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"Role of lysophospholipids in the biological activity of NK cells"

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Abbreviations

18:1 LPA	18:1 Lyso PA 1-Oleoyl-2-Hydroxy-sn-Glycero-3-Phosphate
14:0 LPA	14:0 Lyso PA 1-myristoyl-2-Hydroxy-sn-Glycero-3-Phosphate
Ab	Antibody
ADCC	Antibody-Dependent Cellular Cytotoxicity
Akt/PKB	Protein kinase B
ATX	Autotaxin
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine-3',5'-Monophosphate
Ctx	Cholera Toxin
DGPP	Dioctylglycerol pyrophosphate
ELISA	Enzyme-linked immuno-absorbent assay
ERK	Extracellular signal regulated kinase
FACS	Fluorescence Cytometry
FCS	Fetal Calf serum
FITC	Fluoro-isothiocyanate
GCPR	G protein couple receptor
GROα	Stimulates Growth related oncogene-α
Grz	Granzymes
GSK3β	Glycogen synthase kinase 3β
HLA	Human Leukocyte Antigen
HRP	Horseradish peroxidase
HS	Human Serum
IL-	Interleunkin-
IFN-	Interferon

LPA	Lysophosphatidic acid
LPLs	Lysophospholipids
MHC	Major Histocompatibility Complex
NBD	Nitrobenzooxadiazol
NK cell	Natural Killer cells
PBS	phosphate-buffered saline
p38	mitogen-activated protein kinase p38
PE	Phycoerythrin
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
Ptx	Pertussis Toxin
PVDF	Polyvinylidene difluoride
Raji	Human Burkitt's lymphoma cell line
RANTES	Regulated on activation-normal T cell expressed and secreted
S1P	Sphingosine-1-phosphate
SDS-PAGE	SDS-Polyacrylamide-Gel electrophoresis
SEM	Standard error of the mean
TGF β	transforming growth factor β
Wtm	Wortmannin

Summary

Lysophosphatidic acid and sphingosine 1-phosphate are bioactive lysophospholipids (LPLs) that transmit signals through a family of G-protein-couple receptors to control cellular differentiation and survival, as well as vital function of several types of immune cells. In addition, LPA and S1P are potent inducers of many of the hallmarks of cancer including cell proliferation, survival, migration, invasion and neovascularization. The enzymes that produce these two small lysophospholipids are aberrant in multiple cancer lineages and exhibit transforming activity. Additionally, LPLs levels are increased in patients with several types of cancer including melanoma.

NK cells are critical members of the immunological tumor surveillance machinery. They are able to attack abnormal cells such as virus-infected cells or transformed tumor cells. Enhanced NK cell cytotoxic activity after stimulation with classical chemotaxins such as RANTES/CCL5 is well known. These classical chemotaxins bind to specific G_i protein-coupled receptors linked to activation of phospholipase C and PIP_3–generating type IB phosphatidylinositol 3-kinase. Moreover, LPA and S1P induce chemotaxis of NK cells through pertussis toxin sensitive-G proteins. In this context, the biological functions of LPLs and their influence on the interaction of human NK cells with tumor cells were characterized.

The results presented in this work indicate that LPA and S1P in contrast to classical chemotaxins such as CC chemokines stimulate G_i as well G_s protein-dependent signalling pathways in NK cell. While LPL-induced actin polymerization is dependent on Gi activation, LPL activation of NK cells results in increased cAMP levels and decreased cytotoxic activity against tumor cells. Consequently, cAMP signalling leads to the immediate activation of protein kinase A. Moreover, blocking the regulatory subunits of PKA I abrogates the inhibitory effect of LPLs, whereas the catalytic subunits are not involved. The inhibitory effect of LPLs on NK cell cytotoxicity have been observed with Burkitt's lymphoma cell line Raji and the A2058, HS294T and SK-Mel 23 human melanoma cell lines and at various effector:target ratios.

Therefore one can assume that activities of NK cells are impaired *in vivo* by local production of LPLs. LPA and S1P may block the activity of a major anti-tumor effector cells i.e. NK cells, providing a favourable environment for the growth of tumor cells.

Zusammenfassung

Lysophosphatidsäure und Sphingosin 1-Phosphat stellen bioaktive Lysophospholipide (LPL) dar, die über G-Protein gekoppelte Rezeptoren wirken und somit Einfluss auf die Differenzierung, Überleben und weitere wichtige Lebensfunktionen verschiedener Immunzellen ausüben. Darüberhinaus sind LPA und S1P in der Lage charakteristische zelluläre Eigenschaften von Tumorzellen, wie Proliferation, Überleben, Migration, Invasion und auch Neovaskularisierung zu induzieren. In verschiedenen Krebszelllinien zeigen die Enzyme, die diese zwei Lysophospholipide bilden, eine erhöhte Funktionsrate und haben somit eine transformierende Wirkung. So konnte in Patienten mit verschiedenen Krebstypen, unter anderem auch in Hautkrebspatienten erhöhte LPL-Konzentrationen nachgewiesen werden.

Natürliche Killerzellen (NK-Zellen) sind wichtiger Bestandteil der immunologischen Abwehr von Tumorzellen. Sie sind in der Lage, abnormale Zellen wie zum Beispiel virusinfizierte Zellen als auch Tumorzellen anzugreifen. Es ist bekannt, dass NK-Zellen nach ihrer Stimualtion mit klassischen Chemotaxine wie RANTES/CCL5 eine gesteigerte zytotoxische Aktivität gegenüber ihren Zielzellen aufweisen. Diese Chemotaxine binden an spezifische Gi-Protein gekoppelte Rezeptoren und aktivieren intrazelluläre Signalwege, die spezifische Signalproteine wie die Phospholipase C und PI3Kγ einschließen.

Auch LPA und S1P induzieren in NK-Zellen chemotaktische Reaktion; die über Pertussis Toxin sensitive Gi-Proteine vermittelt werden. In diesem Zusammenhang sind die biologischen Funktionen der LPLs und deren Einfluss auf die Interaktion zwischen NK-Zellen und Tumorzellen gut charakterisiert.

Die Ergebnisse dieser Arbeit deuten darauf hin, dass LPA und S1P im Gegensatz zu klassischen Chemotaxinen wie CC-Chemokine sowohl G_i als auch G_s Protein abhängige Signalwege induzieren. Es konnte gezeigt werden, dass die LPL-induzierte intrazelluläre Aktin-Polymerisierung Gi-Protein abhängig ist. Darüber hinaus führte eine Aktivierung von NK-Zellen aber auch zu einem Anstieg des intrazellurären cAMP-Spiegels und zu einer verringerten ytotoxischen Aktivität gegenüber

Tumorzellen. In Folge der Aktivierung des cAMP-Signalweges kommt/kam es erwartungsgemäß auch zur Aktivierung der Proteinkinase A. Durch die Hemmung der regulatorischen Untereinheit der PKA konnte der inhibitorischen Einfluß der LPLs auf die zytotoxische Aktivität unterbunden werden. Im Gegensatz dazu scheint die katalytische Untereinheit von PKA in diesem Signalweg keine Rolle zu spielen. Der inhibitorische Einfluss der LPLs auf die zytotoxische Aktivität von NK-Zellen konnte gegenüber verschiedener Krebszelllinien, wie dem Burkitt's Lymphom, der Raji-Zelllinie und humanen Hautkrebszelllinien (A2058, HS294T und SK-Mel 23) in verschiedenen Verhältnissen zwischen Effektor- und Zielzellen beobachtet werden.

Somit kann angenommen werden, dass die Aktivität von NK-Zellen *in vivo* durch eine lokale Produktion dieser Lysophospholipide beeinträchtigt werden. Möglicherweise hemmen LPA und S1P die Aktivität wichtiger Effektorzellen der Krebsbekämpfung, so zum Beispiel NK-Zellen, wodurch ein günstiges Mikromilieu für das Wachstum von Tumorzellen geschaffen wird.

1. Introduction

1.1. Immune System

The immune system is the body's defense machinery against infectious organisms and other invaders. Through a series of steps called the **immune response** and using several mechanisms including cell-cell contact and secreting soluble factors, the immune system attacks organisms and substances that invade our systems. The immune system is composed of an intricate network of tissues, cells and molecules that collectively work to protect the body. Pluripotent hematopoietic stem cells give rise both to myeloid lineages that participate in the innate and adaptive immune responses and to the lymphocytes responsible for adaptive immune response.

1.1.1. Innate Immune Response

The innate or nonspecific immune response is the first line of defense against pathogen invasion and does not require previous exposure to pathogens to be activated. The innate immune response includes epithelial barriers, phagocytic cells, NK cells, complement system, defensins and cytokines. The innate immune response provides resistance based on physical, chemical, biochemical and cellular approaches. Through these approaches, innate immunity can prevent the colonization, entry and spread of microbes (Immunobiology, Janeway 5th Ed.).

The innate immune response is immediately activated upon pathogen recognition by dendritic cells (DCs), natural killer (NK) cells, natural killer T (NKT) cells, γδ T cells, macrophages and granulocytes (Münz et al., 2005). The innate immune response depends on germline-encoded receptors that have evolved to recognize structures conserved among many pathogens, for example, lipopolysaccharide (LPS) and CpG rich regions. Toll-like receptors, which exemplify these pattern recognition receptors, are involved in the innate immune response. Engaging innate immune receptors activates effector functions such as cytotoxicity, phagocytosis, and production of

cytokines. Neutrophils, eosinophils and basophils are collectively known as granulocytes; they circulate in the blood unless recruited to act as effector cells at sites of infection and inflammation. Macrophages and neutrophils of the innate immune system provide a first line of defense against many common microorganisms and are essential for the control of common bacterial infections. Macrophages phagocyte bacteria and recruit other phagocytic cells from the blood. Mast cells are exocytic and are thought to orchestrate defense against parasites; these cells recruit eosinophils and basophils, which are also exocytic (Immunobiology, Janeway 5th Ed.).

The innate immune mechanisms are instantly triggered and are followed by early induced responses, which do not generate lasting specific protective immunity. Only if an infectious organism can breach these early lines of defense will an adaptive immune response arise and generate antigen-specific effector cells or antibodies that specifically target the pathogen and generate memory.

1.1.2. Adaptive Immune Response

The adaptive or specific immune response, the second line of defense, refers to responses which are less rapid but more effective than the innate immune response. The delay that occurs is due to expansion of a limited responder pool of pathogen-specific effector cells. In contrast to innate immunity, adaptive immunity can amplify and sustain its responses, and has the unique ability to establish memory. These memory cells produce a vigorous immune response immediately following subsequent encounters with the same agent. The cellular components of the adaptive immune system are white blood cells called lymphocytes. After genes B and T are rearranged, lymphocytes acquire a pathogen-specific response to express B cell (BCR) and T cell (TCR) receptors that recognize pathogenic antigens. The TCR recognizes pathogen-encoded peptides presented on the surface of an infected cell by major histocompatibility complex (MHC) molecules. Engaging TCRs that recognize pathogen antigens results in clonal expansion and MHC-dependent

cytotoxicity and/or cytokine production. Engaging BCRs leads to the production of pathogen-specific antibodies and the presentation of pathogen-derived peptides.

The mechanisms of innate immune response discriminate very effectively between host cells and pathogen surfaces. The ability to discriminate between self and nonself and to recognize broad classes of pathogens contributes to the induction of an appropriate adaptive immune response. Natural killer cells constitute another population of lymphocytes but unlike other lymphocytes subsets, NK cells are components of innate immunity which play a critical role in early innate immune responses to viral infections (Lanier et al., 1986; Moretta et al., 2002).

1.2. Natural killer cells

1.2.1. Background

Natural killer cells are specialized lymphocytes of the innate immune system capable of distinguishing between normal healthy cells and abnormal cells such as virus-infected cells or transformed tumor cells. Like T and B cells, NK cells arise from a pluripotent hematopoietic stem cell in the bone marrow. Upon maturation they migrate out and circulate through the blood and lymphoid organs, where they make up approximately 5% of circulating lymphocytes (Yoon et al., 2007). They are also found in peripheral tissues, including the liver, peritoneal cavity and placenta. They were first identified in the 1970s by their ability to kill cancer cells and virally infected cells without prior sensitization (Kiessling et al., 1975). NK cells are also able to secrete cytokines and chemokines that mediate inflammatory responses and exert a regulatory effect on the adaptive immune responses (Lanier et al., 1986; Moretta et al., 2002).

Phenotypically, NK cells are defined by the expression of CD56 and lack of expression of CD3. Two distinct populations of human NK cells can be identified based on the cell surface density of CD56 (Cooper et al., 2001). $CD56^{dim}$ NK cells constitute the majority (90%) and express CD16

(FcRIII). They possess greater cytotoxic capacity than do the minority subset of $CD56^{bright}$ NK cells. These subsets of NK cells express low amounts of CD16 but produce large amounts of cytokines. NK cell subsets differentially express other structures involved in cytotoxicity, such as chemokine receptors (e.g. CCR7, CXCR3, CXCR1) and adhesion molecules (e.g. CD2, CD44, LFA-3, or ICAM- 1) and may have distinct trafficking patterns during the immune response (Vivier, 2006).

Inhibitory signals keep NK cytotoxicity in check, preventing normal cells from being killed. The NK cell effector functions are regulated by ubiquitously expressed MHC class I molecules. These findings led to the "*missing-self*" hypothesis (Kärre et al., 1986). The first breakthrough in understanding how NK cells recognize target cells was the realization that the loss of MHC class I expression makes NK cells sensitive to lysis (Kärre et al., 1986). According to this hypothesis, NK cells appear to be actively inhibited by receptors that recognize MHC class I molecules. Normal cells express a threshold level of MHC class I molecules that protect them from NK cell-mediated destruction. In contrast, virus-infected or malignant cells express reduced levels of MHC class I molecules and therefore become sensitive to NK cell-mediated attack. Until recently, there has been a common misconception that NK cells will attack any cell lacking MHC expression because such target cells do not provide the necessary inhibitory signal. However, NK cells must also receive positive signals from target cells to be activated. NK cell activation requires cell-cell contact, immune synapse formation and Ca^{2+} mobilization following an encounter with target cells lacking MHC class I (Davis et al., 1999). Additionally, to activate and co-stimulate their effector function, NK cells must recognize many ligands on target cells (Kirwan and Burshtyn, 2007). Several studies determined that NK cells often express multiple receptors simultaneously. This fact allows NK cells to be broken down into subset populations expressing specific combinations of activating or inhibitory receptors (Smyth et al., 2005). The discovery of activating receptors expressed in NK cells adds another layer of complexity to how these cells function and underscores

the need to consider the regulation of NK cells in terms of a balance between inhibitory and activating signals.

1.2.2. NK cell Receptors

First, NK cells bind to potential target cells. Then, activating and inhibitory receptors interact with ligands on the target cells, and the integration of signals transmitted by these receptors determines whether the NK cell detaches and moves on or stays and responds. Human NK cell activity is repressed upon recognition of both classical and non-classical HLA (HLA stands for **h**uman **l**eukocyte **a**ntigen) class I molecules. Humans synthesize three different types of classical class I molecules, designated HLA-A, HLA-B, and HLA-C, and three different types of non-classical class I molecules, HLA-E, HLA-F and HLA-G (López-Botet and Bellón, 1999). Two major families of NK cell receptors have been identified in humans. The killer immunoglobulin (Ig)-like receptors (KIR) constitute the first family and they have extracellular immunoglobulin domains that recognize HLA-B and HLA-C molecules. The second family of receptors are the C-type lectin NKG2 (NK group 2). In each family, some receptors inhibit and some receptors activate NK cells (Moretta et al., 2006; Lanier, 2001; Lanier, 2005) (Figure 1.1.).

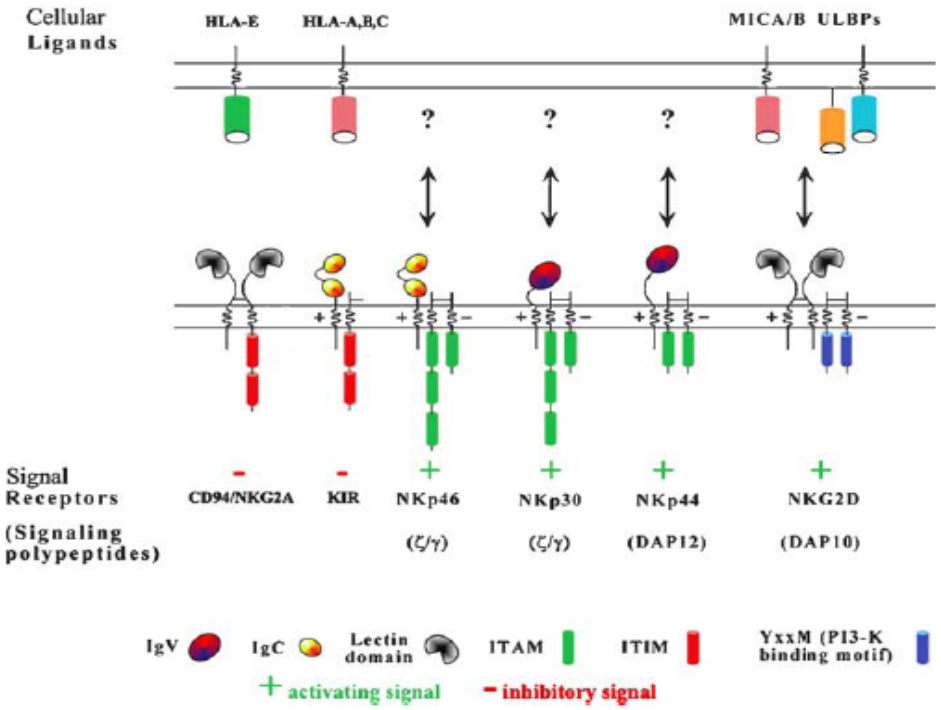


Figure 1.1. The main activating and inhibitory receptors in NK cell triggering or inhibition. Molecules or domains responsible for the generation of activating signals are represented in green. Those mediating inhibitory signals are in red (adapted from Moretta et al., 2006).

1.2.2.1. NK inhibiting receptor

The best characterized inhibitory signals are those transduced by HLA-specific receptors. In humans, killer immunoglobulin-like inhibitory receptors (KIRs) and the lectin-like receptor CD94/NKG2A play a major role as HLA-class I-specific inhibitory receptors (Figure 1.1.). A conserved feature of these inhibitory receptors resides in the presence of one or two intracytoplasmic inhibitory signalling domains, immunoreceptor tyrosine-based inhibition motifs (ITIMs) (Vivier and Romagné, 2007). The expression of ITIMs is restricted to NK cells and a small subset of T cells. Each NK cell expresses at least one receptor specific for self HLA class I molecules. The binding of inhibitory KIRs by their HLA ligands on healthy cells inhibits NK cell activation and protects the HLA^+ cell from NK cell lysis. Ligation of KIRs by their cellular ligands

results in phosphorylation of the ITIMs and the recruitment of phosphatases. In turn, these phosphatases switch off the signalling cascade initiated by various activating receptors (Moretta and Moretta, 2004). Parallel to the KIRs, NK cells express CD94 and NKG2 heterodimer receptors, which recognize the non-classical class I HLA-E molecule (O'Callaghan, 2000). Therefore, KIRs are functionally complemented by CD94/NKG2A reacting to changes in global HLA expression (O`Connor et al., 2005).

1.2.2.2. NK activating receptor

The term 'NK activating receptor' generally refers to those receptors that trigger release of cytolytic granules and induce cytokine production. Established activating receptors on NK cells include the activating killer cell Ig-like receptors (activating KIRs), CD16 (low-affinity Fc receptors for immunoglobin G), the natural cytotoxicity receptors (NCR) and the NKG2 (natural-killer group 2) family (Figure 1.1.). Ligands for several but not all of these receptors are known. In contrast to NCR and NKG2 receptors, they bind to HLA ligands (Moretta et al., 2006). The activating KIR receptors do not contain ITIMs; rather they contain a positively charged residue within their transmembrane domains, which facilitates binding of the adaptor protein DNAX-activating protein (DAP) of 12 kDa. The key feature of DAP 12 which links it to an activating function is the presence of an immunoreceptor tyrosine-based activation motif (ITAM). Upon receptor engagement, the ITAMs of DAP-12 become phosphorylated and subsequently initiate an activating signalling cascade (Tassi et al., 2006).

CD16 is a molecule of the immunoglobulin (Ig) superfamily known to be involved in antibody-dependent cellular cytotoxicity (ADCC) and is a low-affinity receptor for the Fc portion of some IgGs (Mandelboim et al., 1999).

Three different types of natural cytotoxicity receptors (NCR) have been identified: NKp30, NKp44 and NKp46. They can associate with different transmembrane-anchored polypeptides bearing ITAMs including DAP12, FcεRIγ or CD3ζ and then transduce activating signal to NK cells upon ligand recognition (Bottino et al., 2004). Apparently, NCRs may have been co-opted by NK cells to recognize pathogen-specific moieties. NKp46 and NKp44 recognize virus-specific haemagglutinins and facilitate NK cell lysis of virally infected cells. In contrast, NKp30 appears to have been targeted by the pp65 protein of human cytomegalovirus (HCMV) (Lanier, 2001; López-Botet et al., 2000).

NKG2D (natural-killer group 2, member D) is a powerful activating receptor. The NKG2D molecule is a C-type-lectin-like molecule and is expressed by NK cells, by some subsets of T cells and by the recently identified myeloid-lineage interferon-producing killer dendritic cells (IKDCs). Unlike other NKG2 receptors, NKG2D exists as a homodimeric receptor. The stimulus for inducing the expression of NKG2D ligands is widely referred to "cellular stress". This term include tumorigenesis and infection by a variety of pathogens as well as classic-stress stimuli such as heat shock. NKG2D ligands are proteins that are structurally similar to MHC class I molecules. Some class IB genes – for example, the members of the MIC gene family –are controlled by a different regulator that the classical MHC class I genes and are induced in response to cellular stress. There are five MIC genes, but only two, *MICA* and *MICB*, are expressed and produce protein products (Bauer et al., 1999; Li et al., 2001). The cytomegalovirus UL16-binding protein (ULBP, also known as RAET1 proteins) as well as distantly related MHC class I molecules are ligands for the NKG2D receptor (Cosman et al., 2001). In general, NKG2D ligands are expressed at low to undetectable levels in normal cells; however, their expression is upregulated in pathological states such that involving the transformation or bacterial infection leading to the NKG2D-mediated clearance of these aberrant cells. Expression of these ligands is closely tied to activation of DNA repair mechanisms (Smyth et al., 2005; Hayakawa and Smyth, 2006). NKG2D is associated with

the adaptor molecule DAP10, which lacks an ITAM but does contain tyrosine-based motifs that can mediate an activating signal by recruiting PI3K (Rabinovich B, 2006).

NKG2D is known to participate in anti-tumor immune responses, immune surveillance and the suppression of experimentally induced tumors (Hayakawa and Smyth, 2006). NKG2D ligand expression by tumor cells may not be a barrier to tumor growth because many primary tumors and tumor cell lines naturally express NKG2D ligands, and in some cases these ligands may be secreted. The presence of a number of diverse NKG2D ligands that can be expressed in infected or malignant cells make it much more difficult for the virus or tumor to evade NKG2D-mediated immunity. Diversity also allows NKG2D ligands to be switched on by a greater range of stimuli, increasing the number of cell types in which they can be used to alert the host of danger.

Most activating receptors in NK cells couple to one of the primary signalling pathways: DAP12/Syk-ZAP70, DAP10/ Phosphoinositide 3-kinase (PI-3K) or SAP/Fyn. Phospholipase Cγ (PLCγ)-1, PLCγ-2 and Vav family members are subsequently activated to transmit these membrane events to NK cell effector functions (Lanier, 2001).

1.2.3. The mechanisms of NK function

As key members of the innate immune response, NK cells mediate cytotoxicity and cytokine secretion. Natural killing involves exocytosis of perforin and granzyme-containing cytoplasmic granules via a metabolically active process, or signalling via the death receptor pathway, which induces apoptosis in target cells. The second major function of NK cells is cytokine release, which occurs as the result of receptor engagement via cell-cell contact or soluble factors (e.g. IL-12 and IL-18).

1.2.3.1. Cytotoxic Activity

The destruction of antibody-coated target cells by NK cells is called antibody-dependent cell-mediated cytotoxicity (ADCC) and is triggered when an antibody bound to the surface of a cell interacts with Fc receptors on the NK cell. NK cells express the receptor FcγRIII (CD16), which recognizes the subclasses immunoglobulin IgG1 and immunoglobulin IgG3 and triggers cytotoxic attack to antibody-coated target cells. ADCC represents a mechanism through which antibodies can engage an Fc receptor and direct an antigen-specific attack by an effector cell that itself lacks specificity for antigens (Mandelboim et al., 1999).

Lytic granules are specialized 'secretory' lysosomes containing various proteins that are involved in cytotoxic functions, such as perforin and granzymes. Acting together, these proteins generate pores on the plasma membrane and activation of the caspase cascade after cleavage of caspase-3. Caspase activates DNase, which in turn degrades DNA, inducing the apoptosis of target cells (Moretta et al., 2008). Granules contain mediators of a diverse range of cell-death pathways that have evolved to rapidly kill cells harboring intracellular pathogens. Although pathogens have devised ways to prolong an infected cell's life, the death mediated by NK cells remains rapid and powerfully limits pathogen replication and spread (Trambas and Griffiths, 2003). The use of perforin and granzyme knockout mice has shown that the granule exocytosis pathway is the principal mechanism used by NK cells to eliminate virus-infected and transformed cells. Mice deficient in perforin are profoundly immunodeficient and susceptible to viral infection and cancer. Although mice deficient in either granzyme A or B are variously impaired, generally they are able to handle most infections (Kägi et al., 1994).

Major granule proteins include perforin and granule enzymes or 'granzymes' (Grz). Grz comprise about 90% of the mass of cytolytic granules of both cytotoxic T lymphocytes (CTLs) and NK cells. Eleven granzymes have been described (Smyth et al., 1996). Five of these are expressed in humans

(A, B, H, M and tryptase-2, which is also known as granzyme 3). The most abundant granzymes are granzyme A and granzyme B. Redundant, each is able to proteolytically activate independent cell death pathways. Perforin is a Ca^{2+}-dependent pore-forming protein that has homology to complement components. It is the only molecule that can deliver granzymes into the target cells. According to the current model, perforin creates pores in the target cell membrane that transiently allow Ca^{2+} influx into the cell. This Ca^{2+} flux triggers a wounded membrane repair response in which internal vesicles donate their membranes to reseal the damaged membrane. This also triggers rapid endocytosis of granzymes, which then play a critical role in initiating apoptotic cell death directly via the mitochondria by activating cellular caspases or caspase-independent pathways (Lieberman, 2003). Recently, Pipkin and Lieberman have proposed an alternative model that involves active target cell collaboration. They hypothesize that perforin is coendocytosed with granzymes and claim that perforin perturbs the endosomal membrane to release endosomal contents and direct the target cell to an apoptotic rather than a necrotic death (Pipkin and Lieberman, 2007).

However, NK cell are equipped with other destructive arms that can also eliminate target cells: the death receptor pathway, defined as TNF family ligand-mediated apoptotic killing. The tumor necrosis factor receptor (TNFR) family is composed of receptors containing cytoplasmic death domains that, once engaged by their ligands, activate caspase-signalling pathways leading to apoptosis. NK cells can express at least three death receptor ligands from this family: Fas ligand (FasL), TNF-α, and TNF related apoptosis-inducing ligand (TRAIL), all of which induce apoptosis in their targets (Smyth et al., 2005; Vujanovic, 2001). There is evidence suggesting that some of this ligand (e.g. FasL) and granular exocytosis pathways may work together (Tanaka et al., 1995).

1.2.3.2. Cytokine Secretion

An equally important function of NK cells involves their capacity to promptly produce cytokines and chemokines. NK cells can secrete several cytokines and chemokines, including interferon γ (IFN-γ), tumor necrosis factor α (TNFα), transforming growth factor β (TGFβ), granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage inflammatory protein 1α and 1β (MIP-1α, -1β), regulated on activation-normal T cell expressed and secreted (RANTES), interleukin-3 (IL-3), IL-10 and IL-13 (Maghazachi, 2003). These cytokines play diverse roles in immune function. They modulate infected or transformed cells increasing their MHC expression and making them more susceptible to clearance. Finally, they can shape the adaptive immune response. NK cells are the major source of IFN-γ during the innate immune response. NK cells secrete IFN-γ in response to IL-12 and IL-18 produced by macrophages and dendritic cells. Among other functions, IFN-γ directly inhibits viral replication and disrupts viral infectivity. IFN-γ also up-regulates transcription of MHC class I, class II, and peptide transporter molecules, increasing antigen presentation (Young and Hardy, 1995).

NK-cell-secreted cytokines influences not only macrophage and T cell functions but also B cell switch recombination and differentiation. Therefore, NK cells function as critical players in the innate immune response and bridge the gap between innate and adaptive immunity via the cytokines they secrete (Figure 1.2.). Thus, deregulating the NK function may alter the adaptive immune response and help set the stage for developing autoimmunity or disease (Robertson, 2002).

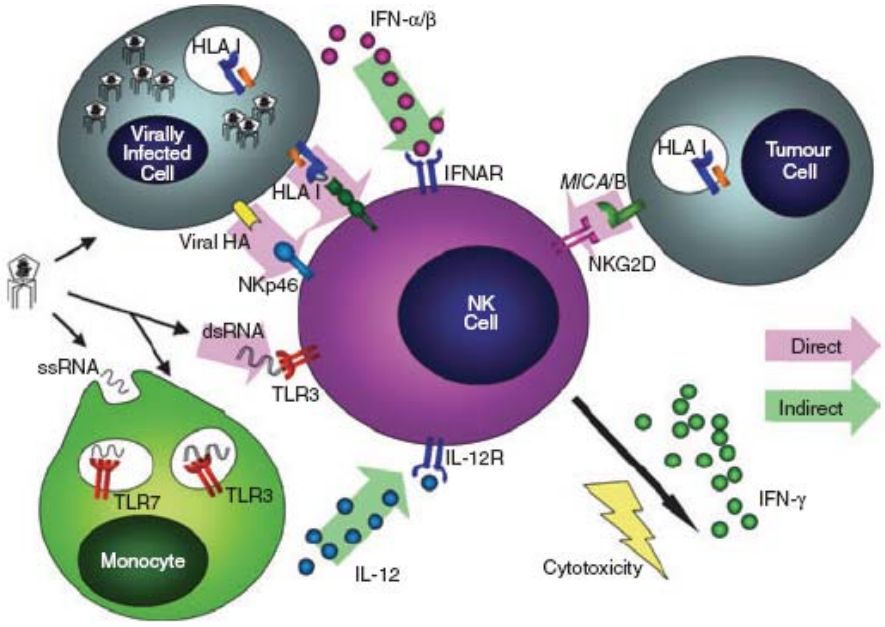


Figure 1.2. NK cells receive activation signals via a variety of direct and indirect mechanisms. NK cells receive a number of signals depending on the immunological challenge, which can be both direct and indirect, and it is the balance of these that culminates in the final NK cell response (O´Connor et al., 2005).

1.3. Chemotaxins and NK cells

1.3.1. Chemokines and NK cells

Chemotactic cytokines or chemokines are small-molecular-weight-secreted proteins (8-10 kDa) that direct the migration of leukocytes to sites of inflammation. As proinflamatory mediators, they recruit various cell types to help counteract inflammation. Chemokines are implicated in allergic disorders, autoimmune diseases and in ischemia associated with the infiltration of leukocytes (Maghazachi, 2000). These mediators also play important roles in the inflammatory conditions associated with autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus.

Chemokines are divided into three subfamilies according to shared structural characteristics including their small size and the presence of four cystein residues in conserved locations that are key to forming their 3-dimensional shape: the CC chemokines (or β chemokines), the CXC

chemokines, and the CX_3C chemokines (Murphy et al., 2000; Maghazachi et al., 1997). Despite their structural homology and ability to induce chemotaxis, different chemokines can elicit other distinct cellular responses and/or activate different pathways to elicit a particular response. The signalling and physiological response downstream of receptor activation can also vary, depending on the chemokine/receptor combination, the cell type and the pathophysiological state (Maghazachi, 2000).

The chemokine receptors are seven-transmembrane G protein-coupled receptors (GPCR). As such, they have been best characterized with respect to signalling through heterotrimeric G proteins, primarily through G_i. Although the α-subunit of G-proteins has traditionally been regarded as the major signalling subunit, the βγ-subunits are crucial for the activation of many chemokine-induced pathways (Figure 1.3.). Two of the major pathways activated by Gβγ are the phosphatidylinositol (3,4,5) tryphosphate PIP_3–generating type IB phosphatidylinositol 3-kinase and PLC β2 (phospholipase C) (Taubb et al., 1996). Additionally, $PtdIns(3,4,5)P_3$ mediates activation of the mitogen-activated protein kinase pathway or Jun kinase (Curnock et al., 2002), whereas Gαi proteins mainly inhibit adenylate cyclase and transduce signals through tyrosine kinases such as Src (Maghazachi, 2000).

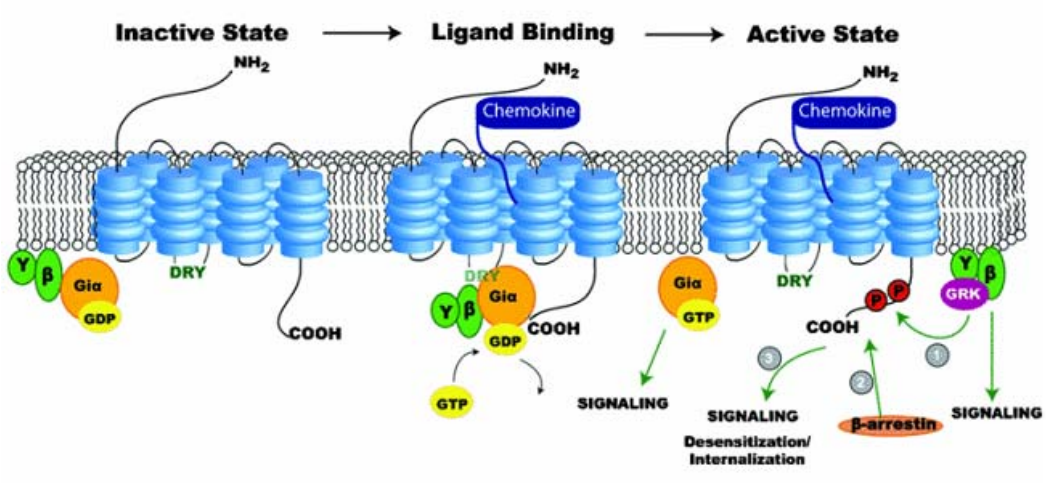


Figure 1.3. Classical activation of a chemokine GPCR. Chemokines bind to the extracellular side of their receptor and activates heterotrimeric G-proteins inside the cell by exposing important motifs. The G-proteins have three subunits: α, β and γ. The Gα subunit interacts directly with the GPCR C-terminal domain and with the G-protein β subunit, forming a tight complex with the γ subunit. In the inactive state, the Gα subunit binds GDP. Upon ligand binding and activation of the GPCR, GDP is then replaced by GTP, Gα-GTP dissociates from the receptor and from Gβγ, and both of these complexes subsequently activate a variety of downstream effectors that ultimately lead to the physiological response (adapted from O´Hayre et al., 2008).

In NK cells, several chemokine receptors are variously coupled to various subtypes of G proteins (al-Aoukaty et al., 1998). Such coupling is important since chemokines induce various activities in NK cells. CXCL1, CXCL8, CXCL10, CXCL12, CCL1, CCL2, CCL4, CCL5, CCL6, CCL7, CCL8, CCL11, CCL17, CCL19, CCL20, XCL1, and CX3CL induce the chemotaxis of human NK cells (Sebok et al., 1993, Maghazachi et al., 1994, 1997; Taub et al., 1995; Loetscher et al., 1996; Godiska et al., 1997; Maghazachi, 1997; Al-Aoukaty et al., 1998; Inngjerdingen et al., 2000, 2001; Fraticelli et al., 2001).

Under pathological conditions where inflammatory cytokines are present, NK cells are induced to express inflammatory chemokine receptors. Subsequently, they migrate into inflammatory, tumor, or virally infected sites where inflammatory chemokines are secreted in large quantities (Inngjerdingen et al., 2001; Maghazachi, 2003). In addition, NK cells recruit other cell types into the injured tissues by secreting chemokines. It turns out that the conditions governing NK cell

chemotaxis are the same as in other cell types where PI3Kγ and pleckstrin homology domain (PH domain) containing proteins play significant roles. Chemoattractants induce activation of various intracellular signalling molecules such as PI3K and lymphocyte-specific protein tyrosine kinase (Lck) that activate members of the GTPases necessary for NK cell orientation toward the chemoattractants, reshaping the cytoskeleton, and forming filopodia and lamellipodia (Maghazachi, 1999).

In addition to chemotaxis, chemokines have been shown to modulate a number of biological responses in leukocytes, including cellular activation, adhesion, enzyme release, degranulation, tumor cell cytolysis, and intracellular microbe death (Taubb et al., 1996). Although the mechanism by which chemokines enhance NK-cell-mediated killing is not completely known, several mechanisms have been proposed. Chemokines may augment NK cell lysis of target cells in part by facilitating the discharge of NK cell cytotoxic granules upon conjugation with sensitive target cells. Several CC chemokines known to augment NK cell cytotoxicity have been found to induce redistribution of adhesion molecules on the cell surface (Robertson, 2002). Thus, an additional mechanism by which chemokines may enhance cytolytic activity involves an increase in the ability of lymphocytes to adhere to their tumor cell targets. In addition, chemokines may alter NK-target cell conjugate formation by altering the avidity of adhesion molecule interactions between the cytolytic cells and the target cell, permitting a more efficacious killing response (Taubb et al., 1996). Cytotoxic activity is enhanced after NK cells are stimulated with chemotaxins such as macrophage-inflammatory protein-1β (MIP-1β/CCL1), thymus and activation-regulated chemokine (TARC/CCL17), regulated upon activation normal T-cell expressed and secreted (RANTES/CCL5) and monocyte chemoattractant protein 1 (MCP-1/CCL-2) is well known (Taubb et al., 1996; Bariagaber et al., 2003).

1.3.2. Lysophospholipids

Like classical chemokines, small phospholipids such as lysophosphatidic acid (LPA) and shingosine-1-phosphate (S1P) induce NK cells chemotaxis (Kveberg et al., 2003). Lysophospholipids (LPLs) are omnipresent bioactive molecules influencing a broad variety of biological processes by binding to cognate G protein-couple receptors. The increasingly well-known LPLs, lysophosphatidic acid and sphingosine 1-phosphate, are responsible for cell signalling in diverse pathways. In addition, the widespread expression of cell surface LPL receptors and coupling to several classes of G proteins regulate various cellular processes, in particular, neurogenesis, vascular development, wound healing, immunity and cancer (Pyne and Pyne, 2000; Mills and Moolenaar, 2003; Anliker and Chun, 2004).

Lysophosphatidic acid is a lysoglycerophospholipid and was originally identified as a key molecule in *de novo* lipid biosynthesis. LPA can be rapidly synthesized by different pathways involving diacylglycerol activation or sphingomyelinase conditioning of cell-derived plasma membrane vesicles followed by a secretory type II phospholipase A_2-dependent metabolism (Clair et al., 2003; Fourcade et al., 1998). Additionally, specific oxidative degradation in minimally oxidized low-density lipoproteins results in the production of LPA. LPA is produced extracellularly from lysophosphatidilcholine by lysophospholipase D (lysoPLD), also called autotaxin (ATX) (Aoki et al., 2004). ATX/LysoPLD is a ubiquitous exo-phosphodiesterease that was originally identified as an autocrine motility factor for melanoma cells and is implicated in tumor progression. Both LPA and lysoPLD are aberrantly expressed in several cancers (Mills and Moolenaar, 2003; Budnik, 2003).

The sphingosine-1-phosphate mediates cellular functions, particularly calcium homeostasis, cell growth, and suppression of apoptosis (Spiegel and Milstien, 2003). S1P levels in cells and tissue are low and tightly regulated. S1P is produced intracellularly in organelles and in the plasma membrane and is then secreted. S1P is produced by phosphorylation of sphingosine by two

sphingosine kinase isoenzymes (SphK1 and 2) and can be dephosphorylated by several phosphatases or cleaved irreversibly by a lyase. The exoenzyme autotaxin also has the ability to produce extracellular S1P from the phosphosphingolipid sphingosylphosphorylcholine (SPC) (Clair et al., 2003).

Each of the lysophospholipid mediators is recognized with high affinity by a family of GPCRs. These receptors transduce signals following lysophospholipid binding by associating with one or more G proteins, thereby allowing for a broad range of possible downstream targets. The receptors that bind LPA are known as LPA_1, LPA_2, LPA_3, LPA_4 and LPA_5; whereas those that bind S1P are known as $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$ (Chun et al., 2002; Anliker and Chun, 2004; Maghazachi, 2005).

NK cells express LPA receptor type-1, -2 and -3 and S1P receptor type-1, -4, and –5 (Jin et al., 2003; Kverberg et al., 2003). Moreover, LPA and S1P mediate chemotaxis and Ca^{2+} release in human NK and these activities are inhibited by pretreatment with pertussis toxin, implicating the involvement of G_i proteins. In contrast to S1P, LPA is also able to stimulate secretion of IFN-γ in NK cells (Jin et al., 2003).

1.4. Tumor cells

1.4.1. Tumor microenvironment

A solid tumor is an ecosystem composed of tumor cells, resident and infiltrating nontumor cells and molecules present in proximity to these cells. This ecosystem can be collectively described as the tumor microenvironment. Both the tumor cells as well as the neighboring nontumor cells take part in establishing the specific conditions existing in the microenvironmental milieu (Witz, 2008). Tumor cells communicate bidirectionally with the surrounding cells, sending and receiving topographical and molecular cues that modulate differentiation, growth, and invasion. The process

by which tumors grow and metastasize is complex, with many steps are required for primary tumor development and the establishment of clinically significant secondary tumors. These steps include, among others, the survival and growth of the primary tumor, the invasion of vascular or lymphatic vessels and the survival, growth and 'organogenesis' of the metastasized cells in their new environment. Growth autonomy appears as the result of multiple mechanisms by which growth regulatory pathways are perturbed and deregulated. It can occur at various steps of signal transduction: (1) malignant cells produce excessive levels of autocrine growth factors, which substitute for exogenous growth factor requirements; (2) resistance to physiologically inhibitory exogenous growth factors; (3) overexpression or altered expression of growth factor receptors; (4) deregulation of growth control at the level of second messengers (Chambers et al., 2002).

Inflammatory cells and molecules are thought to participate in the surveillance of cancer cells by constituting a firewall. But causally they are involved initiating certain types of cancer and promoting tumor progression in essentially all types of cancer. In many ways, the microenvironment of tumors mimics that of tissues during an inflammatory response to injury. For example, both contain a large number of cells from both the innate and adaptive immune system, recruited and activated by a complex profile of chemokines, cytokines, growth factors and proteases. However, unlike the organized morphology of normal tissue, and the ultimate resolution of the inflammation that occurs during healing, tumors exist in a state of chronic inflammation characterized by the presence of malignant cells, development of an aberrant vascular network and the persistence of inflammatory mediators (O'Hayre et al., 2008). Prehn showed that not only may tumor cells resist immune insults, but their propagation may even depend on immune reactivity. As a result, the tumor cell population becomes more aggressive and more resistant to other anti-malignancy factors present in the same milieu (Prehn, 1994). Moreover, tumor cells and their products have the capacity to convert anti-malignant immune functions to activities that promote tumor progression. Several variables such as the tumor type, the progression stage of the tumor, the

status of certain receptors expressed by tumor cells, and other microenvironmental components determine if these factors exert a yin or a yang influence. The role of different inflammatory mediators (cytokines, chemokines, and growth factors) as promoters of tumor progression or tumor rejection may depend on the intracellular signals triggered by these biological agents in the tumor microenvironment (Witz, 2008).

Transforming growth factor β (TGF β) can be considered a prototype for factors playing pro-malignancy and anti-malignancy roles in tumor progression. It is a multifunctional cytokine that controls many cellular functions including proliferation, differentiation, migration, apoptosis, adhesion, angiogenesis, immune surveillance, and cell survival. The growth-inhibitory and apoptotic activities of TGF β characterize its role as a tumor suppressor gene, e.g. TGF β inhibits T cell activation, proliferation and differentiation (Li et al., 2006; Gorelik et al., 2001). Nuclear factor kappa B (NF-κB) is a central coordinator of inflammation, innate immunity, adaptive immunity, and angiogenesis. It is most probably the major mechanistic link in the inflammation-protective tumor immunity interplay. NF-κB is constitutively activated in numerous tumors; it positively regulates cell cycle and manifests anti-apoptotic activity. Nevertheless, in various tumor models, mainly skin cancer, NF-κB plays an anti-malignancy role: its suppression promotes tumorigenesis (Luo et al., 2004). As a central mediator of inflammation, tumor necrosis factor (TNF) might represent other molecular links between chronic inflammation and the subsequent development of malignant disease. Furthermore, deregulated TNF expression within the tumor microenvironment appears to favor malignant cell tissue invasion, migration and ultimately metastasis formation (van Horssen et al., 2006; Mocellin, 2008). The death receptor/TNF-related apoptosis-inducing ligand axis also plays anti-malignancy or pro-malignancy roles. TNF-related apoptosis-inducing ligand (TRAIL) is a microenvironmental ligand of death receptor and induces apoptosis of tumor cells by engaging several death receptors (Witz, 2008). On the other hand, expression of death receptor

ligands (e.g., FasL, TRAIL) by cancer cells may be another mechanism by which tumors escape from immune surveillance (Siegel et al., 2000).

1.4.2. Melanoma tumor cells

Several advances in cancer immunology have come from the study of melanoma. The normal precursor of melanoma is the melanocyte, a pigment-producing cell of neural crest origin. Although melanoma accounts for only 10% of all skin cancers, it is responsible for >90% of skin cancer deaths and the 5-year survival rate for patients with metastatic disease is around 10%. Low oxygen levels (hypoxia) may be essential for melanocyte transformation. The extracellular matrix conditioned by aggressive melanoma cells can epigenetically transdifferentiate normal melanocytes toward an invasive melanoma cell-like phenotype. Recently, Bedogni et al. showed that hypoxia also contributes to melanocyte transformation (Bedogni et al., 2005). Furthermore, the distance of the skin from superficial blood vessels renders it mildly hypoxic (Postovit et al., 2006). Solid tumors, such as melanoma, express tumor-specific antigens that serve as targets for immune effector T cells. In the early phases of solid tumor development, tumor cells do not usually reach lymphoid organs and cannot prime naïve T cells; consequently, immune surveillance is inefficient. In addition, total or partial downregulation of major histocompatibility complex class I expression is frequently found in melanoma cells, which thereby resist killing by MHC-restricted cytotoxic T cells. However, this can make melanoma cells more susceptible to the lysis mediated by NK cells (Solana et al., 2007). It has been demonstrated that melanoma cells can be susceptible to NK-mediated lysis both in murine and human models (Pende et al., 2001). The ability of NK cells to respond to cytokines, such as interleukin-2 (IL-2) and interferons (IFNs), can increase their usefulness in immunotherapy against tumors (Ljunggren and Malmberg, 2007). Thus, activation of NK cells with high-dose IL-2 has been widely used and has been shown to mediate anti-tumor activity in clinical as well as experimental settings (Solana et al., 2007).

However, melanoma cells can evade immune recognition by innate and adaptive immunity. A number of mechanisms have been proposed that allow tumors to avoid NKG2D-medited NK cells attack. Tumors often shed soluble NKG2D ligands from their surface, which can be detected in the blood of patients with cancer. Some cancer cells produce TGF β, which down-regulates NKG2D expression on lymphocytes. In some melanoma, many of the primary tumors are MICA positive, whereas the metastasis lesions did not express this NKG2D ligand. NKG2D-ligand diversity might aid in suppressing the development of cancer (Hayakawa and Smyth, 2006; Eagle and Trowsdale, 2007).

1.4.3. Role of lysophospholipids in the tumor microenvironment

Much evidence suggests that the lysophospholipids LPA and S1P are potent inducers of many of the hallmarks of cancer, including cell proliferation, survival, migration, invasion and neurovascularization *in vitro* and *in vivo* models (Moolenaar et al., 2004; Murph et al., 2006). The enzymes metabolizing LPA and S1P and their receptors are aberrant in multiple cancer lineages (Umezu-Goto et al., 2002). Elevated levels of LPA have been associated with a number of malignancies, including melanoma, ovarian cancer, prostate cancer and multiple myeloma (Murph et al., 2006). mRNA of the LPA-generating ATX is overexpressed in various human malignancies, including lung, breast and renal carcinoma (Tanyi et al., 2003). Through local production of LPA, ATX/lysoPLD might support an invasive microenvironment for tumor cells and therefore contributes to the metastasis cascade. Currently, LPA is known to play an important role in ovarian cancer (Meng et al., 2004; Lee et al., 2006). LPA appears to promote early events in ovarian cancer metastasis dissemination, enhancing matrix metaloproteinase 2 activation, and may be clinically useful as an epithelial ovarian cancer biomarker (Xu et al., 1998). Also, Fishman and co-workers demonstrated that LPA upregulates the FasL expression on the ovarian cancer cell surface, conferring immune-privilege and leading to apoptosis of activated T lymphocytes (Meng et al.,

2004). LPA present in serum and ascites of ovarian cancer patients strongly stimulates Growth-related oncogene-α (GROα) production in ovarian cancer cells through LPA receptor 2 (Lee et al., 2006). GROα, a member of the CXC family, was initially isolated and characterized by its growth stimulatory activity on malignant melanoma cells and it can induce growth, chemotaxis, and metastasis of several other cancer cells (Luan et al., 1997; Bechara et al., 2007).

Like the LPA, S1P is a potent tumorigenic agent released from cancerous tumors into the tumor microenvironment (Sabbadini, 2006). Sphingosine kinase-1 (SphK1), which produces S1P from sphingosine, is also elevated in a variety of human solid tumors, such as those of the breast, colon, lung, ovary, stomach, uterus, kidney and rectum (French et al., 2003, Johnson et al., 2005; Kawamori et al., 2006; Yatomi et al., 1997; Sano et al., 2002). This isoform of the sphingosine kinase is thought to be responsible from S1P release to the extracellular compartment (Milstein and Spiegel, 2006; Payne and Cornelius, 2002). The most direct *in vivo* evidence that S1P contributes to tumor angiogenesis come from a neutralizing S1P-monoclonal antibody (anti-S1P mAb). Anti-S1P mAb treatment effectively retarded progression of deadly and multidrug-resistant cancers in murine xenograft and allograft models (Visentin et al., 2006). Cancerous tumor cells upregulate the expression of SPHK1, which may greatly contribute to the putative increased levels of S1P in the tumor microenvironment. The released S1P is able to act in an autocrine or paracrine manner on tumor cells and vascular endothelial cells (ECs); it promotes DNA synthesis and the resulting proliferation of both and stimulates migration of cells enhancing the metastatic potential of tumor cells while promoting EC-based angiogenesis. As an additional indirect angiogenic effect, S1P is responsible for the release of proangiogenic growth factors (VEFG, IL-6 and IL-8) from tumor cells (Sabbadini, 2006) (Figure 1.4.).

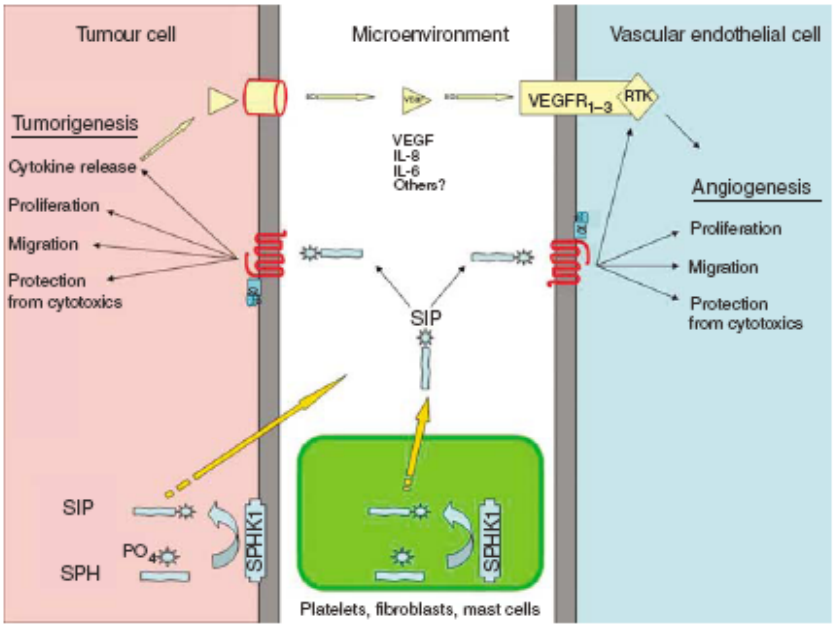


Figure 1.4. S1P is a tumorigenic and angiogenic growth factor produces by blood platelets, mast cells and fibroblast in the tumor microenvironment. Tumor cells upregulate the expression of SPHK1, which may contribute to the putative increased levels of S1P in the tumor microenvironment. The release of S1P is able to acts as a autocrine or paracrine manner in tumor cells and vascular endothelial cells. As an additional indirect angiogenic effect, S1P is responsible for the release of proangiogenic growth factors (VEFG, IL-6, IL-8) from tumor cells (Sabbadini, 2006).

The ability of cancer cells to release LPA and S1P into the tumor microenvironment is consistent with the idea that cancerous tumors release mediators to trick the body into thinking that it has a wound that needs the infiltration of platelets, fibroblasts, mast cells and neutrophils for the purpose of creating an inflammatory response. The infiltrating cells promote further release of LPA or S1P into the tumor microenvironment with the resulting manifestation of the tumorigenic and proangiogenic effects of the lysophosphilipids.

1.5. Aim of the study

Tumor cells might have developed a strategy to recruit cells that secrete cytokines necessary for their growth. However, for the tumor cells to survive, they must downregulate the cytolytic activity of the anti-tumor effector cells. In the last few years, several investigations have pointed out that many functional aspects of tumor cells and their microenvironment play a role in tumor progression; e.g. the relevance of autocrine-secreted products such as CXCL1/GRO-α, lysophosphatic acid (LPA) and sphingosine 1-phosphate (S1P) in the growth response and metastatic process of tumor cells has been demonstrated. In addition, LPA and S1P induce chemotaxis of effector cells like NK cells. Lysophospholipids may recruit NK cells to the sites of tumors where NK cells secrete cytokines such as IFN-γ that in turn provide a suitable environment for the growth of tumor cells. LPLs also may facilitate the interaction of NK cells with other cell types that secrete growth factors such as dendritic cells, which could also facilitate the growth of tumors.

In this context, the present work will address the following biological questions

- role of lysophospholipids in the biological activity of NK cells;
- LPLs in the cross-talk between NK cells and tumor cells;
- LPLs as tumor microenvironmental factors with beneficial effects on tumor development.

2. Materials and Methods

2.1. Materials

2.1.1. Biological Material

2.1.1.1. Cell lines

The A2058, SKMel23 and HS294T human melanoma cell lines and the Human Burkitt's lymphoma cell line Raji were maintained at 37°C in a 5% CO_2 incubator in RPMI 1640 supplemented with 10% fetal calf serum, 10 U/ml penicillin, 10 U/ml streptomycin, and 1 mM L-glutamine (Promocell, Heidelberg, Germany).

2.1.1.2. Natural Killer cells

The use of human cells was approved by the Research Ethics Board of the University of Jena. In this work, the human Natural Killer cells were isolated from Buffy Coats of healthy volunteers produced by the Institute for Transfusion Medicine of the FSU Jena.

2.1.2. Cell culture media

RPMI 10% FCS	RPMI 1640 supplemented with 10% fetal calf serum, 10 U/ml penicillin, 10 U/ml streptomycin, and 1 mM L-glutamine (Promocell, Heidelberg, Germany).
RPMI 2% FCS	RPMI 1640 supplemented with 2% fetal calf serum, 10 U/ml penicillin, 10 U/ml streptomycin, and 1 mM L-glutamine (Promocell, Heidelberg, Germany).

RPMI 10% HS	RPMI 1640 supplemented with 10% human serum AB positive (Lonza), 10 U/ml penicillin, 10 U/ml streptomycin, and 1 mM L-glutamine (Promocell, Heidelberg, Germany).

2.1.3. Reagents

2-mercaptoethanol	Sigma-Aldrich
4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF)	AppliChem
8-Bromoadenosine 3´,5´-cyclic monophosphothioate, Rp-isomer	Alexis, Axxora
14:0 Lyso PA 1-myristoyl-2-Hydroxy-sn-Glycero-3-Phosphate	Avanti Polar Lipids
18:1 Lyso PA 1-Oleoyl-2-Hydroxy-sn-Glycero-3-Phosphate	Avanti Polar Lipids
Acrylamide	Roth
Ammonium persulphate (APS)	Roth
Aprotinin	Bayer
$CaCl_2$	Roth
Cholera toxin	Sigma-Aldrich
Ficoll-Paque PLUS	Amersham, GE Fetal
calf serum	Promocell
Glycin	Roth
Human Serum AB positive	Lonza
Leupeptin	Calbiochem
L-α-Lysophosphatidic Acid, Oleoyl (LPA)	Sigma-Aldrich
Myristoylated PKI_{14-22}	Sigma-Aldrich
$Na_2{}^{51}CrO_4$	Amersham, GE
Nonidet P-40 Alternative	Calbiochem
PBS	Promocell
Pepstatin A	Calbiochem

Pertusis toxin	Sigma-Aldrich
Phenylmethylsulphonyl fluoride (PMSF)	Sigma-Aldrich
Na_3VO_4	Roth
RANTES	Promokine
rHu Interleukin-2 (Proleukine)	Chiron
Sodium Dodecyl Sulphate (SDS)	Sigma-Aldrich
Methanol	Roth
Sphingosine-1 phosphate (S1P)	Sigma-Aldrich
Tris- (hydroxymethyl)-aminomethane	Roth
Triton X-100	Sigma-Aldrich
Wortmannin	Sigma-Aldrich

2.1.4. Equipment

RC-3B Sorvall Centrifuge	Heraeus
5804R Centrifuge	Eppendorf
Flow cytometer FACSCalibur	Becton Dickinson
Electrophoresis gel chambers	Bio-Rad
Power supply, PowePac 300	Bio-Rad
Heating block HB-130	Unitek
Thermomixer comfort	Eppendorf
Incubator CO_2	Sanyo
Laminar-Air HB 2472 workbench	Heraeus
Microscope. Telaval 3	Carl Zeiss
Microplate Reader Emax	Molecular Devices
Vortex	Bender&Hobein
Balance	Sartorius

Autoclave	Varioklav
-20°C Freezer	Siemens
-80°C Hera Freezer	Heraeus

2.1.5. Consumables

PVDF membranes Hybond- ECL	Amersham
96 well-culture plates	CellStar
15 ml, 50 ml tubes	Greiner
5 ml, 10 ml, 25 ml pipettes	Greiner
500 µl, 1000 µl plastic tubes	Eppendorf
Minifac tubes	Costar
10 µl, 100 µl and 1000 µl tips	Greiner
75 cm^2 and 175 cm^2 culture flasks	Nunc
60 ml syringes	Braun, Omnifix
Bottle top filters, Steritop (0.25 µm pore)	Millipore
Filters (0.45 µm and 0.2 µm pore)	Millipore

2.1.6. Antibodies

Mouse anti human CD3	Promokine
Mouse anti human CD4	Promokine
Mouse anti human CD3-FITC	Immunotools
Mouse anti human PE-CD56	Immunotools
Mouse anti human IgG1	Immunotools
Rabbit anti human Akt	Cell Signaling Tech.
Rabbit anti human pAkt [Ser473(193H12)]	Cell Signaling Tech.

Mouse anti human GSK3β	Cell Signaling Tech.
Rabbit anti human pGSK3β [Ser9]	BD Trand Lab
Rabbit anti human pp38 [Thr180/Tyr182(3D7)]	Cell Signaling Tech.
Goat anti human Actin [(I-19:sc-1616)]	Santa Cruz Biotech.

2.1.7. Buffers and solutions

NET-G Buffer (1x)	Tris-HCl pH 8.0 10 mM
	NaCl 150 mM
	EDTA 5 mM
	Tween 20 0.05 % (v/v)
	Gelatine 0.05 % (v/v)
Running Buffer (4x)	Tris-base
	SDS 10% (v/v)
Stacking Buffer (4x)	Tris-HCl pH 6.8 0.5 M
	SDS 10 % (v/v)
Running gel (10%)	H_2Odest. 6.0 ml
	Running buffer (4x) 3.6 ml
	Acrylamide Stock 4.8 ml
Stacking gel (5%)	H_2Odest. 2.8 ml
	Stacking buffer (4x) 1.2 ml
	Acrylamide Stock 0.8 ml

Protein Electrophoresis buffer (10x)	Tris-HCl pH 8.3 250 mM Glycin 2 M SDS 1 % (v/v)
Transfer Buffer	Tris 25 mM Glycin 192 mM Methanol 20 % (v/v)
Stripping Buffer	Tris-HCl pH 6.7 62.5 mM 2-mercaptoethanol 100 mM SDS 2% (v/v)

2.1.8. Kits

Parameter cAMP, Cyclic AMP assay-Quantitative determination of cyclic AMP (cAMP) concentrations, R&D Systems (Wiesbaden-Nordenstadt, Germany).

StressXpress, PKA Kinase Activity Assay Kit (Non-radioactive), Stressgen (Michigan, USA).

2.2. Methods

2.2.1. NK cell Isolation

2.2.1.1. NK cell enrichment by nylon wool nonadherence

Differential adherence properties of T cells, B cells, and accessory cells such as macrophages or other antigen-presenting cells can be employed to enrich NK cells. Nylon wool can be use as column matrices to which B cells and accessory cells will adhere better than T and NK cells do.

2.2.1.2. Preparation of the nylon wool column

Nylon wool was soaked in water for 2 hours and then 1 hour in PBS. Subsequently, 0.5 grams of nylon wool were packed tightly in a 60 ml sterile syringe, autoclaved and stored at 4°C.
The column was washed to equilibrate the nylon wool with 20-30 ml of RPMI 10% FCS media (RPMI 1640 supplemented with 10% fetal calf serum, 10 U/ml penicillin, 10 U/ml streptomycin, and 1 mM L-glutamine), and plugged with a stopper. The column was incubated at 37°C in a 5% $C0_2$ incubator for 45 to 60 minutes. In the meantime, leukocytes were separated by the Ficoll-Paque gradient.

2.2.1.3. Separation of blood leukocytes by the Ficoll-Paque gradient

A 50 ml Buffy Coat was diluted 1:1 with PBS (v/v). Carefully, 25 ml of the cell suspension were layered over 15 ml Ficoll-Paque PLUS solution in 50 ml conical tubes and centrifuged at 1800 rpm for 30 min at 20°C, without braking. Differential migration during centrifugation results in the formation of layers containing different cell types (Figure 2.1.). The bottom layer contains erythrocytes which have been aggregated by the Ficoll-Paque gradient and, therefore, seep completely. The layer immediately above the erythrocyte layer contains mostly granulocytes; at the osmotic pressure of the Ficoll-Paque solution, these granulocytes attain a density great enough to

migrate through the Ficoll-Paque layer. Because of their low density, the lymphocytes are found at the interface between the plasma and the Ficoll-Paque gradient with other slowly sedimenting particles (platelets and monocytes).

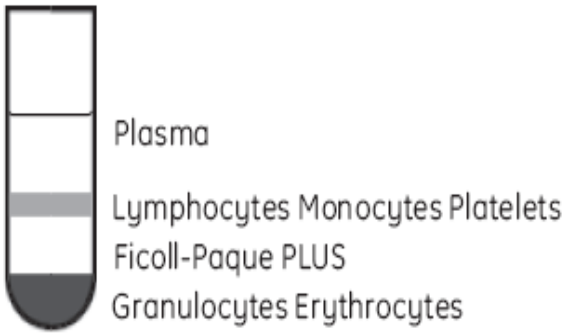


Figure 2.1. Differential migration during centrifugation results in the formation of layers containing different cell types.

The interphase cells (lymphocytes, monocytes and thrombocytes) were transferred by Pasteur-Pipette to a new conical tube and subjected to short washing steps, with a balanced salt solution to remove any platelets, Ficoll-Paque and plasma. After 3 washes (1100 rpm x 10 min), cells were resuspended in 5 ml of RPMI 10% FCS media and overlaid on the prewarmed nylon wool column. The polymorphonuclear leukocytes will adhere tightly to the nylon wool, while T cells, NK cells and any erythrocytes will be eluted in the non-adhering cell fraction. After 1 hour at 37°C incubation, the non-adhering cells were collected by washing the column with 50 ml RPMI 10% FCS media. After centrifugation, cells (> 80% T cells) were resuspended in RPMI 10% HS media (RPMI 1640 supplemented with 10% human serum AB positive, 10 U/ml penicillin, 10 U/ml streptomycin, and 1 mM L-glutamine), according to the total number of cells, and cultured in a 175 cm^2 flask in the presence of 1000 IU/ml Interleukin-2 (IL-2). After 24 h in the presence of IL-2, NK cells adhered to the plastic and non-adherent cells were removed. Adherent NK cells were grown for an additional 7-9 days in RPMI 10% HS media. Fresh media (5-10 ml) was added after the fourth day of culture.

At the end of the culture period, T cells were removed by binding the sample to mouse anti-human CD3 and anti-human CD4 and placing the solution in a rotor at 4°C for 30 min. After washing steps were carried out, cells that bound to the antibodies were incubated with anti-mouse M-450 Dynabeads (Dynal, Oslo, Norway) for an additional 30 min at 4°C. The bead-bounded cells were diminished by placing the tube in a magnetic device for 15 min and then the non-magnetic fraction was collected ($CD3^-$ $CD4^-$ NK cells).

2.2.2. Flow cytometer

The flow cytometer enables a cell population to be acquired on the basis of its cell size and granularity. Additionally, surface molecules, or the purity or vitality of a cell population can also be examined by fluorescence. Purified cells were incubated with PE-conjugated anti-human CD56 (1:200, Immunotools) and FITC-conjugated anti-human CD3 (1:200, Immunotools) monoclonal antibodies for 30 min at 4°C. Isotype matched antibodies (IgG1 control, Immunotools) were used as negative control. Single stained for CD56-PE, CD16-FITC and CD3-FITC were also performed. Analysis was done with a FACSCalibur flow cytometer (BD Biosciences).

2.2.3. Analysis of filamentous (f) Actin content

Actin represents an important component of the cytoskeleton of NK cells. The method, which with the help of NBD Phallacidin can be used to measure the F-actin content of the cell, was described by Howard and Meyer (1984).

The isolated NK cells were adjusted to 1 x 10^6 cell/ml in PBS in 10 ml plastic tubes. Meanwhile, 50 µl Formalin-Buffer (3.7 % formaldehyde) were added to mini fax tubes. The cells became lively in response to the respective concentrations of the stimuli. After 10, 30, 60 and 300 seconds, 50 µl of the stimulated cells were pipetted into prepared Minifac tubes with Formalin to fix the actin polymerization in the respective stage. After 1 h incubation at room temperature, 100 µl NBD

buffers [(7.4% formaldehyde, 0.33 µM NBD-phallacidin (MoBiTec, Göttingen, Germany) and 1 mg/ml lysophosphatidylcholine (Sigma-Aldrich, Taufkirchen, Germany)] were pipetted. Because the contained phallacidin binds specifically to the actin filaments but not to monomers of actin, the Nitrobenzooxadiazol (NBD) could be transferred to a fluorescent connection by coupling. Then the loaded cells were incubated at room temperature in the dark for another hour, after which measurements were made on the cytometer. The fluorescence of the linear channel 1 (FL1) is here proportional to the content of the actin filaments of the NK cells. The relative actin content was indicated as the relationship of channel fluorescence from stimulated to unstimulated cells.

2.2.4. *In vitro* Cytotoxicity assay

Cytotoxicity assays are a fundamental tool for the study of Natural Killer cell function. Life cells will take up but not spontaneously release radioactively labelled sodium chromate ($Na_2{}^{51}CrO_4$). When these ^{51}Cr-labeled target cells are killed, the radioactive chromate is released, and its presence in the supernatant of mixture of target cells and cytotoxic NK cell can be measured on a gamma counter.

Target cells were resuspended in 500 µl of RPMI 2% FCS medium and labelled by incubation with 100 µCi $Na_2{}^{51}CrO_4$ (Amersham, Freiburg, Germany) at 37°C and 5% CO_2 for 1 h. Cells were washed to remove the excess of radioactivity and resuspended at cell density of 1×10^6 cells/ml in RPMI 2% FCS medium. Target cells (T) were added to a dilution series of NK cells, the effector cells (E). Effectors cells and target cells, at the desired E:T ratios ranging from 10:1 to 2.5:1, were placed into individual wells of 96-well U-bottom plates in a total volume of 200 µl, in the absence or presence of stimuli. The effector-target cell mixture was incubated for 4 hours at 37°C and 5% CO_2.

After incubation, 100 µl from the supernatants were removed and mixed with 50 µl of MicroScint-40 cocktail (PerkinElmer, Jügesheim, Germany) in a 96-well Optiplate (PerkinElmer, Jügesheim,

Germany). The plate was then sealed with Packard TopSeal and the amount of intracellular chromium-51 released into the supernatant was analyzed with a gamma counter (TopCount-NXT Packard Instruments). The maximum and spontaneous release were defined as counts from samples incubated with 2% Triton-X or medium alone, respectively. Percentage specific lysis was calculated using the following formula:

$$\frac{\text{(experimental release - spontaneous release)}}{\text{(maximum release - spontaneous release)}} \times 100$$

2.2.5. Measurements of cAMP levels

Adenosine 3´, 5-cyclic monophosphate (cAMP) is a ubiquitous second messenger involved in various cellular activities in many cell and tissue types. It is converted from adenosine triphosphate (ATP) via adenylylcyclases (AC). Intracellular cAMP levels were analyzed using a competitive phase enzyme-linked immuno-absorbent (ELISA) kit.

NK cells were resuspended at 1 x 10^6 /ml in PBS and placed in individual wells of 96-well U-bottom plates in a total volume of 100 µl. After stimulation for 1, 4, 15 or 30 min, the reaction was stopped by a short centrifugation and the cells were lysed by adding 100 µl lysis buffer. The amount of intracellular cAMP in the NK cell preparation was determined using a cAMP assay kit (R&D, Wiesbaden-Nordenstadt, Germany). This assay is based on the competitive binding technique in which the cAMP present in the samples competes with a fixed amount of horseradish peroxidase (HRP)-labelled cAMP for sites on a mouse monoclonal antibody. During the incubation time, the monoclonal antibody becomes bound to the goat anti-mouse antibody coated onto the 96-well plate. Following washes to remove the excess of conjugated and unbound sample, a substrate solution was added to each well to determine the bound enzyme activity. The colour development was stopped and the absorbance was read using a microplate reader set to 450 nm.

2.2.6. Cell Lysis

Equal numbers of cells were washed and resuspended in PBS in 1.5 ml Eppendorf tubes and stimulated at 37°C. Supernatants were removed and cell pellets were transferred to ice and immediately resuspended in an appropriate volume of lysis buffer containing 0.5 % of the detergent NP-40. Cell membranes were dissolved, while the cell cores and remaining cell compartments remained intact and were separated by centrifugation. The different protease inhibitors were added to the lysis buffer directly before use.

Lysis buffer: 20 mM Tris-HCl pH 8.0; 137 mM NaCl; 2.7 mM KCl; 1 mM $MgCl_2$; 1 mM $CaCl_2$; 0.5% NP-40; 0.5 mg/ml BSA; 1 mM Na_3VO_4; 1 mM PMSF; 21 µg/ml Aprotinin; 12 µg/ml AEBSF; 5 µg/ml Leupeptin; 5 µg/ml Pepstatin A.

After 10 min on ice, the lysates were centrifugated (15 min, 14000 rpm, 4 °C) and the NP-40 soluble fraction containing membranes and cytosolic proteins was collected and transferred into 1.5 ml Eppendorf tubes.

2.2.7. Determination of protein concentration

The amount of protein into the lysates was determined using the BCA Protein Assay kit (Pierce, Bonn, Germany). This is a method for colorimetric detection and quantitation of total protein based on bicinchoninic acid (BCA). A calibration standard curve of BSA solutions with concentrations 25 to 2000 µg/ml BSA (in dest. water) was performed. 25 µl each of standard and unknown samples were mixed with 200 µl of the working solution in a 96-well plate and incubated for 30 min 37°C. After incubation, the absorbance at 550 nm was measured on a plate reader. Using the standard curve, the protein concentration of each unknown sample was calculated.

2.2.8. Protein kinase A (PKA) activity assay

PKA activity in NK cell lysates was tested using StressXpress nonradiactive PKA activity kit (Biomol, Hamburg, Germany). This assay is based on a solid phase ELISA assay that utilizes a specific peptide as a substrate for PKA and a polyclonal antibody that recognizes the phosphorylated form of the substrate. The substrate is pre-coated on the wells of a microtiter plate. NK cells (5 x 10^6 per sample) were treated with the stimuli for 1, 4, 15 and 30 min. Thereafter, cells were lysed in lysis buffer and centrifuged at 10,000 rpm for 15 min; supernatants were frozen at - 70°C or immediately quantified. A microtiter plate was soaked with 50 µl of kinase assay dilution buffer. After a washing step, 30 µl (corresponding to 10 µg of whole cell lysate) of each sample were added. The reaction was initiated by adding 10 µl ATP. The plate was incubated for 90 minutes at 30°C and phosphospecific substrate antibody was added. The level of antibody binding was estimated using secondary anti-rabbit IgG-horseradish peroxidase conjugate. This assay is developed with tetramethylbenzidine substrate (TMB) and a color develops in a proportion to PKA phosphotransferase activity. The color development is stopped with acid stop solution and the intensity of the color determined using a microplate reader set at a wavelength of 450nm. Kinase activity in cell lysates was calculated as follows:

$$\frac{\text{mean absorbance (sample) - mean absorbance (negative control)}}{\text{protein in cell lysate}}$$

2.2.9. SDS-Polyacrylamide-Gel electrophoresis (SDS-PAGE)

After cells were lysed, the NP40-soluble fraction contained in the supernatant was removed and diluted 1: 5 with 5x Laemmli sample buffer (Laemmli, 1970) and heated to 95°C for 5 minutes. Samples (20 µg protein/ lane) were electrophoresed in 10% SDS polyacrylamide gels (150 mA). Proteins separated by SDS-PAGE were transferred from the gel to PVDF membranes (HybondTM ECL™, Amersham Biosciences) by electrophoresis (Western blotting) 50V for 90 min. The

primary antibody was diluted 1:2000 with NET G buffer and incubated 12 hr at 4°C. After washing with NET G buffer, membrane was incubated with the secondary antibody (1:10000 in NET G) for 1 hour at room temperature. After washing, the protein of interest was detected by enhanced chemical luminescence utilizing the reaction of Luminol and H_2O_2, which is catalyzed by HRP. For this purpose, the membrane was incubated with the ECL reagents (Perbio, Bonn, Germany) for 1 min and the chemiluminiscense signal was visualized with the Bio Imaging System MF-ChemiBIS 3.2 (Biostep, Germany).

To reprobe the membrane with different antibodies, the previously applied antibodies first had to be removed. For this purpose, the membrane was incubated with 100-200 ml stripping buffer for 45 min at 56°C. The membrane was then washed several times with 1x NETG, until no residual 2-Mercaptoethanol could be detected. The blot was again blocked in 10X NETG for 1 h, and a new primary antibody applied as described.

2.2.10. Statistical analysis

For the statistic analysis of the results, the *t*-test for paired samples was used. Results were considered statistically significant when $p<0.05$. Data were analyzed with the GraphPad Prism™.

3. Results

3.1. Flow cytometry characterization of NK cells

NK cells do not make up a homogeneous cell population; they can be divided into several subsets according to functional and phenotypic differences. Human NK cells are broadly defined as $CD3^-$ $CD56^+$. They can be further subdivided into two main subsets on the basis of their surface expression levels of CD56. $CD56^{dim}$ NK cells have a marked cytotoxic function and also express the low-affinity Fc receptor for IgG CD16 (Fcγ RIII), whereas the minority subset of $CD56^{bright}$ NK cells produces large amounts of cytokines (Lanier et al., 1986; Cooper et al., 2001). To assess how reliable the process of negative selection was flow cytometry analysis was performed. Surface markers present in the cells were assessed, as were levels of CD56 and CD16. The expression of CD3 was analyzed to define the purity of the population obtained. Purified cells were generated by the process described in 2.2.1. After 8-10 days of being cultured, cells were removed from T cells and incubated with PE-conjugated anti-human CD56 and FIT-C anti-human CD3 monoclonal antibodies for 30 min at 4°C. A representative dot plot for double staining is shown in Figure 3.1. Single stainings for CD56-PE, CD16-FITC and CD3-FITC were also performed (Figure 3.2.). Purified cells were found to be negative for CD3, positive for CD56 (up to 90 %) and CD16. Therefore, the depletion method selected in this work is shown to be a trustworthy protocol to obtain a NK-cell enriched cell population.

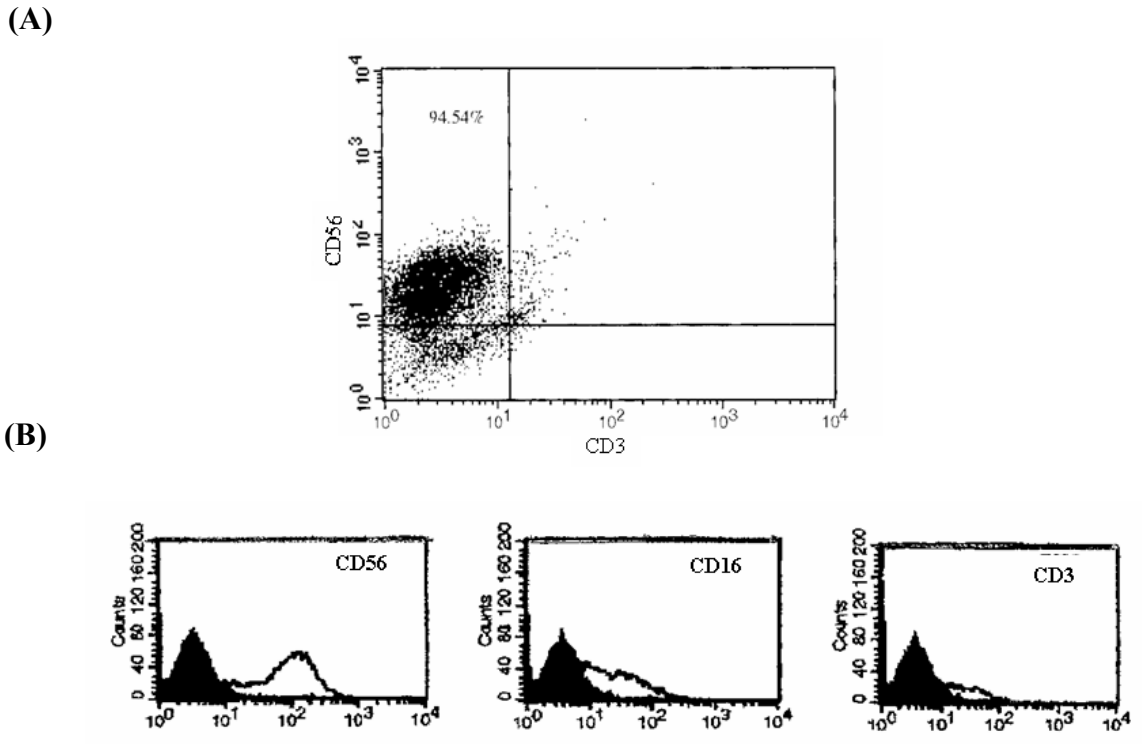


Figure 3.1. Phenotype of activated NK cells. After 8-10 days of being cultured, NK cells were analyzed. (A) Double staining of IL-2-cultured NK cells for CD56 expression (*y*-axis) and CD3 expression (*x*-axis). NK cells are characterized by the $CD3^{-}CD56^{+}$ phenotype. The analysis was performed after gating on $CD3^{-}$ cells, thus excluding contaminated T cells. (B) Histograms show the expression of CD56, CD16 and CD3 in IL-2 activated NK cells. The data are representative of at least three separate analyses.

3.2. Effect of LPA and S1P on the polymerization of actin in NK cells

3.2.1. Lysophospholipids (LPLs) increase actin polymerization in NK cells

In order to characterize the role of LPA and S1P in NK cells, actin polymerization as an intracellular signal transduction event and a chemotaxis-associated intracellular rearrangement was studied in NK cells obtained from different donors. The cells were stimulated for 10, 30, 60 and 300 s as described under 2.2.3 and the relative F-actin content was analyzed. Flow cytometric measurements indicated that LPA caused a rapid and concentration-dependent polymerization of actin molecules within 30 seconds, causing molecules to return to their initial values at around 300 s (Figure 3.2. A). Detailed analyses showed about 70-80 % higher mean fluorescence values in comparison to control cells after stimulation with LPA for 30 s. At 30 s and 60 s, LPA stimulation

(20, 2 and 0.2 µM) had significantly increased fluorescence (p=0.04, 0.02 and 0.03 for 30 s and p= 0.02, 0.02 and 0.01 for 60 s) when compared to the control. Maximal effects were observed after stimulation with 2 µM LPA. Likewise, S1P (20, 2 and 0.2 µM) caused a rapid polymerization of the actin molecules within 30 seconds with a 40-50 % increase in the F-actin content. As shown in Figure 3.2. B, the response was concentration-dependent and maximal effect was observed after stimulation with 2 µM S1P. At 10 s and 30 s S1P stimulation had significantly increased fluorescence compared to the control (p=0.014, 0.0048 and 0.03 for 10 s and p= 0.01, 0.0001 and 0.02 for 30 s).

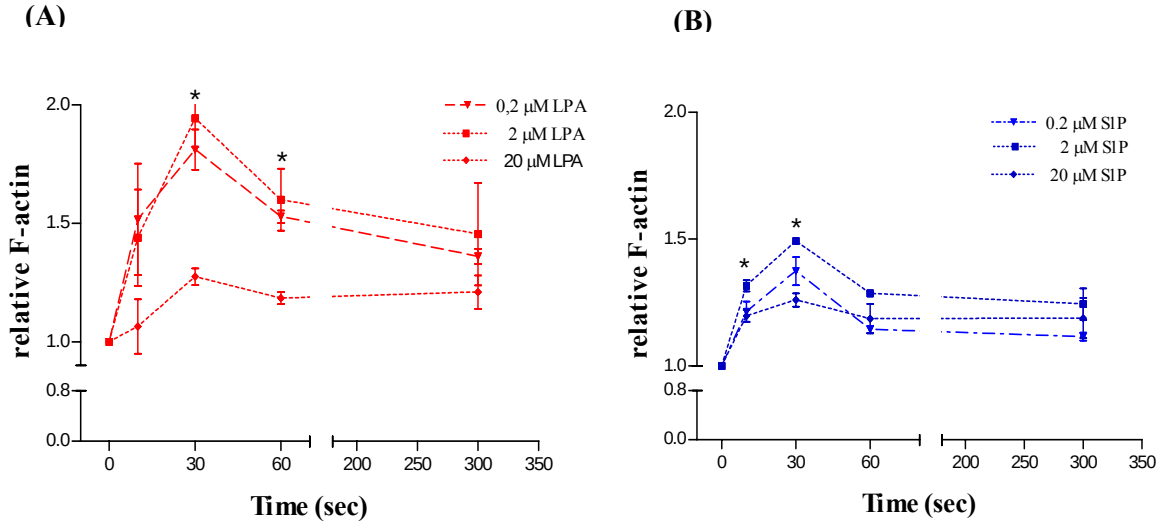


Figure 3.2. Effect of LPA and S1P on actin polymerization in NK cells. Cells were stimulated with LPA 20, 2 or 0.2 µM or S1P 20, 2 or 0.2 µM at the indicated time points. The relative intracellular F-actin content was determined by flow cytometry. (A) At 30 s and 60 s LPA stimulation showed a significant increase of fluorescence when compared to that shown in unstimulated NK cells. (B) At 10 s and 30 s S1P stimulation showed significant differences in fluorescence compared to the control. Data are means of three experiments from different donors ± SEM.

3.2.2. Influence of pertussis toxin on actin polymerization in NK cells

To investigate the involvement of G_i proteins in the actin polymerization response caused by LPA and S1P, NK cells were incubated with 100 ng/ml G_i protein-inactivating pertussis toxin (Ptx) for 1 hr at 37°C. Cells were then stimulated with LPA or S1P and the relative F-actin content was analyzed after 30 s. Because maximal effects on actin polymerization were observed after

stimulation with 2 µM LPA or S1P, this concentration was used to perform the experiments. As shown in Figure 3.3., after 30 s stimulation with LPLs significantly increased the actin polymerization (p= 0.08 for 2 µM of LPA and 0.001 for 2 µM of S1P). Pretreatment with pertussis toxin completely inhibited the LPA-induced actin response (p=0.04) as well the S1P-induced actin response (p=0.04).

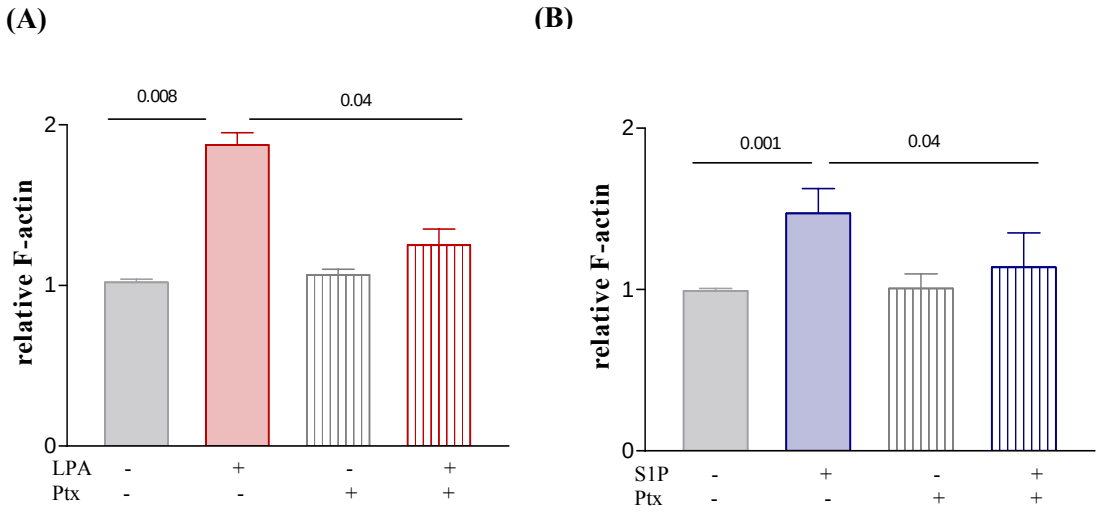


Figure 3.3. Influence of pertussis toxin on the LPLs mediated actin polymerization in NK cells. Cells were incubated with or without 100 ng/ml pertussis toxin (Ptx) for 1 hour at 37°C. Thereafter cells were stimulated with or without 2 µM LPA (A) or S1P (B) and after 30 s of stimulation, actin polymerization was analyzed. The pretreatment with pertussis toxin significantly inhibited the LPA as well as the S1P-increase in the mean fluorescence. Data are means of three experiments from different donors ± SEM.

3.3. LPA and S1P signalling pathways in NK cells

Intracellular signalling pathways induced by chemotaxins such as CCL5/RANTES stimulate phosphatidylinositol 3-kinase and mitogen-activated protein kinase in leukocytes (Maghazachi, 2000; Maghazachi, 2003). To study intracellular signal transduction events downstream of phosphatidylinositol 3-kinase, the activation and/or phosphorylation of different signal proteins were analyzed by Western blot. NK cells obtained from different donors were treated with 2 µM LPA for 30 to 180 s. Probes were analyzed with antibodies against phospho-Akt/protein kinase B[Ser473] (pAkt) and phosphoglycogen synthase kinase-3β[Ser9] (pGSK-3β) as well as phospho-

mitogen-activated protein kinase pp38[Thr180/Tyr182] (p38). The membranes were stripped and reprobed with anti-actin, anti-Akt and anti-GSK-3β to obtain comparable amounts of the analyzed proteins from all samples. Figure 3.4. shows a representative Western blot of Akt, GSK-3β and p38 phosphorylation levels. Basal levels of pAkt, pGSK-3β and pp38 activation were observed in LPA-unstimulated NK cells. Exposing NK cells to LPA further enhancement pAkt, pGSK-3β and pp38. Quantification of the luminescence indicated that LPA induced maximal levels of pAkt and pGSK-3β within 30 s with an increase of about 60 or 35 %, respectively (p=0.0003 for pAkt and 0.002 for pGSK-3β) (Figure 3.5. A and B). The kinetic of the pp38 response peaked more slowly at 90 s, with an increase of about 50 % (p=0.002) (Figure 3.5. C).

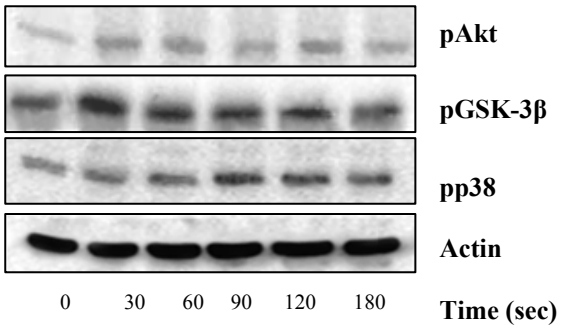


Figure 3.4. LPA induces a transient enhancement of pAkt, pGSK-3β and pp38. NK cells were stimulated at 37ºC with 2 μM LPA for 30, 60, 90, 120 and 180 s. After lysis, the samples were analyzed for pAkt, pGSK-3β, pp38 as well as for actin by Western blot. LPA showed maximal levels of pAkt and pGSK-3β at 30 s and pp38 at 90 s. Data from one representative experiment are shown. The experiment was repeated three times with NK cells isolated from different donors.

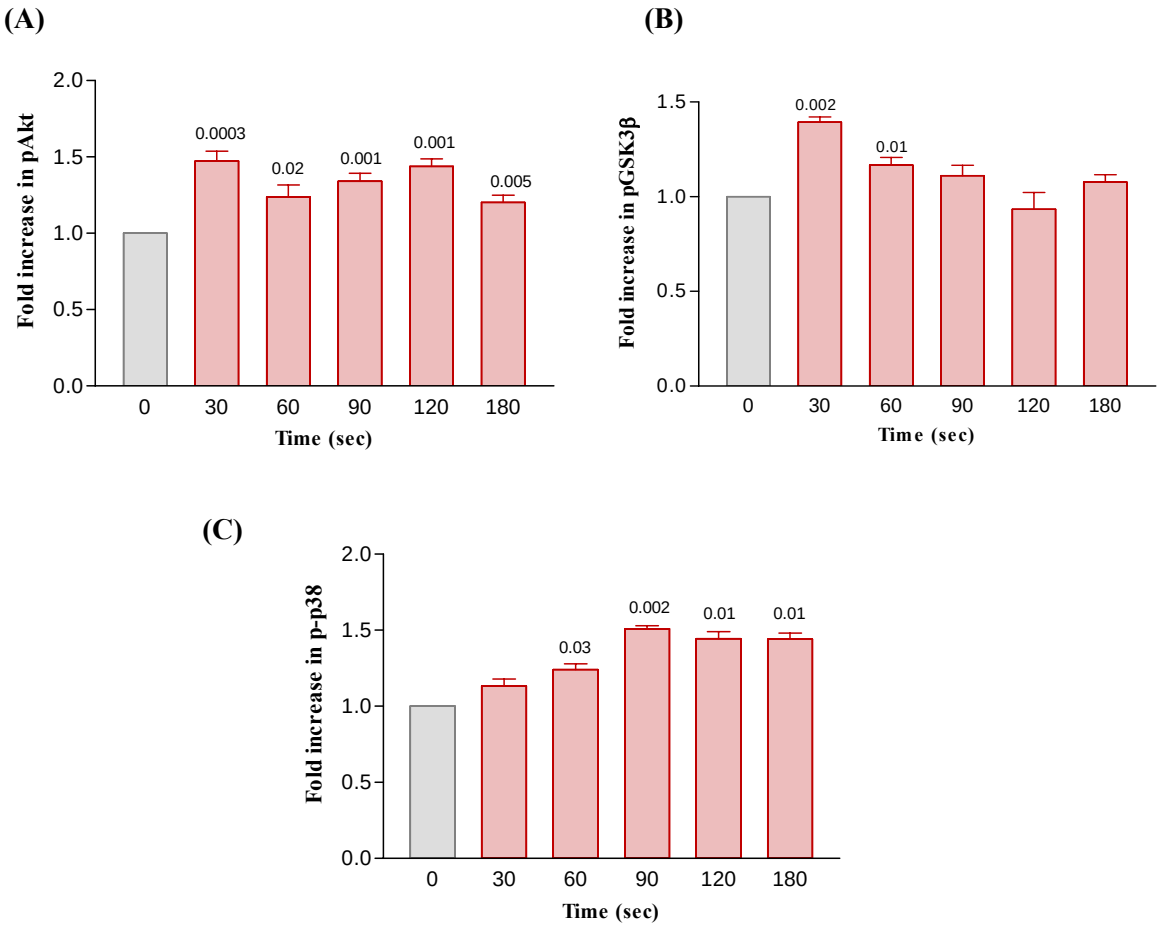


Figure 3.5. Kinetic pAkt, pGSK-3β and pp38 induced by LPA. Quantification of the luminescence indicated that LPA induced maximal enhancement of **(A)** pAkt and **(B)** pGSK-3β within 30 s and **(C)** pp38 within 90 s. Data are means ± SEM of three experiments with NK cell isolated from different donors.

In addition, 1 hr incubation of NK cells with the phosphatidylinositol 3-kinase inhibitor wortmannin (Wtm) reduced the levels of pAkt and pGSK-3β (Fig. 3.6. A and B) in unstimulated NK-cells and inhibited LPA-induced pAkt ($p=0.0003$) and pGSK-3β ($p<0.0001$) responses. In contrast, wortmannin neither influence pp38 levels in unstimulated NK cells nor inhibited LPA-induced pp38 phosphorylation (Fig. 3.6. C).

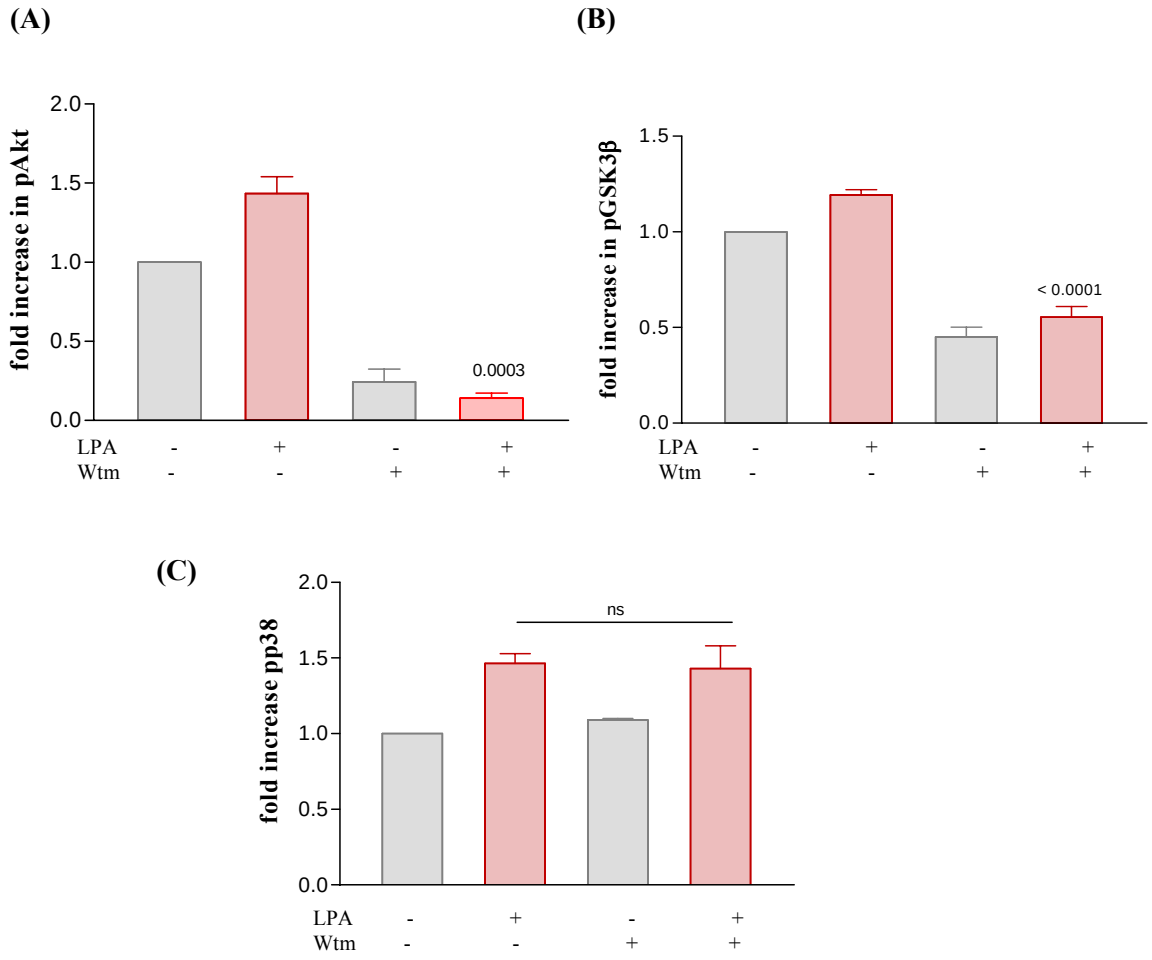


Figure 3.6. Effect of wortmannin (Wtm) on LPA-induced responses. NK cells were preincubated for 1hr with 100 µM wortmannin at 37°C and then stimulated with 2 µM LPA. The effect of Wtm on LPA-induced responses to pAkt at 30 s **(A)**, pGSK-3β at 30 s **(B)** and pp38 at 90 s **(C)** was analyzed. Pretreatment with wortmannin significantly reduced the level of pAkt and pGSK-3β, whereas it did not significantly influence pp38 levels. Data are means ± SEM of three experiments with NK cells isolated from different donors.

Similar results were obtained with the exposure of NK cells to S1P. S1P stimulation also induced a further transient enhancement of pAkt and pGSK-3β. Figure 3.7. shows a representative Western blot of Akt and GSK-3β phosphorylation levels. In this case, the quantification of the luminescence indicated that S1P significantly enhanced pAkt and pGSK-3β within 60 s by about 50 or 75 %, respectively (p=0.01 for pAkt and p=0.02 for pGSK-3β) (Figure 3.8.).

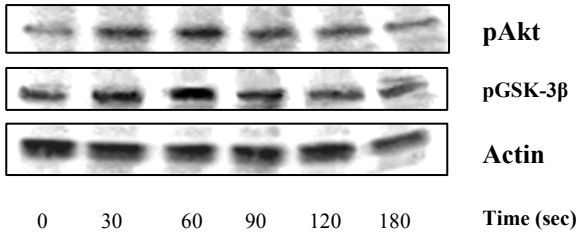


Figure 3.7. S1P induced a transient enhancement of pAKT and pGSK-3β. NK cells were stimulated at 37ºC with 2 µM S1P for 30, 60, 90, 120 and 180 s. After lysis, the samples were analyzed for pAkt, pGSK-3β as well as actin by Western blot. Representative blots from three independent experiments with identical results are shown.

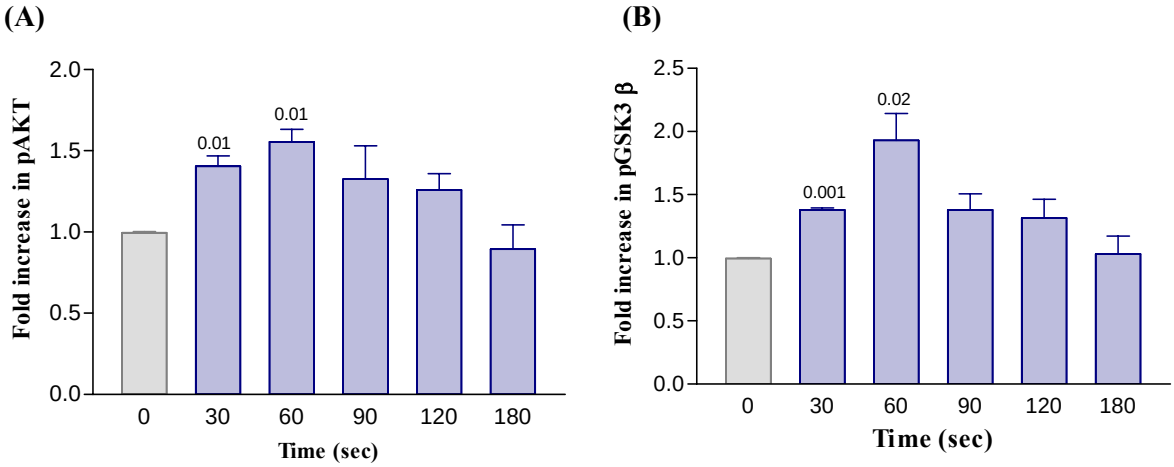


Figure 3.8. Kinetic of S1P-induced pAKT, pGSK-3β Quantification of the luminescence indicated that S1P enhanced **(A)** pAkt and **(B)** pGSK-3β within 60. Data are means ± SEM of three experiments with NK cells isolated from different donors.

Additionally, NK cell were incubated for 1 hr with the phosphatidylinositol 3-kinase inhibitor wortmannin and then the levels of phosphorylation/activation of Akt and GSK-3β were analyzed at 60 s. Wortmannin (Wtm) pretreatment significantly reduced the levels of pAkt and pGSK-3β (Fig. 3.9. A and B) in S1P-unstimulated NK cells and inhibited S1P-induced pAkt ($p=0.0014$) and pGSK-3β ($p=0.0012$) responses.

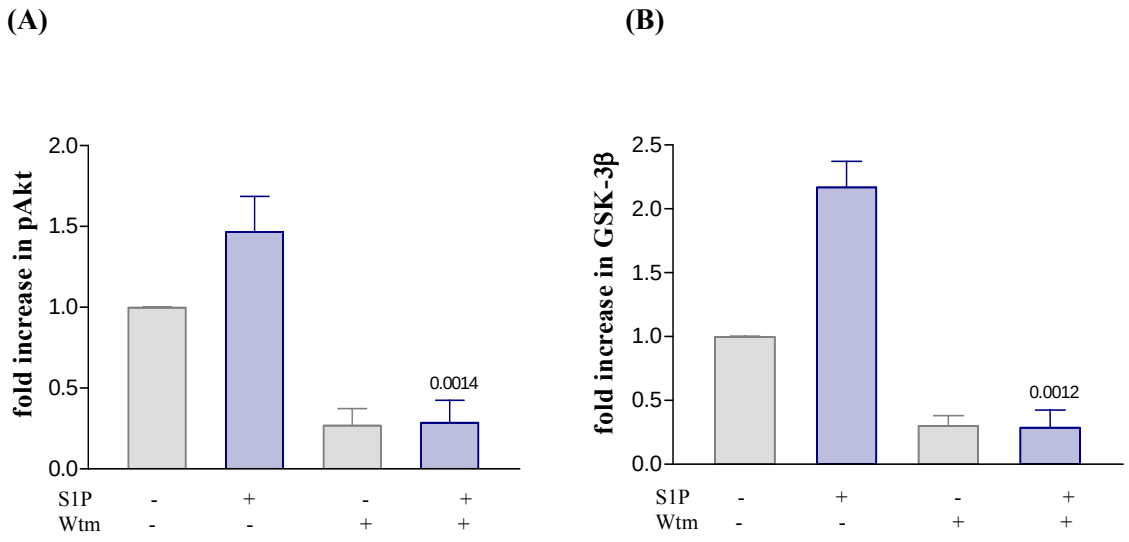


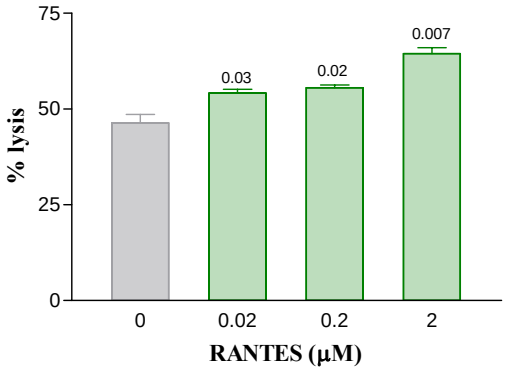
Figure 3.9. Effect of wortmannin on S1P-induced responses. NK cells were preincubated for 1hr with wortmannin (100 μM) at 37°C and then stimulated with 2 μM S1P. The effects of Wtm on S1P-induced responses on **(A)** pAkt at 60 s and **(B)** pGSK-3β at 60 s were analyzed. Pretreatment with wortmannin significantly reduced the level of pAkt and pGSK-3β. Data are means of three experiments from different donors ± SEM.

3.4. Chemotaxins differentially influence the cytotoxic activity of NK cells

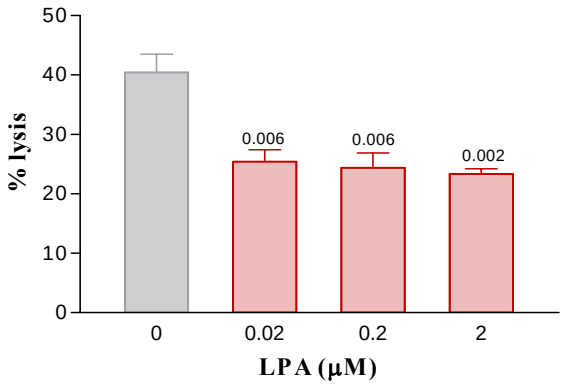
In addition to inducing NK cell recruitment, chemotaxis and the chemotaxis-associated intracellular rearrangements, NK cells exposed to classical chemotaxins such as CCL5/RANTES have an enhanced cytotoxic response. Hence, the effects of CCL5/RANTES and the lysophospholipids (LPLs) on the cytotoxic activity of NK cells against the human Burkitt's lymphoma cell line Raji were analyzed. A standard ^{51}Cr release was performed. Target cells were labelled with 100 μCi $Na_2{}^{51}CrO_4$ for 1 hr at 37°C and were added to a dilution series of NK cells, effector cells. Effector cells (E) and target cells (T), at the desired E:T ratios ranging from 10:1 to 2.5:1, were placed into individual wells of 96-well plates in a total volume of 200 μl, in the absence or presence of stimuli (LPA or S1P). The effector-target cell mixture was incubated for 4 hours at 37°C. Afterwards, the amount of intracellular chromium-51 released into the supernatant was analyzed with a gamma counter and the percentage of specific cytotoxicity was calculated.

As in the literature, CCL5/RANTES increased in a concentration-dependent manner NK cytotoxic activity by about 40 % (Figure 3.10. A). In contrast to CCL5/RANTES, LPA inhibited the cytotoxic activity of NK cells against Raji by about 35-40 % (Figure 3.10. B) and similar results were obtained with S1P-stimulated NK with an inhibition by 40-50 % (Figure. 3.10. C). 2, 0.2 or 0.02 μM of LPA or S1P significantly inhibited NK cell lysis of Raji used at 2.5:1 E:T cell ratio ($p=0.002$, 0.006 and 0.006 for LPA, and 0.002, 0.006 and 0.001 for S1P, respectively). To exclude the possibility that the interaction of LPA and S1P with the Raji target cell might cause a cell-type-specific inhibitory effect on cytotoxicity a second set of experiments with human melanoma cell lines was performed. In these, 2, 0.2 or 0.02 μM of LPA significantly inhibited NK cell lysis of A2058 melanoma cells with a p value of 0.0002, 0.003 and 0.001, respectively (Figure 3.11. A). Additionally, LPA significantly inhibited in a concentration-dependent manner the cytotoxic activity of NK cells against Hs294T and Sk-Mel 23 melanoma cell lines (data not shown). Figure 3.11. B shows similar results obtained with S1P-stimulated NK cells against Hs294T melanoma cell line. S1P (2, 0.2 and or 0.02 μM) significantly inhibited the NK cell lysis of Hs294T melanoma cells with a p value of 0.002, 0.004 and 0.001 respectively. S1P-mediated inhibition of NK cell cytotoxicity was also observed using A2058 and Sk-Mel 23 melanoma cell lines as target cells (data not shown). In addition, the similar inhibitory effects on NK cell cytotoxicity have also been observed at various effector:target ratios (10:1 and 5:1).

(A)



(B)



(C)

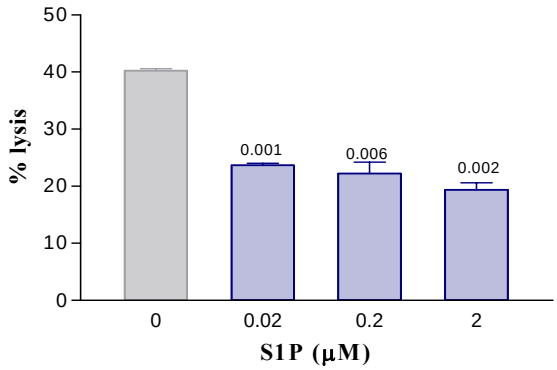


Figure 3.10. Effect of LPLs on the cytotoxic activity of NK cells against Raji cells. NK cells were mixed with ^{51}Cr-labelled Raji at E:T ratio 2.5:1 in the presence of the indicated concentrations of **(A)** RANTES and **(B)** LPA. After 4 hours of incubation, the percentage of ^{51}Cr release was determined. RANTES significantly increased NK cell cytotoxicity, whereas LPA inhibited NK cell lysis of Raji. **(C)** The effect of S1P was also tested. S1P inhibited the NK cell lysis of Raji. Data are from one representative experiment. The experiments were repeated three times with NK cells isolated from different donors.

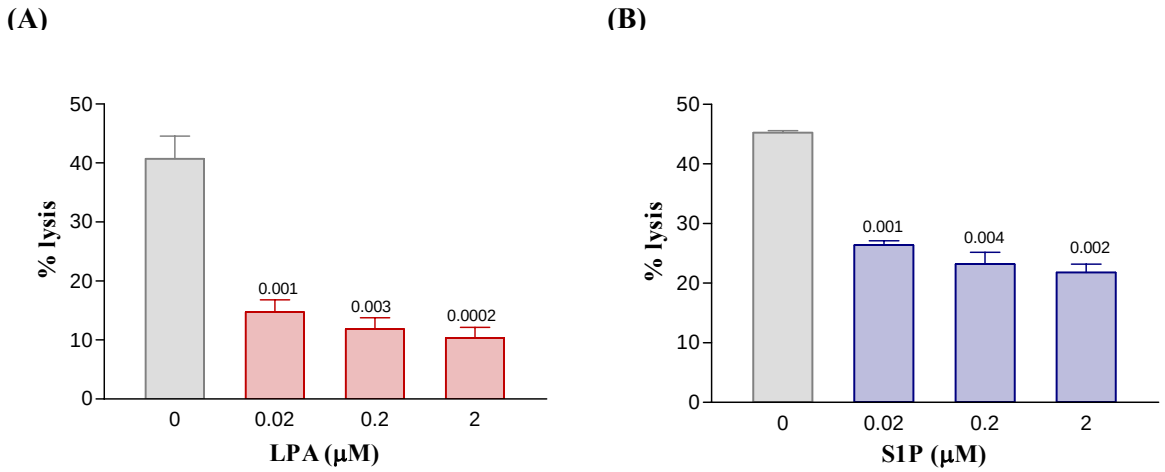


Figure 3.11. Effect of LPLs on the cytotoxic activity of NK cells against melanoma cell lines. The effect of lysophospholipids (LPLs) was also tested with different human melanoma cell lines. **(A)** LPA significantly inhibited the NK cell lysis of A2058 human melanoma cell lines. **(B)** S1P significantly inhibited the NK cell lysis of Hs294T cells. Data are from one representative experiment. The experiments were repeated three times with NK cells isolated from different donors.

3.5. LPA and S1P increase cAMP levels in NK cells

3.5.1. LPLs increase cAMP levels in NK cells

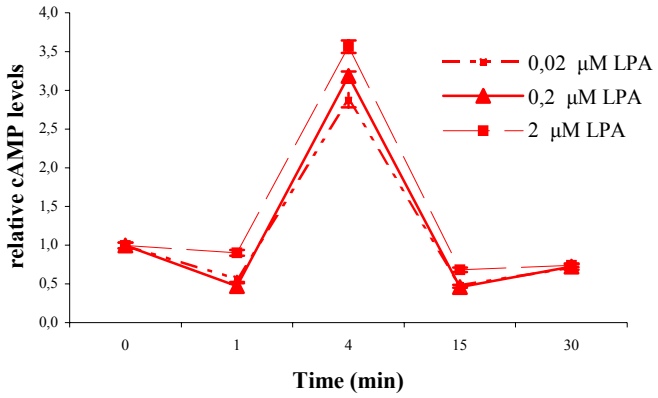
Chemotaxin receptors in leukocytes usually mediate their action by interacting with G_i protein-coupled receptors, which in turn activates Ca^{2+}-transients and phosphatidylinositol-3 kinase (Yin et al., 2003; Maghazachi, 1999). In contrast to the G_i protein-coupled CCL5/RANTES receptor signal, coupling of the different types of LPLs receptors is pleiotropic and includes G_s proteins, which activate adenylylcyclase (Mills and Moolenaar, 2003; Ancellin et al., 1999). To study the influence of the lysophospholipids on intracellular cAMP levels in NK cells, a competitive ELISA was performed. 1 x 10^6 NK cell /ml were stimulated for 1, 4, 15 or 30 min with different concentrations of LPA or S1P. The intracellular cAMP levels were measured using the cAMP direct immunoassay kit.

After NK cells were stimulated with LPA, a significant increase of cAMP was observed [(p= 0.0014 (2 µM LPA), <0.0001 (0.2 µM LPA), <0.0001 (0.02 µM LPA)] (Figure 3.12. A). Maximal

enhancement was observed after 4 min of stimulation. Similarly, NK cells stimulated with S1P also showed a significant increase in cAMP levels compared to unstimulated NK cells. S1P enhancement was also concentration-dependent, showing maximal enhancement at 4 min [(p=0.012 (2 µM S1P), 0.01 (0.2 µM S1P), 0.02 and (0.02 µM S1P)] (Figure 3.12. B).

Due to the high variability in the amount of intracellular cAMP between the donors, results are expressed as the amount of cAMP in relation to the amount of unstimulated NK cells.

(A)



(B)

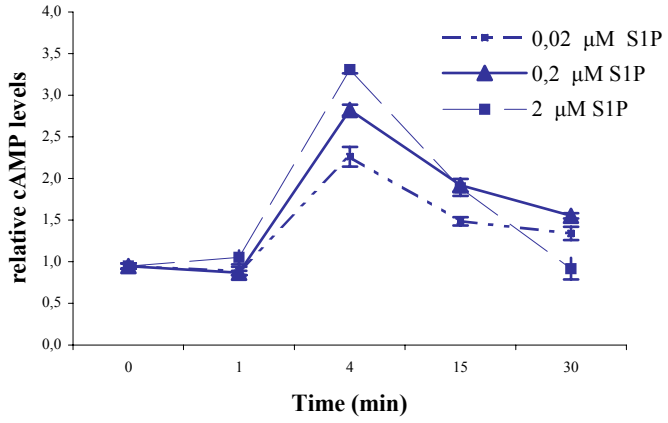
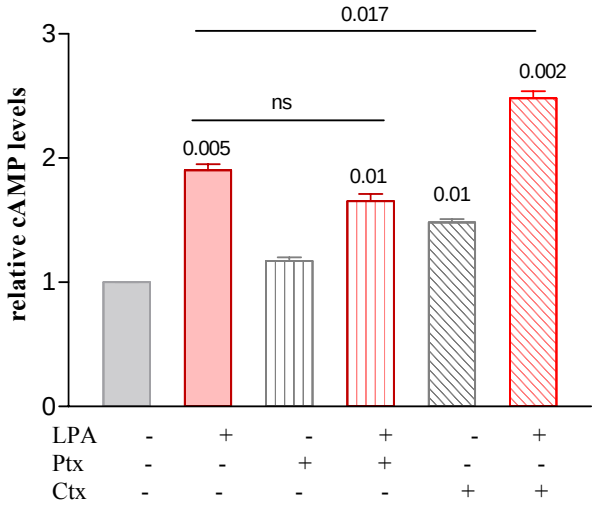


Figure 3.12. LPL stimulation increases cAMP levels in NK cells. NK cells were stimulated for the indicated time points with LPA or S1P. Intracellular cAMP levels were measured with cAMP direct immunoassay kit. **(A)** LPA stimulation significantly increased cAMP levels in NK cells at 4 min in comparison to controls. **(B)** Similarly, S1P stimulation also significantly increased cAMP levels in NK cells, showing maximal enhancement at 4 min. Data are means ± SEM of three experiments with NK cells isolated from different donors.

3.5.2. Influence of pertussis toxins and cholera in the cAMP levels

In light of previous results, only concentrations of 2 μM of LPA or 2 μM of S1P were used to perform this set of experiments. NK cells were incubated for 1 hr with pertussis or cholera toxin, after which the intracellular levels of cAMP were analyzed. As describe above, LPLs significantly increase cAMP levels in NK cells (p= 0.005 for 2 μM LPA and p=0.015 for 2 μM S1P). Pretreating NK cells with pertussis toxin (Ptx) neither influenced the cAMP levels in unstimulated NK cells nor inhibited the LPA and S1P-mediated increase in the cAMP response. In contrast, pretreating NK cells with G_s protein-activating cholera toxin (Ctx) enhanced intracellular cAMP levels in unstimulated NK cells (p= 0.01, 0.004); moreover, NK cells exposed to LPLs further increased cAMP levels with a p value of 0.002 for 2 μM LPA and 0.0004 for 2 μM S1P compared to unstimulated NK cells. Ctx petreatment of NK cells exposed to LPLs further enchanced intracellular cAMP levels compared to LPL-stimulated NK cells with a p value of 0.017 for 2 μM LPA or S1P (Figure 3.13. A and B).

(A)



(B)

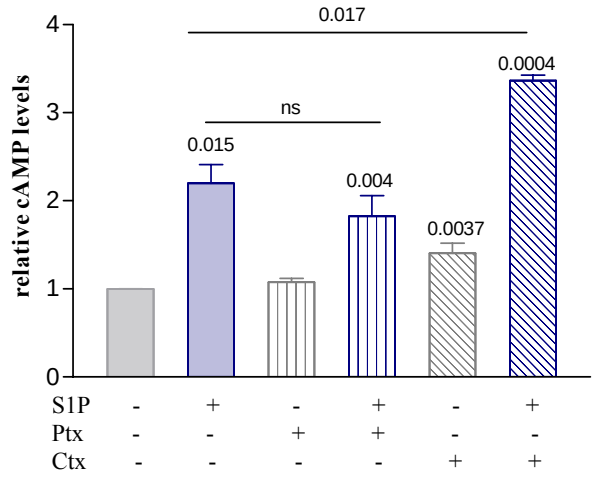


Figure 3.13. Influence of pertussis and cholera toxins on cAMP levels. Cells were preincubated with pertussis toxin (100 ng/ml) or cholera toxin (0.5 µg/ml) for 60 min. cAMP levels were measured after 4 minutes of stimulation with and without **(A)** 2 µM LPA or **(B)** 2 µM S1P. Cells pretreated with pertussis toxin did not differ significantly from untreated cells. In contrast, cholera toxin (Ctx) significantly enhanced the intracellular cAMP levels in unstimulated NK cells and further increased cAMP in LPA- and S1P-stimulated NK cell. Data are means ± SEM of three experiments with NK cells isolated from different donors.

3.5.3. Influence of pertussis and cholera toxins on NK cell cytotoxicity

Studies have suggested that elevated levels of cAMP in NK cells inhibit their cytotoxicity (Ullberg et al., 1983). Reduced cytotoxic activity has been reported after exposure of NK cells to G_s protein-stimulating ligands such as prostaglandin E_2, adenosine and prostaglandin D_2 (Linnemeyer and Pollack, 1993; Raskovalova et al., 2005; Chen et al., 2007). Hence, the influence of pertussis toxins and cholera toxins on the cytotoxic activity of NK cells was analyzed. After 1hr at 37°C with the toxins, NK cells were incubated with target cells in the absence or presence of 2 μM of the stimuli for 4 hr. Thereafter, the percentage of ^{51}Cr released was determined. Pretreatment with pertussis toxin did not influence the cytotoxic activity in either unstimulated or LPA- and S1P-treated NK cells (Figure 3.14.). In contrast, as described in the literature, cholera toxin pretreatment inhibited the cytotoxic activity of unstimulated NK cells ($p=0.005$ and 0.001). The inhibitory effect of the cholera toxin on treated cells was further enhanced by LPA stimulation ($p= 0.003$ compared to untreated cells and $p=0.01$ compared to LPA-stimulated NK cells) and also by S1P ($p=0.0001$ compared to untreated cells and $p=0.001$ compared to S1P-stimulated NK cells).

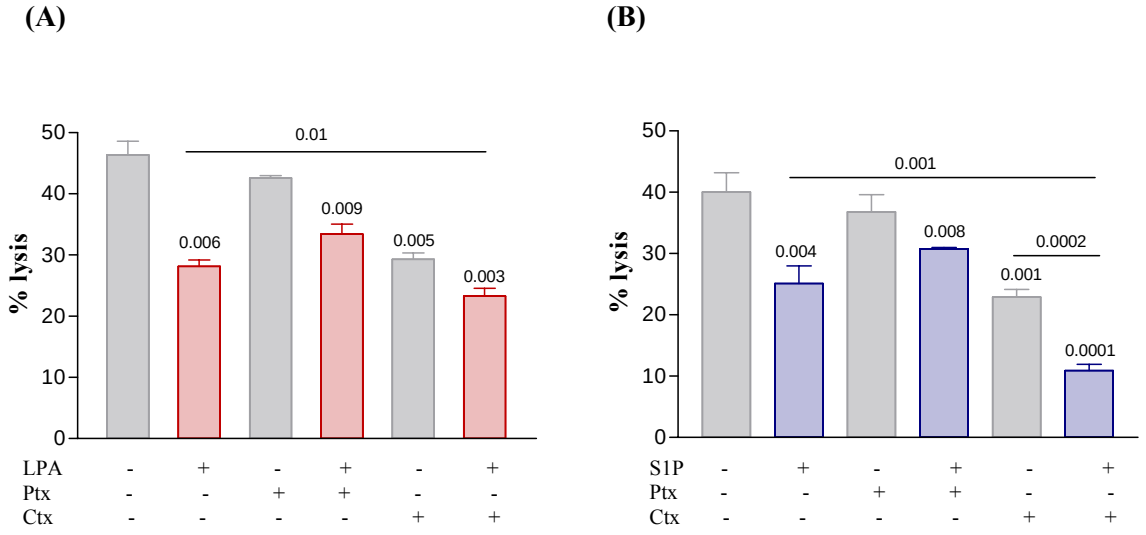


Figure 3.14. Influence of toxins on NK cell cytotoxicity. NK cell were preincubated with Ctx (0.5 µg/ml) or Ptx (100 ng/ml) for 1 hr at 37°C. Thereafter, NK cell were mixed with ^{51}Cr-labelled A2058 melanoma cells at E/T ratio 2.5:1 in the absence or presence of **(A)** 2 µM LPA or **(B)** 2 µM S1P. Pretreatment of NK cells with pertussis toxin did not influence cytotoxic activity in LPA- and S1P-treated NK cells. In contrast, cholera toxin significantly reduced the levels of NK cell cytotoxicity, and this effect was significantly enhanced by LPA and S1P. Data are from one representative experiment. The experiments were repeated three times with NK cells isolated from different donors.

3.6. Activation of PKA in NK cells

3.6.1. LPLs induce activation of PKA in NK cells

Classically, cAMP signalling immediately activates protein kinase A (PKA), resulting in the release of two catalytic subunits; these are able to phosphorylate serine and threonine residues on specific substrate proteins and two regulatory subunits, which are the primary receptors for cAMP. Therefore, PKA activity in NK cells was analyzed. NK cells were stimulated with 2 µM LPA or 2 µM S1P for 1 to 30 min; subsequently the cells were lysed and the PKA activity was tested using the StressXpress nonradioactive PKA activity Kit. Figure 3.15. shows that LPA as well as S1P stimulation increased PKA activity in NK cells. Different concentrations of the LPLs were tested (20-0.02 µM), and a maximal effect was found at 2 µM (data not shown). Additionally, the response was time-dependent: 4 min of stimulation with LPA as well as S1P significantly increased PKA activity by ($p=0.026$ for 2 µM LPA and $p=0.001$ for 2 µM S1P).

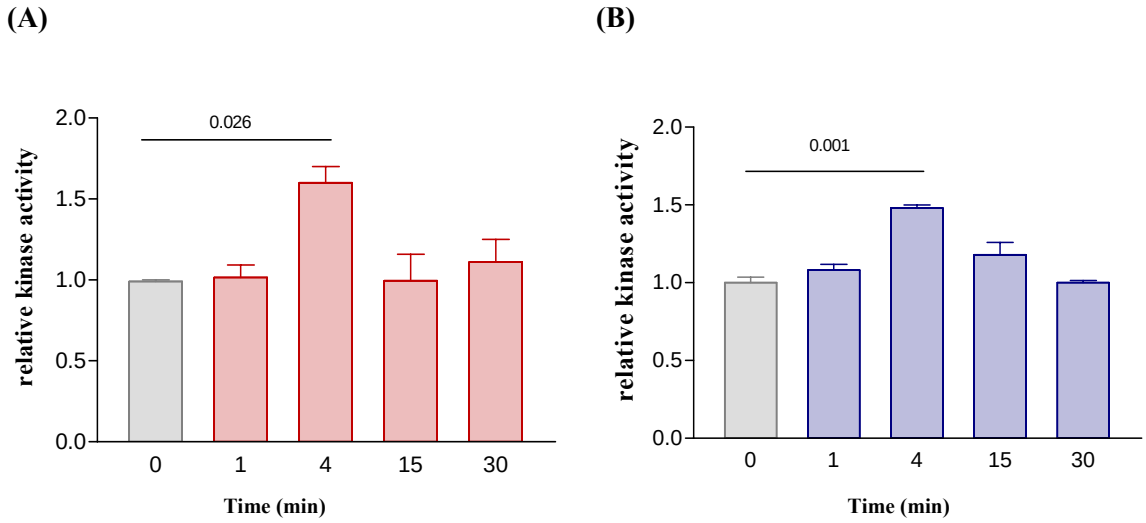


Figure 3.15. LPA and S1P stimulate PKA activity in NK cells. NK cells were stimulated with **(A)** 2 µM LPA and **(B)** 2 µM S1P at the indicated time points; afterward, cells were lysed and PKA activity was tested. After 4 min of stimulation, LPA significantly increased PKA activity in comparison to controls. S1P stimulation of NK cell also significantly increased cAMP levels at 4 min. Data are means ± SEM of three experiments with NK cells isolated from different donors.

3.6.2. Regulatory subunits type I mediate the LPL-mediated inhibitory effect on NK cell cytotoxicity

In order to identify the subunit of PKA that modulates cytotoxic activity, experiments were performed with different PKA inhibitors. Myristoylated peptide PKI_{14-22} (PKI peptide) specifically blocks the catalytic activity of PKA, whereas Rp-8-Br-cAMPS binds to the regulatory subunits I and prevents the PKA I holoenzyme dissociation as well as the release of the regulatory subunits (Gjertsen et al., 1995). If the inhibitory effect of the lysophospholipids is mediated via the PKA pathway, the inhibition of PKA activity will likely block the inhibitory effects of these two lysophospholipids.

NK cells were preincubated with PKI (1 µM) or Rp-8-Br-cAMPS (1 mM) for 30 min at 37°C, after which the cytotoxicity assay was performed as described. Pretreatment with PKI peptide neither affected the cytotoxic activity of unstimulated NK cells nor influenced the LPA and S1P-induced

inhibition (Figure 3.16. A and B). In contrast, pretreating NK cells with Rp-8-Br-cAMPS, a blocker of the regulatory subunits, abrogated the inhibitory effect of the LPLs (p= 0.02 for 2 µM LPA and p=0.001 for 2 µM S1P)(Figure 3.16. C and D). These findings suggest that the RI subunits of PKA mediate the inhibitory effect of LPLs on NK cell cytotoxicity.

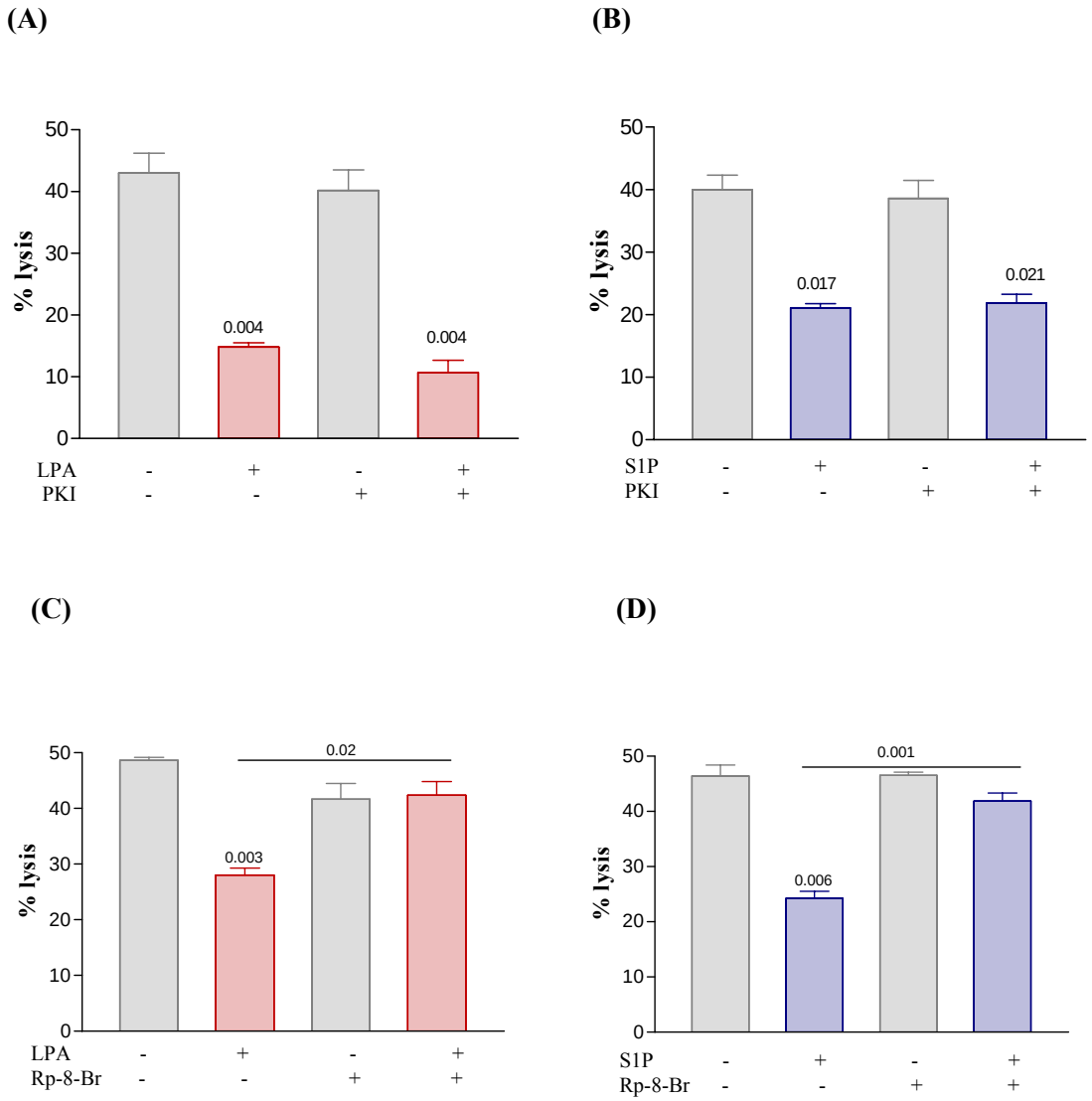


Figure 3.16. Effect of PKA inhibitors on the LPL-mediated inhibition of NK cell cytotoxicity. NK cells were mixed with ^{51}Cr-labelled A2058 melanoma cells at E/T ratio 2.5:1 in the absence or presence of LPA (2 µM) or S1P (2 µM). **(A-B)** NK cells were preincubated for 30 min with PKI peptide (1µM) or Rp-8-Br-cAMP (1 mM) before target cells and LPA or S1P were added. After 4 hr, the percentage of ^{51}Cr was determined. The PKI peptide did not significantly influence the cytotoxicity in control and LPL-stimulated cells. **(C-D)** Rp-8-Br-cAMP treatment showed no significant differences in controls, but prevented the LPA and S1P-induced inhibitory effect. Data are representative from one experiment. Experiments were repeated three times with cells from different donors.

3.7. LPA receptor type-2 mediates the inhibitory effect of LPA

3.7.1. Effect of LPA agonists and antagonists on NK cell cytotoxicity.

NK cells have been reported to express different type of LPA receptors especially LPA1, LPA2 and LPA3 (Jin et al., 2003). To identify the LPA receptor subtype modulating cytotoxicity, experiments with subtype-specific receptor antagonists and agonists were performed. Dioctylglycerol pyrophosphate (DGPP) is an antagonist for LPA1 and LPA3 receptors (Fischer et al., 2001; Tigyi, 2001) and 18:1 LPA is a pan-receptor agonist; in contrast, 14:0 LPA has a selective ability to activate the LPA receptor type-2 (Bandoh et al., 2000). Pretreating NK cells with DGPP did not affect the LPA-induced inhibition of NK cell activity (Figure 3.17. A). Challenging the cells with the LPA2 selective agonist 14:0 LPA reduced cytotoxic activity of NK cells in the same way that LPA did ($p=0.007$ for 2 μM LPA and $p=0.002$ for 2 μM 14:0 LPA). Although there are no significant differences, stimulation with 2 μM 14:0 LPA was more effective than with 2 μM 18:1 LPA in inducing the inhibition, suggesting a more critical role for the LPA2 receptor than for LPA1 or LPA3 in the LPA-mediated inhibition of NK cell cytotoxicity (Figure 3.17. B).

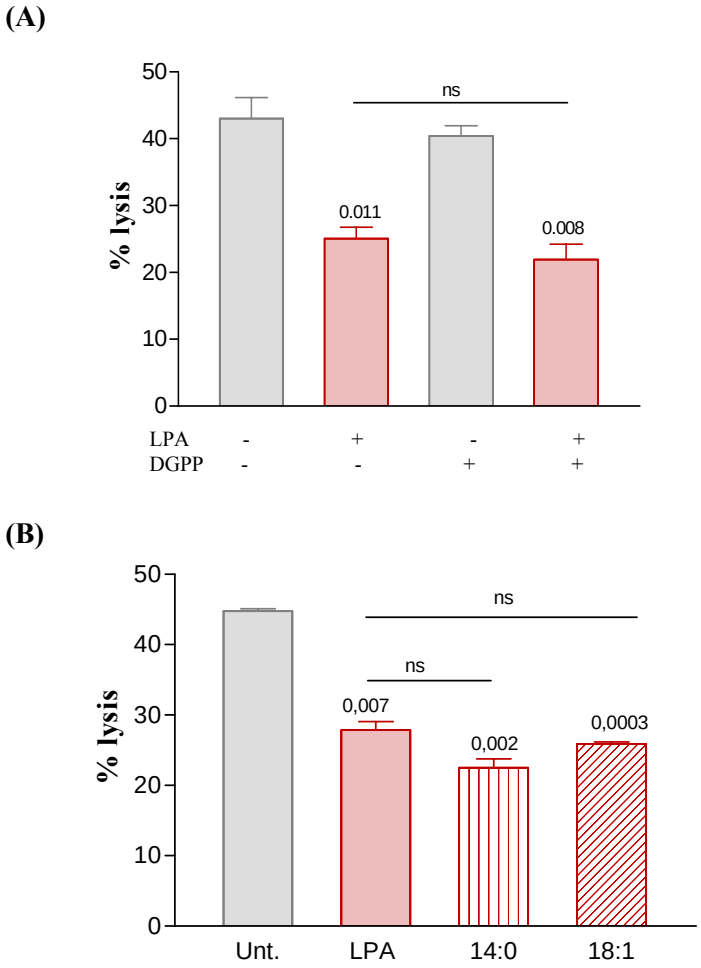


Figure 3.17. Effect of LPA agonists and antagonists on NK cell cytotoxicity. (A) Cells were preincubated with or without 10 µM DGPP for 90 min at 37°C and NK cells were then mixed with ^{51}Cr-labelled A2058 and subsequently stimulated with or without 2 µM LPA. Pretreatment of NK cells with DGPP did not affect either unstimulated or LPA-stimulated. **(B)** Cells were stimulated with the LPA2-selective agonist 14:0 LPA (2 µM). Stimulation with LPA significantly inhibited NK cell cytotoxicity and treatment with the LPA2-selective agonist 14:0 LPA and with the pan-agonist 18:1 LPA had the same effect. One representative experiment is shown; the experiment was repeated three times with NK cells from different donors.

3.7.2. Effect of LPA agonists and antagonists on cAMP levels

NK cells were preincubated with the LPA receptor type-1 and -3 antagonist DGPP or stimulated with the selective LPA receptor type-2 14:0 LPA or with the pan-receptor agonist 18:1 LPA, after which cAMP levels were analyzed. DGPP does not influence the LPA-induced increase of the

cAMP levels. 14:0 LPA as well as 18:1 LPA significantly enhanced cAMP levels in the same way that LPA did (Figure 3.18.). Although 14:0 LPA stimulation of NK cells was more effective, the 14:0 LPA-induced increases of the cAMP levels did not differer significantly from the increase obtained through LPA stimulation.

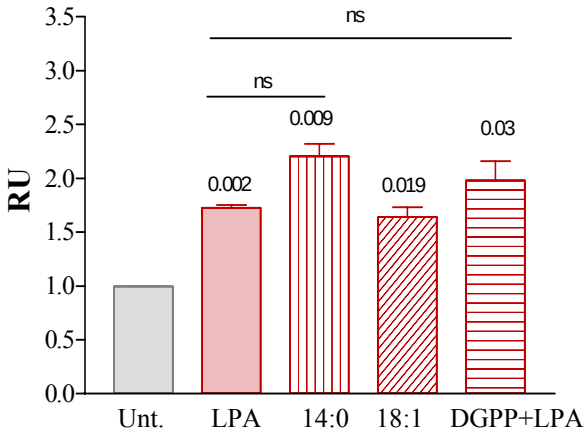


Figure 3.18. Effect of LPA agonists and antagonists on cAMP levels. NK cells were preincubated with DGPP or stimulated with 14:0 LPA or 18:1 LPA and the intracellular levels of cAMP were analyzed. 14:0 LPA-stimulated cells significantly increased cAMP levels compared to cAMP levels in the untreated NK cells. No differences between 18:1 LPA- and LPA-induced increases of cAMP levels were observed. Pretreatment with the LPA type-1 and -3 receptor antagonists DGPP did not differ significantly from the LPA-induced increase of cAMP levels. Data are means ± SEM of three experiments with cells from different donors.

4. Discussion

In the last few years, several investigations have pointed out that many functional aspects of the tumor cells and their microenvironments determine the course of tumor progression, e.g. the relevance of autocrine-secreted products in the growth response and metastatic processes of tumor cells (Umezu-Goto et al., 2002). Lysophospholipids (LPLs) are potent inducers of many of the hallmarks of cancer, including cell proliferation, survival, migration, invasion and neurovascularization (Goetzl et al., 2004; Xie et al., 2002). Cancer cells are able to secrete LPA and S1P into the microenvironment, where LPLs show tumorigenic and proangiogenic effects (Mills and Moolenaar, 2003; Spiegel and Milstien, 2003). Given these results, one can assume that LPLs contribute to the escape of tumor cells from immunological surveillance. Additionally, LPLs are activators and chemoattractants of NK cells, which are critical members of the immunological tumor surveillance machinery. In the context of the above described features, this work characterizes the biological functions of LPLs and the influence of lysophospholipids on the cross-talk between human NK cells and tumor cells.

4. 1. LPA and S1P induce actin polymerization in NK cells

Chemoattractants use $G_{i/o}$-coupled heterotrimeric G proteins to elicit cytosolic changes correlated with actin polymerization and other cytoskeletal events that result in the extension of pseudopods toward the chemoattractant (Maghazachi, 2000). LPA and S1P induce chemotaxis of NK cells through pertussis toxin sensitive-G proteins (Kveberg et al., 2002; Jin et al., 2003). In order to further characterize the role of LPLs with regard to the chemotaxis-associated intracellular rearrangements of NK cells, actin polymerization was analyzed. As expected from substances inducing chemotaxis, LPLs' stimulation of NK cells induced the reorganization of the actin network. Flow cytometric measurements indicated that LPA and S1P caused a rapid and concentration-dependent polymerization of actin molecules within 30 seconds (Figure 3.1). As

described, chemotaxis and calcium release induced by LPLs are inhibited by pretreating NK cells with pertussis toxin, indicating that members of $G_{i/o}$ are involved (Kveberg et al., 2002; Jin et al., 2003; Maghazachi, 2003). To investigate the involvement of G_i proteins in the LPL-mediated actin polymerization, NK cells were incubated with G_i protein-inactivating pertussis toxin. As expected, treatment with pertussis toxin completely inhibited LPL-induced actin response. These data suggest that, like the classical chemokines such as RANTES, pertussis-toxin-sensitive G proteins mediate the LPL-polymerization of actin in NK cells.

4.2. LPLs signalling pathway in NK cells

Chemokine receptors are thought to mainly signal via $G_{i/o}$-coupled heterotrimeric G proteins to inhibit the activity of adenylylcyclase (AC), to stimulate the mitogen-activated protein kinase (MAPK) cascade, and to activate the phosphatidylinositol 3-kinase (PI3K). Similarly, LPLs are able to bind to specific GPCR of the lysophospholipid family and thus activate specific intracellular G proteins. LPLs binding to the receptors activate signalling cascades culminating in different processes such as proliferation, survival, cytoskeletal changes and invasion. In order to characterize the intracellular signalling pathway of LPLs in NK cells, activation and / or phosphorylation of different signal proteins downstream of phosphatidylinositol 3-kinase were analyzed by western blot. Proteins such as Akt/protein kinase B (PKB), glycogen synthase kinase 3β (GSK3β) and p38 MAP kinase were analyzed. Akt plays a critical role in controlling the balance between survival and apoptosis. Akt promotes cell survival by inhibiting apoptosis through its ability to phosphorylate and deactivate several targets. One of the essential functions of Akt is the regulation of glycogen synthesis by phosphorylating glycogen synthase kinase 3α and β (GSK3 α and β). Akt is involved in regulating cell cycles by preventing GSK-3β-mediated phosphorylation and the degradation of cyclin D1 and by negatively regulating the cyclin-dependent kinase inhibitors. GSK3 activity can be inhibited by Akt-mediated phosphorylation. The mitogen-activated protein kinase (MAPK) p38 modulates a variety of cellular functions including proliferation,

differentiation and cell death (Chini et al., 2000). p38 is activated by a variety of cellular stresses including proinflammatory cytokines and growth factors.

Basal levels of pAkt, pGSK3β and pp38 were observed in cells not stimulated by LPLs, probably due to the culture of the NK cells in the presence of interleukin 2. IL-2 promotes survival via phosphatidylinositide 3-kinase/Akt in NK cells (Jiang et al., 2003). Exposing NK cells to LPA further enhanced pAkt, pGSK-3β and pp38. LPA induced maximal levels of pAkt and pGSK-3β within 30 s, increasing levels by about 60 or 35 %, respectively. The kinetic of the pp38 response peaked more slowly, after 90 s, increasing levels about 50 %. Similar results were obtained by exposing NK cells to S1P. S1P stimulation induced also enhanced pAkt and pGSK-3β. In this case, the quantification of the luminescence indicated that S1P significantly enhanced pAkt and pGSK-3β within 60 s. In addition, incubating NK cells with the PI3K inhibitor wortmannin induced a strong decrease in phosphorylation of Akt and GSK-3β in NK cells not stimulated by LPL and inhibited LPL-induced phosphorylation. These data suggest a functional link between GSK3, Akt and PI3K in NK cells. In contrast, p38 phosphorylation occurred in the presence of wortmannin, indicating a PI3K-independent signalling mechanism. Additional studies will be needed to determine which molecules are involved in the p38 activation in NK cells.

Signalling pathways including either MAPK or PI3K/Akt govern the two most important cell processes: proliferation and survival. The MAPK family includes the extracellular signal-regulated kinases (ERK), the c-Jun N-terminal kinase (JNK)/stress-activated protein kinase and the p38 MAPK (p38). PI3K has also been shown to be the upstream activator of ERK and p38 MAPK (Krump et al., 1997; Lopez-Ilasaca et al., 1997). However, the signalling mechanisms leading to p38 activation seem to be highly cell line- and stimulus-specific (Baudhuin et al., 2002). LPA and/or S1P have been shown to activate ERK and PI3K and/or Akt (PKB) via a PTX-sensitive pathway in a number of cell types (Marte and Downward, 1997; Weiner and Chun, 1999; Fang et al., 2000; Lee et al., 2000). In NK cells, ERK activation can functionally modulate the development of cytotoxicity and the expression of INF-γ or TNF. p38 is known to participate in NK cell

cytotoxicity and it is activating on a PI3K-independent pathway (Chini et al., 2000; Trotta et al., 2000).

The work presented here offers novel aspects related to LPA- and S1P-induced Akt and p38 signalling in NK cells. Analysis of signal transduction showed that LPLs induce common signalling pathways of chemotaxins, such as the pathways that activate the MAP-kinase p38 as well as the PI3 kinase-dependent signal molecules (AKT and GSK-3β).

4. 3. LPLs inhibit NK cell lysis of tumor target cells

In addition to inducing NK cell recruitment, chemotaxis and the chemotaxis-associated intracellular rearrangements, classical chemotaxins enhance cytotoxic activity of NK cells. Chemokines such as MIP-1β/CCL1, TARC/CCL17, RANTES/CCL5 and MCP-1/CCL-2 are known to enhance the cytotoxic activity of NK cells (Taub et al., 1995; Maghazachi, 1997; al-Aoukaty et al., 1998; Inngjerdingen et al., 2001). These chemokines bind to G_i protein-coupled receptors linking to activation of phospholipase C and PIP_3–generating type IB phosphatidylinositol 3-kinase (al-Aoukaty et al., 1999).

As described above, LPLs perform functions similar to chemokines in NK cells; these functions also involve using G proteins. Hence, the effect of the LPLs on the cytotoxic activity of NK cells was analyzed. Chromium-51 standard assays were carried out to study and compare the effects of the classical chemokine CCL5/RANTES and the LPLs on the cytotoxic activity of NK cells against tumor cell lines. As the literature, CCL5/RANTES increased NK cells cytotoxic activity in a concentration-dependent manner (Taubb, 1996). In contrast to CCL5/RANTES, LPA and S1P inhibited the cytotoxic activity of NK cells against the human Burkitt's lymphoma cell line Raji. LPLs inhibited the cytotoxicity in a concentration-dependent manner and maximal effects were found with 2 μM LPA or S1P. To exclude the possibility that the LPL-mediated inhibition of NK cell cytotoxicity migh be a specific effect of the Raji target cells, experiments with different human melanoma cell lines were also performed. Similar inhibitory effects on NK cell cytotoxicity have

been observed with A2058, HS294T and SK-Mel 23 human melanoma cell lines and using different effector:target ratios. Although LPLs perform like classical chemokines in NK cells, they inhibit the cells' cytotoxic activity. These data suggest that chemotaxins differentially influence cytotoxic activity in NK cells.

In 1986, Dvorak speculated that tumors are "wounds that do not heal" (Dvorak, 1986). One could argue that the ability of cancer cells to release LPLs into the tumor microenvironment is consistent with the idea that cancerous tumors release mediators to trick the body into thinking that it has a wound which needs to be infiltrated with platelets, fibroblasts, mast cells, neutrophils and NK cells to create an inflammatory response. Infiltrating cells promote the release of LPLs into the tumor microenvironment to further the tumorigenic and proangiogenic effects of LPLs (Sabbadini, 2006). In this way, LPA and S1P may block the activity of major anti-tumor effector cells, i.e. NK cells, which provide a favorable environment for the growth of tumor cells. This fact may also explain how tumor cells can survive *in vivo* and avoid being destroyed by NK cells. Such a conclusion is supported by the fact that lysophospholipid levels are increased in patients with several types of cancer including melanoma (Goetzl and Gräler, 2004; Hla, 2004; Prieschl et al., 1999; Yatomi et al., 1997; Meng et al., 2004).

Nowadays, several approaches involve LPLs as tumor markers, prognostic indicators or indicators of response to therapy. Different strategies have been used to deactivate LPLs to inhibit the growth and metastasis of tumors. Neutralizing extracellular LPLs could markedly decrease the progression of cancer by inhibiting the formation of blood vessel which provides the nutrients needed to support tumor growth (Visentin et al., 2006). The importance of LPL in angiogenic-dependent tumors makes S1P and LPA excellent targets for cancer treatment.

4.4. Involvement of G_s protein-mediated signalling

4.4.1. LPLs increase cAMP levels in NK cells

As described above, chemotaxin receptors in leukocytes usually mediate their action by interacting with G_i protein-coupled receptors; this interaction causes Ca^{2+} mobilization, reorganization of actin, and activation of phosphatidylinositol-3 kinase (Jin et al., 2003; Maghazachi, 1999). Besides coupling to G_i proteins, LPL receptors are also able to interact with other G proteins, including G_s according to heterologous expression studies. In addition, several studies have suggested that the elevation of intracellular levels of cAMP in NK cells inhibits their cytotoxicity (Ullberg et al., 1983). Consequently, the influence of LPLs on cAMP levels was analyzed. After NK cells were stimulated for 4 min with LPA or S1P, a significant and concentration-dependent increase of cAMP levels was observed (Figure 3.4). Moreover, pretreating NK cells with G_s protein-activating cholera toxin enhanced intracellular cAMP levels in NK cells not stimulated by LPL, and exposing cholera toxin-pretreated NK cells to LPA or S1P further increased cAMP levels. In contrast, pretreating NK cells with pertussis toxin did not influence cAMP-levels in LPL-unstimulated NK cells or inhibit LPA and S1P-mediated increases in the cAMP response. These results suggest that the coupling of the different types of LPLs receptors in NK cells is pleiotropic and includes G_s proteins, which activate adenylylcyclase and elevate cAMP levels. Supporting these results, heterologous expression studies demonstrated that LPA receptors are also able to interact with G_q, G_{12}, and G_s proteins (Taubb et al., 1995; An et al., 1998; Bandoh et al., 1999; Mills and Moolenaar, 2003). Similarly, studies with different cell types indicated that S1P is able to induce G_i protein-independent activation pathways (Windh et al., 1999; Ancellin et al., 1999; Pyne and Pyne, 2000; Rosen and Goetzl, 2005).

4.4.2. Influence of pertussis toxin and cholera toxin on the cytotoxicity of NK cells

Adenylylcyclase-activating cholera toxin reportedly increase cAMP levels and inhibit the cytotoxic activity of NK cells (Fuse et al., 1981; Watanabe et al., 1993). In light of the increase of intracellular cAMP levels in response to LPA and S1P, the influence of the toxins on unstimulated NK cells and LPL-stimulated NK cells cytotoxicity was analyzed. The ADP ribosylation of the alpha subunit of the inhibitory G protein of adenylylcyclase ($G_{\alpha i}$) by pertussis toxins did not influence the cytotoxic activity in unstimulated or LPL-treated NK cells. In contrast, pretreating with cholera toxin inhibited the cytotoxic activity of unstimulated NK cells and the cholera toxin-mediated inhibition was further enhanced by LPA or S1P stimulation.

Together with the LPL-mediated increase of cAMP levels, these results suggest that activation of G_s proteins may be responsible of the inhibitory effect of LPLs. Therefore, these data are consistent with the concept that cAMP regulates crucial steps in cytotoxic response machinery of NK cells. Reduced cytotoxic activity has been reported after NK cells were exposed to G_s protein-stimulating ligands such as prostaglandin E_2, adenosine and prostaglandin D_2 (Linnemeyer and Pollack, 1993; Raskovalova et al., 2005; Chen et al., 2007). Moreover, studies have suggested that the elevated levels of cAMP in NK cell inhibit their cytotoxicity due to these cells' decreased ability to conjugate to target cells (Ullberg et al., 1983). Clearly LPLs' influence in the conjugation between NK and target cells needs to be analyzed.

4.5. cAMP-dependent PKA activity inhibits the cytotoxic function of NK cells

The results of the above-mentioned experiments suggest that LPA and S1P modulate the cytotoxic activity of NK cells by G_s protein-dependent signal pathways and involve an increase of the intracellular cAMP levels. Classically, cAMP signalling leads to the immediate activation of protein kinase A (PKA), resulting in the release of two catalytic subunits; these subunits are able to phosphorylate serine and threonine residues on specific substrate proteins and on two regulatory

subunits, which are the primary receptors for cAMP. Two types of isozymes are present in leukocytes: PKA type I and type II. Their catalytic subunits are similar but the regulatory subunits may have distinctive functions. Although both PKAs are present in leukocytes, several reports indicate that the specific activation of PKA type I is sufficient for cAMP-mediated inhibition of T and B cell proliferation (Levy et al., 1996; Skalhegg and Taskén, 2000) and for cAMP-mediated inhibition of the cytotoxic activity of antimelanoma-specific $CD8^+$ T cells (Raskovalova et al., 2007), as well as for the inhibition of NK cell cytotoxicity (Torgersen et al., 1997; Raskovalova et al., 2005). Therefore, the influence of LPLs in the PKA activity in NK cells was analyzed. LPL treatment increased PKA activity in NK cells within 4 min of the cells being stimulated.

Additionally, the idea that NK cells cytotoxicity is modulated by G_s protein-dependent signal pathways was further corroborated here using protein kinase A inhibitors. Myristoylated peptide PKI_{14-22} (PKI peptide) specifically blocks the catalytic activity of PKA, whereas Rp-8-Br-cAMPS binds to the regulatory subunits I and prevents the PKA I holoenzyme dissociation as well as the release of the regulatory subunits I (Gjertsen et al., 1995). If the inhibitory effect of LPLs is mediated via the PKA pathway, inhibiting PKA activity can be expected to abrogate the inhibitory effects of LPA and S1P. However, pretreating NK cells with PKI peptide neither affected the cytotoxic activity of unstimulated NK cells nor influenced the LPL-induced inhibition. These results indicate that the inhibitory effect of LPA and S1P on NK cell cytotoxicity may not be mediated via the catalytic subunits of PKA. In contrast, pretreatment of NK cells with Rp-8-Br-cAMPS, a blocker of the regulatory subunits type I, completely prevented the inhibitory effect of LPLs. These findings suggest that the RI subunits mediate the inhibitory effect of LPLs on NK cell cytotoxicity. These inhibitory effects depend on the involvement of RI and are independent of the activity of catalytic subunits of PKA.

The data presented in this work extend and confirm previous reports using the cAMP-inducing agent adenosine in murine, rat and human NK cells (Torgensen et al., 1997; Raskovalova et al., 2006; Lokshin et al., 2006). Tumor-produced adenosine could be another example of a tumor

microenvironmental factor inhibiting the functional activity of tumor-infiltrating immune cells. Like the results collected here, the inhibitory effect of adenosine involves a mechanism that includes cAMP induction and activation of cAMP-dependent protein kinase A type I (Torgensen et al., 1997; Raskovalova et al., 2006; Lokshin et al., 2006). Therefore, one could assume that the regulatory subunit I has signalling functions in NK cells. In this context, it is worth mentioning that mice whose genes for encoding regulatory subunits have been selectively knocked out (RIα, RIβ or RIIα, RIIβ) reveal distinctive phenotypic changes (Chin et al., 2002). Transfection experiments of tumor cells show that the RIα subunit promotes cell growth, whereas the RIIβ subunit inhibits growth and induces cell differentiation (Cho-Chung et al., 2002). Mutation in RIα causes the Carney complex tumor syndrome, according to which multiple tumors and cardiac and extracardiac myxomatosis develop (Kirschner et al., 2005). These data suggest that PKA has biological significance beyond its catalytic activity and that the regulatory subunits of PKA have a functional activity. However, the mechanisms by which RI subunits might mediate this inhibitory effect need to be further analyzed.

4.6. LPA type-2 receptor mediates the LPA inhibitory effect of NK cytotoxicity

NK cells have been reported to express different types of LPA receptors especially LPA1, LPA2 and LPA3 (Jin et al., 2003). To identify the LPA receptor subtype modulating the cytotoxicity of NK cells, experiments with subtype-specific receptor antagonist or agonist were performed. Certain species of LPA, such as 14:0 LPA, are able to activate the LPA2 receptor over other receptor subtypes (Bandoh et al., 2000), whereas dioctylglycerol pyrophosphate (DGPP) is an antagonist for LPA1 and LPA3 receptors (Fischer et al., 2001; Tigyi, 2001). Pretreating NK cells with DGPP did not affect the LPA-induced inhibition of NK cell activity. If the LPA2 receptor is indeed physiologically more critical than LPA1 and LPA3 for inhibiting the cytotoxicity of NK cells, challenging cells with the LPA2-selective agonist 14:0 LPA might lead to the inhibition of NK cell cytotoxicity. To examine this hypothesis, we compared the ability of LPA2-selective agonist 14:0

and the pan-receptor agonist 18:1 LPA to inhibit cytotoxic activity in NK cells. 14:0 LPA-stimulated NK cells reduced the cytotoxic activity of NK cells similarly to the LPA and 18:1 LPA. Parallel cAMP measurements showed that DGPP does not influence the LPA-induced increase of the cAMP-levels. The 14:0 LPA agonist significantly enhanced cAMP levels as well as the pan-receptor agonist 18:1 LPA. Although no significant differences were found, stimulation with the LPA2-selective agonist 14:0 LPA was more effective than with the LPA and 18:1 LPA in increasing cAMP levels and inhibiting the cytotoxicity of NK cells; this finding highlights the importance of the LPA2 receptor. Taken together the data presented suggest that LPA receptor subtype-2 rather than LPA receptor subtypes-1 and -3 may play a critical role in activating adenylcyclase and modulating the cytotoxicty by LPA in NK cells.

Which receptor subtype couples to which G-protein-effector route(s) in a given cellular type is still not completely known. In most systems, LPA2 shows a higher affinity for LPA than for the other family members (Mills and Moolenaar, 2003). Additionally, heterologous expression studies have shown the coupling of LPA 2 receptor to G_s proteins (An et al., 1998; Bandoh et al., 1999 and 2000).

LPA receptor type-2 might play a critical role in the signalling cascade, causing the inhibition of NK cell cytotoxicity. This cascade involves activating adenylcyclase, increasing cAMP levels and activating protein kinase A I (PKAI), in turn these effects are responsible for the modulatory effect of LPA on NK-cell-mediated cytotoxicity.

4.7. Role of lysophospholipids in the tumor microenvironment

The results collected here suggest that LPLs, unlike classical chemotaxins such as CC chemokines, stimulate G_i as well as G_s protein-dependent signalling pathways in NK cells. The lysophospholipids inhibit the cytotoxicity of NK cells, enhancing cAMP levels and activating protein kinase A. In the last few years, several studies have demonstrated that LPLs have an important role as intracellular messengers and extracellular mediators in immunity, and that they

mediate a variety of physiologic effects. LPLs are secreted at the inflammatory sites by several kinds of tumor cells: ovarian, prostate, multiple myeloma and melanoma, among others (Goetzl and Gräler, 2004; Hla, 2004; Prieschl et al., 1999; Yatomi et al., 1997; Meng et al., 2004). LPLs seem clearly to contribute to the ability of tumor cells to escape from the surveillance machinery of NK cells.

LPLs may also protect malignant tissue from immune-mediated destruction. Although S1P is a potent stimulus for T cell migration (Rosen and Goetzl, 2005; Goetzl and Gräler, 2004), this molecule was found to inhibit T-cell proliferation (Yin et al., 2003). LPA regulates the trafficking, cytokine production, and T cell-activating functions of DCs (Panther et al., 2002) and also induces chemotaxis in naïve T cells (Goetzl et al., 2004) and NK cells (Jin et al., 2003). LPA unregulated the FasL expression in some types of cancer cells, leading to T cell apoptosis (Meng et al., 2004). Because LPA and S1P are chemoattractants for NK cells, it was surprising to discover that these molecules inhibit NK cell lysis of tumor cells.

Although the ability of LPLs to inhibit the cytotoxic activity of NK cells was shown only *in vitro*, there are reasons to believe that LPLs may exert similar effects *in vivo*. Elevated levels of LPLs have been associated with a number of malignancies. Importantly, the enzymes responsible for LPA and S1P production – autotaxin (ATX) and sphingosine kinase (SPHK), respectively – are oncogenic proteins, suggesting that the LPLs are potential therapeutic targets for addressing cancer. ATX/lysoPLD mRNA is overexpressed in various human malignancies, including lung, breast and renal carcinoma (Tanyi et al., 2003). Similarly, Sphingosine kinase-1 (SphK1) is also elevated in a variety of human solid tumors, such as those of the breast, colon, lung, ovary, stomach, uterus, kidney and rectum (French et al., 2003, Johnson et al., 2005; Kawamori et al., 2006; Yatomi et al., 1997; Sano et al., 2002). LPLs could inhibit the effector functions of immune cells and thus protect malignant tissues from immune-mediated destruction. Blocking receptor signalling in LPLs might increase the efficacy of immunotherapy. Decreasing the intratumor levels of LPLs or blocking LPL-mediated immunosuppressive signalling might represent a new strategy for cancer

immunotherapy. Visentin et al. (2006) recently reported a promising approach to cancer therapy involving LPLs. A neutralizing S1P-monoclonal antibody treatment effectively retarded progression of deadly and multidrug-resistant cancers in murine xenograft and allograft models (Visentin et al., 2006). The antibody acts as a therapeutic molecular sponge to selectively absorb S1P, thus lowering the effective extracellular concentrations of the tumor-facilitating factor.

The results collected in this work partially explain how chemotactic factors such as LPA and S1P could inhibit the cytotoxicity of anti-tumor effector cells. Considering that several tumor cells secrete LPLs in large quantities, it is plausible that LPLs recruit NK cells to the sites of tumors; there NK cells secrete cytokines such as IFN- γ which may provide a suitable environment for the growth of tumor cells (Figure 4.1.). Regulating the growth of melanoma cells depends on growth factors and cytokines. Some growth factors could have inhibitory effects on early stages of the melanoma but not on advanced stages. Melanoma cells express GRO-α, IL-8 and IL-10 receptors, among others. These growth factors stimulate proliferation of melanoma cells in an autocrine way and so perhaps in a paracrine way (Lázár-Molnár et al., 2000).

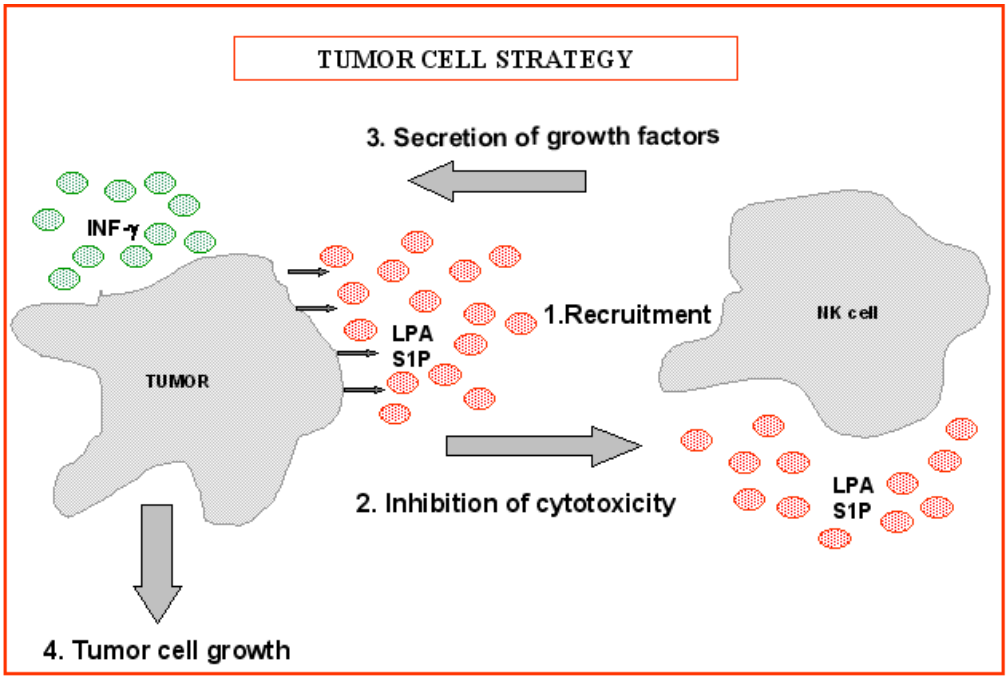


Figure 4. 1. Tumor cells secrete LPA and S1P. Lysophospholipids may recruit NK cells to the sites of tumors and at the same time inhibit their cytolytic function. Once there, NK cells secrete cytokines such as IFN-γ that may provide a suitable environment for tumor cells to grow.

4. 8. Outlook

This work documents the discovery of the inhibitory effect of the lysophospholipids on the cytotoxicity of NK cells against tumor cells. The modulatory effect of LPA and S1P on NK-cell-mediated cytotoxicity needs to be analyzed further. To reinforce the proposed mechanism of inhibition, a dominant negative PKA mutant in NK cells could be transiently expressed to confirm that LPLs mediate their modulatory effect on NK cell cytotoxicity through PKA. In addition, analysis of NK function in LPA receptor subtype-2 -/- NK cells might help to support the 14:0 agonist studies. Similarly, using agonists and antagonists could help to answer the question, which receptor subtype mediates S1P effect on cytotoxicity.

Since LPLs induce an increase in the intracellular cAMP levels in NK cells that inhibit their cytotoxic activity, LPLs' influence on the conjugation between NK and target cells needs to be analyzed.

Interestingly, the inhibitory effect of LPL on NK cell cytotoxicity through PKA may be independent of the catalytic activity of this enzyme and may instead be mediated by the regulatory subunits of the PKA I. The regulatory subunits may have functions other than activating the enzymatic activity of the PKA. The mechanisms by which RI subunits might mediate the inhibitory effect need to be further analyzed.

Additional studies need to be performed to characterize the signalling pathways of LPA and S1P in NK cells. The use of specific inhibitors could help.

Beyond the LPL-mediated inhibitory effect on NK cell cytotoxicity and according to the strategy postulated here, result of special interest the study of the effect of NK cell-secreted cytokines on the proliferation of tumor cells.

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8. Publications

Lagadari M., Lehmann K., Ziemer M., Truta-Feles K., Berod L., Idzko M., Barz D., Kamradt, T., Maghazachi A.A., Norgauer J., 2008. Sphingosine-1-phoshate inhibits the cytotoxic activity of NK cells via G_s protein-mediated signalling (submitted).

Lagadari M., Lehmann K., Truta-Feles K., Berod L., Ziemer M., Idzko M., Barz D., Kamradt T., Maghazachi A.A., Norgauer J.**,** 2008. Lysophosphatidic acid inhibits the cytotoxic activity of NK cells: involvement of G_s protein-mediated signalling (submitted).

Kaatz M., Berod L., **Lagadari M.**, Fluhr J.W., Elsner P., Norgauer J., 2006. Microtubules regulate expression of ICAM-1 in epidermoid cells (KB cells). Skin Pharmacol Physiol 19, 322-8.

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Kaatz M., Berod L., Czech W., Idzko M., **Lagadari M**., Bauer A., Norgauer J., 2004. Interleukin-5, interleukin-3 and granulocyte-macrophage colony-stimulating factor prime actin-polymerization in human eosinophils: a study with hypodense and normodense eosinophils from patients with atopic dermatitis. Int J Mol Med 14, 1055-60.

Lagadari M, Blois S, Margni R, Miranda S., 2004. Analysis of macrophage presence in murine placenta: influence of age and parity status. Am J Reprod Immunol 51, 49-55.

9. Curriculum Vitae

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12.2002.1.1	Master of Science Thesis at the Immunology Department, Faculty of Pharmacy and Biochemistry, University of Buenos Aires(Buenos Aires, Argentina). **Titel:** "Analysis of macrophage presence in murine placenta. Influence of age and parity status" **Supervisor:** Dr. Silvia Miranda.
1996 – 2002	Degree: Graduate in Biological Sciences with an orientation in Molecular Biology and Biotechnology. University of Buenos Aires Faculty of Natural and Exact Sciences.

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10. Selbständigkeitserklärung / Declaration

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel, Literatur und persönlichen Mitteilungen angefertigt habe.

Ich versichere, dass ich diese Arbeit noch an keiner anderen Hochschule eingereicht habe, um ein Promotionsverfahren eröffnen zu lassen. Die Hilfe eines Promotionsberaters wurde von mir nicht in Anspruch genommen. Dritte haben von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit der vorgelegten Dissertation stehen. Die Promotionsordnung der Biologisch-Pharmazeutischen Facultät is mir bekannt.

This Thesis has been written independently and with no other sources and aids than stated

Jena, Juni 2008

Mariana Lagadari