand
TRAILR	TNF-related apoptosis-inducing ligand receptor
U	Units
uNK	Uterin NK cells
UV	Ultraviolet
VCAM	Vascular cell adhesion molecule
VEGFR	Vascular endothelial growth factor receptor
VLA	Very late antigen
vWF	von Willebrand Factor

ABSTRACT

There are several major mechanisms by which tumor angiogenesis may be reduced. Especially, different strategies that could block tumor endothelial cell (EC) activation and proliferation were tested. Among the natural protection mechanisms, natural killer (NK) cells play an important part. It appears now that stimulated NK cells possess a whole armamentarium for rapid elimination of potentially "dangerous" cells. Hence, we hypothesized that, in pathological conditions, ECs involved in tumor angiogenesis may be recognized as "dangerous" cells and, subsequently, killed by NK cells.

In the first part of this study the adhesive interactions between NK cells and ECs were investigated. Using human *in vitro* cell lines, molecular mechanisms involved in NK cells adhesion to ECs under static as well as flow *in vitro* conditions were examined, showing that IL-2 activated NK cells were able to recognize and adhere to endothelial cells. Observed NK cells adhesive capacity was different depending on endothelial cells tissue origin. The molecular mechanism was shown not to be selectin - dependent but either integrin and/or non-C type lectin like - dependent.

Subsequently, using the same human cell lines, cytotoxicity of NK cells toward ECs was analyzed. Molecular mechanism of this cytotoxicity was mainly perforin-granzyme dependent. IL-2 stimulated NK cells were shown to induce in target ECs Bid translocation as well as cytochrome c releasing. Human ECs were found to express death receptor on their surface which might be alternative pathway inducing apoptosis of target ECs. In order to validate the *in vitro* cellular model which had been used, most of these experiments were next repeated with freshly isolated NK cells from peripheral human blood.

The in vivo approach necessitated that, in the second part of this study, adhesion as well as cytotoxicity were performed using murine ECs lines and freshly isolated NK cells from murine spleen. The efficiency of the killing (or susceptibility to cytotoxicity) of murine ECs by IL2-activated murine NK cells was directly correlated to their adhesion capacity. Murine NK cells adhesion and killing efficiency were shown to depend on endothelial cells tissue origin. Furthermore, it was found that IL-12 (known to inhibit tumor angiogenesis) synergizes with IL-2 to stimulate NK cells killing activity toward ECs.

The demonstration that, under specific conditions stimulated NK cells are able to recognize and kill ECs *in vitro* allows to hypothesized that *in vivo* NK cells may participate in tumor angiogenesis inhibition.

In the series of experiments concerning the antitumor effect of IL-12 gene transfer in mice, besides the expected reduction in tumor growth dynamics, the group of dr. Chouaib have demonstrated diminution in the density of blood vessels in IL-12 treated animals, both by Doppler ultrasonography as well as by histological examination* [1]. Further analysis of histological data revealed that tumors in IL-12 treated mice were characterized with increased number of NK cells grouped closely in perivascular regions (Fig. 1).

A **B**

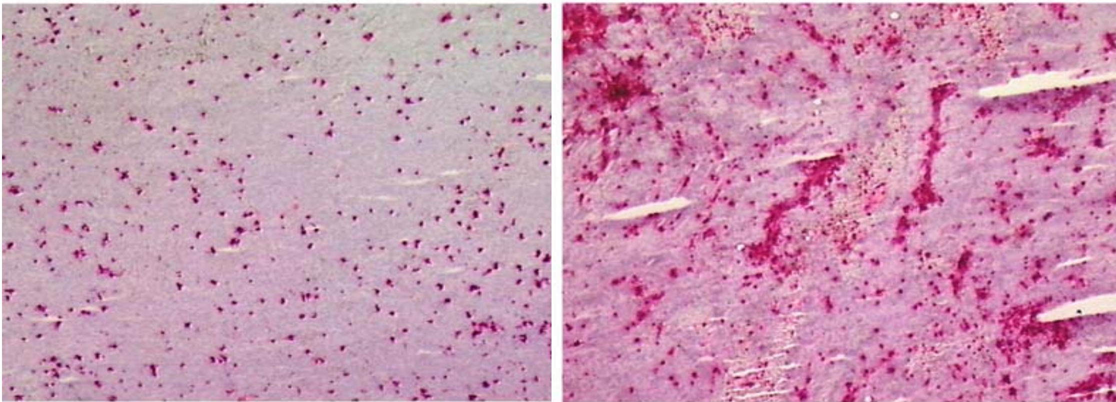


Figure 1. Murine IL-12 influence on NK cell population in murine B16 melanoma s.c. tumor. Visualized infiltration of NK cells (anti-asialo-GM1 - red labeling) around microvessels (anti-Pecam-1 - light brown labeling). ***A***, NK in control, non-IL-12-treated mice tumor; ***B***, NK perivascular localization in tumor of SFV-mIL-12 treated mice.

This observation prompt us to hypothesized, that in tumor microenvironment, NK cells are able to actively recognize the tumor vasculature. The next question was, if NK cells would be also able to kill tumoral endothelial cells thus counteracting the angiogenesis.

Consequently, because we have at our disposal a panel of human and murine endothelial cell lines [2, 3] we next elaborated the *in vitro* model presented below, aimed to answer these key questions for future cellular immunotherapy of cancer.

*B16 melanoma cells were injected s.c. into C57Bl/6JRj mice. Animals with progressively growing tumors received a single intratumoral injection of SFV-mIL-12 vector [1].

INTRODUCTION

WHAT NATURAL KILLER CELLS ARE?

Lymphocytes circulating in blood are constituents of the immune system defending the host against an attack of viral, bacterial or parasitic pathogens as well as against cancer. Lymphocytes are generally divided into T, B and NK (natural killer) cells. T and B lymphocytes are effectors of the adaptive immunity, while NK cells are involved in the nonspecific immune response. Hence, NK cells differ from T and B lymphocytes by the permanent presence of a substantial fraction of educated and primed cells [4, 5].

NK cells have the ability to recognize and destroy a broad array of pathological cells, including neoplastic cells, virus-infected cells, antibody coated cells, foreign transplants as well as "stressed" cells, without damaging normal healthy 'self' cells belonging to the organism [6]. NK cells are therefore characterized by several important effector functions, initiating and amplifying the inflammatory response, producing cytokines and chemokines and lysing susceptible target cells [7].

NK cells are now defined as a subset of $TCR^{-}BCR^{-}$ lymphocytes, which means that they do not express genes encoding the T cell receptor (TCR) nor the B cell receptor (BCR) [6]. The most reliable molecular markers which define human NK cells, are the presence of CD16 and/or CD56 and the absence of CD3 cell surface molecules [7]. CD16 (FcγRIII) is an immunoglobulin heavy chain receptor, that recognizes antibody-coated targets and mediates antibody-dependent cellular cytotoxicity (ADCC) [8]. The direct evidence demonstrating the biological function of CD56 (*neural cell adhesion molecule*; N-CAM) has not been provided yet [4]. Based on the CD16 and CD56 molecules expression levels, NK cells from normal adults can be subdivided into two subsets, with distinct functions and different homing properties. The majority of NK cells express low levels of CD56 (CD56dim) and high levels of CD16 (CD16bright). These CD56dim/CD16bright NK cells are highly cytotoxic. The second subset of NK lymphocytes contains CD56bright cells lacking or expressing low levels of CD16 molecule. They are less effective in cytotoxicity, as compared with CD56dim/CD16bright cells, but are very potent cytokine producers [9, 10]. Moreover, these NK cell subpopulations differ in their trafficking properties: the CD56bright NK cells are located mainly in lymphoid organs, while the CD56dim NK cells are the most prominent subset of blood circulating NK cells.

RECEPTORS TURNING NATURAL KILLER CELLS ''ON'' AND ''OFF''

Advancement in the knowledge on NK cell mediated cytotoxicity began with ascertaining their role in the multiple mutual molecular interactions. NK cells distinguish between normal, healthy and pathologically changed cells using a sophisticated repertoire of cell surface receptors. The list of human NK cells adhesion, inhibitory and activatory receptors and their known ligands is presented in Table 1. Target cell recognition, which involves initial binding, mainly due to cell adhesion molecules, is followed by interactions between NK cell activating and inhibitory receptors, with their appropriate ligands available on the target cell. The combination of signals transmitted by these receptors decides whether the NK cell detaches from the target cells or stays and responds [11].

According to previously accepted "missing-self" hypothesis, the function of NK cells was to recognize and eliminate cells that fail to express 'self' major histocompatibility complex (MHC) class I molecules [12]. The recent experimental evidence shows that NK cells provide immune surveillance not only for cells that lack MHC class I, but also for those overexpressing ligands for NK cell activating receptors [11]. NK cell, whose inhibitory/activatory receptors neither recognize MHC class I molecule nor an activating ligand on the target cell surface, does not respond (Fig. 2A). Similarly, no answer is observed when inhibitory NK cell receptors suppress NK cell cytotoxicity, after binding to their proper ligand (MHC class I molecule) on target cell (Fig. 2B). NK cell executes its cytotoxicity toward target cell when NK cell activatory receptor recognizes its ligand on the surface of target cells and receives no signal from inhibitory NK cell receptors (Fig. 2C). Upon interaction with target cells expressing ligands for both inhibitory and activating receptors, the outcome is determined by the summation of the antagonistic signals strength. The dynamic equilibrium regulates NK cell activation and dictates whether or not NK cells will be able to kill target cell (Fig. 2D) [11, 13].

Receptor	Family	Ligand
ADHESION RECEPTORS		
CD2 (LFA-2)	immunoglobulin	CD48, CD58 (LFA-3)
CD11a (LFA-1)	immunoglobulin	CD54 (ICAM-1), CD102 (ICAM-2)
CD11b (Mac-1)	immunoglobulin	CD54 (ICAM-1)
CD43 (sialoadhesin SIGLEC 1)	sialic acid binding immunoglobulin like lectin	CD227 (MUC1), CD206 (Man Receptor) Alpha 2,3 sialyl
CD44	immunoglobulin	Hyaluronic acid
CD56 (N-CAM)	immunoglobulin	?
CD223 (Lag3)	immunoglobulin	HLA class II
INHIBITORY RECEPTORS		
CD161 (NKR-P1A)	C-type lectin like	LLT-1
KLRG-1	C-type lectin like	Cadherins
SIGLEC 7,9	sialic acid-binding immunoglobulin-like lectin	Sialic acid
CD94/NKG2A	C-type lectin like	HLA-E
LAIR	immunoglobulin	Collagens
CD85 (ILT-2)	immunoglobulin	HLA class I
KIR-L	immunoglobulin	HLA-C, B and A
TGF-βR	Cytokine receptor	TGF-β family
ACTIVATORY RECEPTORS		
CD16	immunoglobulin	Fcγ
CD25 (IL-2Rα)	Cytokine receptor	IL-2
CD122 (IL-2Rβ)	Cytokine receptor	IL-2, IL-15
CD27	TNF receptor	CD70
CD28	immunoglobulin	CD80, CD86
NKp30,NKp44, NKp46	immunoglobulin	Viral hemagglutinins, NKp44L
KIR-S	immunoglobulin	HLA-C
CD94/NKG2C	C-type lectin like	HLA-E
CD94/NKG2E	C-type lectin like	HLA-E
CD69	C-type lectin like	?
NKG2D	C-type lectin like	ULBP/RAET, MICA, MICB
CD160 (BY55)	immunoglobulin	HLA-C
CD244 (2B4)	immunoglobulin	CD48
NTBA	immunoglobulin	NTBA
CS1	immunoglobulin	CS1
CRACC	immunoglobulin	?
α4β1	β1 integrin	VCAM-1, Fibronectin
α5β1	β1 integrin	Fibronectin
α6β1	β1 integrin	Laminin
CR4 (CD11c/CD18)	β2 integrin	LPS, CD23, ICAM-1, Fibrinogen
CD226 (DNAM-1)	immunoglobulin	CD112, CD155
CD96 (Tactile)	immunoglobulin	CD155
CRTAM	immunoglobulin	Ncl2
ILT-1 (Ig-like transcript 1)	immunoglobulin	?
NKp80	immunoglobulin	?
IFN-α/Br	Cytokine receptor	Type I interferons

Table 1. Expression of cell surface markers on human NK cells, and their known ligands [5, modified]

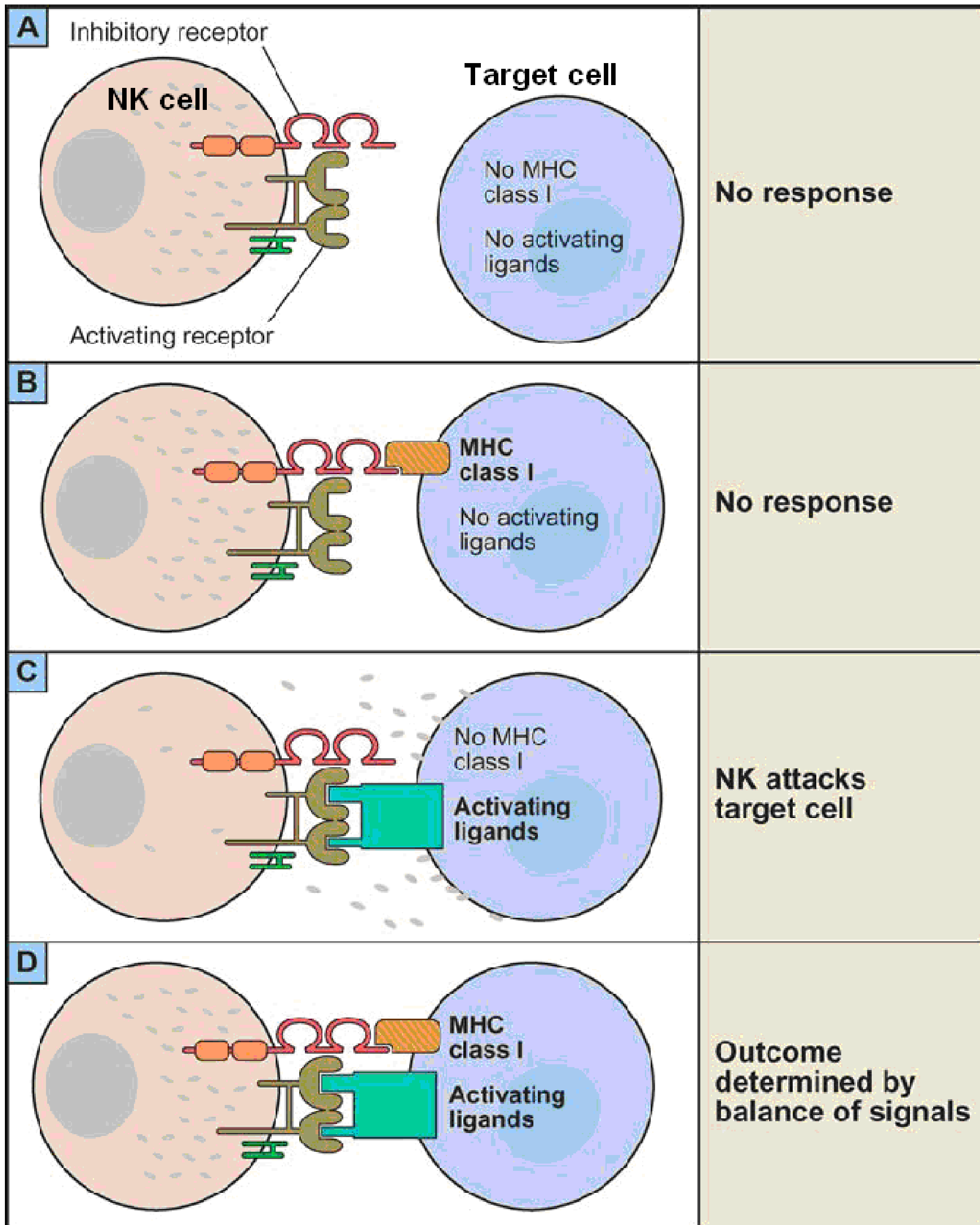


Figure 2. Inhibitory and activating signals which determine NK cell cytotoxicity [11, modified].

NK cells development and their further functional activation are influenced by multiple cytokines such as: interleukins IL-2, IL-12, IL-15 and IL-18, type I interferons (IFNs) as well as chemokines [4, 7, 14]. These activating mediators are not produced by NK cells themselves, therefore they are dependent on other cell types to provide these factors. Upon cytokine stimulation NK cells upregulate their cytolytic activity, demonstrating increased proliferation and production of their own immunoregulatory cytokines and/or chemokines such as: interferon gamma (IFN-γ), tumor necrosis factor (TNF-α), granulocyte macrophage colony stimulating factor (GM-CSF), interleukin 10 (IL-10), interleukin 3 (IL-3), monokine induced protein 1 α and β (MIP-1α and –β), or RANTES.

Among cytokines, particularly IL-2 and IL-15 are known to augument NK cells cytotoxicity and are also involved in interactions between NK cells and other immune cells during development of adaptive immune response [14, 15]. However, it is also known that high doses of IL-2 may directly inhibit NK cells effector functions, which seems to be an inhibitory system which regulates NK cells activity. Reaching the inflammation site, activated T lymphocytes ($CD4^{+}CD25^{+}$) secrete high amount of IL-2 which may reduce further NK cells migration [16]. An enhancement of cytokine secretion by NK cells was observed when submitted to the combination of IL-2/IL-15 with IL-12. IL-12 synergizes with IL-15 or IL-2 in the synthesis of other cytokines, such as TNF-α, IFN–γ and GM-CSF [7, 14, 17, 18]. Interestingly, IL-12 alone induces very weak proliferation of resting NK cells, it may even inhibit NK cell proliferation induced by IL-2. Nevertheless, in response to suboptimal concentrations of IL-2, IL-12 upregulates IL-2 receptor expression on NK cells and enhances NK cell proliferation [19]. Another interleukin modulating NK cell activity is IL-21. It acts synergistically with IL-2 to induce cytolytic NK cell activity [20]. NK cell cytotoxicity may be also stimulated by IFN-α/β. These cytokines upregulate NK cell proliferation [21, 22].

In order to mediate their cytolytic function effectively, NK cells must be recruited to the pathologically changed site. NK cell migration and trafficking processes are mainly regulated by chemokines. Chemokines are a family of small cytokines exerting their biological effects by binding to specific cell-surface receptors, and a given receptor can interact with several chemokines. In the case of circulating cells chemokines are attracting them to the site of inflammation after having been set as a gradient by endothelial cells of the vessels. At that point, interactions between endothelial cells presented chemokines and chemokine receptors expressed by NK cells (CCR2, CCR5, CX3CR1 and CXCR3) allow them to be recruited, establish strong interactions with the vicinal cells adhesion molecules and thus participate in diverse types of inflammatory reactions [5, 23, 24].

Another molecules which are postulated to be involved in recognition of target cells and delivery of signals modulating NK cell cytotoxicity are C-type lectin-like receptors (CTLR), such as CD161, CD69 and CD94 [25, 26]. These molecules, which are not cytokines, belong to lectin family and may recognize a carbohydrate ligand on target cells as well as interact with their proteinacious ligands [27, 28].

THE WAY NATURAL KILLER CELLS KILL

Recent investigations demonstrate that NK cells exert their biological activity by a triad of functions: cytotoxicity, cytokine secretion and co-stimulation of other immunocompetent cells. Consequently, NK cells may eliminate their target cells using different pathways:

Perforin/ granzyme/ granulysin pathway

NK cells contain cytoplasmic granules where proteolytic enzymes, such as perforin, granzymes and granulysin, are stored. Upon activation, NK cells release their granule components. Perforin is a cytolytic protein that, in the presence of calcium cations, can permeabilize a target cell membrane, directly causing target cell death. Perforin may also facilitate effective delivery of granzymes (exogenous serine proteases) to the target cell (Fig. 3) [29, 30].

Recently, mannose 6-phosphate receptor (MPR) has been hypothesized to be the cell surface receptor for granzymes [31]. It has been proposed that under physiological conditions granzyme B bound in complex with glycosaminoglycans is finally endocytosed by a mannose 6-phosphate receptor, and receptor binding is enhanced by cell surface heparan sulfate [32].

Using one of the above pathways, granzymes are delivered into the target cell to induce apoptosis, either by activation of cellular caspases (directly or indirectly, *via* mitochondria), or *via* not well described yet caspase-independent pathway [31, 33-36].

Granulysin is a member of saposin-like protein family. Its structure suggests a potential mechanism of action whereby granulysin functions as a lytic molecule [37]. Positively charged granulysin binds to negatively charged membrane of the target cell. Upon binding, granulysin induces an increase in intracellular calcium and intracellular potassium efflux. These changes induce rapid induction of lysis involving caspases activation and mitochondrial pathway [37].

a) Activation of cellular caspases pathway

Enzymes involved in target cell apoptosis are proteases and nucleases. The major proteases are caspases. These aspartate-specific proteases are expressed in most cell types. In order to preclude unwarranted cell death, executioner caspases (caspase-3, caspase-6 and caspase-7) are maintained as zymogens. Zymogens must be previously proteolytically cleaved by initiator caspases (caspase-8 and caspase-9) to activate executioner caspases. Each initiator caspase has a long pro-domain, and activation is triggered by dimerization of the zymogen on a dedicated adaptor or scaffold protein [35, 36]. Initiator caspase-8 cleaves and activates directly caspase-3 or, alternatively, initiates a mitochondrial apoptotic pathway (Fig. 3). Executor caspases in turn activate caspase-activated deoxyribonuclease (CAD) that degrades DNA.

b) Mitochondrial pathway

As it was mentioned above, granzymes may promote cell death through two main pathways: either through direct caspase activation or through mitochondrial pathway. In this second trail, granzyme B mediates proteolysis of pro-apoptotic protein Bid (*BH3 domain interacting death agonist*; Bcl-2 family member) and targets it to mitochondrion, where Bid recruits Bax and Bak proteins, which change the mitochondrial membrane permeability. This results in the release of cytochrome c and other pro-apoptotic factors from mitochondria. Released cytochrome c initiates the formation of heptameric signaling complex (apoptosome) consisting of Apaf-1 (*apoptotic protease activating factor*) and procaspase-9. This in turn activates caspase-3 (Fig. 3). Activated caspase-3 cleaves nuclear lamins, inducing nucleus breakdown which finally results in internucleosomal fragmentation of genomic DNA [35, 36].

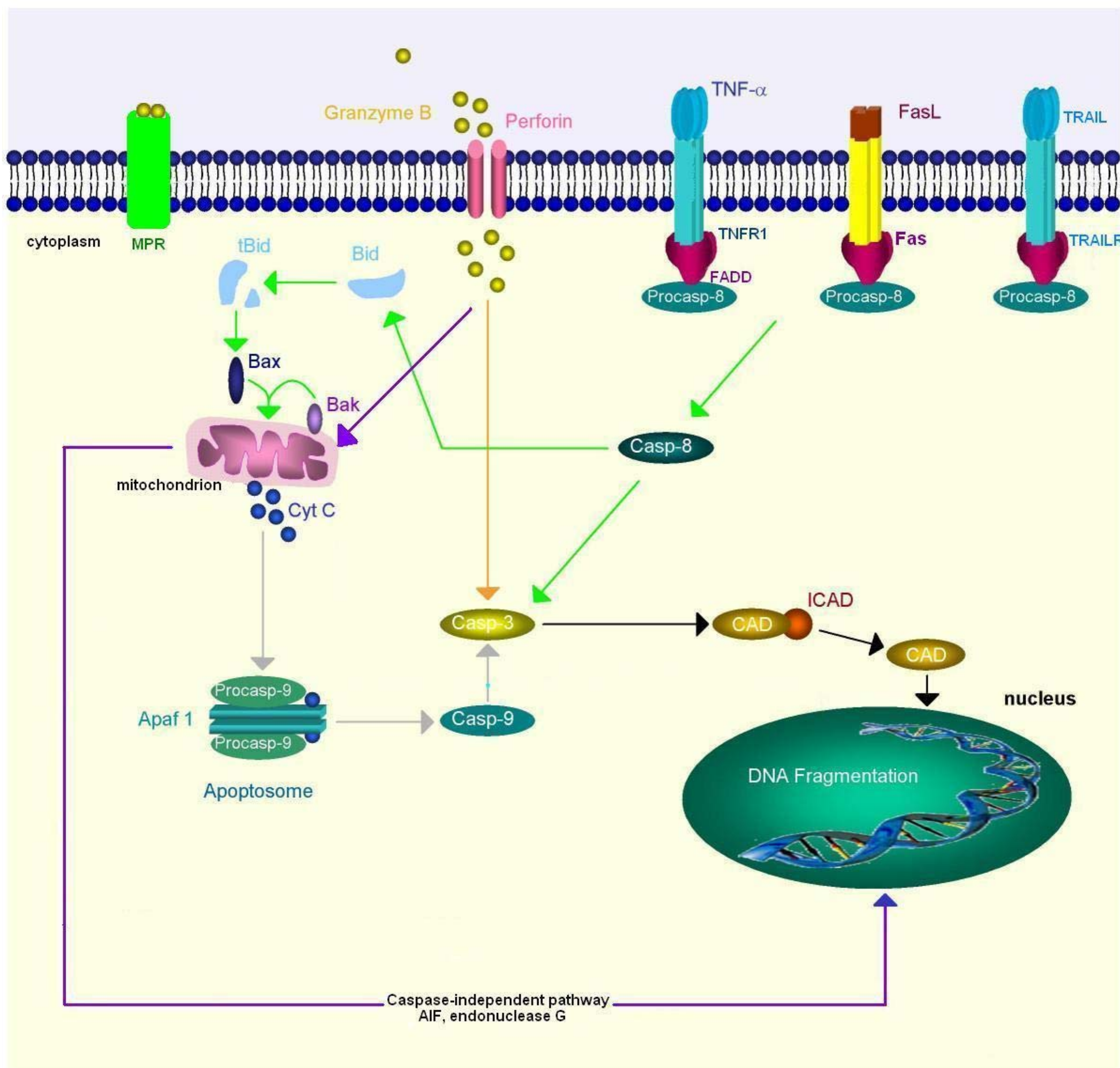


Figure 3. Schematic representation of different pathways involved in target cell apoptosis [38, modified]

c) Caspase - independent pathway

Pro–apoptotic proteins: apoptosis inducing factor AIF and endonuclease G released from mitochondria into the cytoplasm as well as granzymes, have been proposed to contribute to a caspase-independent cell death pathway. These factors have nucleolytic activities sufficient to induce apoptosis, involving chromatin condensation and further large-scale DNA fragmentation or generation of single stranded DNA nicks [31, 35].

Death receptor-mediated apoptosis

This pathway involves the involvement of death receptors such as: Fas, TNFR (*tumor necrosis factor receptor*) or TRAILR (TNF-*related apoptosis-inducing ligand receptor*) expressed on target cell

surface. These molecules contain an intracellular globular protein interaction domain, termed death domain (DD). Upon binding of cognate ligands, namely: FasL, TNF or TRAIL, the caspase-dependent apoptosis machinery is triggered leading to target cell apoptosis [39]. FasL, TNF and TRAIL are members of TNF cytokines family, that may be expressed by effector lymphocytes. It is known that a highly cytotoxic, IL-2-dependent human NK cell line (NK-92) expresses high levels of these cytotoxic effector molecules [40]. Although there are differences in the signaling pathways activated by different death receptors, it is possible to outline a general apoptotic signaling pathway. Receptor-ligand interaction activates signaling through a cytoplasmic receptor death domain, which further interacts with signaling adaptors FADD (Fas-*associated protein with death domain*), finally activating initiator caspase-8. Apoptosis machinery is further induced either *via* the direct activation of cellular caspases or indirectly *via* the mitochondria (Fig. 3).

Antibody Dependent Cell Cytotoxicity (ADCC)

CD16 antigen is a low-affinity immunoglobulin receptor that recognizes antibody-coated target cells [8]. It is one of the best-characterized membrane receptors involved in antibody-dependent cellular cytotoxicity. Cross-linking of CD16 on NK cells with antibody-coated target cells results in release of regulatory cytokine (IFN-γ) as well as cytotoxic granules containing perforin, and granzymes, whose promote target cell death by triggering apoptosis [11]. Certain other cell types, e.g. macrophages and polymorphonuclear leukocytes, may also mediate ADCC, however, these cell types require higher levels of immunoglobulin expression on target cells than NK cells [41].

Cytokine induced cytotoxicity and effector functions

In addition to being cytotoxic, NK cells may also produce a range of cytokines [42]. As opposed to the previously described killing pathways NK cells do not require direct contact with target cells to induce cytokine mediated apoptosis. Upon stimulation, NK cells may rapidly start to produce numerous cytokines and chemokines, including INF-γ, TNF-α, IL-3, GM-CSF, IL-10 and MIP-1. Through cytokines release, NK cells initiate other components of the innate, as well as adaptive immune system: dendritic cells, or T and B lymphocytes [43]. It appears that in viral as well as bacterial infections, IFN-γ produced by NK cells is recognized as a key mediator in defense against these infections [44].

Murine NK cells resemble their human counterparts in many regards, including their cytotoxic ability and cytokine production. Initially, several antigens were described as murine NK cell specific, including CD161 antigen (NK1.1) in C57BL/6 mice or CD49b (integrin α2) recognized by the DX5 antibody in most inbred mouse strains. However, now it is known that these cell surface antigens may be also expressed on T cells and granulocytes. Moreover, murine NK cells also bear signatures of other cell types, expressing low levels of antigens present on dendritic cells, B cells as well as T cells. Hence, a truly NK specific antigen has not been so far identified in mice, and for mouse NK cell identification, a combination of several antibodies recognizing pan-NK cell determinants should be used. Murine NK cells should be identified in mouse strains that are CD161 positive as $CD3^{-}$ $CD161^{+}$ $CD122^{+}$ cells. In mouse strains that are CD161 negative NK cells can be detected as $CD3^{-}$ $CD49b^{+}$ $CD122^{+}$ cells [15].

Murine NK cells functions are regulated, similarly as in humans, by a balance of positive and negative signals provided by a variety of cell surface receptors. Murine NK cells also appear to be strongly inhibited by receptors that recognize MHC class I molecules and must as well receive positive signals from target cells in order to be activated [45, 46]. This balance in the signaling strength could very well dictate the nature of NK - mediated effector functions. Adhesion, activatory and inhibitory receptors on mouse NK cells and their known ligands are presented in Table 2.

Receptor	Family	Ligand
ADHESION RECEPTORS		
CD2 (LFA-2)	immunoglobulin	CD48, CD58 (LFA-3)
CD11a (LFA-1)	immunoglobulin	CD54 (ICAM-1), CD102 (ICAM-2)
CD11b (Mac-1)	immunoglobulin	CD54 (ICAM-1)
CD43 (sialoadhesin SIGLEC 1)	sialic acid binding immunoglobulin like lectin	CD227 (MUC1), CD206 (Man Receptor) Alpha2,3 sialyl
CD44	immunoglobulin	Hyaluronic acid
CD49b (DX5)	immunoglobulin	?
INHIBITORY RECEPTORS		
CD94/NKG2A	C-type like lectin	Qa-1b
CD161 (Nkrp1d)	C-type like lectin	Clr-b (NKR-P1D)
Ly49 A-C, E-G, I-O	C-type like lectin	H-2 classI
KLRG1	C-type like lectin	?
TGF-βR	cytokine	TGF-β family
CD244 (2B4)	immunoglobulin	CD48
ACTIVATORY RECEPTORS		
CD16 (FcγRIII)	immunoglobulin	Immune complexes
CD27	TNF receptor	TRAF CD70
CD28	immunoglobulin	CD80, CD86
CD69	C-type like lectin	?
CD94/NKG2C, E	C-type like lectin	Qa-1b
CD122 (IL-2Rβ)	cytokine	IL-2, IL-15
CD161 (Nkrp1c,f)	C-type like lectin	Clr-g (NKR-P1F)
CD244 (2B4)	immunoglobulin	CD48
NKG2D	C-type like lectin	H60
Ly49D, H, P	C-type like lectin	H-2 class I, MCMV m157 (Ly49H)
NKp46	immunoglobulin	Viral hemagglutinins
IFN-α/βR	cytokine	Type I interferons
gp49A	C-type like lectin	?

Table 2. Expression of cell surface markers on murine NK cells and their known ligands [5, modified]

ENDOTHELIUM: CHARACTERISTICS AND FUNCTIONS

Endothelium is the name of ECs monolayer lining the lumen of the entire vascular system. Endothelium is actively involved in many vital functions of the cardiovascular system, e.g. fluids and solutions exchange, haemostasis and coagulation, intercellular interactions, as well as inflammatory response. The strategic location of endothelium allows it not only to “sense” changes in hemodynamic forces and blood-borne signals, but also to be involved in a network of interactions between cells, cellular factors, humoral factors and matrix components [47]. It should be emphasized that there are phenotypic differences among endothelium located in the particular areas of vascular tree as well as between arterial and venous endothelium [48, 2].

Endothelium is directly involved in the vessel wall integrity and circulatory functions. As a semipermeable barrier they control the transfer of small and large molecules. Another important

function of endothelium is to serve as a point of exit for leukocytes from the blood stream into underlying tissue [2, 48]. This extravasation process occurs at specialized postcapillary vascular sites called high endothelial venules (HEVs). In humans, HEVs are found in all secondary lymphoid organs, including numerous lymph nodes dispersed in the body, tonsils, Peyer's patches in small intestine, appendix, and small aggregates of lymphoid tissues in stomach and large intestine [49].

The orchestration of systemic immune responses is critically dependent on coordinated lymphocyte migration and recirculation. Lymphocyte homing is a multistep process that requires chemotaxis and cell adhesion coupled with strategies to overcome physical barriers. At the molecular level, it is regulated by adhesion molecules and chemokines allowing highly effective lymphocyte traffic between different tissue compartments. In case of malignant transformation homing permits rapid tumor dissemination irrespective of the conventional anatomic boundaries limiting early spread in most types of cancer. Understanding the molecular mechanisms underlying this behavior may provide novel targets for treatment of cancer patients [50].

ENDOTHELIAL CELL: CHARACTERISTICS AND FUNCTIONS

Common markers identifying endothelial cells (ECs) are: von Willebrand Factor (vWF), CD143 *(angiotensin converting enzyme; ACE*), CD31 - PECAM-1 (*platelet endothelial cell adhesion molecule-1*); CD144 - VE-cadherin (*vascular endothelial cadherin*), CD34 and CD102 - ICAM-2 (*intramolecular cell adhesion molecule-2*) [51]. Several inducible molecules: CD54 - ICAM-1 (*intramolecular cell adhesion molecule-1*), CD - 106 VCAM-1 (*vascular cell adhesion molecule-1*), CD62E - E-selectin, CD62P - P-selectin and CD309 - VEGFR-2 (*vascular endothelial growth factor receptor-2, Flk-1*) appear, or their expression level increases, on ECs after activation by inflammatory cytokines or growth factors. Pro-inflammatory cytokines generated early during inflammation up-regulate the expression of ECs adhesion molecules. In addition to cytokine regulation, angiogenic factors may also modulate endothelial adhesion molecule expression. Finally, small bioactive chemical compounds (e.g. nitric oxide) as well as local level of oxygen may also induce these activation-dependent endothelial adhesion molecules [52]. This suggests that, in addition to organ-specific adhesion molecules profile, a population of inducible endothelial adhesion molecules may be modulated and further involved in several ECs functions. These temporal and spatial expressions of various cell adhesion molecules results in specificity and selectivity of different endothelium functions. This selectivity is regulated also by a specific arrangement of cytokines, chemokines and adhesion receptors that guide circulating cells to

specific locations. Chemokines, which are presented on the surface of endothelial cells *via* glycosaminoglycans, seem to play an essential role in the selective regulation of lymphocyte homing [24].

INTERACTIONS BETWEEN LEUKOCYTES AND ENDOTHELIAL CELLS

Leukocytes (including NK lymphocytes) circulating in the blood protect us against blood-borne pathogens and are rapidly recruited from blood to sites of inflammation and tissue damage. During this process circulating lymphocytes selectively bind to HEVs, while ignoring normal vascular endothelium. Inflammation results in hemodynamic changes at the site of injury, where resident cells start to communicate with circulating effector cells by cytokines and direct cell-cell contact. The irritated tissue triggers enhancement of adhesive properties in the local microvascular ECs [53]. Hence, extravasation needs first a recognition step which involves active mechanisms of lymphocyte-endothelial-cell interactions.

Transendothelial migration into sites of inflammation is generally referred to as a 'multistep adhesion cascade'. Most adhesion molecules involved in these interactions are from one of the three molecular superfamilies: selectins, integrins or immunoglobulin-like molecules as well as chemokines [54-56].

The cascade is initiated by inflammatory endothelium activating signals, which in turn displays upregulated E- and P-selectins expression (Fig. 4). Interactions between endothelial selectins and some of their glycosylated ligand, e.g. CD162 - PSGL-1 (*P-selectin glycoprotein ligand-1*) expressed by leukocytes, slow down their velocity in the bloodstream and induce transient rolling on ECs surface under hemodynamic shear forces. According to their site and organ specificity ECs may also express selectin ligands, such as GlyCAM-1 (*glycosylation dependent cell adhesion molecule-1*), CD34, podocalyxin and/or MAdCAM-1 (*mucosal addressin cell adhesion molecule-1*), which are recognized and bound by leukocyte L-selectin. Subsequently, activated ECs adhesion molecules are recognized by integrins expressed on activated lymphocytes that have been recruited to the site of inflammation [54]. *Via* integrins, interacting with molecules belonging to immunoglobulin superfamily, leukocytes adhere firmly to endothelium. ICAM-1 and ICAM-2 molecules expressed by ECs are recognized by LFA-1 (*lymphocyte function-associated antigen-1*) integrin, whose expression is augmented on activated lymphocytes, while VCAM-1 molecule expressed by ECs interacts with VLA-4 (*very late antigen-4*) receptor on lymphocytes.

The next step of the transmigration process involves also attraction and stabilization of leukocyte adhesion by chemokines that are immobilized on the surface of vascular ECs by various means, among which the glycosaminoglycans play the key role as displaying the chemoattracting gradient by binding specifically chemokines in such a way, that the active site is available for binding to the receptor on an another cell. Thus, chemokines can bind to leukocytes *via* a variety of receptors expressed on a given leukocyte population. In the case of NK cells, subsets chemokines receptors: CXCR1, CXCR3, CXCR4, CCR7 are expressed in various combinations. Therefore, the regulated expression of chemokines and their receptors is a critical determinant for differential, tissue oriented trafficking of NK cell subsets [23].

The coordination of these molecular interactions between ECs and leukocytes results in their effective transendothelial migration. Diapedesis through the vessel wall is the final step in the transmigration process. Subsequently, bound leukocytes can cross the endothelial layer, using either a paracellular pathway, through intercellular junctions, or a transcellular pathway through the EC body [54, 55]. Finally, leukocytes perceiving and accumulating signals from multiple chemoattractant sources can migrate to their destination site [55]. During this process, NK cells do not behave aggressively toward ECs.

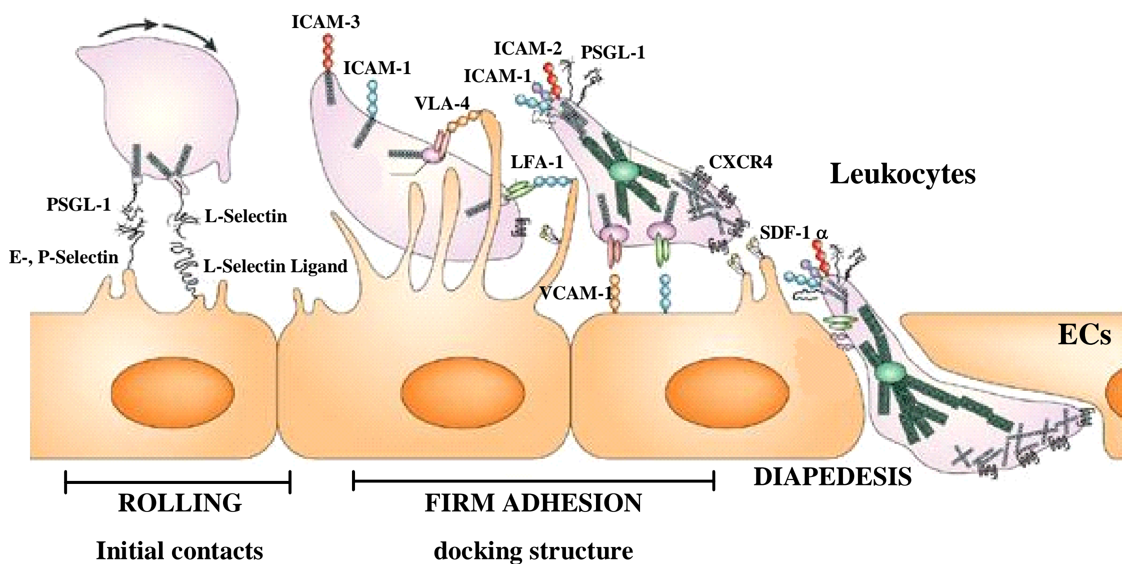


Figure 4. The multistep leukocyte adhesion cascade [57, modified]

Interactions between NK cells and ECs

Under physiological conditions NK cells interact with ECs present at local vascular endothelium but do not kill them. During pregnancy NK cells are even engaged in promoting angiogenesis. Angiogenesis is the process of the formation of new blood vessel by sprouting of pre-existing ones [58]. It has been observed that uterine natural killer (uNK) cells, which are a major (~70%) leukocyte population within the early pregnant uterus, are an important source of angiogenic growth factors, including angiopoietins and vascular endothelial growth factor-C (VEGF-C), which affect the migration and proliferation of endothelial cells. A fundamental enigma of pregnancy is that the fetal cells constitute an allograft but, in normal pregnancies, they are not perceived as foreign and are not rejected by the maternal immune system. Uterine NK cells remain non-cytotoxic to the fetal ECs. In spite of expressing the whole panel of activatory receptors, the presence of non-classical MHC class I molecule (HLA-G) on fetal ECs inhibits the uNK cells cytotoxicity. However, women with recurrent miscarriage have more activated uNK cells accumulated at the endometrium than control ones, and a possible participation of uNK cells in spontaneous abortion is still a matter of discussion [59-61].

It has been observed that human NK cells can exert cytotoxicity against porcine ECs in xenografts. These cytotoxic interactions are induced by the expression of 'non-self' MHC class I molecules by porcine ECs. Apart from induction of direct lysis *via* perforin/granzyme pathway, human NK cells were also able to mediate ADCC [62, 63]. Presented by NK cells C-type lectin-like receptors (CTLR), such as CD161, CD69 and CD94 are also supposed to be involved in recognition of target cells and delivery of signals modulating NK cell cytotoxicity [25, 26]. It was demonstrated that interaction between CD94/NKG2A receptor expressed on NK cells with its ligand, human leukocyte antigen (HLA-E) on porcine ECs may inhibit NK cell mediated cytotoxicity [64]. According to the current paradigm, chemokines also play role in NK cells interactions with xenotransplanted ECs. Inflammatory chemokines recruit those lymphocytes that express their proper chemokines receptors. But apart from chemoattractant function, soluble chemokines immobilized on ECs surface participate in cell-to cell adhesion process. Therefore, membrane-bound chemokines activate NK cells and initiate NK cell-mediated damage of ECs [65].

SPECIAL FEATURES OF TUMOR ENDOTHELIAL CELLS – TARGETING ANGIOGENESIS IN TUMORS

The relationship of angiogenesis with cancer is of special relevance, since angiogenesis is one of the hallmarks of cancer, playing an essential role in tumor growth, invasion and metastasis. Cumulative studies on tumor vasculature show its unique features depending on tissue specificity, angiogenic micromilieu, tumor grade and stage, host immunity level, and others [66]. Tumor environment is capable to regulate/alter gene expression in ECs mainly due to hypoxia and thereby shape their phenotype. Tumor angiogenic niche induces faster ECs proliferation than that in normal tissue, consequently specific ECs angiogenic switch is observed [67]. It is known that tumor cells under the hypoxic signal secrete angiogenic factors - most prominently VEGF - which initiates tumor vascularization [67]. Moreover, significant differences have been shown in the transcriptome of tumor ECs in comparison to endothelium in the surrounding normal tissue. *In vivo* experiments indicate that created tumor blood vessels are often tortuous and dilated, with excessive branching and numerous dead ends, which results in nonuniform blood flow within the tumor. In solid tumors hypoxia is the pathophysiological consequence of structurally and functionally disturbed microcirculation. Tumors respond to low oxygen tension by enhancing the hypoxia-inducible factor (HIF) – mediated response [68, 69]. Recent insights into cellular and molecular crosstalk suggest a model in which hypoxia, HIF and numerous HIF regulated genes participate in the coordinated collaboration between tumor, endothelium and inflammatory cells, to enhance and promote tumor vascularization [69].

Since tumor vessels display many differences from normal vessels and their vessels wall is composed mainly by ECS that are not genetically unstable, they are potential targets for anticancer therapy. Until now, the efforts were devoted to development of agents that could block ECs activation and proliferation by inhibition of angiogenic growth factors [70]. The effectiveness of the first generation angiogenesis inhibitors is still far from satisfying, due to the fact that tumor vasculature has been understood in an oversimplified manner. It is now known that different tumor types may also acquire their blood supply by mechanisms different from angiogenesis. These include neovasculogenesis realized by recruitment of circulating endothelial progenitor cells, or so called vascular mimicry: the generation of pseudomicrovascular channels by genetically deregulated and aggressive tumor cells. Further experimental efforts and clinical trials were undertaken. The analysis of the past approaches revealed a failure of inhibitors of neovascularization used as a monotherapy against developed tumors. Alternative approaches propose vascular targeting against established, non-proliferating tumor vessels. Finally, the potential of angioprevention (prevention of vascularization) should be also analyzed [70].

Many differences were shown between normal and tumor blood vessels, not only at the level of their morphology but also their physiology as well as the molecules they express. In order to optimize the antiangiogenic therapy, reliable biomarkers of tumor angiogenesis are needed to be used as a target in view of a specific therapy. Overall, how much angiogenic therapy will be engaged in the future to the treatment of cancer patients depends on further advances in the understanding of molecular mechanisms involved in tumor angiogenesis as well as of tumor microenvironment.

AIM OF THE STUDY

The aim of this study was to investigate the possible activation conditions and molecular mechanisms involved in cellullar interactions of human and mouse NK cells with ECs of different tissue origin, and study how they lead to killing of target ECs. This was approached at the level of the attraction/ recruitment as well as the recognition/ adhesion and killing processes.

Considering the fact that ECs are proven to be organ-specific and that their phenotype greatly reflects the state of their microenvironment, it is prompting to imagine that it would be possible to target the tumor environment-modified ECs and further induce their killing by NK cells.

To participate in the elaboration of this new antitumor immunotherapy strategy, a detailed identification of this particular NK cells – ECs cytotoxicity phenomenon is necessary.

MATERIALS AND METHODS

MICE AND CELLS

Mice

FVB/N mice were kept under standard pathogen-free conditions and used at 9 to 10 weeks of age.

Murine endothelial cells for establishing *in vitro* cell lines were isolated from animals bred in the Transgenesis and Archiving of Animal Model laboratory (TAAM, CNRS, Orléans, France).

For the adhesion experiments murine NK cells were isolated from FVB/N mice 6 to 8 weeks of age, bred in Polish Academy of Sciences Medical Research Center (Warsaw, Poland). All manipulations were performed in accordance with the institutional animal care guidelines.

Cells

a) Cell lines and cell culture conditions

Endothelial cell lines were established in Dr. Claudine Kieda's « Cell Recognition » Laboratory in the Center for Molecular Biophysics, National Center for Scientific Research, Orléans, France. Human and murine microvascular ECs were isolated and immortalized as described previously [2, 3] and patented (patent 99-16169). Human microvascular endothelial cell lines used in this work were isolated from: peripheral lymph node (**HPLNEC.B3**), mesenteric lymph node (**HMLNEC**), brain (**HBrMEC**), lung (**HLMEC**) and skin (**HSkMEC.2**). Murine microvascular endothelial cell lines, from FVB/N mouse, were isolated from: bone marrow (**mBMMEC FVB**), brain (**mBrMEC FVB**), peripheral lymph nodes (**mPLNMEC FVB**) and Peyer's patches (**mPPMEC FVB**). Cells were cultured in OptiMEM-I/Glutamax-I medium (Invitrogen Life Technologies) supplemented with 3% heat-inactivated fetal bovine serum (FBS, Invitrogen Life Technologies), 40 mg/mL gentamicin (Panpharma) and 0.5 mg/mL fungizone (Invitrogen Life Technologies). When routinely passaged using 0.05% trypsin/0.02% EDTA (w/v) solution (Biochrom), ECs displayed an approx. 20 hr doubling time.

Human natural killer cell lines: **NKL1** and **NKL2** were established in Dr. Salem Chouaib's laboratory (Human Tumor Immunology Laboratory, French National Institute for Health and Medical Research, Villejuif, France) from peripheral blood of patients with large granular lymphocytic leukemia, according to the method previously described [71]. They were characterized as CD16 positive, while CD56 and CD3 negative cells.

NKL1 cells were cultured in OptiMEM I/Glutamax-I medium, 1% penicillin/streptomycin (Biochrom) and 0.5 mg/mL fungizone, supplemented with 2% heat-inactivated FBS. These lymphocytes were adapted to growth in the absence of IL-2 up to one month. After this time spontaneous apoptosis of NKL1 cells were observed.

NKL2 cells were cultured in OptiMEM I/Glutamax-I medium, 1% penicillin/streptomycin (Biochrom), and 0.5 mg/mL fungizone, supplemented with 200 U/mL rhIL-2 (Aventis Pharma, France). Because these cells did not tolerate FBS for a longer period of time, the medium had to be supplemented with 3% human AB serum (Institut Jacques Boy, France). To avoid a medium adaptation reaction, culture medium was replaced 48 hr before experiments by the medium in which ECs were maintained.

K562 human chronic myelogenous leukemia cell line (ATCC number CCL-243) cells were cultured in RPMI 1640 medium (Biochrom) supplemented with 10% heat-inactivated FBS, 1% HEPES buffer (Invitrogen Life Technologies), 1% penicillin/streptomycin and 1% sodium pyruvate (Biochrom). Cells were used as a target cells in human NK cells cytotoxicity test.

Jurkat human T cell leukemia cell line (ATCC number CRL-8131); **MCF-7** human breast carcinoma cell line (ATCC number HTB-22) and **IGR-Heu** human lung carcinoma cell line [72] were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1% HEPES buffer, 1% penicillin/streptomycin and 1% sodium pyruvate. These TRAIL, TNF and Fas-sensitive cells, respectively, were used as positive controls in the experiments where endothelial cell susceptibility to death receptor ligands was investigated.

A549 human lung carcinoma cell line (ATCC number CCL-185) was maintained in OptiMEM I/Glutamax-I medium, 1% penicillin/streptomycin and 0.5 mg/mL fungizone supplemented with 2% heat-inactivated FBS. Cells were used as a negative control in the experiments where CTLRs expresssion was investigated.

YAC-1 mouse lymphoma cells (ATCC number TIB-160) were maintained in OptiMEM I/Glutamax-I medium, 1% penicillin/streptomycin and 0.5 mg/mL fungizone supplemented with 2% heat-inactivated FBS. Cells were used as target cells in murine NK (**mNK**) cells cytotoxicity test.

All cell cultures were incubated at 37°C, 5% CO_2/ 95% air, humidity saturated atmosphere and medium was changed every 2 - 4 days. All cell cultures were observed at regular time intervals using an inverted microscope (Zeiss) equipped with a UV module and were monitored regularly for mycoplasma contamination (DAPI test - DNA staining with 4',6-diamidino-2-phenylindole dye) and for viability, by Trypan blue dye exclusion test.

b) Primary cells and their culture conditions

Human NK cells (**NKp**) were isolated from peripheral blood of healthy donors using Human NK Cell Enrichment Cocktail (StemCell Technologies) according to the manufacturer's instruction. These CD16 and CD56 positive, CD3 negative ($CD16^{+}$ $CD56^{+}CD3^{-}$) cells used in further experiments. They were cultured in RPMI 1640 medium supplemented with 8% human AB serum, 1% HEPES buffer, 1% penicillin/streptomycin and 1% sodium pyruvate. Even though, 1000 U/mL rhIL-2 was added every two days, these cells died within one month time *in vitro* culturing.

Human peripheral blood mononuclear cells (**PBMC**) were isolated from healthy donors using Ficoll-Hypaque density gradient centrifugation method [73]. Cells were kept in culture in OptiMEM I/Glutamax-I medium, 1% penicillin/streptomycin and 0.5 mg/mL fungizone, supplemented with 2% heat-inactivated FBS. Cells were used as a positive control in the experiments where C-type lectin like receptors expression was investigated.

Murine splenocytes were isolated from homogenized spleen of FVB/N mouse. Erythrocytes were separated on a Ficoll 400 gradient (density 1.081 mg/mL at 21°C). Then, mNK cells were isolated with an R-phycoerythrin (PE) Selection Kit (StemCell Technologies) and CD49b/Pan-NK PE antibody (Becton Dickinson - BD) according to the manufacturer's instruction. The purity of mNK cells was ≥ 85% as determined by flow cytometry (FACS) analysis. CD49b positive cells were used in further experiments. MNK cells were cultured in OptiMEM I/Glutamax-I medium supplemented with 10% heat-inactivated FBS, 1mM non-essential amino acids (NAA; Gibco), 1% HEPES buffer, 1% penicillin/streptomycin, 1% sodium pyruvate and $5x10^{-5}$M β-mercaptoethanol (Sigma). 1000 U/mL rhIL-2 was added every two days unless differently specified. Spontaneous apoptosis of mNK cells was observed after keeping them longer than one month in culture.

FLOW CYTOMETRIC ASSAY

The phenotypes of cell lines used and isolated primary cells were identified using flow cytometry.

Cell staining procedures

Single-cell suspensions were stained using one of the following protocols:

a) Indirect staining procedure

Cells were mixed with appropriate concentrations of different monoclonal antibodies, or their respective isotypic controls, and incubated for 30 min. at 4°C. After washing with PBS, staining by the corresponding fluorochrome labeled secondary antibody was performed for additional 30 min. at 4°C.

b) Direct staining procedure

Cells were mixed with appropriate concentrations of different fluorescent dye-conjugated monoclonal antibodies, incubated for 15 min. at 4°C and finally washed with PBS prior to flow cytometry analysis.

Flow cytometry measurement

Cells were analyzed using different FACS flow cytometers (FACSort, FACSLSR and/or FACSCalibur, Becton Dickinson). Upon excitation at 488 nm with an argon laser, the emission of different fluorochromes such as fluorescein isothiocyanate, phycoerythrin and phycoerythrin - *Cyanin*-5 tandem were measured (their fluorescence emitted at 520 nm - FL-1, 575 nm – FL2 and 670 nm - FL3, respectively). Upon excitation at 650 nm with helium-neon laser, the emission of allophycocyanin was measured at 660 nm – FL-4. Propidium iodide (PI, Sigma Aldrich) was used for dead cell exclusion (emission at 617 nm – FL-2 or FL-3). In each sample at least 5,000 cells were acquired in the analysis region of viable cells (PI negative cells). Data, presented as dot plots or histograms, were processed using CellQuest® software (BD Biosciences).

NATURAL CYTOTOXICITY ASSAY

^{51}Cr-release test

Cytotoxic activity of human NK cells was measured by standard 4 hr ^{51}Cr-release assay. Trypsin/EDTA-detached ECs ($6x10^5$) or K562 cells ($6x10^5$) were labeled for 45 min. with 100 µCi of $Na_2(^{51}Cr)O_4$ (Amersham Biosciences). After incubation, cells were washed three times with medium (RPMI 1640/10% FBS) and $3x10^3$ target (T) cells in 0.1 mL volume per one well were distributed in 96-well microtiter plates (Falcon; BD Biosciences). Different numbers of effector (E) cells (at the ratio E/T = 30/1, 20/1, 10/1 and/or 1/1) were added to a final volume of 0.2 mL. After 4 hr incubation at 37°C in a 5% CO_2 atmosphere, plate was centrifuged at 2000 x g for 2 min., the supernatants (approx. 0.15 mL) were harvested, and their radioactivities were counted on a gamma counter (Cobra 5000 Parkard, Doowners Groove III, USA). Spontaneous ^{51}Cr release was determined by incubating the target cells with 0.1 mL of medium (10% FBS/RPMI 1640) and maximum release was determined by incubating the target samples with 100 µl of 1 N HCl. The spontaneous release was usually less than 25% of the total release. The percentage of lysis was calculated using the following equation:

$$\text{lysis (\%)} = \frac{\text{(experimental release – spontaneous release)}}{\text{(maximum release – spontaneous release)}} \times 100$$

In the blocking experiments EGTA (4 mM) and $MgCl_2$ (2 mM) were added just before the incubation of ^{51}Cr-labeled target cells with effector cells.

Flow cytometric measurement of the cytotoxic activity

The numerous disadvantages of ^{51}Cr release cytotoxicity test led to evaluate cytotoxicity functions of NK cells by using non-invasive flow cytometry assay. Briefly, target murine endothelial cells (T) were labeled with PKH67GL green fluorescent dye [74] and then incubated with effector (E) mNK cells for 4 hr at 37°C at the different E/T ratio (10/1 or 1/1). Triplicate samples were set up for each E/T ratio. As a positive control YAC-1 cells were used. Before acquisition PI (1 µg/mL) was added. Next, flow cytometry analysis was performed. Upon excitation with an argon laser (at 488 nm), PKH67GL green fluorescent dye and PI fluorescence were detected in the FL-1 (green fluorescence emission at 530 nm) and FL-2 channel (red fluorescence emission at 617 nm) of flow cytometer, respectively. In order to distinguish between murine ECs and mNK cells, the gating of fluorochrome-labeled mECs and non-labeled mNK cells was done. Next in each sample at least 5,000 target fluorochrome-labeled mECs were acquired and percentage of PI positive fluorochrome-labeled mECs (double positive target cells) was calculated. Data were processed using CellQuest® software (BD Biosciences).

APOPTOTIC CELL DEATH DETECTION

Apoptosis of ECs induced by NK cells was detected by two-color fluorescence staining. Trypsin/EDTA-detached ECs ($3x10^5$) or positive control cell lines were incubated with the transmembrane potentiometric fluorescent marker 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$, 50 µM, (Sigma-Aldrich) for 30 min. at 37°C to reveal mitochondrial membrane potential [75]. $DiOC_6(3)$ is staining living cells, but not apoptotic cells. After two washings in PBS to distinguish early apoptotic cells, which show a low $DiOC_6(3)$ staining from dead cells, PI was added (1 µg/mL). PI late apoptotic cells are positive, PI and $DiOC_6(3)$ -negative are cells in early apoptotic state, while PI negative and $DiOC_6(3)$ positive are alive cells. Samples were read within 1 hr time with a FACSort (BD) flow cytometer. $DiOC_6(3)$ and PI fluorescences were detected in FL-1 (green fluorescence emission at 501 nm) and FL-2 flow cytometer channel (red fluorescence emission at 617 nm). Data were processed using CellQuest® software (BD Biosciences).

ADHESION TESTS

Static conditions

a) Normoxic conditions

Human ECs were seeded into 24-well plate (Falcon) at a desired concentration and cultured until they reach semiconfluency (~48 hr). Human NK cells were labeled with PKH26GL red fluorescent dye [74] and were then overlaid onto the ECs monolayer at five lymphoid cells to one EC ratio (5:1) in OptiMEM I/Glutamax-I medium. At the day of experiment, number of ECs placed in one well was calculated as a mean number of ECs coming from at least three separate wells. NK cells were allowed to adhere at 37°C for defined time period, under static conditions. Nonadherent cells were removed by at least three gentle washings with PBS.

In the murine cells adhesion tests in order to be distinguished from mNK cells, murine ECs were labeled with PKH67GL green fluorescent dye.

b) Hypoxic conditions

In the adhesion test performed under hypoxic conditions human ECs were cultured during different time periods (0 hr, 3 hr, 6 hr and 24 hr) in 1% oxygen/ 94% N_2/5% CO_2 gas mixture (Air Liquide, Paris, France) introduced under the control of the PROOX sensor (model 110; BioSpherix, Redfield, NY) in an automated PROOX *in vitro* chamber (C-174; BioSpherix). To establish hypoxia, the medium was first carefully degassed, then balanced by being kept under hypoxic atmosphere for 24 hr before being applied to the cell culture. The same medium was used during adhesion tests. Human NK cells labeled with PKH26GL red fluorescent dye were resuspended in hypoxia balanced medium (for at least 72 hr) and overlaid onto the monolayer of hypoxia treated ECs at five lymphoid cells to one EC ratio (5:1).

c) Blocking test performed under normoxic conditions

In the blocking adhesion experiments human ECs were labeled with PKH67GL green fluorescent dye and seeded into 24-well plate (Falcon) at a desired concentration and cultured until they reach semiconfluency (~48 hr). Before the adhesion test human NKL2 cells were preincubated with IgG PE-Cy5, or anti CD161 PE-Cy5 antibodies for 20 min. at RT (20µL Ab/ 100µL PBS). Next, after one washing in PBS, NKL2 cells were overlaid onto the ECs monolayer at five lymphoid cells to one EC ratio (5:1) in OptiMEM I/Glutamax-I medium. Number of ECs placed in one well was calculated as a mean number of ECs coming from at least three separate wells. NKL2 cells were allowed to adhere at

RT for 30 min. under static conditions. Nonadherent cells were removed by at least three gentle washings with PBS.

Adhesion data analysis

The adhesion was detected by fluorescence microscopy (Axiovert 200M; Zeiss) and then the results were analyzed using AxioVision 3.1 software. Alternatively, cells were quantified by flow cytometry (FACSLSR or FACSort, BD). Before flow cytometric analysis, cells (ECs and adhered lymphocytes) were detached using trypsin/EDTA solution to obtain single cell suspension. Samples were read on FACSLSR or FACSort (BD) flow cytometer. FL-2 flow cytometer channel (red fluorescence emission at 617 nm) for PKH26GL red fluorescent dye labeled cells and FL-1 flow cytometer channel (green fluorescence emission at 530 nm) for PKH67GL green fluorescent dye labeled cells were detected. In order to distinguish different cells, the gating of fluorochrome-labeled and non-labeled cells was performed. Percentages of both cells populations in the sample were calculated by CellQuest® software (BD Biosciences), for (R1) gate representing fluorochrome labeled cells and (R2) gate representing non-labeled cells (Fig. 5). Results were re-calculated and presented as the ratio indicating the number of NK cells adhered per one EC [76] (R). When indicated, divalent cations chelators, EDTA (4 mM) or EGTA (4 mM)/$MgCl_2$ (2 mM), were added to the medium just before overlaying NK cells onto ECs. In the adhesion blocking tests NKL2 cells were previously preincubated with proper antibodies.

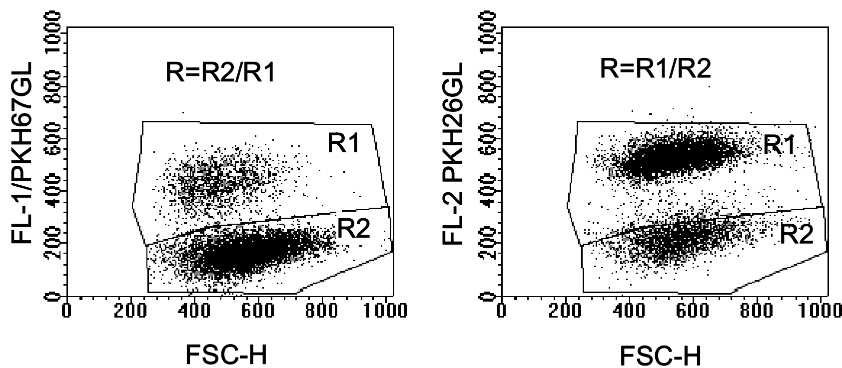


Figure 5. Flow cytometric quantification of adhering cell populations. Dot-plot analysis of a mixture of fluorochrome labeled cells and non labeled cells collected after adhesion test, as assessed by CellQuest® software. Results were re-calculated and presented as the ratio indicating the number of NK cells adhered per one EC (R).

Flow normoxic conditions

Human ECs were seeded onto polystyrene tissue culture slides (Nalgene Nunc International) at the desired concentration in OptiMEM I/Glutamax-I medium without serum. In co-culture experiments, one of the EC lines was first labeled with 5(6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE, Sigma) fluorescent dye. Briefly, trypsin/EDTA-detached ECs ($2x10^6$) were washed with PBS and incubated in CFSE (5 μM) solution in PBS for 10 min. at 37°C. After three washings with 0.5% (w/v) BSA in PBS, cells were mixed in the desired proportions to be seeded together with non-labeled ECs of another tissue origin cell line and were co-cultured in OptiMEM I/Glutamax-I medium without serum at 37°C for 72 hr.

The cell adhesion flow chamber (Immunetics, France) was used to perform dynamic cell adhesion assay in a laminar flow conditions, under semi-physiological shear stress (Fig. 6). Polystyrene tissue culture slides (Nalge Nunc International) with growing target ECs were placed inside the flow chamber. Then, previously labeled with PKH26GL red fluorescent dye NK cells - NKL ($1x10^6$) were injected, under a laminar flow, at the fixed flow rate of 50 μL/min., which correspond to shear stress of 0.75 dyne/cm^2, for 5 min. Recognition, rolling, and adhesion of the NKL cells to ECs were allowed for 5 min., then washing with medium was performed for another 5 min., under the same flow conditions. During the acquisition phase contrast microscopy (Axiovert 200; Zeiss) with Axio Vision software (Zeiss) were used. CFSE green fluorescent dye (green fluorescence emission at 517 nm) and PKH26GL red fluorescent dye (red fluorescence emission at 617 nm) were detected in the FL-1 and FL-2 channel, respectively. Image acquisition was performed using CCD camera (Hamamatsu, Japan) in order to obtain high gain images in very low light. Images were analyzed with the Axio Vision Image Analysis program (Zeiss).

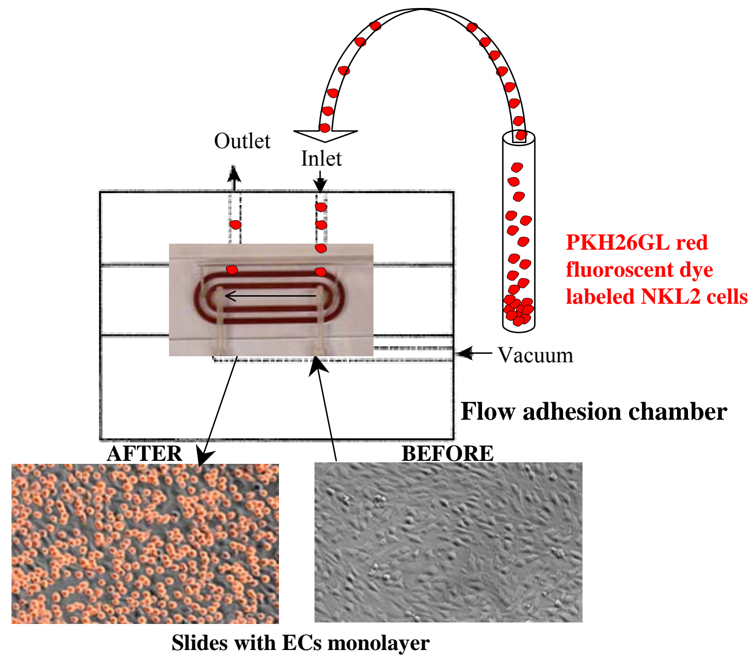


Figure 6. Scheme of PKH26GL red fluorescent dye labeled NKL cells adhesion to ECs monolayer, performed under flow conditions.

CONFOCAL MICROSCOPY ANALYSIS

Human ECs were seeded onto gelatin-coated micro-coverglasses (0.2% gelatin in distilled water; Erie Scientific) at the desired concentrations and were allowed to grow, in culture conditions, for 48 hr. PKH26GL-labeled NKL cells were overlaid onto the ECs in a ratio approx. 10 lymphoid cells to one EC. After 4 hr incubation at 37°C, coverglasses were washed once with PBS, fixed with 4% paraformaldehyde (w/v) solution (Sigma-Aldrich) in PBS for 60 min. at RT, and washed three times with PBS. SDS (0.1% solution in PBS; Bio-Rad) was used to permabilize the cells for 10 min. at RT. After three washings with PBS, free aldehyde residues were blocked by incubation with 10% FBS solution (v/v) in PBS for 20 min. Then cells were allowed to react with antibody against cytochrome c (5 μg/mL, BD Pharmingen) or Bid (4 μg/mL, Santa Cruz Biotechnology) for 60 min. at RT. Cells were washed three times with PBS and incubated with Alexa 488-conjugated goat anti-mouse IgG or Alexa 488-conjugated goat anti-rabbit IgG secondary antibody (Molecular Probes), respectively. Micro-coverglasses were washed and mounted on slides using a drop of Vectashield antiphotobleaching reagent (Vector Laboratories). Cells were analyzed with a confocal microscope imaging system (MRC-1024; Bio-Rad) equipped with an Optiphot epifluorescence microscope (Nikon) and a Planapo objective

(numerical aperture, 1.4). A krypton/argon laser was tuned to produce both 488 nm (fluorescein excitation) and 568 nm (rhodamine excitation) wavelengths. The images were recorded under a Kalman filter (an average of seven images) and were analyzed using the Photoshop software (Adobe Systems).

REVERSE TRANSCRIPTION - POLYMERASE CHAIN REACTION

In order to evaluate an expression pattern of C-type lectin like molecules genes, reverse transcription-polymerase chain reaction (RT-PCR) was used. Total RNA was isolated from $1x10^6$ cells with the use of RNeasy Protect Mini Kit (Qiagen, Germany). First strand cDNA synthesis was performed by reverse transcription of 1µg of total RNA using the Omniscript Reverse Transcription Kit (Qiagen, Germany) according to the manufacturer's instructions. The primer used for the RT reaction was an oligo(dT) and the primers used to amplify C-type lectin-like molecules in the PCR were:
CD69 (203 bp) s:TGATTCTGCTGATCCTGTGC as:ACAGGGGCTGCATTTATGAC CD94 (184, 277 bp) s:TCTCCAGCTCAGCTTCAACA as:TTGGCAAGAACAGCAGTCAG CD161 (172 bp) s:GAGCCGTTTATCCACTTCCA as:AGAATCCAGCCTGCTGCTTA.
The PCR reaction conditions were: 94°C, 5 min.; 30 cycles of (94°C, 1 min.; 60°C, 1 min.; 72°C, 1 min.); followed by final extension at 72°C for 7 min. After amplification, PCR products were separated by electrophoresis on a 1% agarose gel containing ethidium bromide (0.01%) and visualized under UV light illumination.

Results presentation

Data were calculated using MicroSoft™ Excel. The results are expressed as the mean value ± standard deviation (SD) or standard error of the mean (SEM) from at least three independent experiments performed in triplicate (unless differently specified).

RESULTS AND DISCUSSION

THE ORGAN SPECIFICITY DEMONSTRATED BY THE IN VITRO HUMAN ENDOTHELIAL CELLS MODEL

NK CELLS TO ENDOTHELIAL CELLS ADHESION ASSAYS

Human NK cells adhere to human ECs after IL-2 activation

In order to approach the mechanism of recognition between NK cells and ECs the adhesion process was examined first by a static *in vitro* adhesion assay that allowed quantitative flow cytometry estimation, then in the blood flow conditions reconstitution, which allowed to decipher the molecular partners of these interactions.

a) static normoxic conditions

It is documented that IL-2 stimulation is necessary for proper functional activation of NK cells [77, 78]. NK cells can be maintained in *in vitro* culture only in the presence of rhIL-2. However, NKL1 cells isolated from peripheral blood of a patient with large granular lymphocytic leukemia were able to grow in the absence of rhIL-2, while NKL2 cells were kept in culture in the continuous presence of rhIL-2. Consequently, NKL1 and NKL2 cells represent resting and activated NK cells, respectively. The data in Fig. 7 show the resulting adhesion of NKL cells toward ECs lines, when the experiment was done in static conditions. It appears that in those conditions only negligible amount of NKL1 cells was able to adhere to monolayer of any of five human ECs tested; they were found to adhere best to HLMEC originating from the lung (Fig. 7 A). NKL2 cells were in each case able to adhere much more efficiently to the ECs (Fig. 7 B). The difference, as compared to the NKL1 adhesion efficiency indicates that IL2 activation is necessary for this recognition effect. This, indeed, is mimicking the *in vivo* process; consequently, the model designed is significant, as far as the cytokine regulation is concerned. Moreover, as far as the organ specificity is concerned, it was clearly displayed by NKL2 cells toward the HPLNEC.B3 which are derived from the peripheral lymph nodes. A still quite efficient adhesion was displayed toward the HMLNEC and HLMEC as compared to the skin and brain derived ECs. It suggests that NKL2 cells recognize ECs from lymphoid organ preferentially to those from non lymphoid tissues, which means a tissue (organ) specificity of these interactions [2].

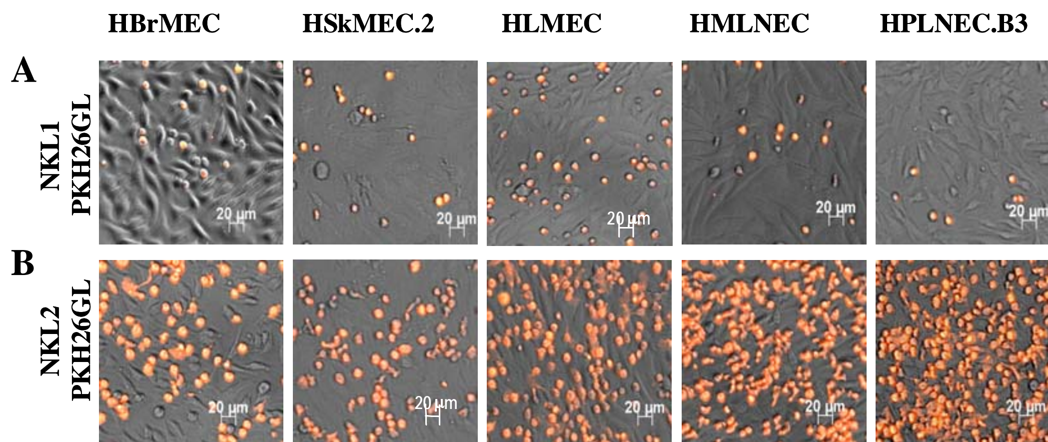


Figure 7. NKL adhesion to monolayers of human microvascular ECs isolated from different organs. NKL1 (cultured without rhIL-2) or NKL2 cells (cultured with 200 U/mL of rhIL-2) were PKH26GL red fluorescent dye labeled. Five NKL1 (***A***) or NKL2 (***B***) cells were overlaid per one EC and incubated at 37ºC for 30 min. under static conditions. Before taking photographs non-adherent NKL cells were removed by gentle washing with PBS. HBrMEC – brain, HSkMEC.2 - skin, HLMEC - lung, HMLNEC - mesenteric lymph node and HPLNEC.B3 - peripheral lymph node-derived human ECs.

In order to quantify the adhesion capacity of NK cells toward ECs flow cytometry was used. This method was chosen because it allows for counting the numbers of adherent NKL cells as well as ECs in the tested sample and further calculation of NKL/EC ratio. As shown in Fig. 8, the adhesion test was performed for three incubation periods (1, 15 and 30 min.). Taking into account the qualitative data seen in the previous figure obtained by fluorescence microscopy, it appears that NKL cells after being labeled by the red fluorescent dye PKH26GL are clearly distinguished and counted by flow cytometry. The data analysis was done as explained in Materials and Methods. It allowed for quantifying the numbers of fluorescent adhering NKL cells (FL-2). The ECs were not labeled and appeared as events with low fluorescence intensity (FL-2), besides the differences in Side Scattered and Forward Scattered signals due to the physical properties (size and granularity, respectively) of the cells.

The data presented in Fig. 8 confirm the microscopic observations. NKL1 cells adhered in low numbers to ECs, while NKL2 cells demonstrated efficient adhesion. After 15 min. almost all ECs (except HSkMEC.2) have bound a comparable number of NKL2 cells as after 30 min. therefore, the adhesion ratios calculated for both, 15 and 30 min. were similar.

As noticed before, the adhesion of NK cells to ECs is specific in an organ restricted manner. Indeed, lymphoid organ-derived ECs: these from peripheral (HPLNEC.B3) and mesenteric (HMLNEC) lymph nodes, bind more NKL2 cells (over 2-fold) than remaining, peripheral tissue-derived ECs tested.

The data obtained clearly showed that rhIL-2 augments NKL adhesion to ECs, which corroborates with experimental results reported by others, where IL-2 rapidly induced NK cell adhesion to human ECs [79]. As it was mentioned before, it is also known that high doses of IL-2 may directly inhibit NK cell effector function: reaching the inflammation site, activated T lymphocytes secrete high amount of IL-2, which results in the reduction of NK cells extravasation [16]. This seems to be an enhancer/inhibitory system which regulates NK cells activity.

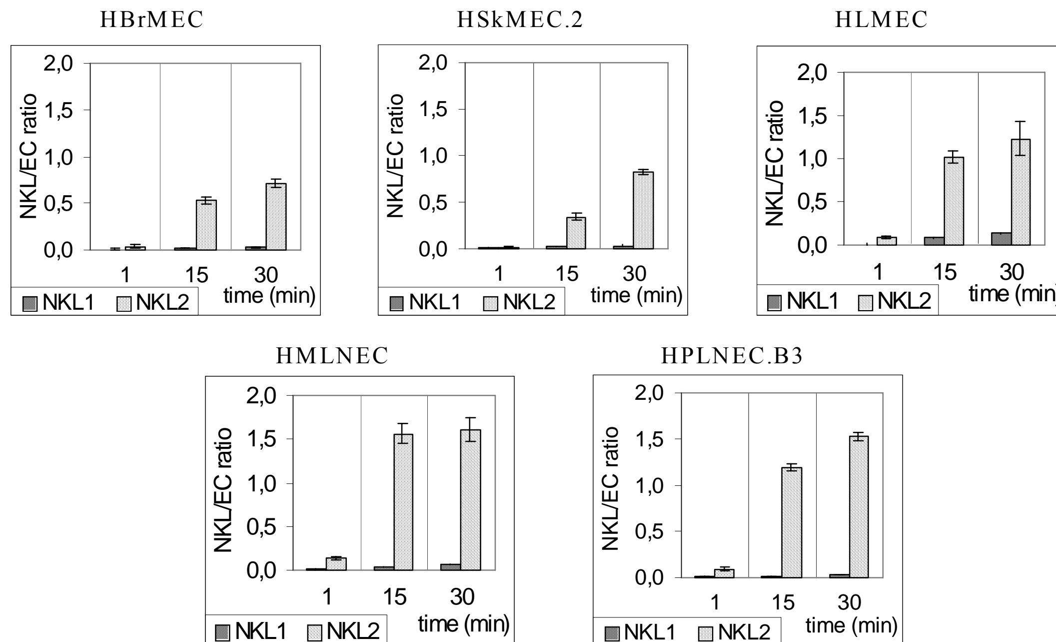


Figure 8. Adhesion of NKL cells to human endothelial cells of different organ origin as a function of time. NKL1 (cultured without rhIL-2) and NKL2 cells (cultured with 200 U/mL of rhIL-2) were labeled with PKH26GL red fluorescent dye. Five NKL cells were overlaid per one EC and incubated at 37ºC for indicated time period, under static conditions. Before acquisition, non-adherent NKL cells were removed by gentle washing with PBS. Then the mixture of ECs and adhering NKL cells was collected with the use of trypsin/EDTA and analyzed by flow cytometry. The bars represent mean ratio numbers ±SD of adherent NKL cells per one EC (n=5). HBrMEC – brain, HSkMEC.2 - skin, HLMEC - lung, HMLNEC - mesenteric lymph node, and HPLNEC.B3 - peripheral lymph node derived human ECs.

This quantitative method has been proven to be highly reproducible in other interaction systems as for the example of the model tumor cells interacting with ECs [76]. In both cases: the tumor phenotype as well as the NK cells recruitment, especially in this microenvironment, is a fundamental factor modulating the ECs biological properties. To take this into account, the general feature of the tumoral milieu, namely the poor level of oxygen (hypoxia) was applied to the cells before and during the adhesion experiments.

b) Static hypoxic conditions

It has been found that along the progression of tumor growth, the tumor cells are in decreasing oxygen levels conditions. In order to restore growth permissive conditions the tumor recruits ECs that undergo proliferation and migration to form capillary tubules within tumor tissue [69]. Therefore, the question was whether these physical conditions present in the tumor microenvironment may have a direct impact on NK - ECs adhesion and their further interactions. To answer this question, static adhesion test under hypoxic conditions was performed (Fig. 9). It was found that the exposure of ECs to hypoxia during different time periods (3 hr, 6 hr and 24 hr) did not modulate significantly NKL2 cells adhesion to ECs of brain and lung origin. As a control, the adherence of NKL2 cells to normoxia kept ECs was evaluated.

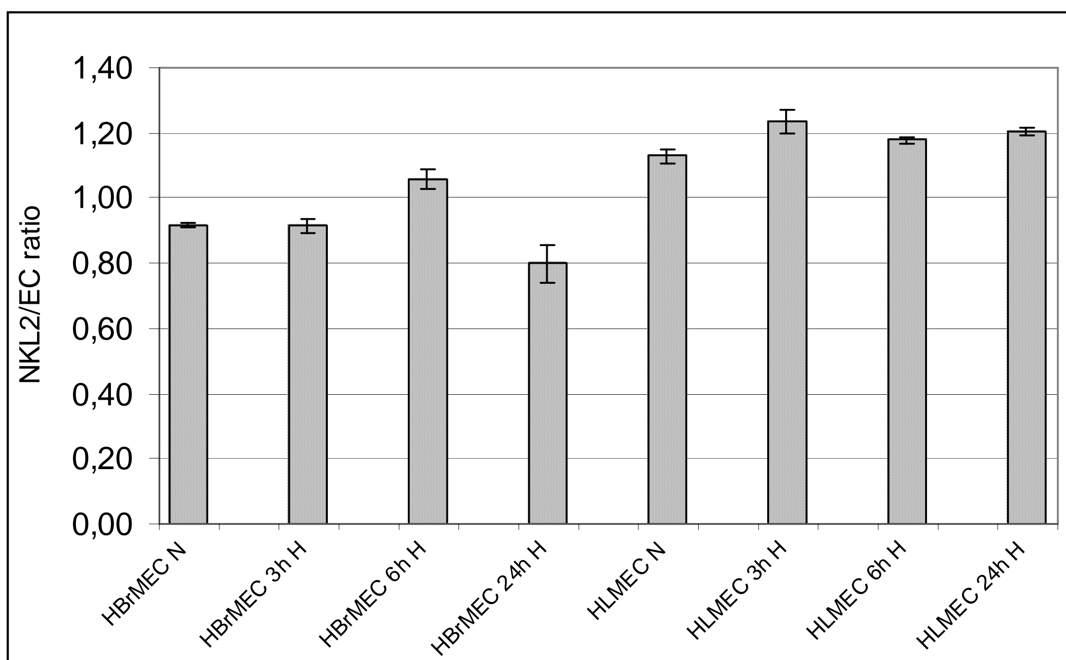


Figure 9. Influence of hypoxia on the adhesion profile of NKL2 cells to human endothelial cells under static conditions. NKL2 cells (cultured in the presence of 200 U/mL rhIL-2) were PKH26GL red fluorescent dye labeled. Five NKL cells were overlaid per one EC and incubated at 37ºC for 30 min. under static conditions. Endothelial cell lines isolated from brain (HBrMEC) or lung (HLMEC) were kept in hypoxic (H) conditions (1% oxygen) for indicated time period (hr). As a control adhesion between NKL2 and ECs kept under normoxic (N) conditions (24 hr in culture) was evaluated. Each bar represents mean ratio value ±SD of NKL2 cells per one EC (n=5).

Next, adhesion profile of NKL1 cells (unstimulated by rhIL-2) was investigated (Fig. 10). NKL1 cells did not increase significantly their adhesion efficiency to hypoxia -treated endothelial cells, as compared to these kept in normoxia conditions. It confirmed the previous reports that hypoxia alone

does not display any effects on NK cells adhesion to human ECs [80]. However, augmented adhesion of NKL2 cells to hypoxia treated ECs was observed under flow conditions [Kieda *et al.* unpublished data].

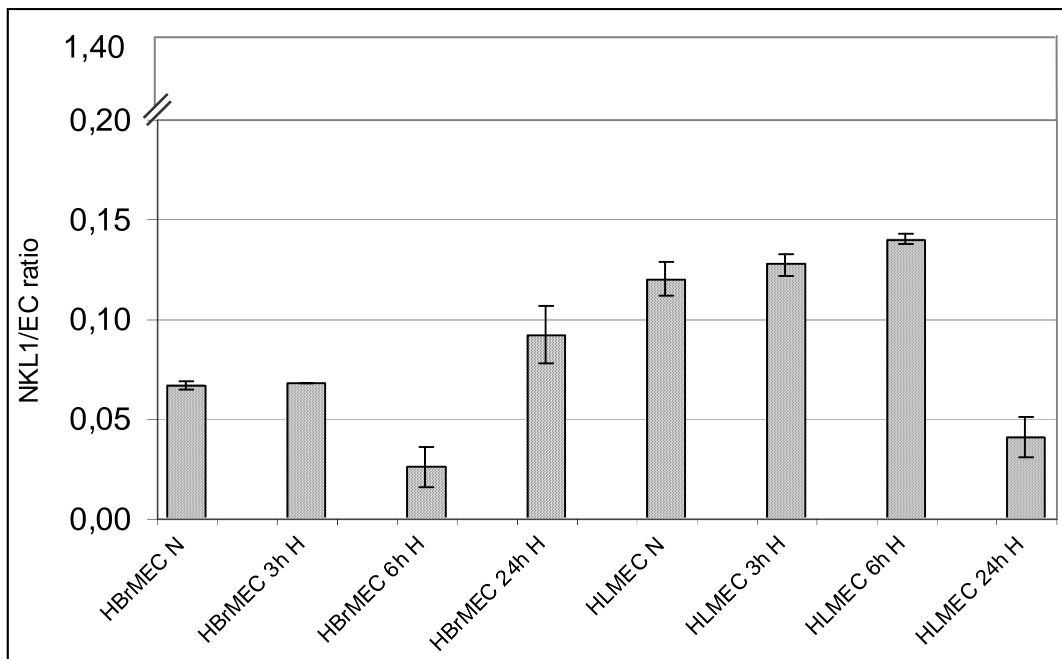


Figure 10. Adhesion profile of NKL1 cells to human endothelial cells under hypoxic conditions. NKL1 (cultured without rhIL-2) were PKH26GL red fluorescent dye labeled. Five NKL cells were overlaid per one EC and incubated at 37ºC for 30 min. under static conditions. Human endothelial cell lines isolated from brain (HBrMEC) or lung (HLMEC) were kept in hypoxic (H) conditions (1% oxygen) for indicated time period (hr). As a control adhesion between NKL1 and ECs kept under normoxic (N) conditions (24 hr in culture) was evaluated. Each bar represents mean ratio value ±SD of NKL1 per one EC (n=5).

It is tempting to speculate that in combination with other factors present in the tumor microenvironment, hypoxia may augment NK cells adhesion. This scenario should be further investigated, in spite of the study which reports, that combined treatment of endothelial cells with TNF-α and hypoxia neither increased the effect of TNF-α treatment [81].

Considering the *in vivo* recruitment of cells at the microvascular bed level, it has been shown that among the major parameters of the microenvironment, the dynamics of the flow that regulates the blood pressure and velocity can modulate expression of many genes of the ECs lining the vessel wall. Consequently, further experiments in flow mimicking conditions were performed.

c) Normoxic flow conditions

This setting allowed for study the interactions of NK cells and ECs in dynamic conditions, applying environmental parameters comparable, as much as possible, to the *in vivo* situation.

Indeed, *in vivo* interactions between circulating NK cells and ECs lining the vessel are a process where various receptor-ligand interactions take place under blood shear stress conditions. Studies performed *in vitro,* but under defined flow conditions, are more informative as compared to those done in static conditions, and may allow for clarifying the conceptual framework of the adhesion process [82]. Therefore, adhesion tests where human NKL cells were allowed to roll and adhere to different ECs monolayers under defined semi-physiological flow conditions were performed. Obtained results confirmed the previously presented data obtained under static conditions, where NKL2 cells were able to efficiently adhere to ECs, whereas NKL1 cells adhesion was negligible. Adhesion of NKL1 and NKL2 cells to HBrMEC are visualized in Fig. 11.

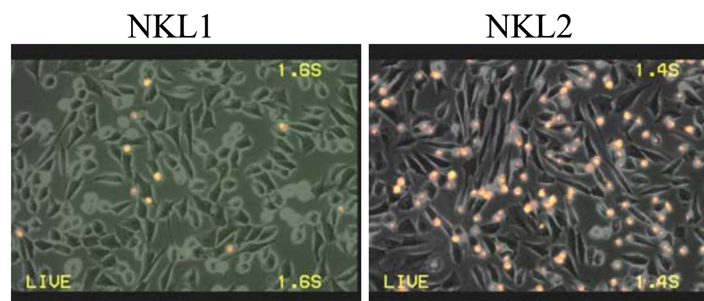


Figure 11. Adhesion under flow conditions of NKL1 (cultured without rhIL-2) or NKL2 (cultured in the presence of 200 U/mL rhIL-2) cells to human microvascular endothelial cell line isolated from brain (HBrMEC). PKH26GL red fluorescent dye labeled NKL cells were flewed over monolayer of HBrMECs under defined shear stress conditions for 5 min. (see Materials and Methods).

The data obtained indicate that our *in vitro* model is a proper tool to use in the purpose to design antiangiogenic cell-based strategies. This also legitimates the further approach of the study of the mechanisms of the NK cells to ECs interactions at the molecular level.

Most of the previous *in vitro* studies which provide information about NK cells interactions with ECs involved human umbilical cord derived endothelial cells - HUVEC [65, 83]. Such studies are clearly limited by the lack of an evident ECs organ specificity, demonstrated by previous works of Dr. Kieda group [2, 3, 84]. In order to look closer for the interactions that govern organ specific adhesion of human NK cells under shear stress, endothelial cells of different tissue origin were used, first in a separate way. Then, we attempted to demonstrate the organ specificity of the adhesion of NKL2 cells

toward the endothelial cells wall by an assay using mixing culture of two EC lines of different tissue origin.

d) Confirming endothelial cells organ specificity in *in vitro* targeting model

An attempt to demonstrate, that ECs lines coming from distinct organs can be representative enough to allow the design of an *in vitro* targeting, had been undertaken. This was done by setting co-culture of two EC lines of different tissue origin. Cells were labeled in order to be distinguished. One of the EC lines was labeled by CFSE (green fluorochrome) before the co-culture. NKL2 cells (red fluorochrome) were allowed to roll and adhere to ECs monolayers, in dynamic flow conditions. It occurred that rolling NKL2 cells were able to discriminate the mesentery-derived ECs (HMLNEC) from the skin-derived ones (HSkMEC.2) (Fig. 12). Consequently, NKL2 cells adhere better to the ECs isolated from mesenteric lymph node than to those from skin. These findings were well in line with the previous data, obtained in static adhesion assay. In these experiments, for the first time a specific organ dependent adhesion was demonstrated by using *in vitro* model under shear stress conditions.

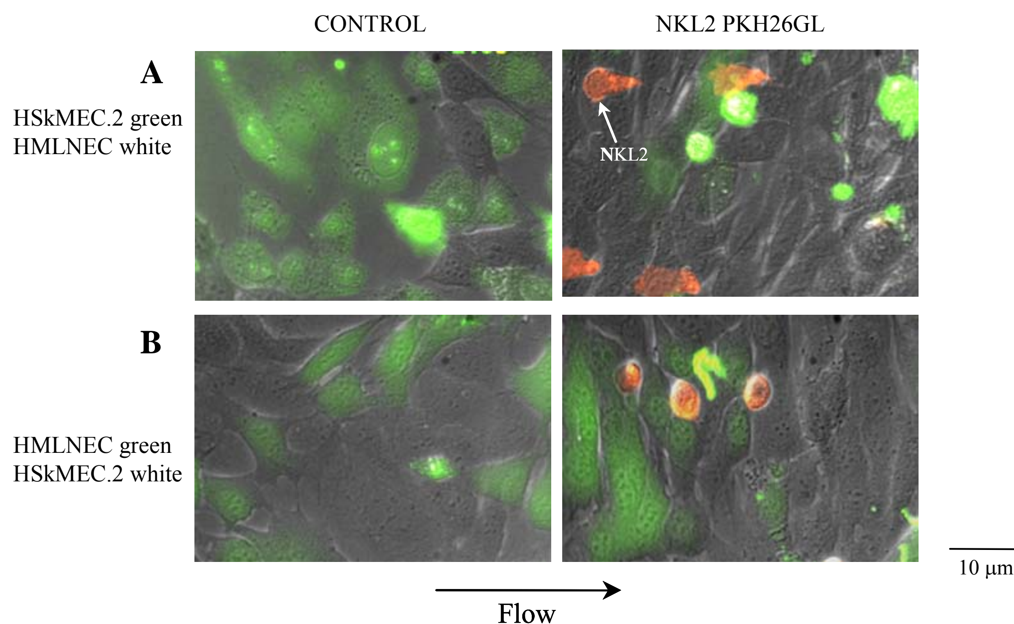


Figure 12. Specificity of human endothelial cell recognition by NKL2 cells under flow conditions. ***A***, left picture - monolayer of co-cultured EC lines isolated from skin (HSkMEC.2, CFSE-labeled) and from mesenteric lymph nodes (HMLNEC, nonlabeled); right picture - PKH26GL red fluorescent dye labeled NKL2 cells were moved over co-culture of ECs under defined flow conditions; ***B,*** left picture - monolayer of co-cultured EC lines isolated from mesenteric lymph nodes (HMLNEC, CFSE-labeled) and from skin (HSkMEC.2, nonlabeled), right picture - PKH26GL red fluorescent dye labeled NKL2 cells were moved over co-culture of ECs subjected the same flow conditions (see Material and Methods).

Molecular mechanisms of adhesion

Leukocyte adhesion cascade is realized in a sequence of adhesion, regulation and activation events involving several different molecules. Integrins and selectins are very important adhesion molecules, while cytokines/chemokines and other co-receptors are necessary for leukocyte effective activation, chemoatraction, recognition and further transmigration [54, 55]. The purpose of the following approaches was to decipher the adhesion molecules that are responsible for the first event that leads to the possibility of killing endothelial cells by activated NK cells.

a) Integrins pathway

Knowing that divalent cations are crucial for the proper activity of some adhesion molecules [85], adhesion tests were performed under static normoxic conditions, during which the effect of cations chelators: EDTA or EGTA/$MgCl_2$, was examined. Concentrations of cations chelators have been previously determined in a separate set of experiments. EDTA is a known chelator of calcium and magnesium ions with almost the same affinity at neutral pH: log K = 7.27 and logK = 5.37, respectively, whereas EGTA is used for selective binding of calcium cations, due to the its higher association constant for calcium: log K = 6.68 as compared with that for magnesium: log K = 1.61 at pH 7 [86]. These chelators were used to assess the participation of integrin molecules in the process of NKL2 adhesion to endothelial cells [87]. EDTA strongly inhibited adhesion of NKL2 cells to ECs monolayers (Fig. 13 B) which indicates that ions are involved, but does not permit to distinguish among Ca^{2+} and Mg^{2+}. Then the total reversal of the inhibition was observed after the incubation with EGTA/$MgCl_2$, (Fig. 13 C) which indicates for the role of Mg^{2+} in the adhesion process.

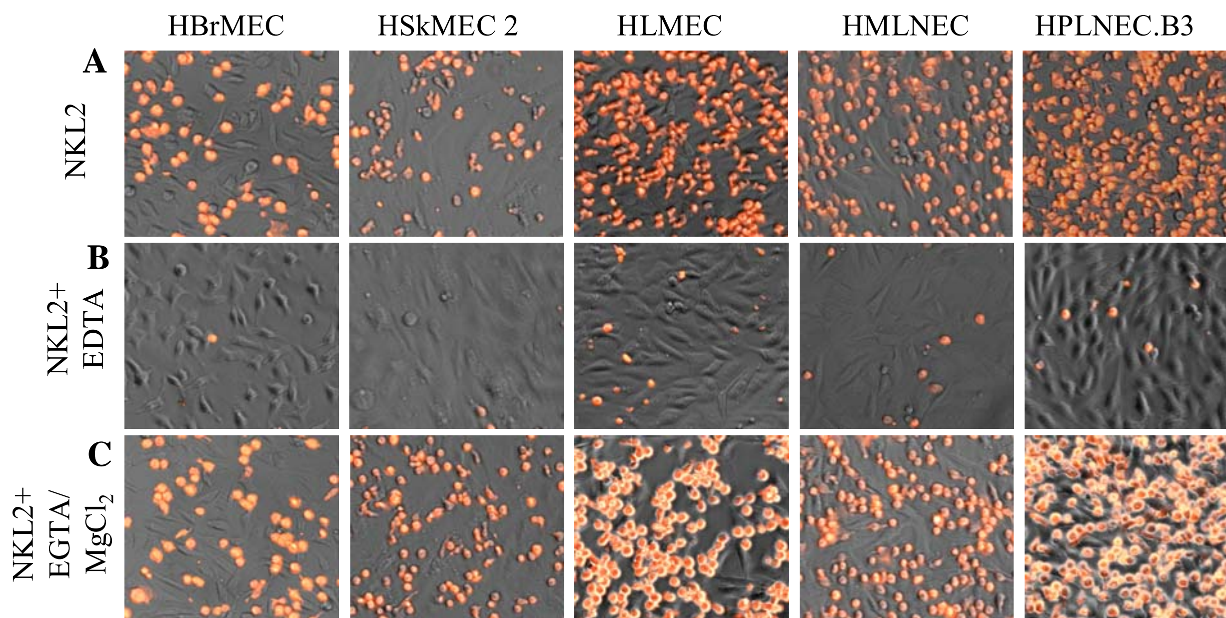


Figure 13. EDTA inhibition of NKL2 cells adhesion to human endothelial cells of different organ origin - microscopic observation. NKL2 cells (cultured with 200 U/mL of rhIL-2) were PKH26GL red fluorescent dye labeled. Five NKL2 cells were overlaid per one EC and incubated at 37ºC for 30 min. under static conditions in medium alone (***A***), in medium with EDTA (***B***) and in medium with EGTA/$MgCl_2$ (***C***). HBrMEC – brain, HSkMEC.2 - skin, HLMEC - lung, HMLNEC - mesenteric lymph node and HPLNEC.B3 - peripheral lymph node derived human ECs.

Similar results were obtained in the adhesion test performed under flow conditions. The representative adhesion results are shown in Fig.14. PKH26GL red fluorescent dye labeled NKL2 cells adhere to HBrMECs monolayer, whereas the adhesion was totally inhibited in the presence of EDTA.

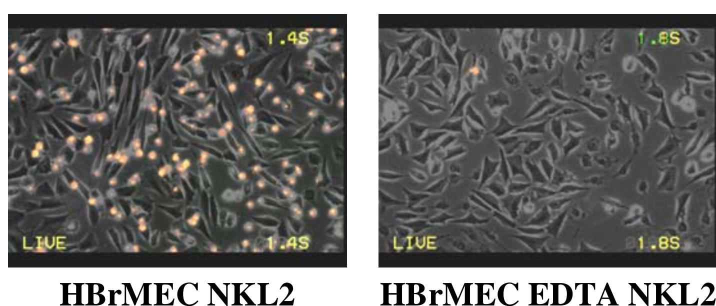


Figure 14. EDTA inhibition of NKL2 cells adhesion, under flow conditions, to human ECs isolated from brain (HBrMEC). NKL2 cells (cultured with 200 U/mL of rhIL-2) were PKH26GL red fluorescent dye labeled and moved under defined flow conditions over HBrMEC monolayer in medium alone or in medium with EDTA.

Microscopic observations (Fig. 13) were further confirmed and quantified using flow cytometry method. Consequently, strong inhibition of NKL cells adhesion was observed in the presence of EDTA (Fig. 15). NKL2 cells in presence of EDTA adhere 10-fold less efficiently than NKL2 cells kept in medium alone.

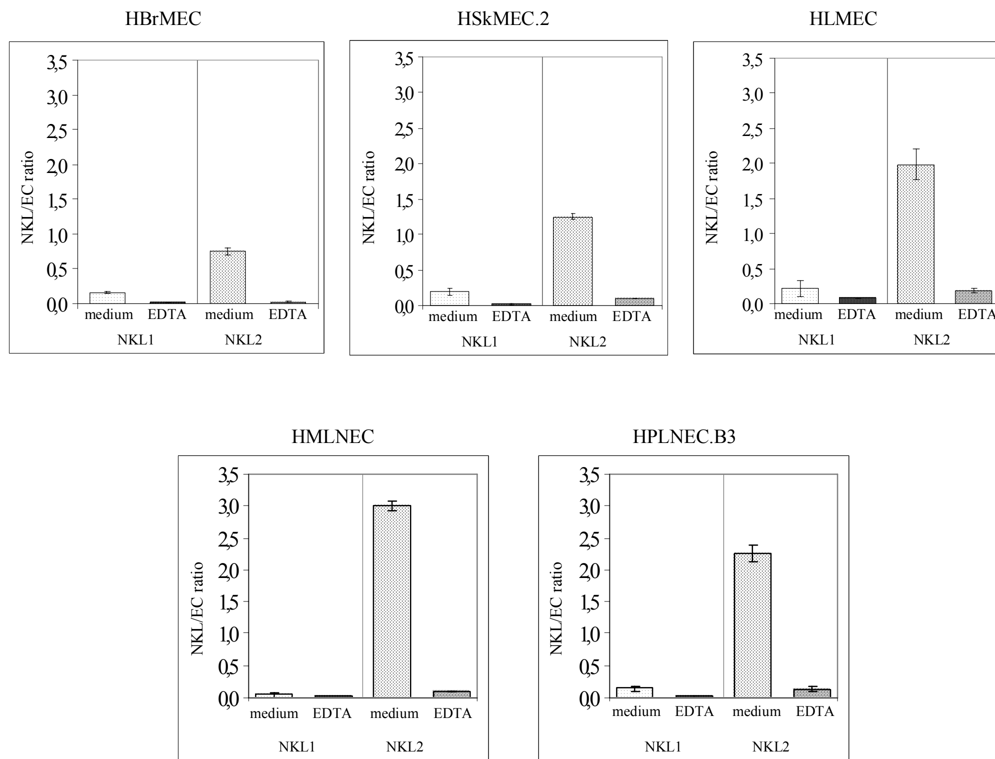


Figure 15. EDTA inhibition of adhesion of NKL cells to human endothelial cells of different organ origin – flow cytometry analysis. NKL1 (cultured without rhIL-2) or NKL2 cells (cultured with 200 U/mL of rhIL-2) were PKH26GL red fluorescent dye labeled. Five NKL cells were overlaid per one EC and incubated at 37ºC for 30 min under static conditions in medium alone or in medium with EDTA (4mM). As a control PKH26GL red fluorescent dye labeled NKL or ECs analyzed alone were used. Each bar represents mean ratio value ±SD of NKL cells per one EC (n=3). HBrMEC – brain, HSkMEC.2 - skin, HLMEC - lung, HMLNEC - mesenteric lymph node and HPLNEC.B3 - peripheral lymph node derived human ECs.

Moreover, number of adhered NKL2 cells per one EC did not significantly change in the presence of EGTA/$MgCl_2$ chelator as compared with the control (Fig. 16). Insignificant increase of adhering NKL1 cells number to HLMEC as well as HMLNEC was observed in the presence of EGTA/$MgCl_2$ chelator. This can be caused by the presence of additional magnesium cations from EGTA/$MgCl_2$, which may activate the integrin molecules. Result obtained using flow cytometry method corroborates with previous microscopic observations (Fig. 13).

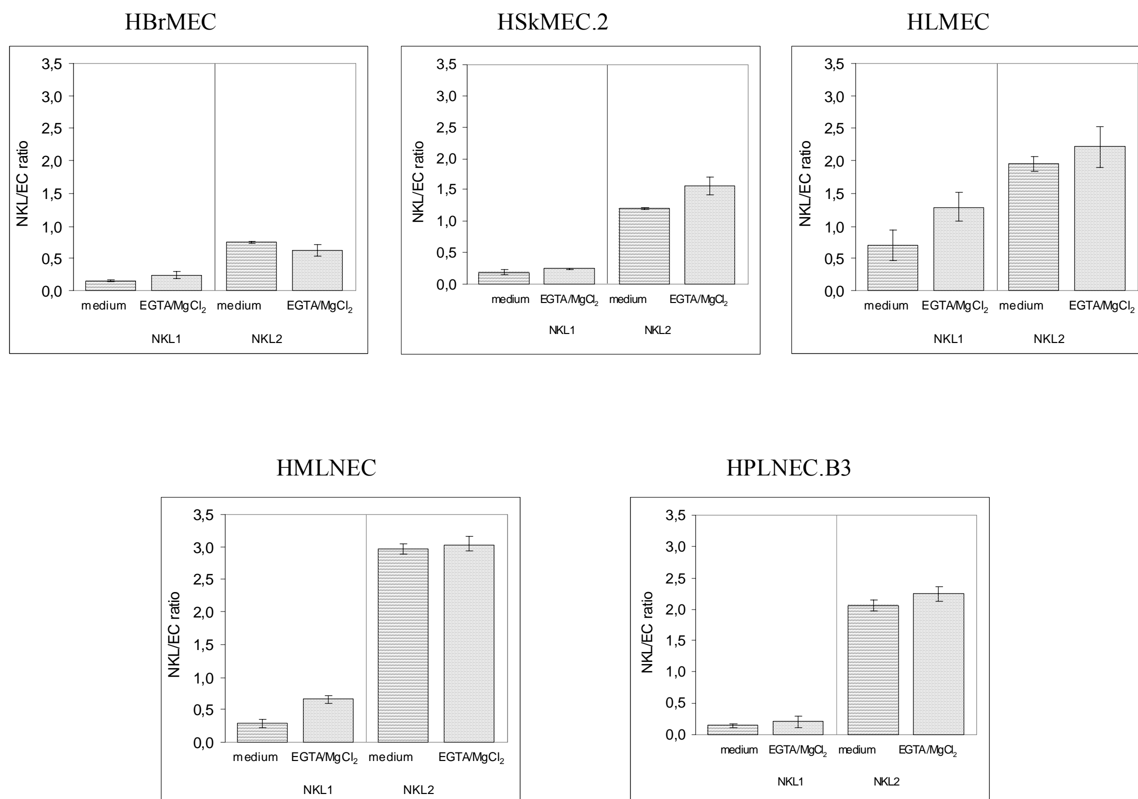


Figure 16. EGTA/$MgCl_2$ inhibition of adhesion of NKL cells to human endothelial cells of different organ origin – flow cytometry analysis. NKL1 (cultured without rhIL-2) or NKL2 cells (cultured with 200 U/mL of rhIL-2) were PKH26GL red fluorescent dye labeled. Five NKL cells were overlaid per one EC and incubated at 37ºC for 30 min. under static conditions in medium alone or in medium with EGTA/$MgCl_2$. As a control PKH26GL labeled NKL or ECs analyzed alone were used. Each bar represents mean ratio value ±SD of NKL cells per one EC (n=3). HBrMEC – brain, HSkMEC.2 - skin, HLMEC - lung, HMLNEC - mesenteric lymph node and HPLNEC.B3 - peripheral lymph node derived human ECs.

The accumulated literature has demonstrated that lymphocyte adhesion process is a phenomenon in which selectins and integrins play a crucial role [54, 55]. The experiments performed indicate that the adhesion process was dependent upon the presence of magnesium ions, consequently it suggests the participation of the integrin dependent adhesion mechanism [88].

As far as integrins are concerned, the EC lines used in this work have been proved to express CD29 integrin (beta1 integrin unit associated with very late antigen receptor) [2].

NKL2 cells were found to express CD18 molecule, which belongs to the integrin family (Fig. 17). It is the beta subunit of three different structures: LFA-1 (paired with CD11a), macrophage-1 antigen (paired with CD11b) and integrin alphaXbeta2 (paired with CD11c), which are known to participate in cell adhesion as well as in cell-surface mediated signalling [89].

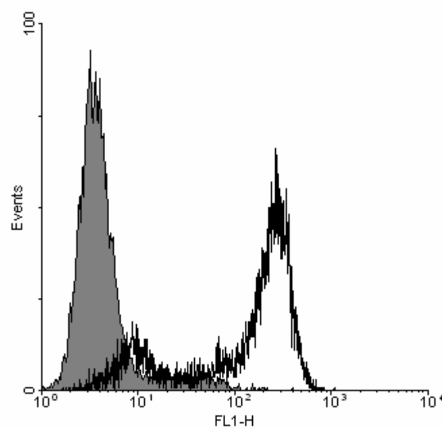


Figure 17. Cell surface expression of CD18 by NKL2 (cultured in the presence of 200 U/mL rhIL-2) cells. Flow cytometry analysis of NKL2 cells labeled with monoclonal antibody (mAb) recognizing human CD18 antigen. Results are expressed as a logarithm of fluorescence intensity (arbitrary units) *versus* number of events. Green fluorescence intensity FL1-H (horizontal axis) represents reactivity to the mAb (empty black histogram). As an isotypic control proper immunoglobulin was used (shaded grey histogram).

As NKL1 cells were CD18 molecule negative (data not shown), it could be hypothesized that their adhesion to ECs involved other integrin molecules. It remains to be estimated if blocking of CD18 molecule by a specific neutralizing antibody may inhibit NKL2 cells adhesion.

b) C-type lectin-like pathway

Since NK cells receptors: CD69, CD94 and CD161 belong to the C-type lectin-like family, it was conceivable that they may participate in NK cells adhesion to ECs. Because it was shown that even though they are C type lectin-like molecules in terms of sequence, they lack the residues that are required for the coordinate binding of the calcium ion and that is necessary for a real C-type lectin [90, 91]. Therefore, these molecules are candidates for being participants to the NK – ECs interactions. Therefore, the expression of C-type lectin-like molecules by NKL cells was examined. RT-PCR reaction showed high expression levels of CD94 and CD161 mRNA in NKL2 cells, wheareas NKL1 cells were found to be negative (Fig. 18).

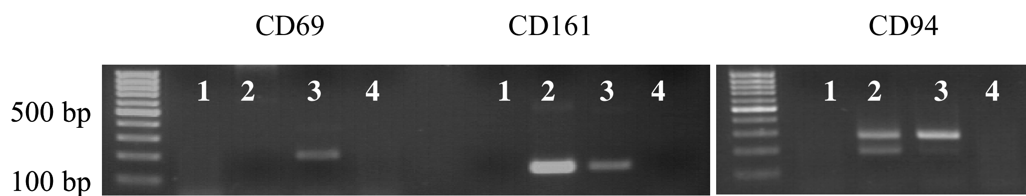


Figure 18. Detection of the C-type lectin-like molecules mRNA in human NKL cells. RT-PCR analysis of mRNA isolated from NKL1 (cultured without rhIL-2) and NKL2 (cultured in the presence of 200 U/mL rhIL-2) cells. PCR products obtained using sets of primers for CD69 (203 bp), CD161 (172 bp) and CD94 (184, 277 bp). PBMC were used as a positive control and A549 cells as a negative control; 1-NKL1, 2-NKL2, 3-PBMC,4-A549.

Both NKL cell populations tested were CD69 mRNA negative. Consequently, our attention was focused on CD94 and CD161 molecules. The results obtained in the RT-PCR reactions were verified using flow cytometry analysis (Fig. 19).

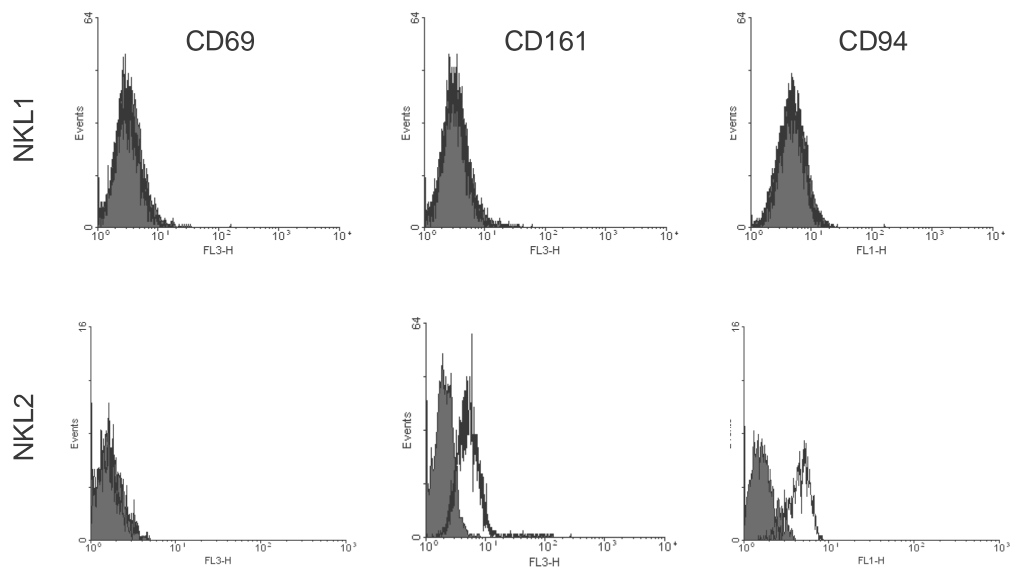


Figure 19. Cell surface expression of C-type lectin-like molecules by NKL1 (cultured without rhIL-2) and NKL2 (cultured in the presence of 200 U/mL rhIL-2) cells. Flow cytometry analysis of NKL cells labeled with monoclonal antibodies (mAbs) recognizing human CD69, CD161 and CD94 antigens. Results are expressed as the logarithm of fluorescence intensity (arbitrary units) *versus* number of events. Green fluorescence intensity FL1-H (horizontal axis) or red fluorescence intensity FL3-H (horizontal axis) represent reactivity to the indicated mAbs (empty black histograms). As an isotypic control proper immunoglobulins were used (shaded gray histograms).

Knowing that NKL2 cells are CD161 positive, adhesion blocking tests were further performed using monoclonal antibody against CD161 molecules. No significant changes in the adhesion ratio of NKL2 cells per one EC were observed (Fig. 20). However, these data do not permit to conclude about the activity of that receptor because the monoclonal antibody, which has been used, probably has not neutralizing/blocking ability.

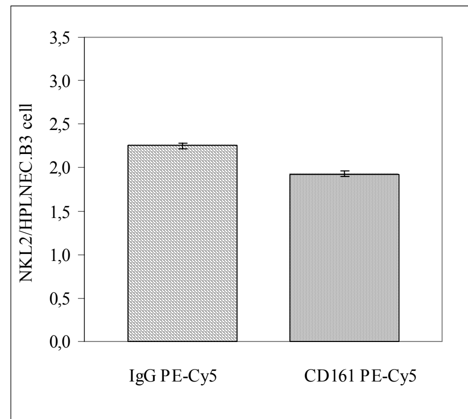


Figure 20. Effect of anti CD161 PE-Cy5 treatment of NKL2 cells on their adhesion to PKH67GL green fluorescent dye labeled HPLNEC.B3 cells. NKL2 cells were preincubated with monoclonal antibody (20µL CD161PE-Cy5/100 µL PBS) for 20 min. at RT. Next, five NKL2 cells were overlaid per one EC and incubated at RT for 30 min. under static conditions. Before acquisition, non-adherent NKL2 cells were removed by gentle washing with PBS. Then the mixture of ECs and adhering NKL2 cells was collected with the use of trypsin/EDTA solution and analyzed by flow cytometry. As a control NKL2 cells preincubated with proper isotypic control (IgG PE-Cy5) were used. The bars represent mean ratio numbers ±SD of adherent NKL cells per one HPLNEC.B3 cell (n=3).

Lanier *et al.* have recently identified lectin-like transcript-1 (LLT-1) as a physiological ligand for human CD161 [92]. Therefore it still needs to be verified if EC lines used in the test express LLT-1 molecule, and if this molecule is involved in NK - ECs adhesive interactions. These data suggest that the mechanism of the NK - ECs interaction may be glycosaminoglycan and chemokines dependent.

c) Chemokines role

Chemokines are a family of proteins which play important role in the recirculation of lymphocytes and their local recruitment in response to inflammatory stimuli. They participate in the adhesion and recruitment, controlling the circulation of lymphocytes [23]. It has been shown that chemokines may also modulate cytolytic activity and proliferation of NK cells [23, 65], therefore it has been tempting to look for the molecular mechanisms involved in this process.

The study recently done in Dr. Kieda's laboratory demonstrated that CCL21 (6Ckine) is able to bind and to be presented by ECs, forming a gradient [24]. Moreover, it has been shown that this chemokine was also able to stimulate ECs, which resulted in a considerably increased adhesion capacity of NKL2 cells (Fig. 21 C).

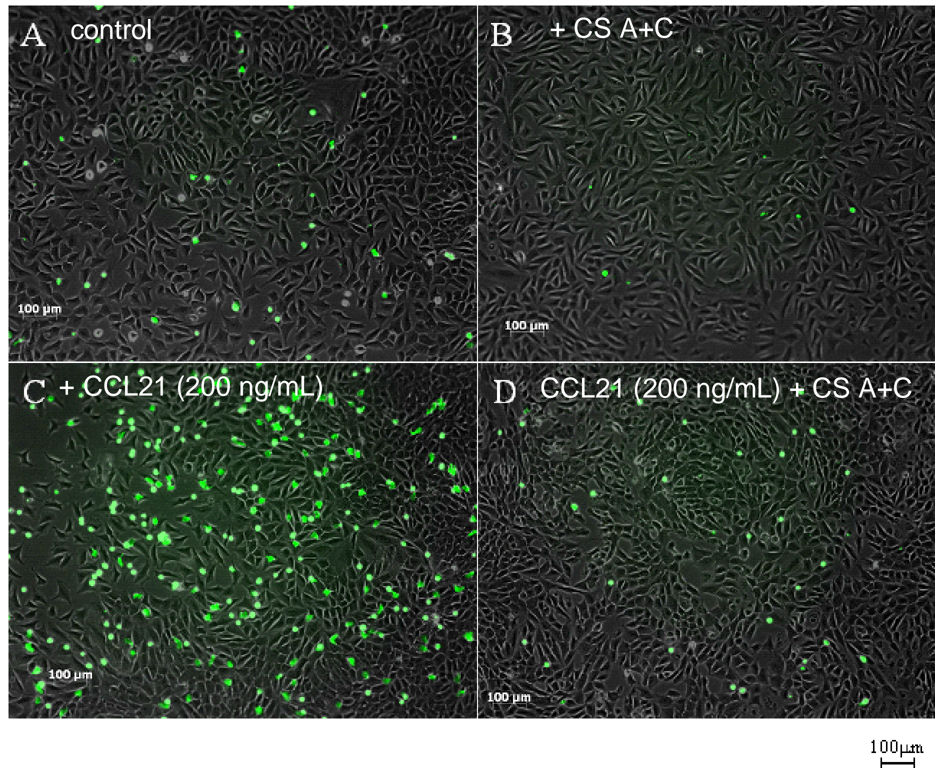


Figure 21: Endothelial cell activation by CCL21, glycosaminoglycans dependent NKL2 cells adhesion to ECs. Under flow conditions adhesion of PKH67GL green fluorescent dye labeled NKL2 cells was allowed toward: ***A***, HPLNEC.B3 cells in medium alone; ***C***, HPLNEC.B3 cells preincubated for 1hr with 200 ng/mL of CCL21; ***B,*** HPLNEC.B3 cells preincubated for 1 hr with chondroitin sulfate A+C (CS A+C); ***D***, HPLNEC.B3 cells preincubated for 1 hr with chondroitin sulfate A+C (CS A+C) and for 1 hr with 200 ng/mL of CCL21 (Kabala P. and Mleczko K., master thesis, 2007).

This means that the chondroitin sulfate A (chondroitin-4-sulfate, CS A) + chondroitin sulfate C (chondroitin-6-sulfate, CS C) prevents the CCL21 binding, consequently its presentation. The resulting activation of the ECs is also inhibited if the cells are incubated in the presence of the CCL21 co-incubated with chondroitin sulfate A+C: the GAG inhibits the CCL21 binding to ECs. This suggests that CCL21 presentation occurs via the binding of the chemokine to the GAG present on the cell surface, in this case the chondroitin sulfate. To assess this it was necessary to check for the presence of this GAG on the ECs. The chondroitin sulfate indeed was preferentially detected on the HPLNEC.B3 [Dr. Lamerant-Fayel, personal communication].

All these data suggest that the interaction between ECs and circulating lymphocytes can be directly dependent on the specific attraction and recognition mediated by chemokines presented in a GAGs - specific manner by the endothelium, acting toward the circulating attracted cells by binding them through their chemokine receptors (G-protein signaling) [93, 94].

In order to further confirm the possible role of chemokines in NK - ECs interactions, the expression of different chemokines receptors on NKL2 cells were investigated using flow cytometry assay. As it is shown in Fig. 22, NKL2 cells expressed CXCR4, CXCR3 and CCR7 molecules. Positive for CXCR4 were approx. 70 % of NKL2 cells. Approximately 50 % of NKL2 cells exhibit CCR7 expression and only 36 % of them were CXCR3 positive.

NKL2 cells were found to be negative for CCR5, CCR6, CX3CR1 (Fig. 22) and CCR4, CCR1, CXCR6 receptors (data not shown).

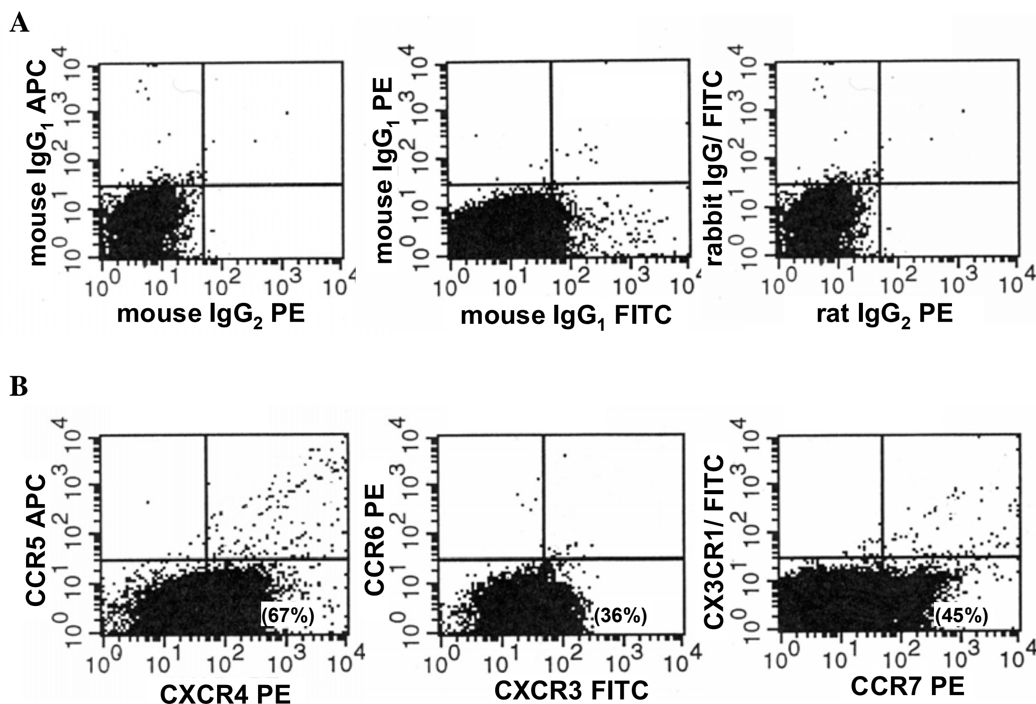


Figure 22. Cell surface expression of chemokines receptors on NKL2 cells (cultured in the presence of 200 U/mL rhIL-2). Flow cytometry analysis of NKL2 cells labeled with isotypic controls (***A***) or monoclonal antibodies (mAbs) recognizing human CCR5, CXCR4, CCR6, CXCR3, CX3CR1 and CCR7 antigens (***B***). Percentages of NKL2 positive cells were quantified by quadrant analysis and are given in brackets. As an isotypic control appropriate immunoglobulins were used.

Peripheral blood NK cells expressing receptors CXCR4, CXCR3 and CCR7 are heterogeneous in their expression of chemokine receptors, and thereby may vary in their abilities to migrate into

different tissues [23, 95]. CXCR3 and CXCR4 have been shown to stimulate migration of NK cells and regulate leukocyte trafficking in a very specific manner. As CCR7 expression is critical for the entry of leukocytes into secondary lymphoid organs, such as lymph nodes, its role in the NK – ECs interactions was further investigated.

In collaboration with master students (Pawel Kabala and Katarzyna Mleczko; Erasmus stays in the CBM, Orleans), it has been shown that CCR7, a receptor actively expressed on the membrane of activated NKL2 cells, participates in NKL2 adhesion to HPLNEC.B3 (Fig. 23). The inhibition of NKL2 cells binding may be obtained upon preincubation of the NKL2 cells with the CCR7 specific neutralizing antibody [Kieda *et al.*, submitted].

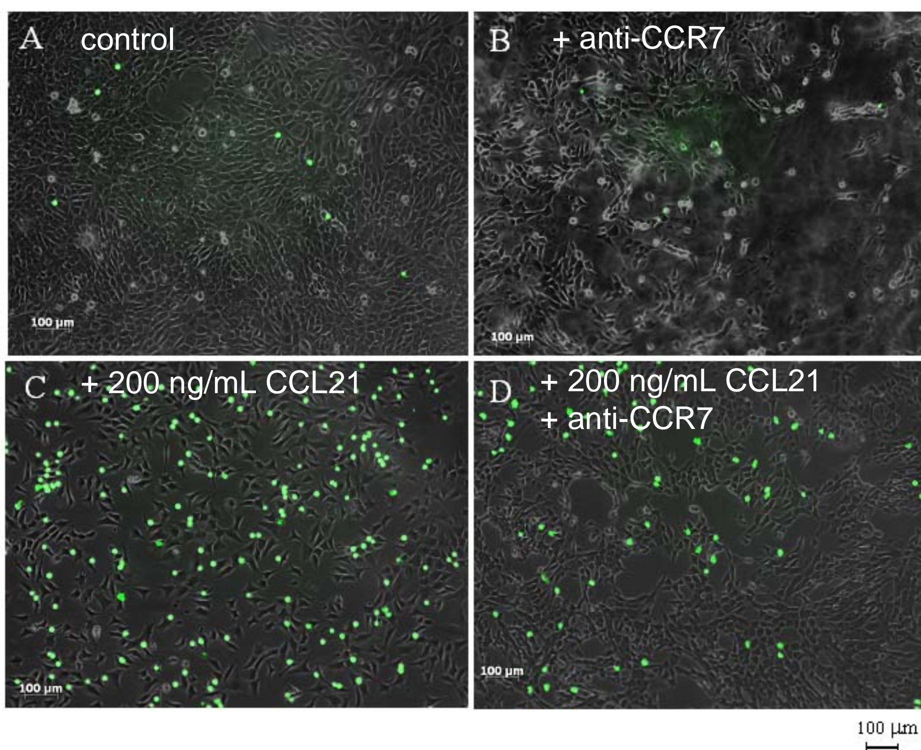


Figure 23: Endothelial cell activation by CCL21, CCR7 dependent NKL2 cells adhesion to ECs. Under flow conditions adhesion of PKH67GL green fluorescent dye labeled NKL2 cells was allowed toward: ***A***, HPLNEC.B3 cells in medium alone; ***C***, HPLNEC.B3 cells preincubated for 1hr with 200 ng/mL of CCL21; PKH67GL green fluorescent dye labeled NKL2 cells preincubated with neutralizing anti CCR7 antibody ***B,*** HPLNEC.B3 cells; ***D***, HPLNEC.B3 cells preincubated for 1hr with 200 ng/mL of CCL21 (Kabala P. and Mleczko K., master thesis, 2007).

Further investigation is required to dissect the contribution of integrins, C-type lectin like molecules as well as chemokines to the migration and effector functions of NK cells but at this point, our participation in the attempt to decipher the molecular mechanism of the NK cell recognition of endothelial cells is represented in the following Fig. 24.

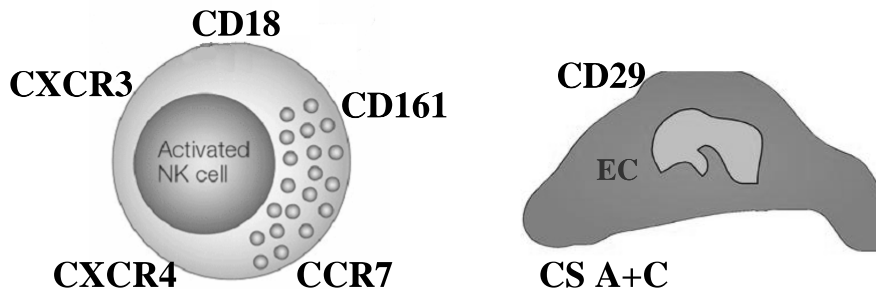


Figure 24. Adhesion molecules participating in the mutual recognition between human NK cells and ECs.

Analysis of the cytotoxicity of human NK cells toward human ECs

Having demonstrated that NK cells are able to adhere specifically to endothelial cells in an activation dependent manner, further question was whether the observed positive effect of rhIL-2 activation on NKL2 adhesion to endothelial cells was meaningful for their killing mechanism. Further, the cytotoxicity of rhIL-2 activated NKL2 cells for ECs was examined. Sensitivity of endothelial cells of different tissue origin to NKL2 cytotoxicity was tested using the standard chromium release assay. K562 human chronic myelogenous leukemia cell line was used as a positive control. All endothelial cell lines tested were equally killed by NKL2 cells. The percentage of lysed endothelial target cells was approx. 35%, whereas for the control K562 cells the mean percentage of lysed cells was up to 80% (Fig. 25). However, no direct correlation between the previously observed adhesion level and the susceptibility of ECs to cytolytic effect of NKL2 was observed.

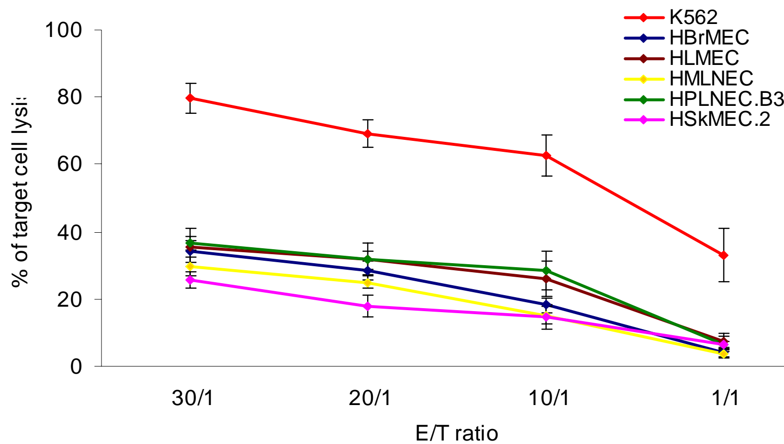


Figure 25. Cytotoxicity of NKL2 cells toward human endothelial target cells of different organ origin. A 4 hr ^{51}Cr-release assay was performed in order to asses cytolytic activity of NKL2 cells (cultured in the presence of 200 U/mL rhIL-2) against endothelial cells isolated from different organs. Data are expressed as a percentage of specific target cell lysis obtained for different effector-to-target (E/T) ratio ±SEM. As a control NKL2 cells were tested for cytotoxicity against K562 cells (n=5). HBrMEC – brain, HSkMEC.2 - skin, HLMEC - lung, HMLNEC - mesenteric lymph node and HPLNEC.B3 - peripheral lymph node derived human ECs.

In order to rule out the possibility that the NKL2 line might represent only one of NK cell subpopulations, the same type of experiments were undertook with freshly isolated human NK cells. Cytotoxicity test was performed using NK cells from peripheral blood of healthy human donors (NKp). In a general way, NKp cytotoxicity was almost twice higher than that of NKL2 cells (Fig. 26). Maximal cytotoxicity level observed was in this case similar to control K562 cells (approx. 70%). The organ specificity of that interaction was confirmed by the difference in the susceptibility displayed by the brain ECs, where NKp cytotoxicity was lower (approx. 40%).

These results indicate that freshly isolated NK cells, probably containing several different subpopulations, have the capacity for different cytotoxicity pathways, while NK cell line, being a selected NK cell subset, possess poorer repertoire of means to mediate target cell death. Nevertheless, both results are a direct proof that NK cells indeed are able to kill ECs.

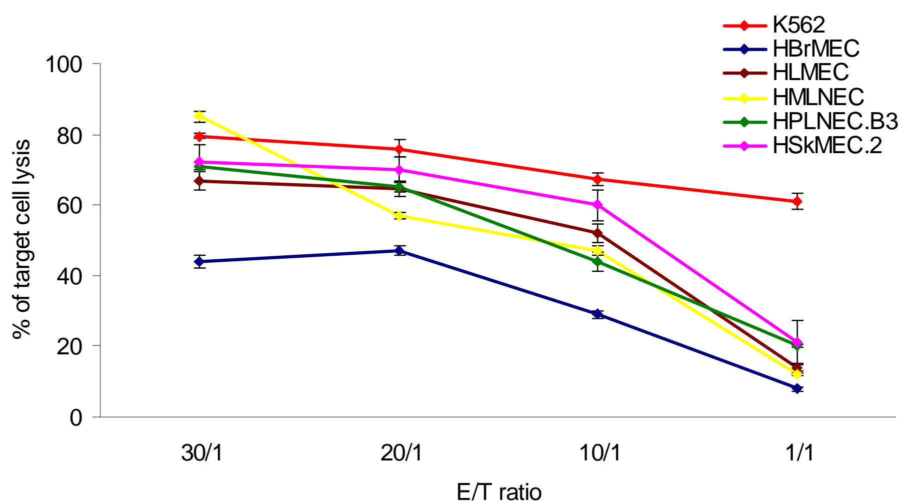


Figure 26. Cytotoxicity of NKp cells toward human endothelial target cells of different organ origin. A 4 hr ^{51}Cr-release assay was performed in order to asses cytolytic activity of NKp cells (cultured in the presence of 1000 U/mL rhIL-2) against endothelial cells isolated from different organs. Data are expressed as a percentage of specific target-cell lysis obtained for different effector-to-target (E/T) ratio ±SEM. As a control NKp cells were tested for cytotoxicity against K562 cells (n=3). HBrMEC – brain, HSkMEC.2 - skin, HLMEC - lung, HMLNEC - mesenteric lymph node and HPLNEC.B3 - peripheral lymph node derived human ECs.

Study of NK cells cytotoxicity toward endothelial cells has been started only during the past decade. Among others it has been demonstrated that vascular leak syndrome and xenograft rejection are processes where endothelial cells are primary targets for NK cells attack [79, 96]. Concluding, among their various complex effects, NK cells may damage normal vessel ECs (leak syndrome) and may exert cytotoxic activity toward pathological cells. It is important to take these into account considering their possible influence on the angiogenesis process [97]. This is consistent with hypothesis that stimulated NK cells can migrate into solid tumors, by being attracted through the specific chemokine gradient toward the tumor, recognize the tumor vessels and subsequently cause their death [98].

On the other hand, there are also data showing that LAK (*lymphokine-activated killer*) cells can die by apoptosis upon contact with vascular endothelium [99]. Apoptosis of interleukin-2-activated human natural killer cells upon binding to tumor cells was also reported [100, 101]. But such an activation-induced death of NK cells may rather represent a mechanism for NK cells regulation.

Molecular mechanism of NK cells cytotoxicity to human ECs

Perforin/granzyme pathway

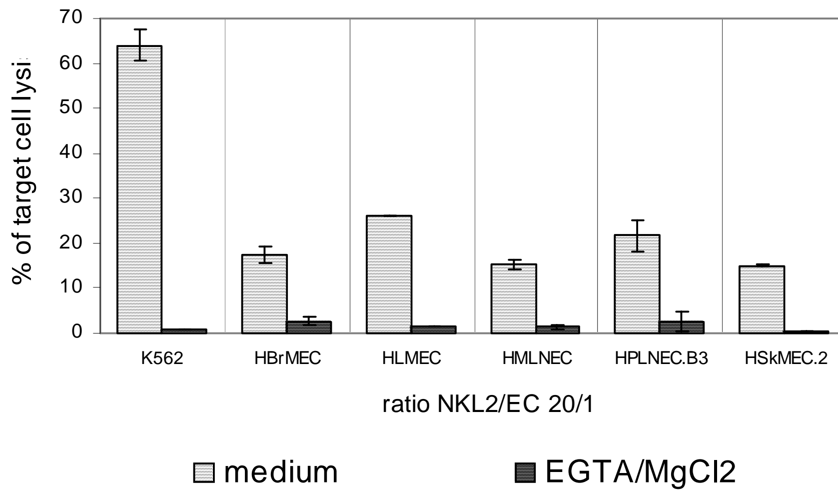
To examine the mechanisms used by NK cells to execute their cytotoxic activity toward ECs, further *in vitro* cytotoxicity tests were performed. To check whether they used the granzyme performing pathway, the assay in the presence of Ca^{2+} chelator EGTA/$MgCl_2$ was used to inhibit the Ca^{2+} -

dependent perforin/granzyme-mediated lysis. K562 cell line was used as a positive control. As shown in Fig. 27 A, the NKL2 cells cytotoxicity was almost totally inhibited after Ca^{2+} chelation. Remarkably, this indicates for a clear distinction between the adhesion step and the subsequent killing step by these cells, because the previous observations showed that the NKL2 cells adhesion was not inhibited by the Ca^{2+} chelators, but was dependent on the Mg^{2+} presence.

Consequently, one may conclude that the pathway used by NKL2 cells to exert killing toward ECs involves the granzyme granules exocytosis. This is in agreement with other experimental data indicating that xenogeneic human NK cytotoxicity against porcine endothelial cells is perforin/granzyme B dependent [62, 102].

When freshly isolated from human peripheral blood NK (NKp) cells were used as effectors in the test instead of NKL2 cell line, the presence of EGTA/$MgCl_2$ (chelating specifically calcium cations) only partially inhibited their cytotoxicity to human ECs (Fig. 27 B). Therefore, it could be concluded that freshly isolated human NKp cells do kill human ECs not exclusively on perforin/granzyme pathway.

A



B

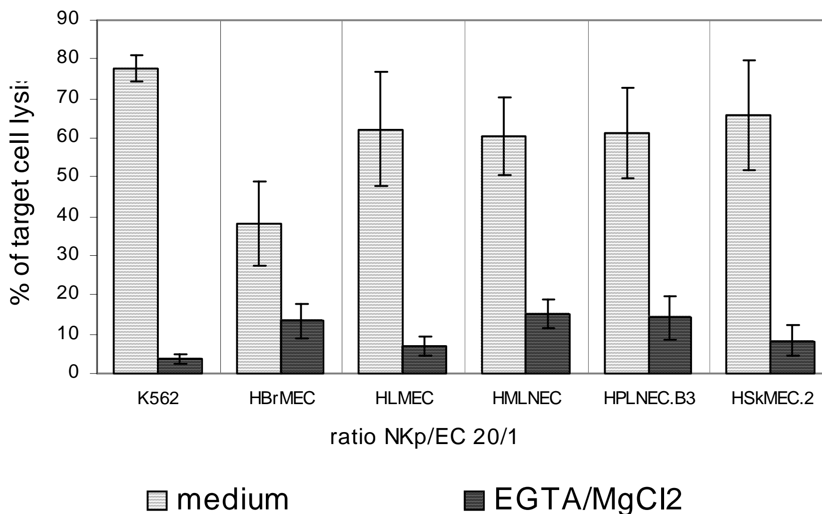


Figure 27. Cytolytic activity of NK cells toward human endothelial cell lines isolated from different organs *via* perforin/granzyme pathway measured in 4-hr ^{51}Cr-release assay. ***A,*** Cytolytic activity of NKL2 cells (cultured in the presence of 200 U/mL rhIL-2) was assessed in medium alone or in presence of EGTA/$MgCl_2$ to chelate calcium cations; ***B,*** Cytolytic activity of NKp cells (cultured in the presence of 1000 U/mL rhIL-2) was assessed in medium alone or in presence of EGTA/$MgCl_2$ to chelate calcium cations. Data are expressed as a percentage of specific target-cell lysis obtained for effector-to-target (E/T) ratio 20/1 ±SD (n=3). As a positive control NK cells were tested for cytotoxicity toward K562 cells. HBrMEC – brain, HSkMEC.2 - skin, HLMEC - lung, HMLNEC - mesenteric lymph node and HPLNEC.B3 - peripheral lymph node derived human ECs.

There are data that NK cells cytotoxicity is also triggered by natural killer cell receptors such as NKp44 (belonging to *natural cytotoxicity receptor* – NCR family) and NKG2D [103]. It occurred that NKL2 cells were negative for NKp44 antigen (Fig. 28 A), however NKp cells were positive for NKp44 receptor (Fig. 28 B). Therefore, it could be speculated that NKp cells are able to kill target ECs also using NCR. It was shown that a cellular ligand for NKp44 receptor is NKp44L [104]. In order to find out whether this receptor is involved in NKp cells cytotoxicity, neutralizing antibody against NKp44 molecule should be used. That would provide the clue for this hypothesis and confirm that NK cells could kill ECs using several pathways.

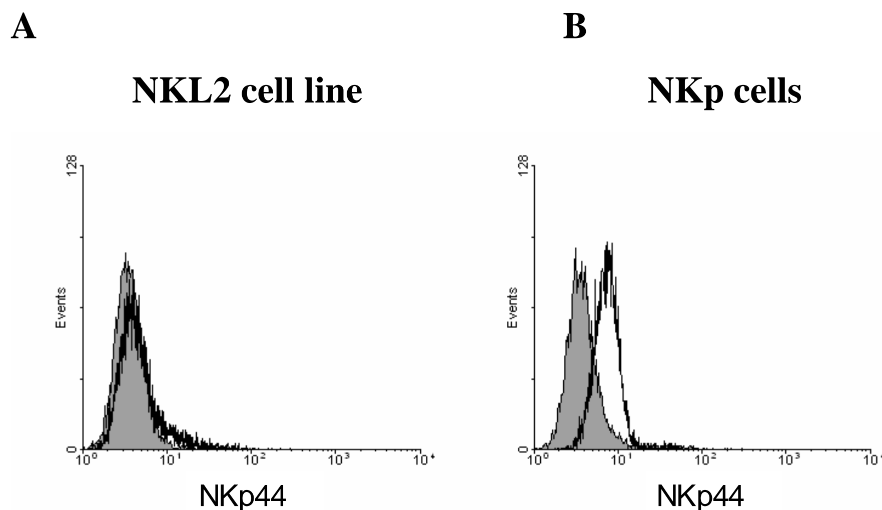


Figure 28. Cell surface expression of NKp44 on NK cells. Flow cytometry analysis of NKL2 (***A***) cells or NKp (***B***) cells labeled with monoclonal antibody (mAb) recognizing human NKp44 antigen. Results are expressed as a logarithm of fluorescence intensity (arbitrary units) *versus* number of events. Green fluorescence intensity FL1-H (horizontal axis) represents reactivity to NKp44 mAb (empty black histograms). As an isotypic control proper immunoglobulins were used (shaded grey histograms).

The results presented above clearly indicate for a role of chemokines in NK cells recruitment and adhesion. This is a fact that chemokines direct the lymphocyte homing into secondary lymphoid organs and also participate very importantly in the recruitment of cells into inflammatory sites [24, 105]. Moreover, chemokines are reflecting the microenvironment state, and it could be hypothesized that they may also participate in NK cells cytotoxicity. It has been reported that chemokines were able to mediate the vascular ECs damage by NK cells [65]. The concept that chemokines participate not only in the binding of NK cells to ECs, but also in NK cell-mediated endothelium damage, raises the necessity of further studies on the role of chemokines in NK cells cytotoxicity toward ECs.

Triggering of cytochrome c release and Bid translocation in ECs after interaction with NKL2 cells

It is known that programmed cell death (apoptosis) is achieved by several pathways. It may begin with activation of initiator caspases, which further stimulate the formation of activated form of Bid (tBid). It redistributes from cytosol to mitochondria and initiates mitochondrial apoptotic pathway by releasing cytochrome c [106-108]. To determine the possible involvement of mitochondria in NK cells induced ECs killing, subcellular localizations of Bid and cytochrome c were assessed by confocal microscopy.

Human ECs isolated from brain (HBrMEC) or from mesenteric lymph nodes (HMLNEC) were incubated with NKL2 cells (4 hr incubation at the 10/1 NKL2/EC ratio) and subjected to immunostaining for Bid and cytochrome c. As shown in Fig. 29, a punctuated cytoplasmic staining pattern showed mitochondrial localization of cytochrome c, whereas a diffuse staining pattern indicated that cytochrome c was released from mitochondria. After endothelial cells co-culture with NKL2 cells, cytochrome c was released from mitochondrial intermembrane space to the cytoplasm of target ECs (Fig. 29). This was observed in all endothelial cell lines tested.

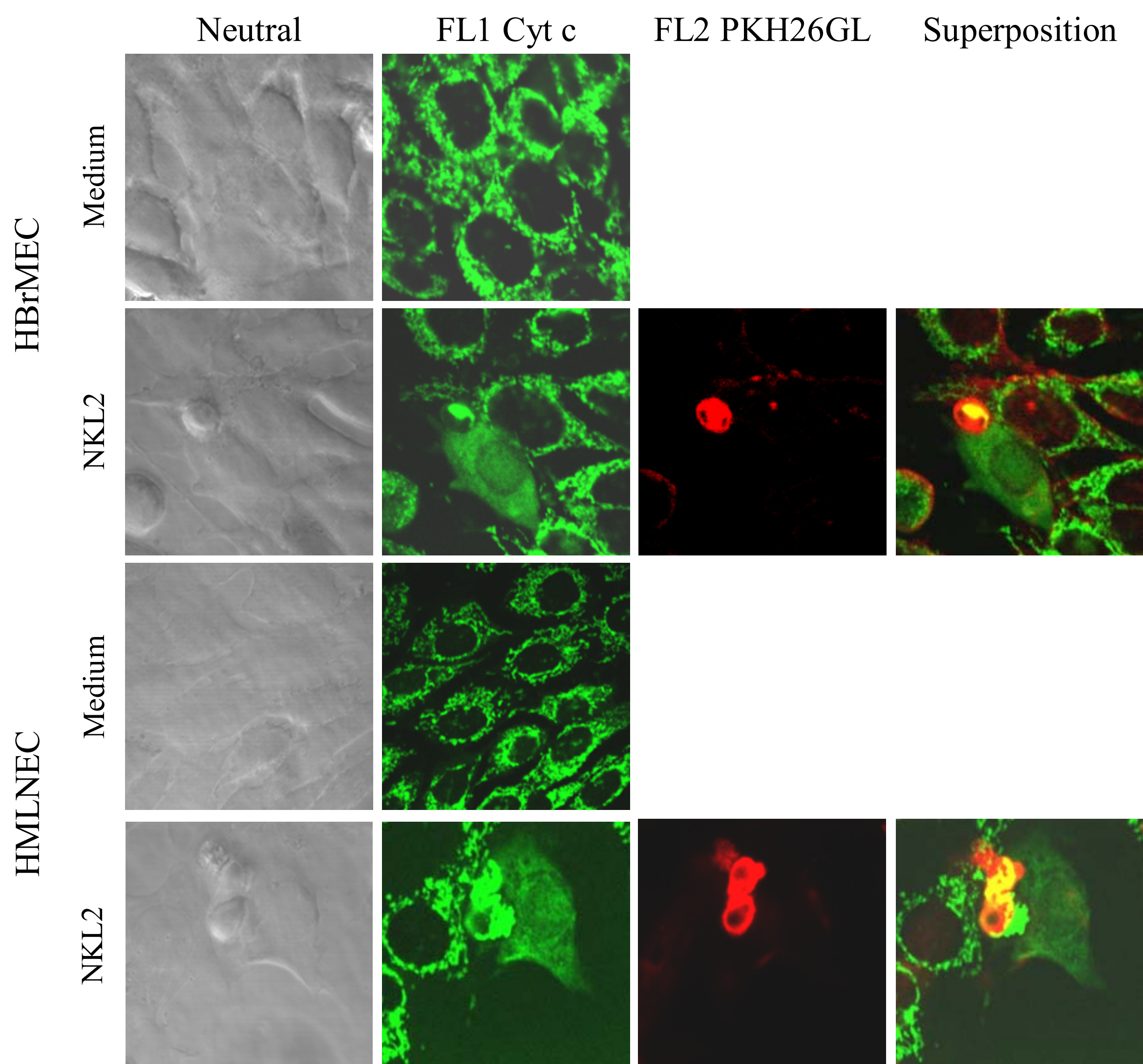


Figure 29. Translocation of cytochrome c in ECs after interaction with NKL2 cells. Human ECs isolated from brain (HBrMEC) or mesenteric lymph node (HMLNEC) were incubated for 4 hr at 37°C with PKH26GL red fluorescent dye labeled NKL2 cells (cultured in presence of 200 U/mL rhIL-2) at the effector-to-target cell E/T ratio 10/1. Cytochrom c translocation was evaluated using monoclonal antibody against cytochrome c and Alexa 488-conjugated goat anti-mouse IgG antibody (green fluorescence). As a control ECs kept in culture medium alone were stained with the same antibodies.

Moreover, after incubation of human ECs with NKL2 cells, Bid translocation from the cytosol of HBrMEC and HMLNEC cells to mitochondria was observed (Fig. 30).

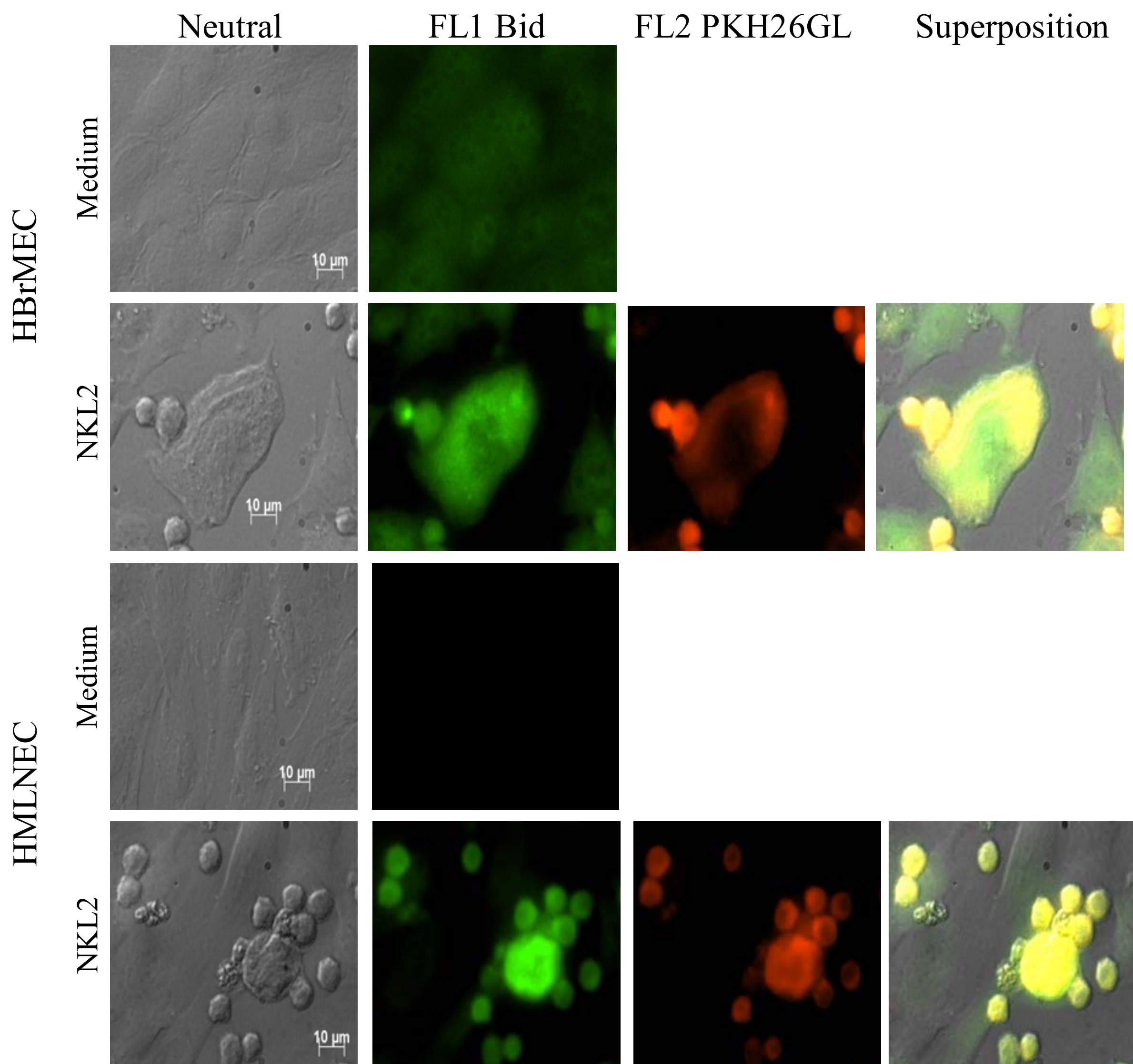


Figure 30. Translocation of Bid molecule in endothelial cells after interaction with NKL2 cells. Human ECs isolated from brain (HBrMEC) or mesenteric lymph node (HMLNEC) were incubated for 4 hr at 37°C with PKH26GL red fluorescent dye labeled NKL2 cells (cultured in presence of 200 U/mL rhIL-2) at the effector-to-target cell E/T ratio 10/1. Bid redistribution was evaluated using polyclonal antibody against Bid and Alexa 488-conjugated goat anti-rabbit IgG antibody (green fluorescence). As a control ECs kept in culture medium alone were stained with the same antibodies.

These data clearly showed that NKL2 cells, upon binding to ECs, are able to activate mitochondrium-dependent apoptotic pathway in EC target cells. Endothelial cells apoptosis, including cytochrome c release and Bid cleavage, was also observed after photodynamic therapy of HUVEC cells [109].

Therefore it could be hypothesized that NK cells can be activated to kill ECs, consequently the study was continued to explain various mechanisms possibly used to achieve that aim.

Reactivity of ECs to TNF - related molecules

Many studies, including studies in perforin deficient animals, corroborate with the above experimental results, leading to the conclusion that perforin/granzyme-mediated induction of target EC death is a principal pathway used by NK cells. However, as revealed by more recent reports, NK cell mediated cytotoxicity may be also executed through a family of TNF-related death receptors (TRAILR, Fas or TNFR) expressed on target cells [108, 110]. It has been proven that stimulated NK cells remarkably upregulate TRAIL expression and can produce TNF, active ligands for death receptors [111, 112]. Target cell apoptosis may be induced after their binding to a family of TNF-related death receptors.

In order to find out whether the TNF induced death may be one of the means used by NK cells to kill the ECs, the presence of death receptors on the ECs surface was assessed. Indeed, flow cytometric analysis revealed the expression of TNFR type 1 on all ECs tested, whereas Apo2-TRAIL and Fas were not found. Representative histograms of TNFR1, Fas, TRAILR1 and TRAILR2 expression analysis on microvascular endothelial cells of skin origin (HSkMEC.2) are shown in Fig. 31.

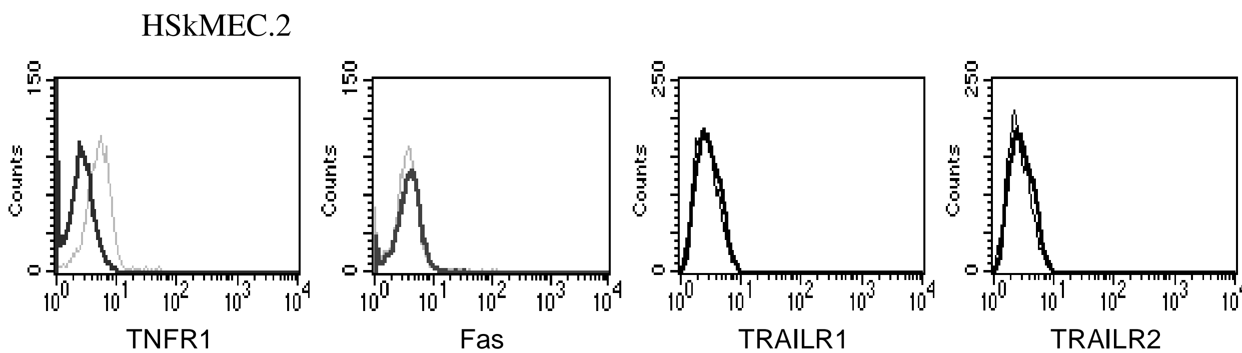


Figure 31. Surface expression of death receptors on human skin endothelial cells (HSkMEC.2). Flow cytometric analysis of HSkMEC cells using monoclonal antibodies (mAbs) for human TNFR1, Fas (UB2), TRAILR1 and TRAILR2 antigenic epitopes. Results are expressed as a logarithm of green fluorescence intensity (arbitrary units) *versus* number of events. Green fluorescence intensity (horizontal axis) represents reactivity to the indicated mAbs (empty gray histograms). As a control, proper negative isotypic control mAbs were used (empty black histograms).

TNFR1 cell surface expression was rather low on all endothelial cell lines examined. However, it is known that signals elicited by TNF are largely dependent on interaction of TNF with TNFR1 [113, 114]. According to reports by others, unstimulated microvascular ECs express relatively low levels of Fas or its presence has been not found at all. However, Fas expression may be upregulated after TNF-α or IFN-γ treatment [115]. It is also known that HUVECs express TRAILR1 and TRAILR2 on the cell surface [116].

Consequently, knowing that ECs express TNFR1, it was still to be determined whether TNF really mediates ECs apoptosis. Therefore flow cytometry analysis was performed where ECs were incubated with ligands for death receptors. Using double (DiOC/PI) staining, where PI positive cell population were late apoptotic/dead cells, PI and DiOC6(3) - negative cells in early apoptotic state, while PI negative and DiOC6(3) positive were alive cells, TNF-α cytotoxic activity toward all ECs lines was checked after 72 hr incubation. As it is shown in Fig. 32, in the case of TNF-α treated probes approx. 30% of cells were found dead (panel ***B***) *versus* approx. 12% of dead cells in controls (panel ***A***). As a positive control for the effective TNF-α induction of target cell apoptosis, MCF-7 cells were used (data not shown).

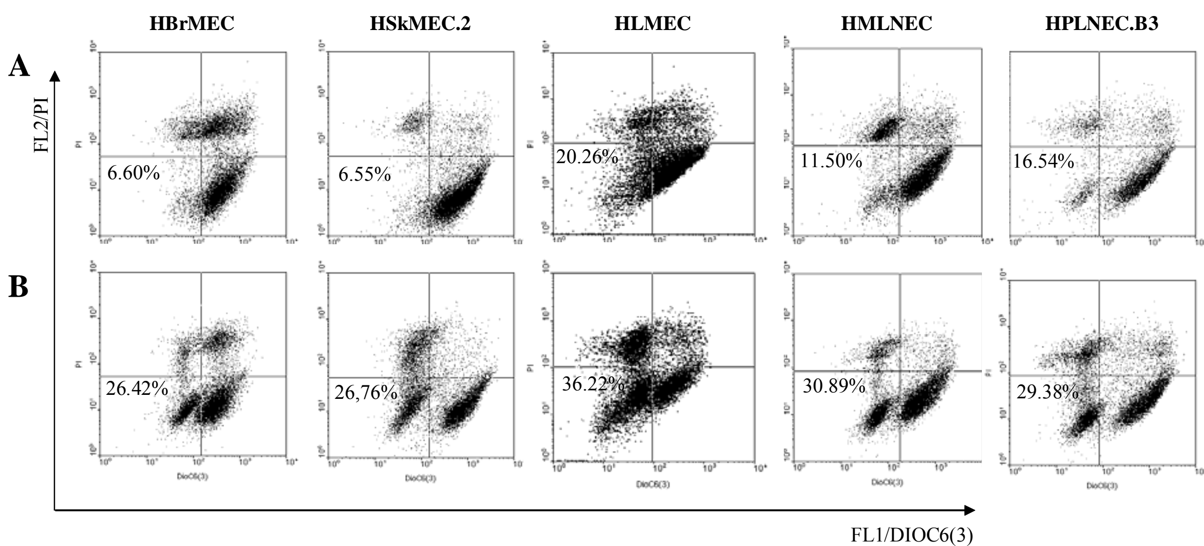


Figure 32. TNF-α induced apoptosis in ECs. Endothelial cells of different organ origin were incubated with: ***A***, medium alone; ***B***, 250 ng/mL TNF-α for 72 hr. After DiOC6(3) and propidium iodide (PI) double staining, flow cytometric analysis was performed on endothelial cells. The figure shows fluorescence cytograms of endothelial cells (expressed as dot plots). Percentage of apoptotic cells (DiOC6(3)-, PI+ cells) are given. HBrMEC – brain, HSkMEC.2 - skin, HLMEC - lung, HMLNEC - mesenteric lymph node, and HPLNEC.B3 - peripheral lymph node derived human ECs. As a control endothelial cells treated with culture medium alone was used (***A***).

These results confirmed the presence of functional TNFR1 death receptor on all human ECs lines examined.

TNF may elicit a broad spectrum of biological effects in ECs including proliferation, differentiation and/or apoptosis. The TNF effect depends not only on TNF concentration but also depends upon their growth status [117]. However, whether NK cells may kill ECs by simple TNF-α releasing has yet to be determined.

TRAIL (1 μg/mL) and agonistic anti-Fas antibody (7C11, 100 ng/mL) exerted no cytotoxic effect on ECs after 24, 48 and/or 72 hr incubation, whereas Jurkat (TRAIL sensitive positive control cell line) and HeuN (FasL sensitive, positive control cell line) were efficiently killed (data not shown).

As ECs do not express Fas or express it at a low level, they are known to be resistant to FasL mediated apoptosis [115]. TRAIL, although generally associated with the induction of apoptosis in transformed cells, has been reported to exert diverse effects *in vitro* on ECs, depending on the concentrations used [116]. In the range of hundreds of nanograms per millilitre TRAIL induces apoptosis in a variety of other cells including vascular smooth muscle cells and HUVEC [118, 119]. In experiment presented above the intracellular death program was not induced by TRAIL nor FasL, as organ specific ECs examined do not express TRAIL receptors nor Fas on their surface.

Further investigation is required to dissect the contribution of Natural Cytotoxicity Receptors, cytokines as well as chemokines to the effector functions of NK cells but at this point, our participation in the attempt to decipher the molecular mechanism of the ECs killing by NK cells is summarized in the following Fig. 33.

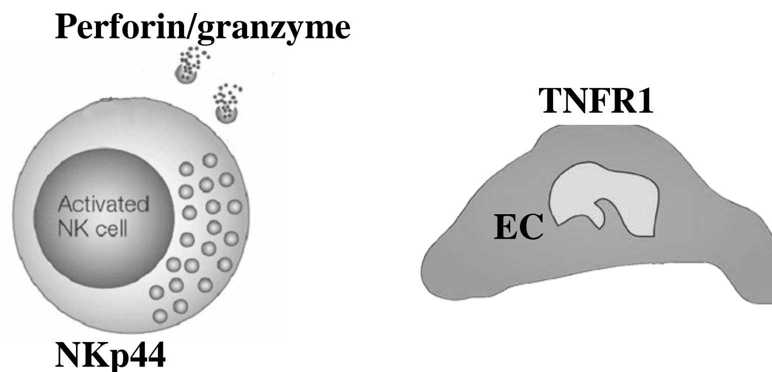


Figure 33. Molecules found on both partners' cells, participating in the mutual recognition between NK cells and ECs resulting in ECs apoptosis.

IN VITRO MURINE MODEL

Adhesion of murine NK cells to murine ECs

As mentioned above the validation of the *in vitro* experiments performed on the model human endothelial cells had to be repeated using a murine cell model. Elaboration of the *in vitro* conditions for interactions between murine endothelial and isolated murine NK cells (mNKp), may further allow planning the *in vivo* inhibition of tumor angiogenesis experiments. Therefore, the adhesion test under static conditions with murine NK cells and mouse ECs of different tissue origin was performed. Murine NK cells (mNK; CD49b^{+}, CD3^{-} ; of approx. 90% purity) isolated from spleen of FVB mouse were cultured in the presence of rhIL-2.

As it is shown in Fig. 34, mNK cells adhesion levels to murine endothelial cells from: bone marrow, peripheral lymph node and Peyer's patches were comparable, with the exception of endothelial cells of brain origin, where it was a little lower.

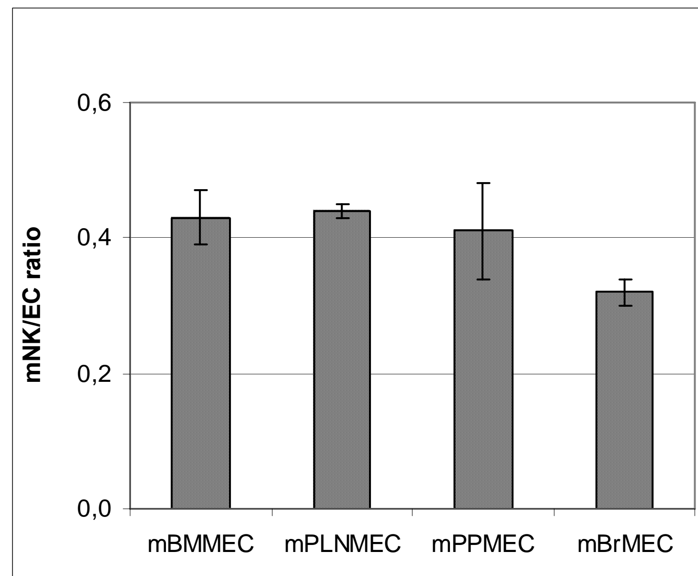


Figure 34. Adhesion of murine mNK cells to murine ECs of different organ origin. mNK cells were isolated from FVB spleen mouse and cultured with 1000 U/mL of rhIL-2 for 3 up to 25 days. Five mNK cells were overlaid per one EC and incubated at 37ºC for 30 min under static conditions. Each bar represents mean ratio value ±SD of adherent mNK cells per one mEC (n=5). mBMMEC - bone marrow, mPLNEC - peripheral lymph node, mPPMEC - Peyer's patches and mBrMEC - brain derived murine ECs.

These data sustained the previously obtained results for human NKL2 cells, which also adhered less efficiently to endothelial cells isolated from brain. Interestingly, relatively low adhesion of mNK kept in culture *in vitro* for 2-3 weeks as compared to human NKL2 cells was observed. It has been found that longer than three weeks activation of mNK cells by rhIL-2 may increase their adhesion (Fig. 35), however, during long lasting culture in the presence of rhIL-2 a number of undesired mNKT cells

(CD49b$^+$, CD3$^+$) significantly increased (Fig. 36). Knowing that human NKT cells are able to recognize and adhere to ECs [120], all the experiments with isolated murine NK cells were done within 14 days time in order to minimize number of NKT cells, hence their influence for the results.

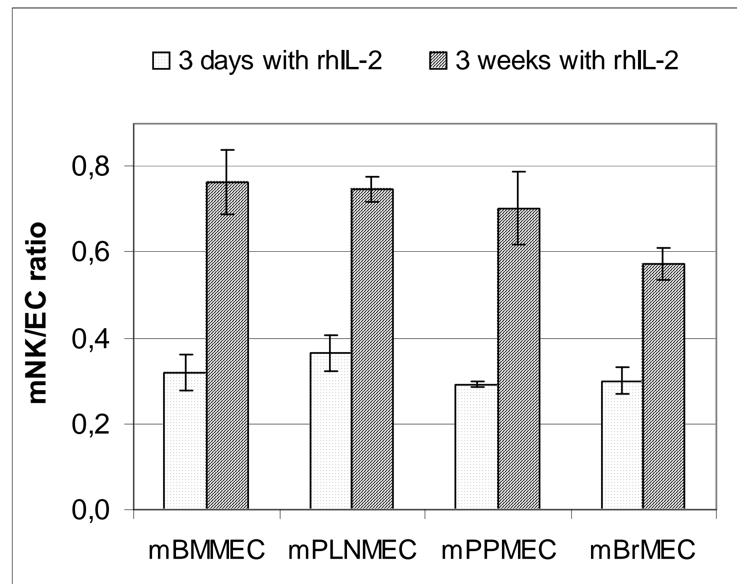


Figure 35. Adhesion of murine mNK cells activated with rhIL-2 for a different time to murine ECs of different organ origin. mNK cells were isolated from FVB spleen mouse and cultured with 1000 U/mL of rhIL-2 for 3 days or 3 weeks. Five mNK cells were overlaid per one EC and incubated at 37ºC for 30 min. under static conditions. Each bar represents mean ratio value ±SD of adherent mNK cells per one mEC (n=3). mBMMEC - bone marrow, mPLNEC - peripheral lymph node, mPPMEC - Peyer's patches, and mBrMEC - brain derived murine ECs.

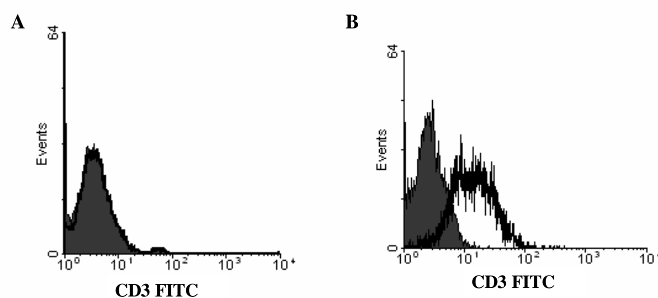


Figure 36. Changes in surface expression of CD3 antigen on mNK cells during long lasting culture in the presence of rhIL-2. Flow cytometric analysis of CD3 expression on mNK cells kept in culture with 1000U/mL rhIL-2 for 3 days (***A***) and for 3 weeks (***B***). Results are expressed as a logarithm of green fluorescence intensity (arbitrary units) *versus* number of events. Green fluorescence intensity (horizontal axis) represents reactivity to the indicated mAb (empty black histograms). As a control, proper negative isotypic control mAb was used (shaded gray histograms).

Cytotoxicity of murine NK cells toward murine ECs

Proving that mNK cells were able to adhere to murine endothelial cells, their cytotoxicity toward murine endothelial cells was investigated next. YAC-1 mouse lymphoma cells were used as a positive control for mNK cytotoxicity. The four above murine EC lines derived from bone marrow, peripheral lymph nodes, Peyers'patches and brain were tested. The killing was assessed with mNK cells applied in various proportions versus the target cells (either 1 to 1 or 10 to 1). Figure 37 demonstrates that all endothelial cell lines tested were significantly but differently killed by mNK cells. At high E/T ratio (10/1) there was an increase in the susceptibility of target cells as compared to lower E/T ratio (1/1) and only murine ECs derived from bone marrow did not display this dose susceptibility effect (Fig. 37).

A direct correlation between the adhesion level and differential susceptibility of mECs to the cytotoxic effect of mNK cells was observed. Murine endothelial cells isolated from: bone marrow, peripheral lymph nodes and Peyer's patches were equally killed by mNK cells (35%-50% of apoptotic target cells, depending on E/T ratio). The lowest cytotoxicity of mNK cells was observed toward murine endothelial cells isolated from brain, approx. 20% apoptotic target cells at 10/1 E/T ratio. NK cell-mediated cytotoxicity for murine cells as evaluated by flow cytometry worked as well as the standard ^{51}Cr release assay that was previously used for human cells [121].

Consequently, one may conclude that mNK cells are able to kill murine ECs at a similar level as human NKp cells kill human ECs. As it is shown in Fig. 26 and Fig. 37, approximatively 25% of apoptotic target cell for brain ECs and approximately 50% lysis for peripheral lymph node ECs were observed (for E/T ratio 10/1).

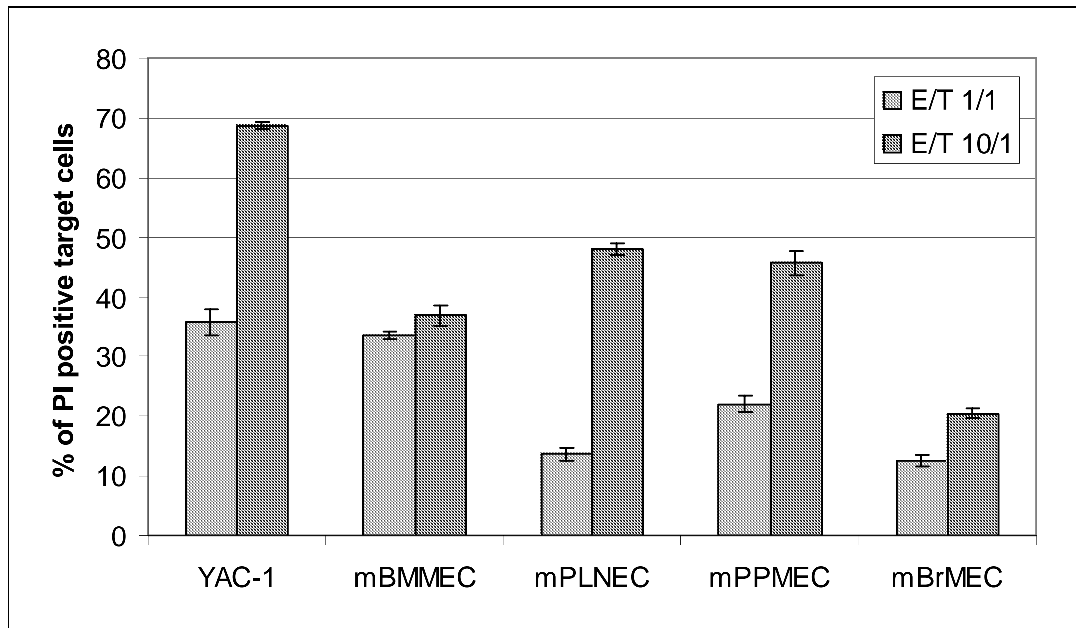


Figure 37. Cytotoxicity of mNK cells toward murine ECs of different tissue origin. A flow cytometric assay was performed in order to asses cytotoxic activity of mNKp cells (cultured in the presence of 1000 U/mL rhIL-2) against murine endothelial cells isolated from different organs. Data are expressed as a percentage of propidium iodide positive target cells obtained for different effector-to-target (E/T) ratio ±SD (n=5). As a positive control mNKp cells were tested for cytotoxicity against YAC-1 cells. mBMMEC - bone marrow, mPLNEC - peripheral lymph node, mPPMEC - Peyer's patches, and mBrMEC - brain derived murine ECs.

It is known that murine NK cells do not express the murine homolog of human CD56 molecule and therefore it was difficult to relate the knowledge on mouse NK biology to that on human NK cell biology. However, analogous results obtained for human NKp and murine NK cells investigated under parallel *in vitro* conditions, validate experimental models that have been used in these series of experiments.

Killing of murine ECs by IL-12 primed spleen NK cells

It is established that IL-12 exerts a variety of immunomodulatory antitumor effects, including induction of IFN-γ secretion by T and NK cells as well as promotion of cytotoxic T lymphocytes maturation [122]. As IL-12 alone induces very weak proliferation of resting lymphocytes, it should be used in combination with suboptimal doses of IL-2, to enhance lymphocyte proliferation and activation [17]. Moreover, *in vivo* IL-12 may inhibit tumor angiogenesis [123]. The accepted antiangiogenic effect of IL-12 may be mediated by IFN-γ, which in turn induces the production of chemokines acting as a chemoattractants for lymphocytes. It has been also demonstrated that *in vivo* mIL-12-stimulated inhibition of tumor growth and neovascularization were dependent almost entirely on NK cells activity

[124]. A potential mechanism by which IL-12 can suppress neovascularization may be NK-cell mediated cytotoxicity toward ECs [98]. Having all these data in mind, *in vitro* cytotoxicity of rhIL-2/mIL-12 activated mNK cells toward mECs has been explored. Data summarized in Fig. 38 indicate that stimulation by rhIL-2/mIL-12 may induce a cytotoxic response against endothelial as well as control YAC-1 cells, comparable to that obtained by high doses of rhIL-2. These data indicate that IL-12 may be the second key cytokine required for NK cells activation to recognize and kill murine endothelial cells.

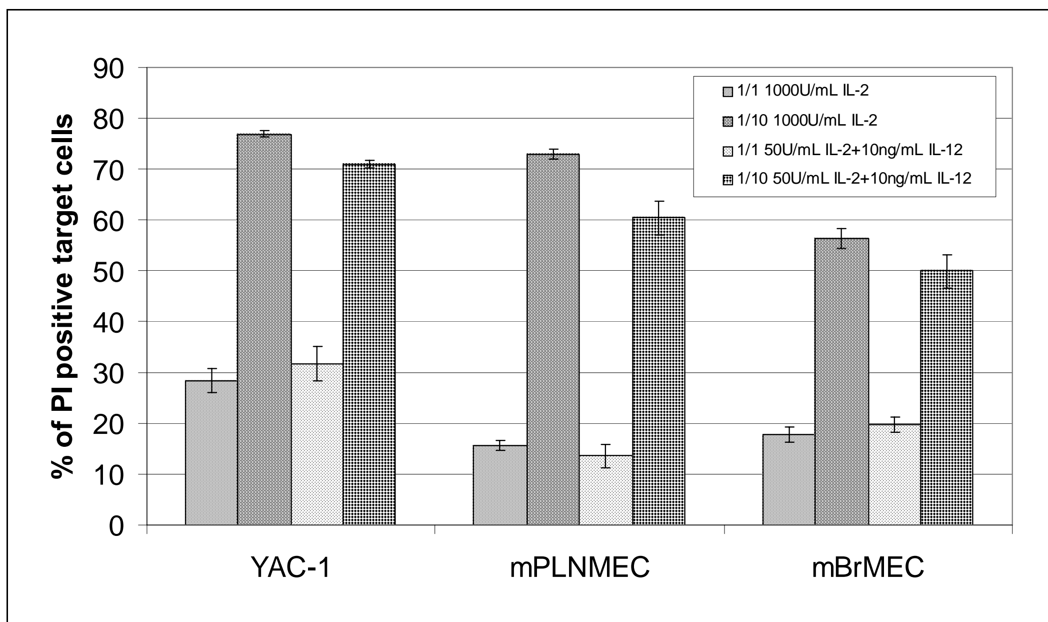


Figure 38. Cytotoxicity of mNK cells toward murine ECs isolated from peripheral lymph node (mPLNEC) or brain (mBrMEC). Flow cytometric assay was performed in order to asses cytotoxic activity of mNK cells (cultured in the presence of 1000 U/mL rhIL-2, or 50 U/mL rhIL-2 with 10 ng/mL mIL-12) against murine endothelial cells. Data are expressed as a percentage of propidium iodide positive (dead) target-cells obtained for different effector-to-target (E/T; 1/1 and 1/10) ratio ±SD (n=5). As a positive control mNK cells were tested for cytotoxicity against YAC-1 cells.

Obtained results are preliminary data for planned further *in vivo* investigations of the NK cells – ECs interactions. Knowing that under specific conditions stimulated NK cells are able to recognize and kill ECs *in vitro,* consequently *in vivo* experiments were planned in which IL-12 antiangiogenic activity may be realized by NK cells cytotoxicity toward ECs [Chouaib *et al.,* unpublished data]. *In vivo* experiments should take into consideration more of the possible NK-EC interactions mechanisms leading to recognizing and killing tumor ECs by activated NK cells.

CONCLUSIONS

CONCLUSIONS AND PERSPECTIVES

Natural killer cells were initially identified on the basis of their capacity to destroy susceptible target cells *via* granule-mediated cytotoxicity. Subsequently, production of cytokines (IFN-γ, TNF-α) by NK cells was shown to be critical in restricting pathogen infection, defining a non-cytotoxic role for NK cells in host defense. Recently, specialized NK cells subsets with biased effector functions have been described in mice and man [125]. Nowadays, it has been considered that diverse NK cells effector functions, which could not be realized by a single multifunctional cell, could be achieved in an alternative way through NK cell diversification [125, 126]. Taking into account the functional pleiotropy exhibited by NK cells, the experiments presented in this thesis were designed with different experimental cellular models of NK cells. Human NKL cell lines, as well as human and murine purified NK cells were used in order to estimate the cellular interactions between NK cells and endothelial cells.

The activity of NK cells is determined by the balance between activatory and inhibitory receptor molecules expressed on the cell surface [11]. It is known, that several cytokines and chemokines can significantly modulate NK cells activity. NK cells, after activation, can differentiate into regulatory NK cells (producing cytokines) or cytotoxic NK cells (releasing granzyme and perforin), depending on cytokine environment [8, 9, 127]. Among cytokines, particularly IL-2 is known to augment NK cells cytotoxicity [78]. Moreover, it has been demonstrated that IL-2 activated NK cells induce their adhesion to ECs [79]. Consequently, it was proposed that, under specific conditions, IL-2 activated NK cells would be able to recognize and adhere to ECs. Adhesion test performed under static conditions reveal that only IL-2 activated NK lymphocytes were able to adhere effeciently to ECs, preferentially to ECs isolated from lymphoid organs, as compared to ECs isolated from non lymphoid tissues. It has been previously demonstrated, that immortalized human and murine microvascular ECs preserved their organ specificity [2, 3; 84]. The organ specificity of EC interactions with NK cells demonstrated in static conditions was verified and confirmed with flow adhesion assay. The first method used for the evaluation of lymphocyte adhesion to endothelium was the Stamper–Woodruff assay [128]. Different approach is used now, involving the study of adhesion under physiological shear flow conditions in order to apply environmental parameters comparable to *in vivo* conditions [129]. In these conditions it was confirmed that IL-2 stimulated NK cells adhere better to ECs isolated from lymphoid organs than to ECs isolated from non lymphoid tissue. Moreover, organ specificity of ECs was also demonstrated by using the *in vitro* assay where NK cells adhere under flow conditions to the monolayer mixture of two EC lines of different tissue origin. This was the first direct demonstration that *in vitro* organ targeting can be achieved and further used for diagnostic purposes.

Physiological changes distinctive for the tumor microenvironment are characterized by hypoxia, low nutrient levels, low extracellular pH, and high interstitial fluid pressure [130]. However, it is still not known, how the immune system is modulated in response to such a stress. Therefore experiments include hypoxia as well as low pH influence on all lymphocyte subtypes, especially NK cells activity should be performed to clear the subject. Targeting the tumor microenvironment to awaken or re-awaken immune cells will require a deep understanding of these interactions mechanisms. It is known that tumor ECs are proliferating in response to the tumor hypoxic signals. Therefore, adhesion experiments of NK cells toward ECs performed in a hypoxia under static conditions were performed. Hence, the exposure of ECs to hypoxia did not modulate significantly the NK cells adhesion to ECs, it could be hypothesized that not hypoxia alone, but in combination with other tumor factors, may augment NK cells adhesion or at least induce ECs phenotypic switch in such a way that tumor ECs would be recognized by NK cells as target cells.

It is known that selectins, integrins and immunoglobulin gene superfamily of adhesion receptors mediate different steps of lymphocytes migration from the bloodstream toward the inflammatory foci. In general, lymphocyte/endothelial cell interactions such as capture, rolling, and firm adhesion can no longer be viewed as occurring in discrete steps mediated by individual families of adhesion molecules but rather as a series of overlapping synergistic interactions among adhesion molecules, resulting in an adhesion cascade [131]. Therefore it was necessary to investigate the molecular mechanisms that could be involved in NK - ECs interactions. It has been demonstrated that NK cells adhesion to ECs is mainly an integrin-dependent process. However, it was also shown that chemokines, which are presented on the surface of ECs, *via* glycosaminoglycans [24], as well as C-type lectin like molecules [92, 132], may contribute in these interactions by activating ECs and/or NK cells.

Further, the physiological functions of NK cells suggest that these cells may play a potentially important role in pro- or anti-angiogenesis regulation within the tumor microenvironment. It is also well known that many angiogenic factors may influence the activity and function of immune cells that generally favors tumor survival and tolerance [133]. For example, NK cells cytotoxicity is enhanced *in vitro* in the presence of IL-2. However, when used in immunotherapy, this cytokine has not consistently augmented NK activity or even not caused antitumor effect, as NK cells activity was suppressed by various tumor tissues factors. Therefore current conventional therapies, which target tumors or tumor cells are clearly affected the physiological characteristics of the tumor microenvironment. Remembering that activated NK cells were able to recognize and further kill target cells, it was hypothesized that tumor endothelium may be lysed by IL-2-stimulated NK cells. NK cells may thus limit or exacerbate tumor progression. Therefore *in vitro* experiments, where ECs susceptibility to NK cells lysis was analyzed, were prepared. Currently, biological conditions for the *in vitro* cytotoxicity tests are only

experimental approaches which exploit the targeted cytolytic activity of lymphocytes. Too high effector-to-target cell ratio as well as an arbitrary time of cellular mixture incubation are not reflecting *in vivo* conditions at all. Nevertheless, only those preliminary data obtained by such tests allow extrapolating *in vitro* results to *in vivo* situation.

Knowing that *in vivo* NK cells cytotoxic activity is executed mainly *via* a granule-mediated pathway, it was necessary to investigate if NK cells are able to kill ECs by releasing perforin and granzymes. Obtained results show that it was the main cytotoxic pathway for the NK cell line used. Human purified NK cells were shown to induce not only this pathway. Knowing that purified NK cells are positive for NKp44 (*N*atural *C*ytotoxicity *R*eceptor) it seems that NKp44 may be involved in NK cells cytotoxicity. It is known that this receptor, upon engagement by their ligand NKp44L, induces a strong activation of NK-mediated cytotoxic activity [104, 134, 135].

Target cell apoptosis may be also induced by death receptors (membrane-bound protein complexes), which bind their cognate ligands and activate an intracellular signaling cascade. More recently, signaling from these receptors has been shown to activate multiple other processes, including cell survival and proliferation [136]. Therefore it was necessary to determine, whether death receptors ligands could really mediate ECs apoptosis. It was shown that only TNF–α may induce apoptosis of ECs used in the tests. It has to be verified if TNF-α produced by stimulated NK cells could induce ECs apoptosis, as it was described that this cytokine may elicit a broad spectrum of biological effects in target ECs [117]. Additionally, it was demonstrated that NK cells upon binding to ECs are able to activate their cytochrome c releasing and bid translocation, main executor molecules in mitochondrial apoptosis pathway.

Knowing that several cytokines may augment NK cells cytotoxic activity *in vitro,* such an activation could be proposed as a therapeutic approach to apply *in vivo* against a variety of cancers. The immunomodulating and antiangiogenic functions of IL-12 were documented in several preclinical studies, however, clinical trials with IL-12 have shown its limited efficacy, in most instances [137]. It was found that IL-12 exerts an antiangiogenic program mediated by IFN-gamma-inducible genes and by lymphocyte-endothelial cell cross-talk. Transfer of the murine IL-12 gene *in vivo* induced tumor regression through inhibition of mouse tumor blood vessel formation [1]. Based on this observation it was hypothesized that stimulated simultaneously by IL-2 and IL-12 murine NK cells could lyse ECs involved in tumor angiogenesis. Experiments reported above showed that *in vitro* murine NK cells are able to recognize and kill murine ECs.

Taking all these facts into consideration it could be proposed an alternative antitumor therapy with activated NK cells. Instead of direct killing of tumor cells by stimulated NK cells, targeting of tumor proliferating endothelial cells under specific microenvironmental conditions will be investigated.

SUMMARY

NK cells as well as endothelial cells are easily phenotypically and functionally adapted to different biological situations, being greatly submitted to influence of local microenvironment.

To solve the puzzle of NK - ECs interactions, adhesion and cytotoxicity of NK cells toward human and murine model endothelial cell lines were investigated using established NK cell lines, as well as freshly isolated NK cells. The data revealed by this work are listed below:

ECs – NK mutual recognition and adhesion:

- Activated by IL-2 NK cells are able to recognize and adhere to ECs,
- Adhesion of human NK cells takes place under static as well as flow conditions,
- Adhesion process seems to be achieved mainly by integrin molecules (magnesium, not calcium dependent),
- The adhesive interactions efficiency is different depending on ECs organ origin.

Mechanisms of ECs killing by NK cells:

- After activation by interleukins IL-2 (human and mouse) and IL-12 (mouse) NK cells are able to kill ECs,
- The NK cells killing efficiency is different according to ECs organ origin,
- Perforin and granzyme are the major proteins involved in NK cells cytotoxicity toward ECs,
- ECs apoptosis is induced mainly *via* mitochondrial pathway: NK cells induce Bid translocation, as well as cytochrome c releasing from mitochondrion,
- ECs express also TNF death receptor (TNFR1); consequently ECs are sensitive to TNF-α induced cell apoptosis.

Although there are differences between murine and human NK lymphocytes regarding their surface molecules expression profile, our work shows that in both species NK cells have the ability to recognize and to kill endothelial target cells. The differential reactivity of organ specific endothelial cell lines used, added new information to knowledge on human ECs biology.

However, the prospective murine model offers exciting opportunity to investigate some of NK cells mediated cytotoxicity mechanisms in *in vivo* conditions.

It could be *a priori* hypothesized, that NK cells, being a lymphocyte subpopulation, will be able to recognize and to interact with endothelial cells. This phenomenon occurs commonly in NK cells trafficking to pathologically changed sites. However, in such a situation NK cells do not any harm to

encountered ECs. But it is also well known that NK cells are able to recognize pathologically changed cells. Particularly, interactions between immune system cells and pathologically changed cells within tumor still need exploration.

The above presented work has proven that in specific *in vitro* conditions, NK cells may kill endothelial cells. The next question to be answered is: *How to target specifically NK cells at tumor vascular endothelial cells, taking advantage of the tumor immunological environment?*

APPENDIX

PRODUCTS REFERENCE LIST

Name of the product	Company/Country
CFSE	Sigma, Germany
DAPI	Sigma, Germany
$DiOC_6(3)$	Molecular Probes, USA
DMEM	Invitrogen Life Technologies, Germany
dNTP	Qiagen, Germany
BSA	Sigma, Germany
EDTA	Sigma, Germany
EGTA	Sigma, Germany
fetal bovine serum	Invitrogen Life Technologies, Germany
Ficoll 400	Institute of Immunology and Experimental Therapy, Wroclaw, Poland
fungizone	Invitrogen Life Technologies, Germany
chymotrypsin	Sigma, Germany
gentamicin	Panpharma, France
$CaCl_2$	Sigma, Germany
HCl	Sigma, Germany
HEPES buffer	Invitrogen Life Technologies
human AB serum	Institut Jacques Boy, France
$MgCl_2$	Sigma, Germany
$Na_2(^{51}Cr)O_4$	Amersham Biosciences, France
NAA (non-essential amino acids)	Gibco, Germany
NK Cell Enrichment Cocktail	StemCell Technologies, USA
Oligo d(T)	Sigma, Germany
Omniscript Reverse Transcription Kit	Qiagen, Germany
OptiMEM I/Glutamax-I	Invitrogen Life Technologies, Germany
PBS	Gibco, Germany
PE Selection Kit	StemCell Technologies, USA
penicillin/streptomycin	Biochrom, Germany
Paraformaldehyde	Sigma, Germany
PKH26GL fluorescent green dye	Sigma, Germany
PKH26GL fluorescent red dye	Sigma, Germany
polymerase DNA Taq	Qiagen, Germany
propidium iodide	Sigma, Germany
recombinant human soluble TRAIL	Genentech, USA
R-phycoerythrin Selection Kit	StemCell Technologies, USA
rhIL-2	Aventis Pharma, France
RNeasy Protect Mini Kit	Qiagen, Germany
RPMI 1640	Biochrom, Germany
SDS	Bio-Rad, Germany
sodium pyruvate	Biochrom, Germany
TNE buffer	Sigma, USA
TNF-α	R&D Systems, France
Trypan Blue	Sigma, Germany
Trypsin/EDTA	Biochrom, Germany
Vectashield antiphotobleaching reagent	Vector Laboratories, United Kingdom
β-mercaptoethanol	Sigma, Germany

ANIMALS

FVB/N mouse	Polish Academy of Sciences Medical Research Center, Warsaw, Poland

ANTIBODIES

a) Anti-human

Name of the antibody	Host species/conjugate	Company
Bid	rabbit polyclonal/unconjugated	Santa Cruze Biotechnology
CCR1	mouse IgG_2/PE	R&D Systems
CCR4	mouse IgG_1/PE	BD Pharmingen
CCR5	mouse IgG_1/APC	R&D Systems
CCR6	mouse IgG_1/PE	BD Bioscience
CCR7	rat IgG_2/PE	BD Bioscience
CD161	mouse IgG_1/PE-Cy5	BD Pharmingen
CD18	mouse IgG_1/unconjugated	BD Pharmingen
CD3	mouse IgG_1/FITC	BD Pharmingen
CD56	mouse IgG_1/PE	BD Pharmingen
CD69	mouse IgG_1/PE-Cy5	BD Pharmingen
CD94	mouse IgG_1/FITC	BD Bioscience
CX3CR1	rabbit IgG/unconjugated	Serotec
CXCR3	mouse IgG_1/FITC	R&D Systems
CXCR4	mouse IgG_2/PE	BD Pharmingen
CXCR6	mouse IgG_2/PE	BD Pharmingen
Cytochrome c	mouse IgG_1/unconjugated	BD Pharmingen
Fas (UB2)	mouse IgG_1/unconjugated	Immunotech
Fas (7C11)	mouse IgM/unconjugated	Immunotech
Isotypic control	mouse IgG_1/unconjugated	Sigma
Isotypic control	rabbit polyclonal IgG/unconjugated	Sigma
Isotypic control	mouse IgM/unconjugated	Sigma
Isotypic control	mouse IgG_2/unconjugated	Sigma
Isotypic control	mouse IgG_1/APC	BD Pharmingen
Isotypic control	mouse IgG_1/PE	BD Bioscience
Isotypic control	rat IgG_2/PE	BD Bioscience
Isotypic control	mouse IgG_2/PE	R&D Systems
Isotypic control	mouse IgG_1/FITC	BD Bioscience
Isotypic control	mouse IgG_1/PE-Cy5	BD Pharmingen
NKp44	mouse IgG_1/PE	BD Pharmingen
Secondary	goat anti mouse IgG_1/Alexa Fluor 488	Molecular Probes
Secondary	goat anti rabbit/Alexa Fluor 488	Molecular Probes
Secondary	goat anti mouse IgG_2 FITC	Sigma
TNFR1	mouse IgG_2/unconjugated	Serotec
TRAIL-R1	mouse IgG_2/unconjugated	Immunex
TRAIL-R2/R3/R4	mouse IgG_1/unconjugated	Immunex

b) Anti-mouse

Name of the antibody	Host species/conjugate	Company
CD3	rat IgG_2/FITC	BD Pharmingen
CD49b	rat IgM/PE	BD Pharmingen
Isotypic control	rat IgG_2/FITC	BD Pharmingen
Isotypic control	rat IgM/PE	BD Pharmingen

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Cytotoxicité des cellules tueuses naturelles vis à vis des cellules endothéliales organospécifiques : vers une immunothérapie tumorale

Résumé : Plusieurs mécanismes peuvent réduire l'angiogenèse tumorale d'où les stratégies visant à bloquer les cellules endothéliales (CE). Les cellules tueuses naturelles (NK) (natural killer cells) stimulées, s'arment pour l'élimination des cellules « dangereuses ». Notre hypothèse est qu'en conditions pathologiques (tumeur), les CE, acteurs de l'angiogenèse tumorale seraient reconnues comme telles et candidates à l'attaque par les NK. Les interactions entre les NK et les CE sont abordées à l'aide de CE humaines *in vitro,* quant aux mécanismes moléculaires de l'adhésion des NK en conditions statiques et conditions de flux. Ceci montre que les NK activées par l'IL-2 reconnaissent et adhérent aux CE selon leur origine tissulaire. Ce mécanisme est indépendant des sélectines mais dépend soit des intégrines, soit des co-récepteurs similaires aux lectines de type C. La cytotoxicité des NK vis-à-vis des CE s'exerce par la voie perforine-granzyme. En outre, stimulées par l'IL-2, les NK induisent la translocation de Bid et libération du cytochrome C dans les CE cibles lesquelles expriment les récepteurs de "mort", voie alternative d'apoptose. Ce modèle *in vitro* est validé avec des NK du sang humain. A visée *in vivo*, les expériences réalisées avec des CE murines et des NK de la rate de souris indiquent que l'efficacité des NK activées par l'IL-2 est directement reliée à leur adhésion, laquelle dépend de l'origine tissulaire des CE. Nous démontrons que l'IL-12 (interleukine connue pour inhiber l'angiogenèse tumorale) active les NK en synergie avec l'IL-2. Les NK reconnaissant et tuant les CE *in vitro* suggère l'hypothèse *qu'in vivo* elles inhibent l'angiogenèse tumorale.

Mots clés : angiogenèse, cellules endothéliales, cellules tueuses naturelles, cytotoxicité.

Study on NK cells cytotoxicity toward endothelial cells

Résumé en anglais: There are several mechanisms by which tumor angiogenesis may be reduced. They are addressed to tumor endothelial cell (EC) to block their activation and proliferation. Among the natural protection mechanisms, natural killer (NK) cells play an important role. Stimulated NK cells may eliminate potentially "dangerous" cells. Hence, we hypothesized that ECs involved in tumor angiogenesis may be recognized as "dangerous" cells and killed by NK cells. First, the adhesive interactions between NK cells and ECs were investigated. Using human *in vitro* cell lines, molecular mechanisms of NK cells adhesion to ECs under static and flow in vitro conditions were examined, showing that IL-2 activated NK cells were able to adhere to ECs. The molecular mechanism was shown not to be selectin - dependent but integrin and/or C type lectin like - dependent. Subsequently, cytotoxicity of NK cells toward ECs was analyzed. Its molecular mechanism was mainly perforin-granzyme dependent. NK cells were shown to induce in target ECs Bid translocation and cytochrome c releasing. To validate *in vitro* cellular model most of these experiments were repeated with freshly isolated human blood NK cells. The *in vivo* approach necessitated that adhesion and cytotoxicity were performed using murine ECs lines and freshly isolated NK cells from murine spleen. It was found that IL-12 synergizes with IL-2 to stimulate NK cells killing activity toward ECs. The demonstration that, under specific conditions stimulated NK cells are able to kill ECs *in vitro* allows hypothesizing that *in vivo* NK cells may participate in tumor angiogenesis inhibition.

Key words: angiogenesis, endothelial cells, natural killer cells, natural cytotoxicity.

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