**Charles University in Prague**

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# NKR-P1C RECEPTOR – CARBOHYDRATE INTERACTION CONTRIBUTES TO ANTITUMOR IMMUNE RESPONSE VIA ACTIVATION OF NK AND B CELLS

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**Ph.D. dissertation thesis**

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Prague, May 2010 Katarína Hulíková

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# SUMMARY

Lectin – saccharide interactions and the involved receptors are currently intensively studied for their important role in antimicrobial as well as antitumor immunity. The major cell types participating in carbohydrate recognition are NK, NKT, and B cells. The differentiation of B lymphocytes could be induced by activated NK cells via direct intercellular contact and/or IFN-γ release.

Our research is focused on NK cell receptors of C-type lectin-like family recognizing carbohydrate epitopes of glycoproteins. Synthetic glycoconjugates with terminal N-acetyl-D-glucosamines on polyamidoamine (GN8P) or calix[4]arene (GN4C) scaffold used in this study, exerted the highest binding affinity to activating isoforms of rodent NKR-P1 (A and C) receptor. The aim of the presented dissertation thesis was to elucidate, how *Nkr-p1c* gene divergence, between C57BL/6 (NK1.1-positive, NKR-P1C$^{B6}$) and BALB/c (NK1.1-negative, NKR-P1C$^{BALB/c}$) mouse strains, could affect NK cell activation and subsequent triggering of B lymphocyte effector functions using GN8P and GN4C as NKR-P1C prototype ligands.

We demonstrated, that GN8P increased mRNA expression for NKR-P1C receptor, IFN-γ synthesis and lytic activity of NK cells, antigen-specific (anti-KLH, anti-DNP, and anti-B16F10) IgG (particularly IgG2a) formation, number of CD138+ plasma cells, and antigen presenting B lymphocytes in C57BL/6 mice. The GN8P-induced enhancement in IFN-γ synthesis correlated with elevated anti-B16F10 melanoma antibodies triggering ADCC reaction. The NK cell activation as well as up-regulation of IgG2a levels after GN8P administrations to BALB/c and DBA/2 mice (NKR-P1C$^{BALB/c}$) was not demonstrated. However, in F1 hybrids (BALB/c x C57BL/6, DBA x C57BL/6) expressing both *Nkr-p1c* gene alleles, the stimulatory effect of GN8P was restored. Similarly, GN8P-mediated increase in IgM formation *in vitro* was not detected, when BALB/c, DBA/2, or NK1.1-depleted C57BL/6 spleen mononuclear cells were used. The newly synthesized GN4C slowed the tumor growth and prolonged the survival time of B16F10 melanoma-bearing animals. Beside the stimulatory effect on NK and B cells, as in the case of GN8P, GN4C activated also the tumor specific cytotoxic T cells.

In conclusion, the glycoconjugate-mediated NK cell activation and subsequent (tumor) antigen-specific IgG2a formation can be triggered by NKR-P1C$^{B6}$ receptor engagement. The introduction of calix[4]arene scaffold further potentiates the *in vivo* antitumor efficacy of the glycoconjugate.

# THE LIST OF ABBREVIATIONS

**ADCC**: antibody-dependent cell-mediated cytotoxicity

**AID**: activation-induced cytidine deaminase

**APCs**: antigen-presenting cells

**Bcl-$X_L$**: B cell leukaemia/lymphoma $X_L$

**BCR**: B cell receptor

**BSA**: bovine serum albumin

**B2M**: β-2-microglobulin

**CA 125**: cancer antigen 125

**C-C**: chemokine with adjacent cysteine residue

**CCR7**: C-C chemokine receptor type 7

**CD**: cluster of differentiation

**CEA**: carcinoembryonic antigen

**CEACAM-1**: carcinoembryonic antigen-related cell adhesion molecule 1

**c-FLIP**: cellular FLICE-like inhibitory protein

**CTL**: cytotoxic T cell

**Cys**: cysteine

**DC**: dendritic cell

**DC-SIGN**: DC-specific ICAM-3 grabbing non-integrin

**DNP-LPS**: 2, 4-dinitrophenylated-lipopolysaccharide

**EDTA:** ethylenediaminetetraacetic acid

**FasL**: Fas receptor ligand

**Fc**: fragment crystallizable

**FITC**: fluorescein isothiocyanate

**FSC/SSC**: Forward scatter/Side scatter

**GalNAc**: *N*-acetyl-galactosamine

**GlcNAc**: *N*-acetyl-glucosamine

**GlcNAc-TV**: *N*-acetyl-glucosaminyl-transferase V

**GM-CSF**: granulocyte-macrophage colony-stimulating factor

**GvL**: graft versus leukaemia

**HIF**: hypoxia inducible factor

**HLA**: human leukocyte antigens

**HSP**: heat-shock protein

**IDO**: indoleamine 2, 3-dioxygenase

**IFN**: interferon

**IFN-γR**: interferon-γ receptor

**Ig**: immunoglobulin

**IKDC**: IFN-γ-producing killer dendritic cell

**IKK-β**: IκB kinase-β

**IκB**: inhibitor of NFκB

**IL**: interleukin

**iNOS**: inducible nitric oxide synthase

**i.p.**: intraperitoneally

**ITAM**: immunoreceptor tyrosine-based activation motif

**ITIM**: immunoreceptor tyrosine-based inhibitory motif

**KIR**: killer cell immunoglobulin-like receptor

**KLH**: keyhole limpet hemocyanin

**KLRG1**: killer cell lectin-like receptor G1

**Lck**: lymphocyte-specific protein tyrosine kinase

**LIR/ILT**: leukocyte immunoglobulin-like receptor/immunoglobulin-like transcript

**LLT-1**: lectin-like transcript 1

**LMP**: low molecular weight protein

**LPS**: lipopolysaccharide

**LRC**: leukocyte receptor complex

**ManNAc**: N-acetyl-manosamine

**mAb**: monoclonal antibody

**MAPK**: mitogen-activated protein kinase

**MCMV**: mouse cytomegalovirus

**MCP-1**: monocyte chemotactic protein 1

**MDC**: macrophage-derived chemoattractant

**MFI**: mean fluorescence intensity

**MGL**: macrophage-galactose type lectin

**MHC**: major histocompatibility complex

**MIC**: MHC class-I-chain-related protein

**MIP-1α**: macrophage inflammatory protein 1α

**MULT-1**: mouse UL16-binding protein-like transcript 1

**NCR**: natural cytotoxicity receptor

**NF-κB**: nuclear factor kappa-B

**NK**: natural killer

**NKC**: natural killer gene complex

**NKG2**: natural killer group 2

**NKR-P1**: natural killer cell receptor protein 1

**NKT cell**: natural killer T cell

**NP-Ficoll**: nitrophenol-Ficoll

**NSAIDs**: non-steroidal anti-inflammatory drugs

**Ocil/Clr**: osteoclast inhibitory lectin/C-type lectin related

**OD**: optical density

**PAMAM**: polyamidoamine

**PBCs**: peripheral blood cells

**PBMCs**: peripheral blood mononuclear cells

**PBS**: phosphate buffered saline

**PD-1**: programmed death 1

**PDL-1**: programmed death ligand 1

**PFC**: plaque forming cell

**PI**: propidium iodide

**PLGF**: placental growth factor

**Poly (I:C)**: polyriboinosinic polyribocytidylic acid

**Pro**: proline

**PSA**: prostate-specific antigen

**PWM**: pokeweed mitogen

**Rae1**: retinoic acid early transcript 1

**RT-PCR**: reverse transcription polymerase chain reaction

**s.c.**: subcutaneously

**SCID**: severe combined deficiency

**SD**: standard deviation

**SDS**: sodium dodecyl sulfate

**Ser**: serine

**SHP-1**: SH2-domain-containing protein tyrosine phosphatase 1

**Siglec**: sialic acid binding immunoglobulin-like lectin

**SMCs**: spleen mononuclear cells

**SOCS-1**: suppressor of cytokine signalling 1

**SRBCs**: sheep red blood cells

**Src**: sarcoma tyrosine kinase

**STAT1**: signal transducer and activator of transcription 1

**TAAs**: tumor-associated antigens

**TACAs**: tumor-associated carbohydrate antigens

**TalNAc**: *N*-acetyl-talosamine

**TAP**: transporter associated with antigen processing

**TARC**: thymus and activation-regulated chemokine

**TCR**: T cell receptor

**TD**: T cell-dependent

**TGF**: transforming growth factor

**Th**: T helper

**Thr**: threonine

**TI**: T cell-independent

**TLR**: Toll-like receptor

**TMB**: tetramethylbenzidine

**TNF**: tumor necrosis factor

**TNFR**: tumor necrosis factor receptor

**TRAIL**: tumor necrosis factor-related apoptosis-inducing ligand

**TRAILR**: tumor necrosis factor-related apoptosis-inducing ligand receptor

**Treg**: regulatory T cell

**TSAs**: tumor-specific antigens

**ULBP**: UL16-binding protein

**u-NK cells**: uterine NK cells

**VEGF**: vascular endothelial growth factor

# 1. INTRODUCTION

Natural killer (NK) cells constitute a heterogeneous lymphocyte subpopulation belonging to the innate immunity that is primarily involved in surveillance against virus-infected and malignant transformed cells. Apart from exerting the cytotoxic activity, they are able to influence various microenvironmental niches (e.g. tumor, autoimmune, reproductive, developmental, etc.) and functions of other cell types, including B cells, via cytokine secretion and/or intercellular contact.

NK cells express wide repertoire of carbohydrate-recognizing surface receptors, members of the C-type lectin-like family (e.g. NKR-P1, NKG2D, CD69, etc.). Rodent NKR-P1 receptors exist in several isoforms. Namely, activating rat NKR-P1A and mouse NKR-P1C present orthologous molecules. In addition, individual mouse strains possess distinct *Nkr-p1c* gene forms encoding NKR-P1C$^{B6}$ and NKR-P1C$^{BALB/c}$ proteins, differing from each other in amino acid sequence, which is responsible for binding of anti-NK1.1 monoclonal antibody (NK1.1-positive C57BL/6, while NK1.1-negative BALB/c mice). The physiological ligands for NKR-P1C receptor remain unknown.

The research in our laboratory is focused on characterization of the carbohydrate ligands for NK cell C-type lectin-like receptors in rat, mouse, and human systems. We showed that *N*-acetyl-D-glucosamine based oligosaccharides bound to the rat recombinant NKR-P1A molecule. Structural studies of NK cell receptors and their carbohydrate recognition domains brought results indicating that multiple copies of saccharide epitopes presented on a suitable scaffold (molecular, dendritic, polymeric) exerted higher binding affinity to the receptor, which contributed to the synthesis of more effective NKR-P1 ligands. In this dissertation thesis, the potential immunomodulatory properties of synthetic *N*-acetyl-D-glucosamine-substituted glycoconjugates with the polyamidoamine (GN8P) or calix[4]arene (GN4C) scaffold were investigated.

Malignant diseases still represent a considerable health and socio-economic problem, and therefore further analysis of anticancer immune mechanisms is needed and currently being investigated. We demonstrated previously that GN8P activated natural killing of tumor targets *in vitro*, reduced the incidence and growth of tumors, and prolonged the survival time of treated

animals. Our approach using synthetic compounds mimicking physiological NKR-P1 ligands could contribute to better understanding of NK cell receptor biology and/or NK cell functions in general as well as in cancer immunosurveillance.

# 2. AIMS OF THE STUDY

1) to analyze the effects of *N*-acetyl-D-glucosamine-substituted polyamidoamine dendrimer (GN8P), the potential ligand for mouse NKR-P1C receptor, on NK cell functions

2) to verify whether *Nkr-p1c* gene divergence between prototype C57BL/6 and BALB/c mouse strains might play a role in response to GN8P treatment

3) to investigate whether GN8P is able to modulate antibody formation in C57BL/6 mice via NK cell activation

4) to find out whether the newly synthesized *N*-acetyl-D-glucosamine-substituted calix[4]arene (GN4C) has the capacity to stimulate NK cells and exert anticancer properties using mouse B16F10 melanoma model

# 3. GENERALITIES AND BACKGROUND

## 3.1. NATURAL KILLER CELLS

NK cells were firstly described more than three decades ago as large granular lymphocytes that are capable of killing leukaemia cells *in vitro* without previous host exposure to the tumor (hence the term "natural killers"), *(Kiessling et al., 1975a; 1975b).* Although these cells are primarily known to serve as the first line of defence against malignant transformed and virus-infected cells, they also exert regulatory function via direct intercellular contact (e.g. with macrophages, dendritic, T, and B cells) and/or cytokine production (IFN-γ, TNF-α, β, IL-10, IL-13, IL-8, MIP-1α, GM-CSF, etc.). Thus, upon activation, NK cells modulate inflammatory responses, haematopoiesis, antigen presentation, and antibody formation *(reviewed in Yuan, 2004; Hayakawa et al., 2006; Vivier et al., 2008).*

It is generally accepted that NK cells originate from CD34+ precursors in the bone marrow, eventually in the liver. However, recent studies showed that there were other sites of NK cell development including the thymus in mice, and lymph nodes in humans *(reviewed in Freud and Caligiuri, 2006; Di Santo et al., 2006).* Mature NK cells recirculate not only in the blood but also in the lymph, and are widespread throughout lymphoid as well as non-lymphoid tissues, where they represent a minor fraction of lymphocyte population (2 – 5% in the spleen, 5 – 7% in the blood of mice; 5 – 15% in the human blood). By means of flow cytometry, NK cells are usually identified as CD3-/CD56+, CD3-/DX5+ (CD49b+) or CD3-/Nkp46+ cells in humans, mouse spleen and blood, respectively *(Yokoyama and Plougastel, 2003; Vivier et al., 2008; Di Santo, 2008).* Further, they can be divided on the basis of CD56 expression density into two distinct functional subsets, which also differ from each other in homing properties. The conventional $CD56^{dim}$ subpopulation represents about 90% of human blood and splenic NK cells that display high cytotoxicity and secrete IFN-γ after *in vitro* incubation with tumor cells. In contrast, $CD56^{bright}$ NK cells of the liver, lymph nodes, tonsils, and placenta are more potent producers of IFN-γ (in response to stimulation with IL-12, IL-15, or IL-18), whereas they are characterized by poor cytotoxic activity *(Cooper et al., 2001).* Although mice lack CD56 cell surface marker, recent observations suggest that their $CD27^{high}$ NK cells, which are enriched in lymph nodes and show enhanced cytokine secretion, could constitute a potential counterpart to

human $CD56^{bright}$ ones *(Hayakawa et al., 2006)*. In addition, a very recent study has demonstrated that the B cell co-receptor CD72 is also expressed on mouse NK cells, and that $CD72^{high}$ NK cell subpopulation, upon activation, produces significantly lower levels of IFN-γ than $CD72^{low}$ one, whereas both of them are equally cytotoxic *(Alcon et al., 2009)*. These findings might provide an important tool to understand the role of individual NK cell subsets e.g. in antitumor response and within inflammatory microenvironment that accompanies cancer development.

Unlike B and T cells, which are able to generate almost infinite repertoire of antigen-specific receptors (BCRs and TCRs, respectively) in one host as a consequence of somatic gene rearrangement, NK cells are limited to the receptors encoded by genes pre-existing in the genome. A single B or T cell, however, expresses only one type of antigen specific receptors, whereas each NK cell disposes with a range of inhibitory as well as activating receptors binding mainly to MHC class I molecules or carbohydrate structures. According to the "missing-self" hypothesis, down-regulation or absence in MHC class I molecule expression on the target cell results in the "loss" of the signal from inhibitory receptors and permits NK cell activation (in addition, "stress molecules" and products of aberrant glycosylation can be recognized by activating receptors). Thus, the NK cell function is regulated by a balance of positive and negative signals triggered after the receptor ligation, which enables killing of tumor or virus-infected cells while maintaining self tolerance *(Karre et al., 1986; Ljunggren and Karre, 1990; reviewed in Lanier, 2005; Gasser and Raulet, 2006; Cheent and Khakoo, 2009)*.

It is worth noting that not undergoing of receptor gene rearrangement along with rapid response to the infection are the main arguments, why NK cells are considered to form components of the innate (natural) immunity. However, in the light of the latest findings showing that NK cells possess nearly all the features of the adaptive (specific) immunity such as clonal expansion, long-living progeny, receptor specifity for one ligand (e.g. Ly49H NK cell receptor for m157 glycoprotein of the mouse cytomegalovirus), including memory-like functions, some authors propose the placement of NK cells as an "evolutionary bridge" between innate and adaptive immunity, and warn against tendency to partition the immune cells in a strictly binary fashion *(Sun and Lanier, 2009; Cooper et al, 2009)*.

NK cells, like cytotoxic T cells (CTLs), contain cytoplasmic granules (specialized lysosomes) with perforins and granzymes. These cytotoxic proteins are released after the formation of the

immunological synapse with the target cell, which is subsequently lysed (necrosis) or killed by apoptosis. Other possibility how to trigger apoptotic pathways in the target cell is based on the "death" receptor – ligand interactions (e.g. Fas receptor (CD95) – FasL, TRAILR – TRAIL, TNFR – TNF), when the ligand expressed by NK cell binds to its receptor on the target cell. In addition, most mouse as well as human NK cells possess low-affinity Fc receptor recognizing Fc fragment of IgG (FcγRIIIA, CD16), which allows them to participate in the elimination of specific antibody-coated target cells (antibody-dependent cell-mediated cytotoxicity (ADCC)), *(reviewed in Colluci et al., 2003; Vivier et al., 2008).*

The intensity and the quality of NK cell cytotoxic as well as cytokine response are influenced by microenvironmental context (nature of initiating event, presence of other cells, reciprocal co-operations with them, cytokine milieu), *(reviewed in Di Santo, 2008).* The NK cell function is enhanced by a number of soluble factors such as IFN-α, β, IL-2, IL-12, IL-15, and IL-18 *(Walzer et al, 2005; reviewed in Vivier et al., 2008).* On the other hand, it is inhibited e.g. by regulatory T cells (CD4+/CD25+) or malignant transformed cells in TGF-β and IL-10 dependent manner *(Ghiringhelli et al., 2005a; Gajewski et al., 2006).* There are also several reports indicating that NK cells cultured in the presence of IL-12 or IL-4 develop into NK1 or NK2 subtype, respectively, differentiating in the cytokine profile (IFN-γ vs. IL-4, IL-5, and IL-13) similarly as Th1 and Th2 cells *(Peritt et al., 1998; Katsumuto et al., 2004).* Recently, NK-22 cells that are programmed to secrete IL-22, upon selective activation by IL-23, have been discovered in the mucosa-associated lymphoid tissue both in mice and humans. The cytokine IL-22 up-regulates proliferation of epithelial cell as well as their production of anti-apoptotic molecules, bactericidal proteins, and anti-inflammatory IL-10. Therefore, NK-22 cells are probably important for maintenance of mucosal homeostasis and protection of the barrier between the host and the pathogen during infection and/or inflammation *(Aujla and Kolls, 2009; Cella et al., 2009).* In contrast to currently often discussed Th17 cells that were reported to be involved in pathogenesis of several autoimmune diseases such as psoriasis or multiple sclerosis through IL-17 and IL-22 secretion *(Iwakura et al., 2008; Zenewicz and Flavell, 2008),* NK-22 cells do not produce IL-17. So that, dual role of IL-22 could be explained by its different cellular source and dependence on the local cytokine milieu *(Cella et al., 2009).* Finally, a functionally specialized subset of NK cells occurs in the uterus (u-NK cells). These cells accumulate at the site of embryo implantation during pregnancy, where they secrete pro-angiogenic factors such as VEGF or PLGF. In this way, they participate in vascular

remodelling, which is necessary for endometrial decidualization and subsequent placenta development *(Hanna et al., 2006; Riley and Yokoyama, 2008; Murphy et al., 2009).*

All these data confirm that NK cells constitute a heterogeneous lymphocyte population, which is on one hand important for shaping innate as well as adaptive immunity, and on the other one strongly influenced by distinct environmental niches (e.g. tumor, inflammatory, etc.). Understanding of NK cell biology, including functional diversity between their individual subsets is of great interest, so that we could reveal new strategies how to use NK cell manipulation in transplantation or for therapy of malignant, infectious, and autoimmune diseases, infertility, etc. *(reviewed in Vivier et al., 2008; Terme et al., 2008), (Figure 1).*

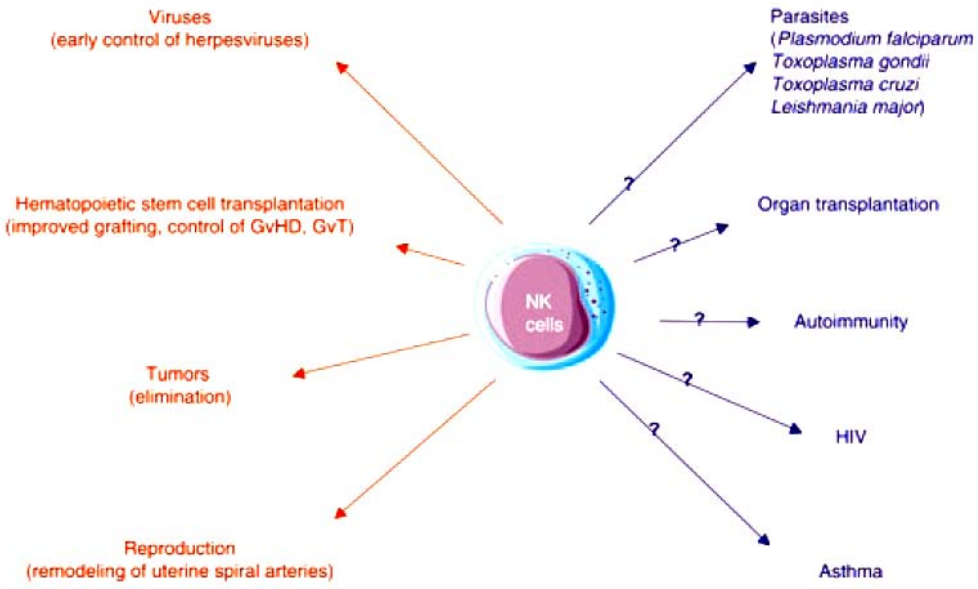


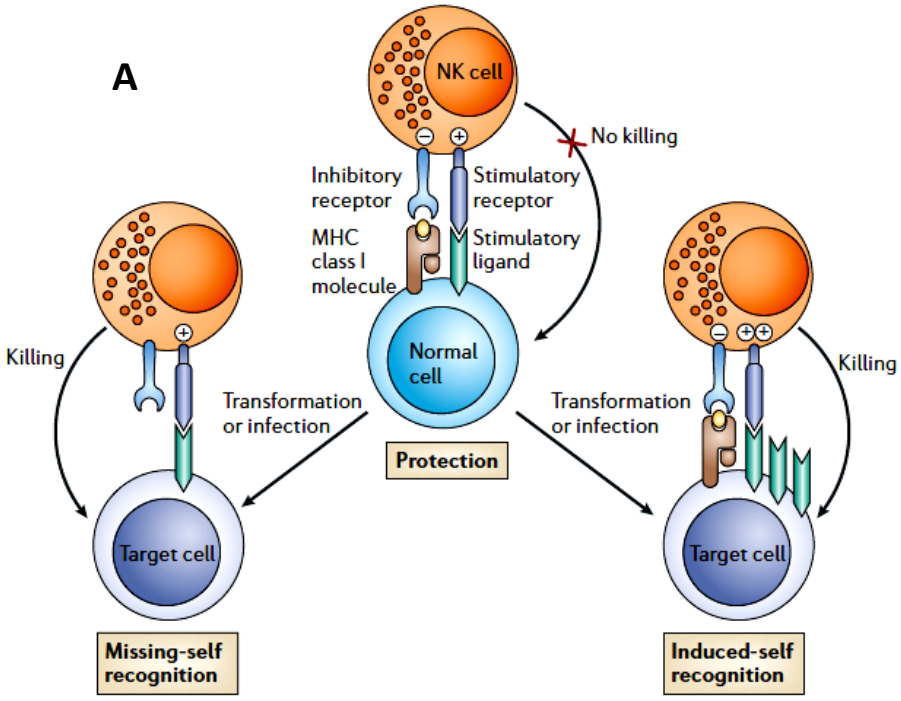

**Figure 1: NK cell functions.** The known and suspected biological roles of NK cells are indicated in red and blue, respectively (GvHD = graft versus host disease; GvT = graft versus tumor; adapted from *Vivier et al., 2008*).

### 3.1.1. NK CELL RECOGNITION AND SELF-TOLERANCE

It is well known that the outcome of NK – target cell interaction ("killing or not killing") is governed by integration of signals from numerous receptors, some activating, others inhibitory. But the question how these receptors exactly interrelate is not completely answered yet, and needs further studies. Initially, NK cells were considered to be independent of MHC molecules during their "self" versus "nonself" recognition process. Later on, as implied above, *Karre et al. (1986)* found out that rather than ignore MHC class I molecules, NK cells appear to be actively inhibited, when they encounter cells expressing them. On the other hand, they attack cells with absent or down-regulated MHC class I expression (e.g. tumor or virus-infected cells "escaping" from killing by CTLs) as these cells cannot effectively engage inhibitory NK cell receptors specific for MHC class I molecules, which results in activation signal overriding ("missing-self" recognition), *(reviewed in Lanier, 2005; Gasser and Raulet, 2006; Raulet and Vance, 2006).* There is, however, experimental evidence documenting that MHC class I inhibitory receptors may serve only to dampen, rather than completely terminate NK cell function. In case, when multiple activating NK cell receptors are involved simultaneously, or when a sufficiently potent one is stimulated, NK cell is capable of eliminating the target cell even if its inhibitory receptors for MHC class I molecules are ligated ("induced-self recognition"), *(Lanier et al., 1997; Diefenbach et al., 2001; Cerwenka et al., 2001; Raulet and Vance, 2006), (Figure 2A).*

The self-tolerance of NK cells is intensively discussed issue that is closely connected to (or overlapping with) their recognition. Originally, it was believed that NK cell self-tolerance was established if each NK cell was endowed with at least one inhibitory receptor specific for self-MHC class I molecules ("at least one model"), and if occupancy of that receptor was adequate to overcome activating signals *(Raulet et al., 1997).* However, the inhibitory receptors are inherited independently of their highly genetically polymorphic MHC class I ligands. In addition, they are expressed on NK cells in a largely random fashion, which means that individual subsets of NK cells typically bear 3 – 5 such receptors out of 10 – 20 encoded in the germ line *(Kubota at al., 1999; Tanamachi et al., 2001).* From this, it emerges that NK cells not expressing inhibitory receptors specific for self-MHC class I molecules could exist, which was already confirmed in C57BL/6 mice (10 – 15% such NK cells of total). These NK cells were proved to be self-tolerant as they failed to lyse autologous cells *in vitro*. Moreover, they exerted impaired cytotoxicity against several tumor cell lines *(Fernandez et al., 2005).* Similar results

were observed using NK cells from mice with MHC class I deficiency (mutation in the gene for β2-microglobulin or TAP1). To explain this, *Raulet et al. (2006)* proposed the "disarming model" saying that NK cells lacking self-MHC class I specific inhibitory receptors or those interacting with MHC class I-deficient self cells could be induced to enter a state of hyporesponsiveness due to chronic stimulatory signals *(Figure 2B)*. On the other hand, *Lanier (2005)* suggested two following possibilities why NK cells tolerate the red blood cells, although they do not express MHC class I molecules: (i) absence of erythrocyte stimulatory ligands that could trigger threshold activation signals to initiate NK cell response; (ii) involvement of inhibitory receptors recognizing non-MHC ligands such as sialic acid (Siglec NK cell receptors).



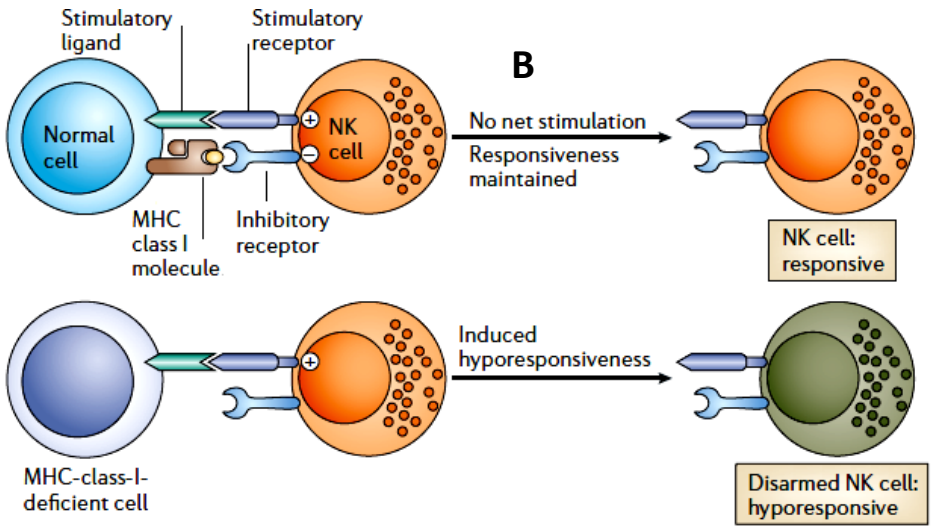



**Figure 2: The classical model of NK cell recognition (A) and "disarming model" explaining NK cell self-tolerance (B),** (adapted from *Raulet and Vance, 2006*).

### 3.1.2. NK CELL RECEPTORS

During the past few years, intensive research on NK cells contributed to the elongation of the list of known NK cell receptors as well as their ligands. In addition, new ones are still continuously described *(Figure 3)*.

NK cell receptors can be divided into two functionally distinct groups, inhibitory and activating ones. Inhibitory receptors recognizing MHC class I molecules are represented by killer cell immunoglobulin-like receptor (KIR) and leukocyte immunoglobulin-like receptor/ immunoglobulin-like transcript (LIR/ILT) family in humans, Ly49 family in rodents, and CD94/NKG2A receptor in both of these species. However, some Ly49 receptors and KIRs (e.g. members of KIR3DS and KIR2DS subfamily) are activating. Furthermore, NK cell response can be inhibited through receptors binding to non-MHC class I ligands such as Siglec-7 (sialic acid), KLRG1 (cadherins), etc., *(reviewed in Yokoyama, 2005; Bryceson et al., 2006; Cheent and Khakoo, 2009)*. The last one is important in relation to cancer as it participates in NK cell-mediated surveillance of invasive and metastatic epithelial tumors that are characterized by the loss of E-cadherin expression *(Ito et al., 2006)*.

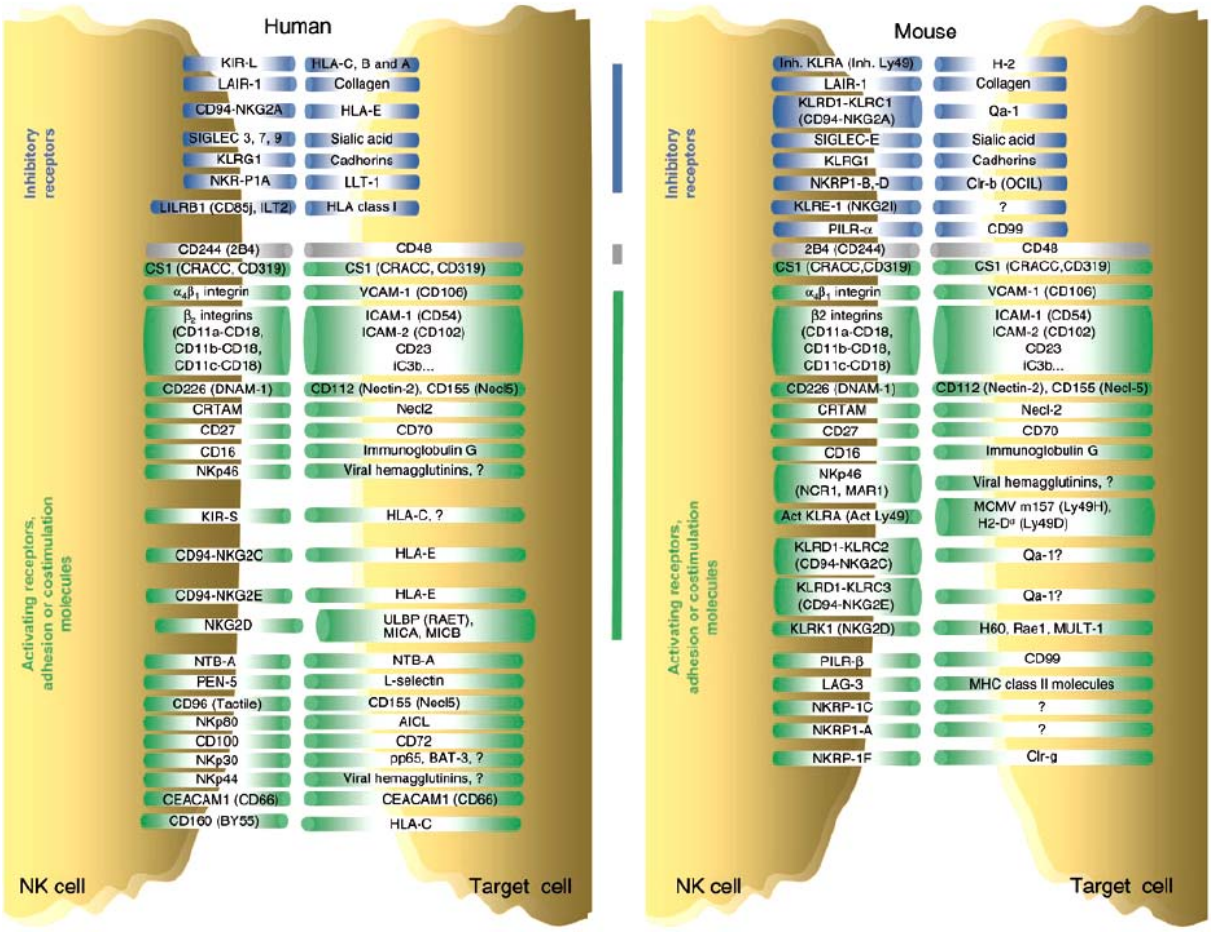



**Figure 3: The NK – target cell "zipper" in humans and mice** (inhibitory receptors are in blue; 2B4, which can act as an activating or an inhibitory molecule, is in gray; other receptors are in green; vertical lines indicate the receptor – ligand pairs conserved between mice and humans; KIR = killer cell immunoglobulin-like receptor; LIR = leucocyte immunoglobulin-like receptor; LAIR = leukocyte-associated immunoglobulin-like receptor; SIGLEC = sialic acid binding immunoglobulin-like lectin; KLRG-1 = killer cell lectin-like receptor G1; NKR-P1 = NK cell receptor protein 1; HLA = human leukocyte antigen; LLT = lectin-like transcript; CRTAM = class I restricted T cell-associated molecule; Necl-2 = nectin-like 2; Tactile (also known as CD96) = T cell-activated increased late expression; CEACAM1 = carcinoembryonic antigen-related cell adhesion molecule 1; PILR = paired immunoglobulin-like type 2 receptor; NTB-A = NK-T-B antigen; CRACC = CD2-like receptor-activating cytotoxic cell; VCAM-1 = vascular cell adhesion molecule-1; ICAM = intercellular adhesion molecule; Ocil = osteoclast inhibitory lectin; adapted from *Vivier et al., 2008*).

The inhibitory receptors contain cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which are phosphorylated, after receptor – ligand interaction, by Src family kinases. These event recruits protein phosphatases (e.g. SHP-1) that dephosphorylate molecules involved in the activation cascade. In this manner, the inhibitory receptors mediate blocking of signalling pathways triggered upon interactions between NK cell activating receptors and their ligands *(Vivier et al., 2004; Long, 2008; Cheent and Khakoo, 2009), (Figure 4).*

Activating receptors, which are more abundant on the NK cell surface than inhibitory ones, include NKG2D (stress-induced ligands), CD94/NKG2C, CD94/NKG2E (HLA-E in humans, Qa-$1^b$ in mice), CD16 (IgG), natural cytotoxicity receptors (NCRs) such as Nkp46 (viral haemagglutinins), etc. Activating receptors usually bind to MHC class I ligands with lower affinity comparing to their inhibitory counterparts *(reviewed in Bryceson et al., 2006; Cheent and Khakoo, 2009)*. The majority of activating NK cell receptors are associated with adaptor proteins (e.g. DAP12, CD3ζ, FcRγ) containing immunoreceptor tyrosine-based activation motifs (ITAMs). The receptor ligation leads to ITAM phosphorylation, which in turn results in recruitment of protein tyrosine kinases of the Syk family (Syk, ZAP-70), and subsequent triggering of downstream signalling pathways *(Underhill and Goodridge, 2007)*. Instead of DAP12, NKG2D receptor can associate with DAP10 adaptor protein lacking ITAMs. Then, different kinases are involved (probably phosphatidylinositol 3-kinase) and NKG2D acts as a co-stimulatory receptor *(Upshaw et al., 2006), (Figure 4).*

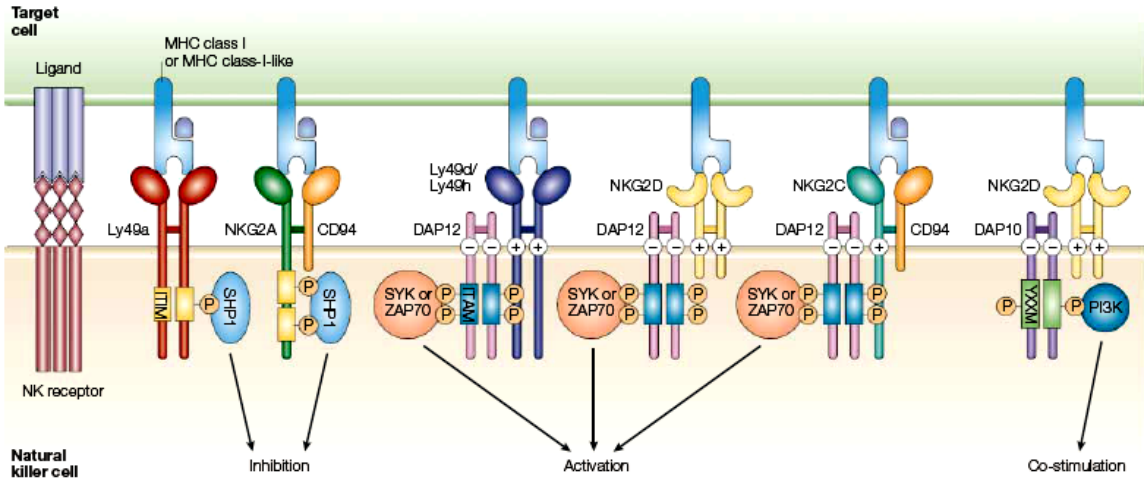



**Figure 4: General paradigm of NK cell receptor signalling** (PI3K = phosphatidylinositol 3-kinase; ZAP70 = ζ-chain associated protein 70kDa; adapted from *Yokoyama and Plougastel, 2003*).

NK cell receptor genes are organized in two main clusters, the leukocyte receptor complex (LRC) and the natural killer gene complex (NKC), located on different chromosomes. Both loci are highly polymorphic. In order of the structure, NK cell receptors form the immunoglobulin-like (e.g. KIRs, NCRs) and the lectin-like (e.g. NKR-P1, Ly49, NKG2D, CD69) family, both of them including activating as well as inhibitory members. Immunoglobulin-like receptors are encoded by LRC, whereas lectin-like ones by NKC *(Yokoyama et al., 1991; Yokoyama et al., 1993; Wende et al., 2000).*

Most genes of NKC are conserved across species, with orthologs identified in mouse and human genome *(Hao et al., 2006), (Figure 5).* There is a following important exception: in humans, only one Ly49 receptor (pseudo)gene is present within NKC, namely *Ly49L* probably not giving a functional product *(Westgaard et al., 1998)*. However, in humans, Ly49 receptors are substituted by KIRs. Despite obviously disparate structure, KIR and Ly49 receptor families share key similarities (e.g. MHC class I ligands, signalling pathways, extensive polymorphism), *(reviewed in Yokoyama, 2008).*

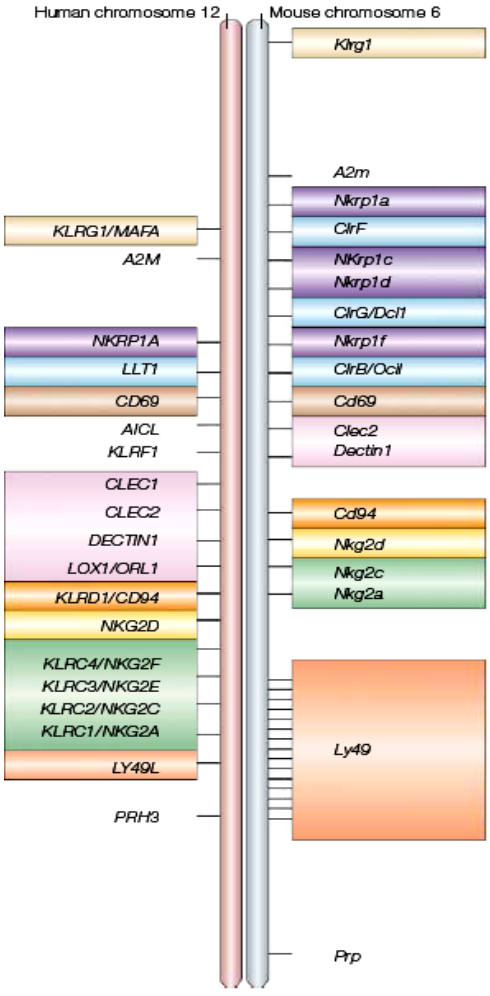



**Figure 5: Lectin-like receptors encoded by the natural killer gene complex.** The genes for lectin-like receptors are located on chromosome 12p13.1 and 6 in humans and mice, respectively. (Coloured boxes indicate gene families that are present both in humans and mice; AICL = activation-induced C-type lectin; A2M = α2-macroglobulin; CLEC = C-type lectin-like receptor; Clr = C-lectin related; DECTIN = dendritic cell-associated C-type lectin; KLR = killer cell lectin-like receptor; LLT = lectin-like transcript; LOX = oxidized low-density lipoprotein; MAFA = mast cell function-associated receptor; NKG2D = natural killer group 2D; NKRP = natural killer cell receptor protein; Ocil = osteoclast inhibitory lectin; PRH = proline rich HaeIII; Prp = proline-rich protein; adapted from *Yokoyama and Plougastel, 2003*).

### 3.1.2.1. C-type lectin-like receptors

The lectins, in general, can be defined as (glyco)proteins of non-enzymatic and non-immunoglobulin nature capable of non-covalent, reversible binding to oligosaccharides or carbohydrate moieties of complex glycoconjugates. The term lectin-like is used for animal lectins to distinguish from earlier identified plant ones (e.g. phytohaemagglutinin). Lectin-like receptors are expressed by various cell types, where they are involved either in cell adhesion (e.g. platelet, leukocyte, and endothelial selectins) or immune recognition (e.g. mannose-binding receptor on dendritic cells), *(reviewed in Sharon and Lis, 2004; Sharon, 2007).*

C-type lectin-like receptors are characterized by recognizing ligands in a $Ca^{2+}$-dependent manner. They are divided into type I and type II depending on the way of anchoring into the cell membrane. The amino (N) terminus of type I receptors is orientated outwards the cell cytoplasm, whereas that one of type II inwards. NK cell C-type lectin-like receptors are type II transmembrane proteins, which consist of extracellular carbohydrate-recognition (C-type lectin-like) domain and prototype lectin fold containing signalling motifs or associating with adaptor proteins *(Weis et al., 1998).*

#### *3.1.2.1.1. Ly49 receptor family*

Ly49 receptors are glycoproteins expressed as disulphide-bonded homodimers on NK cells and some subsets of T cells. In mice, Ly49 receptor family includes at least 23 members from Ly49A to Ly49W *(summarized in Table 1).* The number of Ly49 genes varies in different mouse strains, and there is also evidence for extensive allelic polymorphism. In general, Ly49 receptors consist of extracellular C-type lectin-like domain, transmembrane, and intracellular part, either containing ITIMs (inhibitory receptors) or associating with adaptor protein DAP12 (activating), *(Smith et al., 1998; Wilhelm et al., 2001; Kane et al., 2004; Dimassi and Biassoni, 2005).*

In rodents, Ly49 molecules represent predominant NK cell receptors recognizing MHC class I molecules (H-2 in mice). Despite the fact that Ly49 molecules are referred to as C-type lectin-like receptors, the role of carbohydrate recognition in the receptor – ligand interactions appears to be only minor *(Karlhofer et al., 1992; Tormo et al., 1999; Wang at al., 2002).* As determined by RT-PCR analysis, each NK cell within the population transcribes on average from one to

four *ly49* genes *(Kubota et al., 1999)*. Both the percentage of cells expressing particular Ly49 receptor and the amount of Ly49 molecules on the cell surface are influenced by the level of H-2 expression in the host *(reviewed in Raulet et al., 2001; Veinotte et al., 2003)*.

While inhibitory Ly49 receptors were reported to participate in protection against autoimmunity, the role of activating ones recognizing H-2 molecules is not completely elucidated yet. In H-$2^{b/d}$ heterozygous hosts, the subsets of Ly49D+ NK cells may attack targets that have lost H-$2^{b}$ but retained H-$2^{d}$ molecules due to malignant transformation or viral infection. But it is also possible that the physiological high affinity ligands for activating Ly49 receptors may be not self proteins, and that the interaction with H-2 molecules may represent weak, biologically unimportant cross-reactivity. This notion is supported by the fact, that Ly49H receptor exerts high binding affinity for m157 glycoprotein encoded by mouse cytomegalovirus (MCMV), and thus provides resistance of C57BL/6 mice to such viral infection. On the other hand, in certain MCMV-susceptible mouse strains, m157 protein binds to inhibitory Ly49 receptor (such as Ly49I in 129/J mice) as a consequence of its structural homology to MHC class I molecules *(Daniels et al., 2001; reviewed in Lanier, 2005)*.

As discussed above, mice and humans evolved different receptors (Ly49 receptors vs. KIRs) to serve the same function of preventing NK cell activation upon encounter with self MHC class I molecules. Thus, research on Ly49 receptors might help to find new possibilities how to manipulate human NK cells and their KIRs for therapeutic benefit. In addition, it brought new insights in understanding of NK cell biology (antiviral defence, inhibitory receptor specifities, allelic polymorphism of receptors, etc.), *(Yokoyama, 2005; 2008)*.

| Receptor name | Function | Cellular ligand(s) | Viral ligand |
|---|---|---|---|
| Ly49A | Inhibitory | $D^b$, $D^d$, $D^p$, $D^k$ | |
| Ly49B | Inhibitory | ? | |
| Ly49C | Inhibitory | $K^b$, $K^d$, $K^k$,$D^b$, $D^d$ | |
| Ly49D | Activating | $D^d$ | |
| Ly49E | Inhibitory | ? | |
| Ly49F | Inhibitory | $D^d$ | |
| Ly49G | Inhibitory | $D^d$, $L^d$ | |
| Ly49H | Activating | $D^b$ | MCMV-m157 |
| Ly49I | Inhibitory | $K^d$ | MCMV-m157 |
| Ly49J | Inhibitory | $K^b$ | |
| Ly49K | Activating | ? | |
| Ly49L | Activating | $K^k$ | |
| Ly49M | Activating | ? | |
| Ly49N | Activating | ? | |
| Ly49O | Inhibitory | $D^b$, $D^d$, $D^k$, $L^d$ | |
| Ly49P | Activating | $D^d$ | |
| Ly49Q | Inhibitory | ? | |
| Ly49R | Activating | $D^b$, $D^k$, $L^d$ | |
| Ly49S | Inhibitory | ? | |
| Ly49T | Inhibitory | ? | |
| Ly49U | Activating | ? | |
| Ly49V | Inhibitory | $D^b$, $D^d$, $K^k$ | |
| Ly49W | Activating | $D^d$, $K^k$ | |

**Table 1: Mouse Ly49 receptors and their ligands** (adapted from *Dimassi and Biassoni, 2005*).

### *3.1.2.1.2. NKR-P1 receptor family*

NKR-P1 receptors are glycoproteins expressed predominantly on NK cells as disulphide-linked homodimers. In mice, *Nkr-p1a-g* genes (*Nkr-p1e* and *Nkr-p1g* are probably pseudogenes with no functional transcript) were identified *(Carlyle et al., 2006)*, whereas only one non-polymorphic gene encoding NKR-P1A receptor (CD161) exists in human genome *(Lanier et al., 1994)*. According to the new nomenclature, NKR-P1A, B, F, and G family members are present in rats, where NKR-P1A is an orthologous receptor to mouse NKR-P1C *(Kveberg et al., 2009)*.

NKR-P1C of C57BL/6 (B6), CE, NZB, C58, Ma/My, and ST as well as NKR-P1B of NIH-Swiss (SW), SJL, FVB, and CD-1 mouse strains can be stained using anti-NK1.1 antibody (PK136), (therefore, NKR-P1 is also known in mice as NK1.1). In contrast, other mouse strains including BALB/c, DBA, C3H, AKR, CBA, and 129 are NK1.1-negative. Initially, it was believed that the lack of NK1.1 reactivity was caused by the strain-specific deficiency in the expression of one or more *Nkr-p1* gene family members *(reviewed in Mesci et al., 2006).* Later on, experiments at the genetic level showed that the prototype NK1.1-negative BALB/c strain possessed a full panel of *Nkr-p1* genes including functional *Nkr-p1b* and *Nkr-p1c.* There was only one exception comparing B6 and BALB/c mice: the first ones possessed *Nkr-p1d*, but not *Nkr-p1b* and vice versa. Nevertheless, *Nkr-p1d* appears to represent a divergent allele of *Nkr-p1b* gene with 94.9% homology *(Figure 6). Carlyle et al. (2008)* have suggested *Nkr-p1d* gene to indicate as *Nkr-p1b*$^{B6}$. However, several sequence differences between NKR-P1B$^{BALB/c}$ and NKR-P1B$^{SW}$ as well as between NKR-P1C$^{BALB/c}$ and NKR-P1C$^{B6}$ were observed, and surprisingly, a single amino acid substitution within extracellular domain determined whether anti-NK1.1 mAb was able to bind to these NKR-P1 isoforms *(Carlyle et al., 2006).*

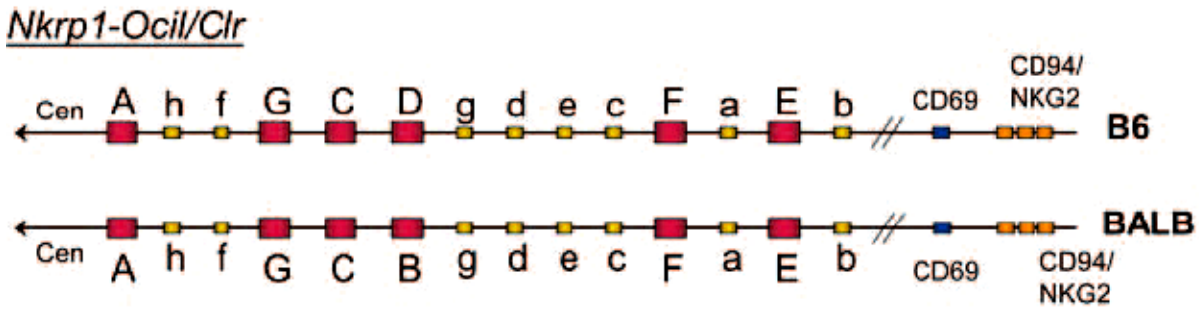



**Figure 6: The *Nkr-p1* – *Ocil/Clr* gene cluster in B6 and BALB/c mouse strains** (*Nkr-p1* genes are drawn as large boxes with capital letters, while *Ocil/Clr* as small boxes with small letters; see text; adapted from *Mesci et al., 2006*).

Recently, *Aust et al. (2009)* have generated a series of novel antibodies specific for individual NKR-P1 receptors (namely for NKR-P1A, D, and F) and showed that NKR-P1F in C57BL/6 mice might be a more suitable NK cell lineage-restricted marker than NKR-P1C (detected by means of anti-NK1.1 antibody), which is also present on the surface of some subsets of NKT,

T, B cells, and monocytes. Moreover, NKR-P1A and F were expressed at low levels by all splenic and bone marrow NK cells, whereas only about 50% of them were identified as clearly NKR-P1D-positive. However, further studies are needed to confirm these results.

In mice, NKR-P1C is activating, NKR-P1A and F seem to be also, while NKR-P1B and D are inhibitory receptors. Ligation of both mouse NKR-P1C and rat NKR-P1A with relevant antibodies (PK136 and 3.2.3, respectively) induced NK cell-mediated cytotoxicity and IFN-γ secretion *(Karlhofer and Yokoyama, 1991; Arase et al., 1996; reviewed in Lanier et al., 2005; Mesci et al., 2006).*

As for NKR-P1-mediated signalling in general, Lck kinase is important for its initiation. Upon receptor cross-linking, Lck associates with Cys-x-Cys-Pro (where x represents any amino acid) motif within the cytoplasmic tail of activating NKR-P1C and phosphorylates ITAMs of adaptor protein FcRγ, which leads to recruitment of Syk kinase and subsequent activation of downstream second messengers. Phosphorylation of ITIMs (in inhibitory receptors), which provides a binding site for SH2 domain of SHP-1, is also performed by Lck, and results in dephosphorylation with inhibition of molecules involved in activation signalling pathways *(Ljutic et al., 2005), (Figure 7).*

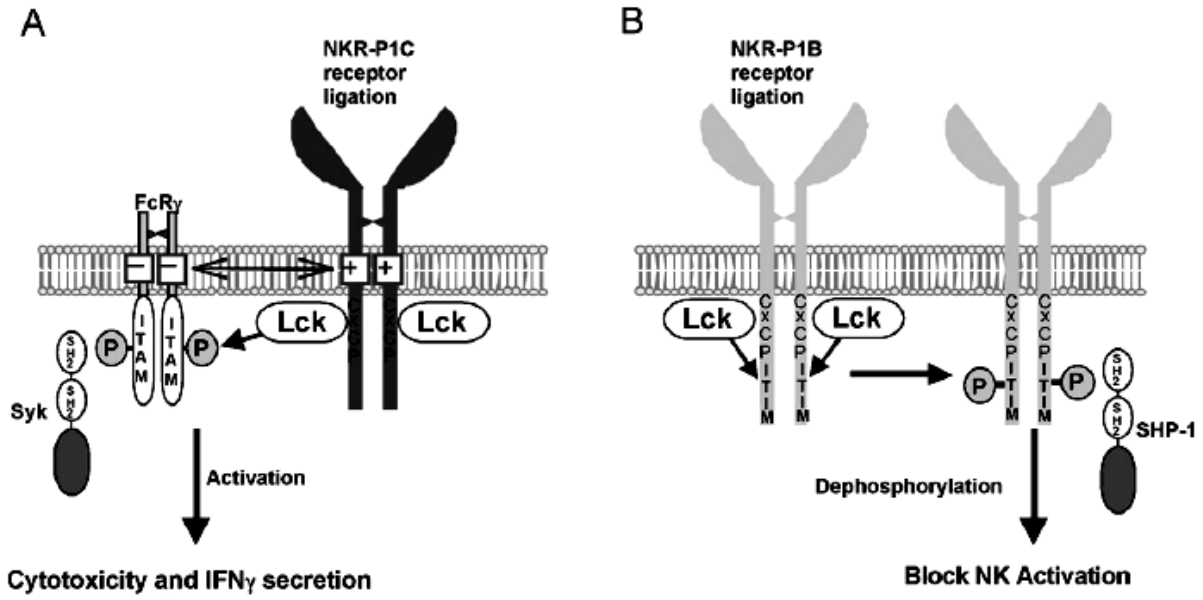



**Figure 7: Signalling pathways triggered upon ligation of the prototype activating (A) and inhibitory (B) NKR-P1 receptor** (adapted from *Ljutic et al., 2005*).

While mouse NKR-P1A and C receptors are orphan (their physiological ligands are unknown), ligands for inhibitory NKR-P1B, D, and activating F were identified as products of the *Ocil/Clr* gene family, which is interspersed among the *Nkr-p1* genes themselves within the natural killer gene complex *(Figure 6)*. Ocil/Clr molecules are type II membrane-anchored glycoproteins structurally related to C-type lectins. It was demonstrated that NKR-P1B and D recognized Crl-b molecules (NKR-P1B with a higher binding affinity), and that transfected cells expressing high Crl-b levels were protected from NK cell-mediated lysis. Furthermore, Clr-b expression on hematopoietic as well as tumor cells obviously patterns that of MHC class I molecules (i.e. low or no levels on erythrocytes, and down-regulated on tumor cells). Finally, an *Ocil/Clr-like* gene was discovered in the rat cytomegalovirus genome, which indicates that viruses evolved strategies to subvert NK cell-mediated immune surveillance. All these findings lead to the suggestion that NKR-P1B/D – Crl-b interactions represent an alternative form of missing-self recognition independent of MHC class I expression *(Plougastel et al., 2001; Voigt et al., 2001; Iizuka et al., 2003; Carlyle et al., 2004)*. NKR-P1F recognizes Crl-g *(Iizuka et al., 2003)* and Crl-x (Clec-2g, OCILrP1, DDV-10) molecules (*Aust et al., 2009)*.

Human NKR-P1A was reported to bind to lectin-like transcript 1 (LLT-1), an ortholog of murine Crls, resulting in inhibition of NK cell activity *(Lanier et al., 1994; Aldemir et al., 2005; Rosen et al., 2005)*. Its inhibitory potential, however, is not single valued as (i) NKR-P1A contains non-canonical ITIM-like motif in its structure; (ii) NKR-P1A cross-linking on certain subsets of T and NKT cells either induced or augmented their proliferation, respectively *(Poggi et al., 1996; Exley et al., 1998; Rosen et al., 2008)*. Furthermore, *Christiansen et al. (2006)* demonstrated that carbohydrate moieties such as *N*-acetyl-lactosamine were also potential ligands for this receptor with probable activating function.

Our previous results showed that the recombinant rat NKR-P1A molecule (NKR-P1C, NK1.1 in mice) bound to *N*-acetyl-manosamine (ManNAc), *N*-acetyl-galactosamine (GalNAc), *N*-acetyl-glucososamine (GlcNAc), and *N*-acetyl-talosamine (TalNAc). Subsequently, it was revealed that glycoconjugates containing multiple copies of saccharide epitopes clustered on a suitable scaffold (e.g. glycodendrimers) exerted higher binding affinity to NKR-P1 and triggered NK cell effector functions *(Bezouska et al., 1994a; 1994b; Krist et al., 2001; Vannucci et al., 2003)*. Testing of these synthetic ligands as mimics of physiological ones could contribute to better understanding of NKR-P1 biology.

### *3.1.2.1.3. NKG2 receptor family*

NKG2 receptors form heterodimers with CD94 molecule (except for NKG2D) expressed predominantly by NK cells and certain subsets of T cells. Both in mice and humans, CD94/NKG2A represents an inhibitory receptor, whereas CD94/NKG2C and CD94/NKG2E are activating ones (NKG2B and NKG2H are generated by the alternative splicing of genes *Nkg2a* and *Nkg2e*, respectively), *(Lazetic et al., 1996; Plougastel et al., 1996; reviewed in Lanier, 2005)*. CD94/NKG2F that, was found only in humans, shows unusual features: it does not contain any C-type lectin-like domain, but possesses cytoplasmic ITIM-like motifs *(Plougastel and Trowsdale, 1997)*.

CD94/NKG2A, C, and E recognize human HLA-E molecules or their mouse homologs Qa-$1^b$ which display mainly peptides derived from the classical MHC class I signal sequence. Thus, by means of these receptors, NK cells can indirectly monitor MHC class I (HLA-A, B, C, and G) expression *(Braud et al., 1998; Vance et al., 1999)*. Activating CD94/NKG2C receptor exerts, however, a six-fold lower binding affinity for its ligands than inhibitory CD94/NKG2A, which correlates with findings on activating KIRs *(Vales-Gomez et al., 1999)*. In addition, peptides bound to HLA-E or Qa-$1^b$ molecules can differentially affect recognition by CD94/NKG2 receptors. Namely, peptides derived from heat shock protein 60 were reported to bind to HLA-E. These HLA-E molecules, however, failed to be recognized by inhibitory CD94/NKG2A receptors, thereby permitting NK cell-mediated elimination of "stressed" cells *(Michaelsson et al., 2002)*.

**NKG2D** is a glycoprotein present as a disulphide-linked homodimer on the surface of NK cells, some subsets of macrophages, NKT, and T cells (e.g. CD8+, γδ). There is only limited sequence homology between NKG2D and other NKG2 receptor family members (28% identity in amino acids of the lectin-like domain), whereas NKG2A, C, and E are closely related to each other (70% identity). Nevertheless, genes for NKG2 receptors are located next to each other within natural killer gene complex *(reviewed in Yokoyama and Plougastel, 2003; Lanier, 2005)*.

In mice, two NKG2D isoforms exist, due to *Nkg2d* gene alternative splicing. The longer protein (NKG2-L) exclusively associates with DAP10, whereas the short one (NKG2-S) is promiscuous and can pair either with DAP12 or DAP10 adaptor protein. Therefore, mouse NKG2D can function either as activating or co-stimulatory receptor. NKG2D provides

co-stimulatory signal mainly in CD8+ T cells to amplify the response triggered via TCR. In contrast, human NKG2D associates only with DAP10 *(Diefenbach et al., 2002; Gilfillan et al., 2002; Billadeau et al., 2003; Rosen et al., 2004; Markiewicz et al., 2005), (Figure 8A)*. Interestingly, resting NK cells from healthy mice lack NKG2D-S isoforms, and so presumably NKG2D – DAP12 complexes, whereas activated NK cells (e.g. by double-strand RNA, IL-2, IFN-α, β) were reported to use predominantly DAP12 for signalling. As IFN-α and β are produced within tumor microenvironment, they might enhance NK cell-mediated anticancer response by "converting" NKG2D to a more active form (*Diefenbach et al., 2002; reviewed in Raulet, 2003)*.

Human NKG2D ligands are members of the MHC class-I-chain-related (MICA, MICB) and UL16-binding protein family (ULBP 1-4, RAET1G). In mice, there are retinoic acid early transcript 1 proteins (Rae1α-ε), the minor histocompatibility protein H60, and mouse UL16-binding-protein-like transcript 1 (MULT-1), *(Figure 8B)*. Despite marked diversity, NKG2D ligands share the following features: (i) they are structurally related to MHC class I molecules; (ii) their expression seems to be inducible e.g. by oxidative or genotoxic stress (DNA damage), viral infection, malignant transformation, and inflammatory responses *(reviewed in Eagle and Trowsdale, 2009; Stern-Ginosssar and Mandelboim, 2009)*. The binding of NKG2D to its various ligands is generally of higher affinity than most immunoreceptor – ligand interactions, and the signal from NKG2D is often strong enough to override those from inhibitory receptors *(Raulet, 2003)*.

Involvement of NKG2D in malignant cell recognition and elimination is supported by the fact that tumor cell lines transfected with NKG2D ligands showed enhanced sensitivity to NK cell killing *(Diefenbach et al., 2001; reviewed in Raulet, 2003)*. In addition, it is obvious that tumors evolved mechanisms how to escape from NKG2D-mediated immune surveillance (e.g. by producing TGF-β or shedding of NKG2D ligands, which results in down-regulation of NKG2D expression), *(Groh et al., 2002; Lee et al., 2004)*.

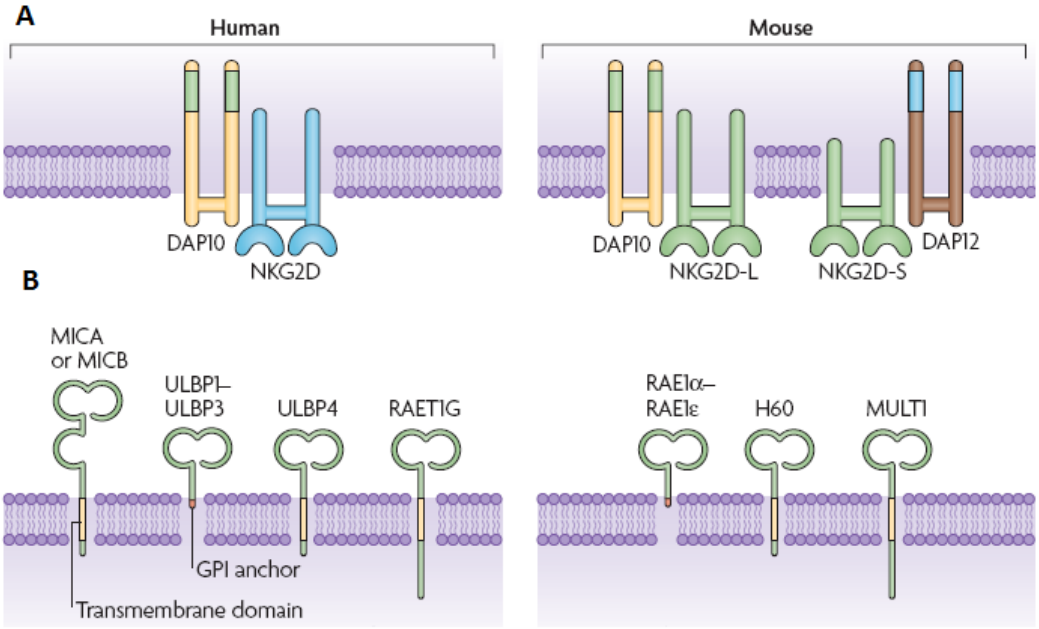



**Figure 8: NKG2D receptor (A) and its ligands (B) in humans and mice.** Mouse NKG2D-S isoform can associate with both DAP10 (not shown) and DAP12 adaptor proteins (MIC = MHC class-I-chain-related protein; ULBP = UL16-binding protein; GPI = glycosylphosphatidylinositol; RAE1 = retinoic acid early transcript 1; MULT1 = murine UL16-binding-protein-like transcript 1; see text; adapted from *Eagle and Trowsdale, 2009*).

### *3.1.2.1.4. CD69 receptor*

CD69 is a constitutively phosphorylated glycoprotein that is expressed as a disulphide-linked homodimer on NK, T, B cells, monocytes, neutrophils, and platelets upon their activation (hence the term "very early activation marker"). The CD69 cross-linking stimulated IL-2 and TNF-α secretion, nitric oxide production, and arachidonic acid metabolism in the relevant cell subpopulations. The CD69 receptor engagement on NK cells induced lysis of tumor targets *(Testi et al., 1994; Marzio et al., 1999; Sancho et al., 2005)*. On the other hand, in NK cell-sensitive tumor mouse models, genetic deficiency of CD69 or therapy with anti-CD69 antibody leaded to reduced TGF-β synthesis, which enhanced antitumor immunity. From these data, it emerges that CD69 receptor appears to behave as immunoregulatory molecule rather than activating receptor, supporting pro-inflammatory as well as anti-inflammatory state depending on the cellular and environmental context *(Esplugues et al., 2003; 2005; Sancho et al., 2005)*.

Investigation on CD69 receptor is bedevilled by the fact that its ligands are not defined yet. However, experiments with recombinant human CD69 molecule revealed that its C-type lectin-like domain bound calcium, which resulted in conformation changes and subsequent formation of high affinity binding sites for GlcNAc and GalNAc *(Pavlicek et al., 2003)*.

## 3.2. NK CELLS IN INTERCELLULAR COMMUNICATION

The NK cell ability to secrete various cytokines and express numerous membrane receptors/ligands confers upon them the potential to regulate the function of other cell types *(Figure 9)* including dendritic cells (DCs), macrophages, T, and B lymphocytes.

NK cells and DCs were reported to reciprocally activate each other under *in vitro* as well as *in vivo* conditions. NK cells secreting IFN-γ an d TNF-α induce DC maturation, which is accompanied by the increased expression of CD80, CD83, CD86, MHC class II molecules, chemokine receptor CCR7, etc. on DC surface. Mature DCs, in turn, enhance NK cell proliferation, cytotoxicity, and IFN-γ production via IFN-α, β, IL-12, IL-15, and IL-18 secretion. Cross-talk between NK cells and DCs also involves the direct intercellular contact based on NKG2D, Ly49H (during MCMV infection) or Nkp30 receptor – ligand interactions *(Fernandez et al., 1999; Gerosa et al., 2002; reviewed in Raulet, 2004; Hamerman et al., 2005; Degli-Esposti and Smyth, 2005)*. Conversely, NK cells can eliminate immature DCs as they express low levels of MHC molecules providing inhibitory signals. In cell culture experiments, DC lysis is favoured, when NK cells are present in excess of immature DCs, while the opposite NK:DC ratio enhances DC maturation *(Carbone et al., 1999; Wilson et al., 1999; Piccioli et al., 2002)*. Thereby, NK cells can influence DC homeostasis, but also potentially limit DC-based vaccination efficacy *(Hayakawa et al., 2004)*.

Monocytes and macrophages as potent producers of IL-12, IL-15, and IL-18, the cytokines inducing IFN-γ secretion, activate NK cells as well. In addition, in response to pathogens e.g. through Toll-like receptor engagement, they were shown to up-regulate NK cell stimulatory molecules such as NKG2D ligands *(Carson et al., 1995; Baratin et al., 2005; Kloss et al., 2008; Lapaque et al., 2009)*. NK cell cytotoxicity, in turn, might play an important role in the elimination of over-stimulated macrophages *(van Dommelen et al., 2006)*. This hypothesis is consistent with the colocalization of NK cells and macrophages in the splenic red pulp and

peripheral tissues *(Gregoire et al., 2007)*, and with *in vitro* cytotoxic activity of human NK cells toward autologous macrophages, observed only if the latter are activated *(Nedvetzki et al., 2007)*. Thus, NK cells seem to be involved in reducing the risk of disorders caused by inflammation via participating to the control of microbial infection as well as killing of over-stimulated immune cells *(reviewed in Vivier et al., 2008)*.

There is obvious evidence that NK cells modulate T cell responses: (i) NK cell depletion resulted in abrogation of tumor-specific CTL and memory T cell reaction *(Geldhof et al., 2002)*; (ii) NK cells as the major source of IFN-γ promote Th1 polarization of immune response *(Martin-Fontecha et al., 2004; Morandi et al., 2006)*; (iii) antigens from targets killed by NK cells are (cross)-presented by DCs to T cells *(reviewed in Vivier et al., 2008)*. Interestingly, activated T cells can limit NK cell activity (e.g. by IL-21 secretion), suggesting a regulatory loop between T and NK cell cytotoxicity *(Kasaian et al., 2002)*.

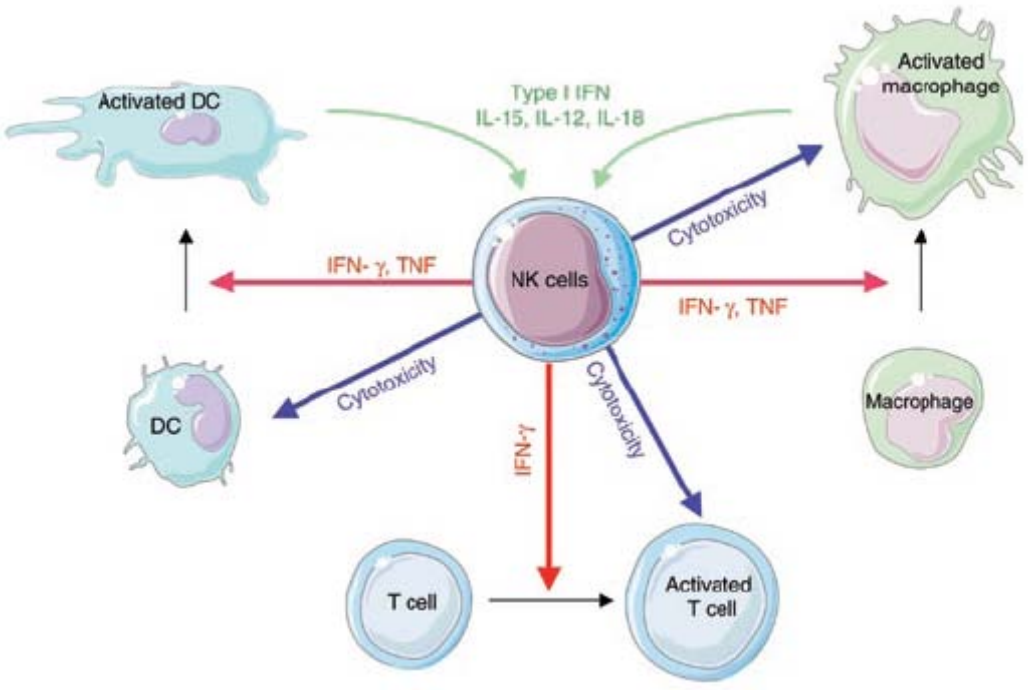



**Figure 9: Regulation of immune responses by NK cells.** Upon priming by various soluble factors (e.g. IL-15, IL-12, etc.), NK cells boost (red arrows) the maturation and activation of DCs, macrophages, and T cells. Conversely, NK cells can also kill (blue arrows) immature DCs, activated CD4+ T cells, and hyperactivated macrophages (adapted from *Vivier et al., 2008*).

## 3.2.1. NK – B CELL INTERACTIONS

### 3.2.1.1. B cell function

B cells are components of the adaptive immunity. Upon activation with the relevant antigen, they differentiate into plasma cells producing specific antibodies or memory B cells. Unlike T-dependent (TD) antigens *(Figure 10)*, T-independent (TI) ones can induce the antibody formation independently of the MHC-restricted T cell help. TI antigens can be further divided into TI-1 (e.g. LPS) and TI-2 (e.g. repetitive polysaccharide units of encapsulated bacteria) classes that require different co-stimulation signals in addition to BCR ligation (B cell activation via Toll-like receptors and T cell-derived cytokines, respectively). Moreover, B cells similarly as monocytes, macrophages, and DCs can serve as antigen-presenting cells (APCs). This is particularly important for T cell activation at the low antigen concentration *(reviewed in McHeyzer-Williams, 2003; Yuan, 2004)*.

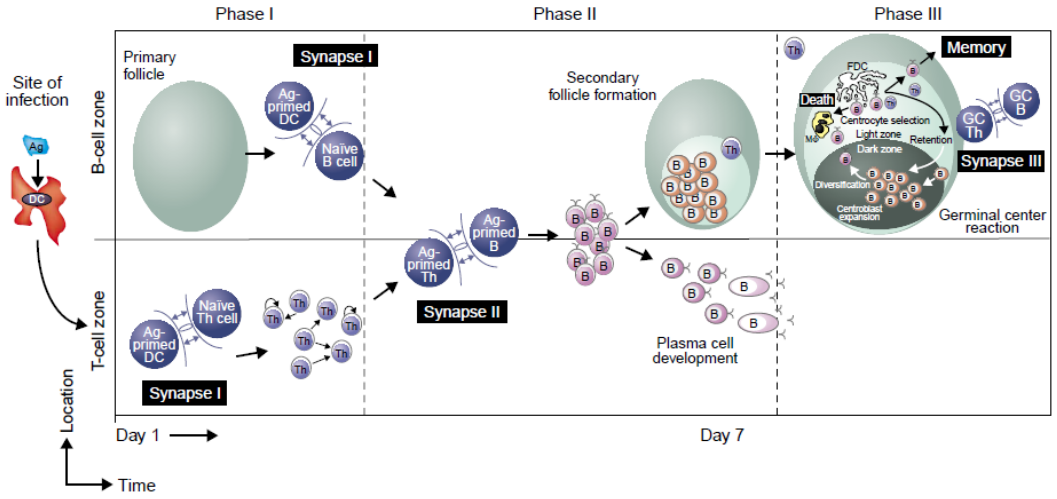



**Figure 10: T helper cell-regulated B cell differentiation.** DCs activated at the site of infection migrate to the T cell zones of lymph nodes. Antigen uptake, processing and presentation within the context of MHC class II molecules enable DCs to contact and stimulate naive Th cells. Following clonal expansion, antigen-activated Th cells migrate to the T – B borders of the lymph node to interact with antigen-primed B cells that subsequently differentiate into short-lived plasma cells or move to secondary follicles, where clonal expansion, somatic hypermutation of the B cell receptors, antigen-specific selection for high affinity variants and memory B cell development occur. Memory B cells can either differentiate into long-lived plasma cells or remain as non-secreting precursors for antigen recall (adapted from *McHeyzer-Williams, 2003)*.

### 3.2.1.2. *In vitro* NK – B cell interactions

Freshly isolated B cells from lymphoid organs or peripheral blood represent a mixture of resting and (pre)-activated lymphocytes. The resting ones, which are mostly double positive for IgD and IgM, express low levels of MHC class II molecules, and do not proliferate extensively, can be separated by the subsequent fractionation on Percoll density gradient *(Layton et al., 1985)*. *Yuan et al. (1992)* demonstrated that both these B cell subsets were stimulated to secrete IgM, when co-cultured with splenocytes or IL-2-activated NK cells from T and B cell-negative SCID mice. This was mediated by soluble factors rather than the direct NK – B cell interaction as NK cell supernatants by themselves exerted the comparable effect. Although the level of induced IgM in cultures of resting B cells with NK cells was quite low, the addition of LPS or cytokines (IL-2 and IL-5) caused its significant enhancement, higher than in those of single B cells primed by the same route. Furthermore, in this B cell subpopulation, the cultivation with IL-2-propagated NK cells up-regulated the expression of activation markers such as CD86 and CD69 as well as the level of mRNA for activation-induced cytidine deaminase (AID) necessary for class-switch recombination. However, switching to downstream Ig isotypes was not observed *(Gao et al., 2001; reviewed in Yuan, 2004)*. Similarly, *Blanca et al. (2001)* showed that human resting B cells were activated by autologous NK cells to form IgM *in vitro* even to a greater extent than by PWM or IL-2 alone, whereas the combination of NK cells with PWM or IL-2 was the most effective. In contrast, these authors also detected IgG as well as IgA in the cultures and suggested that comparing to mitogen-activated B cells, the resting ones required the direct contact with NK cells, and could not be stimulated only by NK cell-derived cytokines. Unlike NK cells, autologous unstimulated T cells failed to activate resting B cells in the absence of exogenous stimuli.

There are several further studies reporting that NK cells increased *in vitro* antibody response (in some of them including IgG2a levels), if they and/or B cells were activated e.g. by IL-2, LPS, poly (I:C), *Trypanosoma cruzi* or *Staphylococcus aureus* infection *(Amirogena et al., 1990; Becker et al., 1990; Michael et al., 1991; Gray and Horwitz, 1995; Vos et al., 1998; De Arruda Hinds et al., 2001)*.

All these data indicate that NK cells, mainly upon activation, can influence B cells *in vitro* in various manners (including elevated antibody formation) depending on the B cell differentiation stage. Thus, unlike pre-activated B lymphocytes, highly purified resting ones

may respond to the direct stimulation by NK cells in the absence of additional B cell-activating signals or NK cell-derived cytokines only to a limited extent *(reviewed in Yuan, 2004).*

Activated B cells, in turn, were found to enhance TNF-α and/or IFN-γ secretion in NK cells (*Becker et al., 1990; Michael et al., 1991; Wyatt and Dawson, 1991).* Similar effect was also exhibited by BCL1-C11 B cell-derived tumor *(Yuan et al., 1994).* In addition, a recent study revealed that B cells (but not macrophages, DCs or T cells) were required for IL-12-induced IFN-γ production by mouse NK cells purified from spleen *(Haddad et al., 2009).* B cells were also shown to stimulate IL-2-propagated NK cells to synthesize mRNA for IL-13 (Th2-type cytokine), which was dependent on interaction between CD244 receptor and its CD48 ligand expressed on NK and B cells, respectively. This finding might explain why under certain conditions (immunization in the presence of some adjuvants), activated NK cells are able to mount predominantly "Th2-like" IgG1 formation, although, in general, they tend to promote the class switch to "Th1-like" IgG2a isotype *(Yuan et al., 2004; Gao et al., 2006).*

### 3.2.1.3. *In vivo* NK – B cell interactions

Despite the data convincing *in vitro* NK – B cell co-operation, it is important to establish its relevance *in vivo*, where productive intercellular interactions take place within a complex microenvironment (presence of other cell types, various soluble factors, antigens, etc.). The most direct method to investigate possible NK cell-mediated modulation of B cell activity *in vivo* is the evaluation of response to TI antigen in order to reduce contributory effects of T cells *(reviewed in Yuan, 2004).*

*Wilder et al. (1996)* reported that the administration of poly (I:C) to mice immunized with TI or TD antigen led to the shift in the distribution of antigen-specific Ig isotypes, selectively increasing IgG2a and IgG1, while leaving IgM and IgG3 unaltered. However, only changes in the primary IgG2a response were dependent on NK cells, as proved by the treatment of NK cell-depleted mice with poly (I:C). In agreement with these findings, resting NK cells failed to regulate the antibody formation, but if activated e.g. by certain tumor cell lines, they up-regulated IgG2a levels *(Koh and Yuan, 1997; 2000).*

Most of the studies showed that NK cell-deficiency or depletion by itself did not markedly influence the *in vivo* antibody response to different antigens *(Amirogena et al., 1990; Wilder et*

*al., 1996; Koh and Yuan, 1997; Wang et al., 1998; Sungjin et al., 2000; Szomolanyi-Tsuda et al., 2001; Yuan et al., 2004).* Other studies uncovered significant effects on isotype switching, from a decrease to absence of IgG2a secretion, whereas IgG1 levels remained unchanged *(Satoskar et al., 1999; Jensen et al., 2004).* These contradictory results could be explained by using different NK cell depletion strategies or NK cell-deficient mouse strains. *In vivo* NK cell depletion can be associated with the following problems: (i) variability and incompleteness; (ii) time-limitation of its efficiency; (iii) used antibodies (e.g. anti-asialo GM1 or anti-NK1.1) can deplete further cell types other than NK cells or elicit an undesired immune response in the host. Some of them can be overcome constructing transgenic animals with defects in NK cell development and/or function. However, genetically modified mice were reported to possess the disturbed B and/or T cell immunity or cytokine profile *(reviewed in Yuan, 2004).*

As immunoglobulins can contribute to protection against pathogens or tumor spreading *(reviewed in Nicodemus et al., 2002; Casadevall, 2003; Brady, 2005),* the involvement of NK cells in regulation of their formation has important clinical consequences. *Koh and Yuan (2000)* identified IgG2a subclass to be the most effective one in mediating ADCC reaction under both *in vitro* and *in vivo* conditions. Therefore, NK cell-induced preferential switching to IgG2a antibodies against the infection agent or tumor cells can augment the target-specific antibody-directed killing.

Co-operation between NK and B cells is supposed to be important during certain parasitic infections such as that with *Trypanosoma cruzi*, which is characterized by enhanced NK cell proliferation and cytotoxic activity as well as IgG2a secretion *(Spinella et al., 1992; Brodskyn et al., 1996; De Arruda Hinds et al., 1999).* This was proved by *in vitro* assays showing that NK cells promoted IgG2a response, when co-cultured with B cells from *Trypanosoma cruzi* infected animals, even in the absence of additional activating signals *(De Arruda Hinds et al., 2001).* Thus, the increase in serum IgG2a levels observed *in vivo* might be a result of NK cell activation *(reviewed in Yuan, 2004).*

### 3.2.1.4. Mechanism(s) involved in NK – B cell interactions

Although the exact mechanisms are not completely elucidated, it is generally accepted that activated NK cells can regulate the antibody formation via the cytokine secretion and/or direct

intercellular contact with B lymphocytes *(reviewed in Yuan, 2004)*. The cytokine milieu is well-known to influence the downstream Ig isotype distribution (*reviewed in Snapper et al., 1997)*. Increased IgG2a levels induced by NK cells were attributed to their IFN-γ secretion (*Wilder et al., 1996; Koh and Yuan, 1997)*. Nevertheless, there is a markedly diminished, but still detectable IgG2a response to infectious agents as well as defined antigens in IFN-$\gamma^{-/-}$ and IFN-γR$^{-/-}$ mice *(Huang et al., 1993; Metzger et al., 1997; Markine-Goriaynoff et al., 2000)* Recently, it has been revealed that NK cells were capable of inducing IgG2a formation in B cells stimulated with TI-2 antigen NP-Ficoll by direct NK – B cell contact independently of IFN-γ *(Gao et al., 2008)*. Receptor – ligand pairs reported to be involved in NK – B cell interactions include CD28 – CD86 *(Cheung et al., 1999; De Arruda Hinds et al., 2001)*, CD40L – CD40 *(Blanca et al., 2001)*, CD244/CD2 – CD48 *(Gao et al., 2005; 2006; 2008)*.

## 3.3. CANCER

Cancer represents a considerable health and socio-economic problem. According to statistics *(Report of the World Health Organization, 2007)*, malignancies are the second leading cause of death, after cardiovascular diseases.

Carcinogenesis is a multistage process, during which genetic mutations and epigenetic changes accumulate in the cell. The critical mutations are those related to protooncogenes and tumor suppressor genes controlling the cell growth and apoptotic pathways. They can be triggered by repeated and/or intensive exposure to carcinogenic agents inducing the DNA damage such are chemicals, radiation or oncogenic viruses. The action of carcinogenes is promoted in individuals with genetic predisposition (the congenital impairment of DNA reparation mechanisms or other inherited mutations in genome), hormonal disturbances (increased level of estrogens in case of the breast cancer or stress hormones in general), and unfavourable immunologic background (e.g. presence of chronic inflammation). Any cell, which has not irreversibly lost the ability to divide because terminally differentiated, can be transformed. However, the intensively proliferating cells are the most prone to transformation as the higher speed of the cell cycle can increase the frequency of somatic mutations and decrease the possibility of successful DNA reparation *(Pike et al., 1993; Renan 1993; Macaluso et al., 2003; Vogelstein and Kinzler, 2004; Lu et al., 2006)*. Subsequently, the transformed cell gives rise to a clone that, while growing, disturbs the local homeostasis and induces the formation of

tumor microenvironment – the complex network involving cancer, immune, and stromal cells, including their soluble factors, and membrane molecules *(Henning et al., 2004)*.

*Hanahan and Weinberg (2000)* stated six basic characteristics of tumor cells: the ability to (i) provide growth signals by themselves in autocrine/paracrine mode; (ii) ignore growth-inhibitory signals by loosing cell – cell and cell – stromal regulations (e.g. contact inhibition); (iii) avoid the cell death due to mutations in tumor suppressor genes such as *p53*; (iv) replicate without limits; (v) sustain angiogenesis (e.g. by VEGF production); (vi) invade tissues through basement membranes and capillary walls in the course of the tumor growth and metastasis (e.g. by release of matrix metalloproteinases). Recently, the avoidance of immune surveillance (tumor escape) has been proposed to constitute the seventh hallmark of cancer *(Dunn et al., 2004; Smyth et al., 2006)*. The latter one is of great importance as it takes into account the complicated interplay between cancer and the host immune system regulating the tumor development.

### 3.3.1. LINK BETWEEN CANCER AND INFLAMMATION

A cancer cell is fundamentally a "self" cell acquiring its special characteristics such as (neo)-expression of stress molecules, tumor specific or associated antigens (TSAs and TAAs, respectively), and aberrant carbohydrate structures in a progressive mode *(Pardoll, 2003)*. Therefore, the initial immune response is addressed to maintenance of the local tissue homeostasis rather than primarily directed against transformed cells. The early tumor – immune system interactions result in acute inflammation supporting the cancer cell elimination. However, if the antitumor immune response is not efficient enough, inflammation becomes chronic and promotes the tumor growth *(Balkwill and Mantovani, 2001; Coussens and Werb, 2002; Balkwill et al., 2005)*.

The hypothesis that cancer originates under conditions of chronic inflammation was originally proposed by Virchow (1863), who observed a "lymphoreticular infiltrate" in neoplastic tissues. In *1986, Dvorak* compared tumors to wounds that do not heal. Apart from several shared parallels, the wound healing usually represents a self-limiting process of tissue remodelling guided by inflammation, whereas the tumor stroma formation proceeds continuously, due to

altered inflammatory network (e.g. via secretion of pro-inflammatory cytokines by cancer cells) and unresponsiveness of the tumor to physiological regulation mechanisms.

The link between cancer and inflammation was also proved by epidemiologic studies: (i) about 15-20% of the global cancer burden is attributable to infections *(Kuper et al., 2000)*; (ii) chronic inflammation of unknown etiology or caused by chemical, physical agents, and autoimmune response was shown to increase the risk of malignancy *(Ekbom et al., 1990; Gulumian 1999; reviewed in Balkwill and Mantovani, 2001), (Table 2)*; (iii) non-steroidal anti-inflammatory drugs (NSAIDs), inhibitors of cyclooxygenase-2, which is induced at sites of inflammation including tumor microenvironment and catalyzes the conversion of arachidonic acid to prostaglandins, decreased the incidence of colorectal, breast, and other cancers *(reviewed in Garcia-Rodriguez and Huerta-Alvarez, 2001; Ulrich et al., 2006; Harris et al., 2009; Wang 2010).*

| Malignancy | Inflammatory stimulus/condition |
|---|---|
| Bladder | Schistosomiasis |
| Cervical | Papillomavirus |
| Ovarian | Pelvic inflammatory disease/tissue remodelling |
| Gastric | *Helicobacter pylori* induced gastritis |
| MALT lymphoma | *Helicobacter pylori* |
| Oesophageal | Barrett's metaplasia |
| Colorectal | Inflammatory bowel disease |
| Hepatocellular | Hepatitis virus (B and C) |
| Bronchial | Silica, asbestos, cigarette smoke |
| Mesothelioma | Asbestos |
| Kaposi's sarcoma | Human herpesvirus type 8 |

**Table 2: Association between inflammation and risk of cancer** (adapted from *Balkwill and Mantovani, 2001*).

New findings that support Virchow's "re-discovered" *(Balkwill and Mantovani, 2001)* theory were revealed due to progress in cell and molecular cancer immunobiology.

Pro-inflammatory cytokines (e.g. TNF-α, IL-1, IL-6), which can be produced either by cancer cells themselves or tumor-infiltrating leukocytes and platelets, were reported to promote the tumor growth and metastasis via mechanisms summarized in *Table 3 (reviewed in Balkwill and Mantovani, 2001).*

| |
|---|
| **DNA damage via reactive oxygen** |
| **inhibition of DNA repair via reactive oxygen** |
| **functional inactivation of tumor suppressor genes** |
| **autocrine/paracrine growth and survival factors for malignant cells** |
| **induction of vascular permeability and extravasation of fibrin/fibronectin** |
| **tissue remodelling via induction/activation of matrix metalloproteinases** |
| **control of tumor cell migration** |
| **control of leukocyte infiltrate** |
| **modulation of cell-cell adhesion molecules** |
| **subversion of host immune responses** |
| **stimulation of angiogenesis** |

**Table 3: Mechanisms, by which pro-inflammatory cytokines/chemokines promote the tumor growth, invasiveness, and metastasis** (adapted from *Balkwill and Mantovani, 2001*).

TNF-α is considered to be a pivotal cytokine in orchestrating inflammatory responses. As its name indicates, this cytokine can cause tumor and/or haemorrhagic necrosis. However, when secreted chronically, it also induces the production of: (i) pro-angiogenic factors; (ii) chemokines (e.g. MCP-1) increasing the content of macrophages in tumor microenvironment, which is often associated with a poor prognosis; (iii) matrix metalloproteinases important for the tumor spreading; (iv) iNOS catalyzing the synthesis of nitric oxide, which can directly oxidise DNA or proteins involved in DNA reparation, and thus amplifying accumulation of genetic mutations in the cell *(Jaiswal et al., 2000; Balkwill, 2002; Bingle et al., 2002; Lewis and Pollard, 2006).* General or cell-selective deletion/inhibition of TNF-α reduced the tumor incidence in animal experimental models. Namely, TNF-$\alpha^{-/-}$ and/or TNFR1$^{-/-}$ mice were resistant to chemically induced skin and liver cancers, as well as the development of metastasis in colon carcinoma *(Moore et al., 1999; Knight et al., 2000; Kitakata et al., 2002; Arnott et al., 2004).*

There is also genetic evidence linking inflammation and cancer as polymorphisms in genes for pro-inflammatory cytokines, mainly present in regions regulating transcription and posttranscriptional modifications, were found to correlate with cancer susceptibility and severity *(El-Omar et al., 2000; Oh et al., 2000)*. Genetic polymorphisms in *TLR* gene cluster encoding Toll-like receptors, the ligation of which stimulates pro-inflammatory cytokine secretion, were shown to be associated with high risk for prostate cancer *(Sun et al., 2005).*

The transcription factor NF-κB involved in signalling pathways during inflammatory responses, was found to trigger the expression of genes encoding inhibitors of apoptosis (e.g. c-FLIP, Bcl-$X_L$), pro-angiogenic and growth factors, proteins regulating the cell cycle progression (e.g. cyclin D1), and proteases. All these molecules are crucial for the tumor development *(Micheau et al., 2001; Luo et al., 2004; Pikarsky et al., 2004; reviewed in Karin et al., 2002; Karin and Lin, 2002; Karin, 2006), (Figure 11).* Blocking of the classical NF-κB activation pathway by selective deletion of IκB kinase-β (IKK-β) in either enterocytes or myeloid cells of mice with colitis-associated cancer reduced the tumor incidence and growth. This was mediated via distinct cell type-specific mechanisms: (i) enhanced apoptosis in enterocytes as a consequence of defective induction of Bcl-$X_L$ gene expression; (ii) down-regulated secretion of pro-inflammatory cytokines (e.g. IL-6) in myeloid cells functioning as cancer growth factors *(Greten et al., 2004; Karin, 2006).*

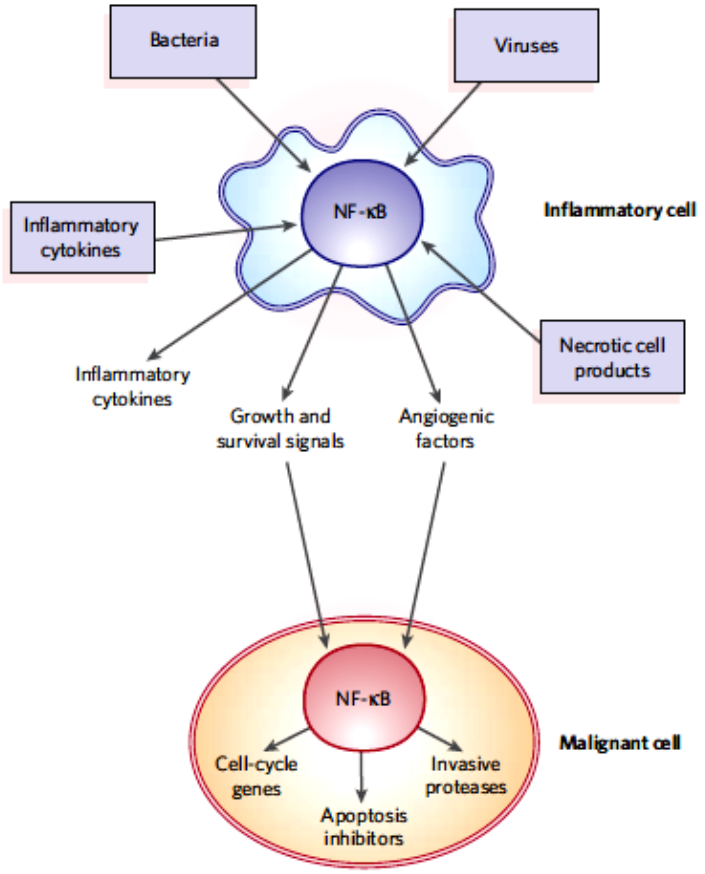



**Figure 11: NF-κB in cancer immunoediting.** NF-κB activation in inflammatory and/or malignant cells can promote malignant transformation and progression (see text; adapted from *Karin, 2006*).

## 3.3.2. CANCER IMMUNOEDITING

The hypothesis of cancer immunosurveillance originally formulated by *Burnett (1957)* and *Thomas (1959)*, the validity of which was intensively discussed during the last five decades, predicted that one of the immune system functions was the early recognition and elimination of transforming cells, and thus protection against development of the clinically evident tumors of non-viral origin. Currently, it is generally accepted that the anticancer immune response is indeed elicited, but tumors are able to escape it either via its active suppression (immunosubversion) or selection of non-immunogenic tumor cell variants (immunoselection). In addition, some immune cell subpopulations can support the tumor growth e.g. by releasing pro-tumor mediators. This combination of host-protective and tumor-promoting mechanisms applying during the tumor development is termed “cancer immunoediting” and envisaged as a

dynamic process composed of three periods: elimination, equilibrium and escape *(Figure 12)*. Elimination embodies the classical concept of cancer immunosurveillance, equilibrium the immune-mediated latency after the incomplete tumor destruction, and escape the final outgrowth of tumors that have overcome immunological restraints of two previous phases (*Dunn et al., 2004; Smyth et al., 2006; Zitvogel et al., 2006; Swann and Smyth, 2007).*

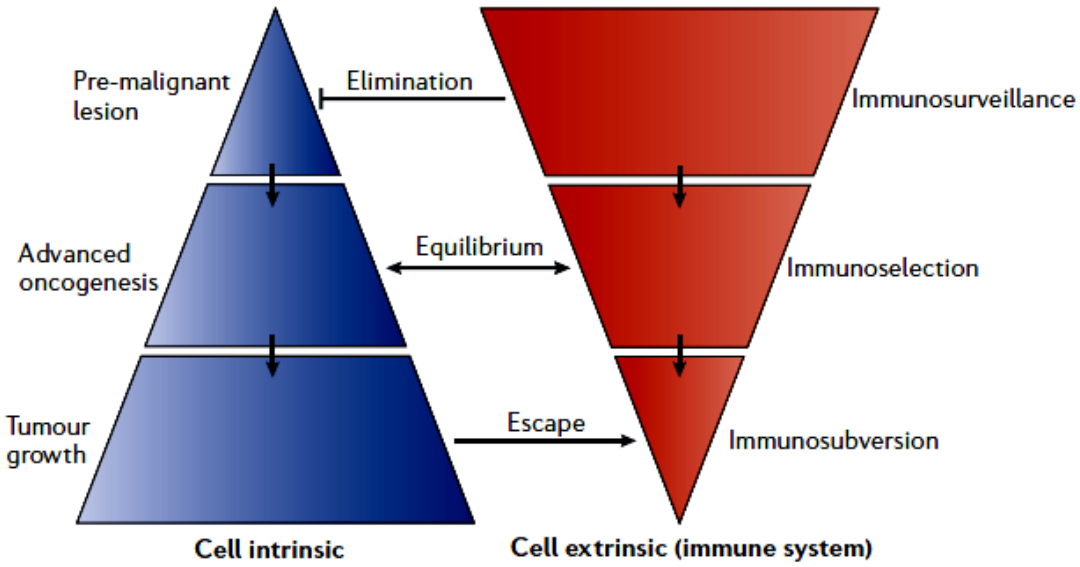



**Figure 12: Crosstalk between tumor cells (intrinsic factors) and the host immune system (extrinsic effects) during multistep carcinogenesis** (adapted from *Zitvogel et al., 2006*).

### 3.3.2.1. Cancer immunosurveillance

The efficient antitumor immune response is based on the tight co-operation between components of the innate and adaptive immunity that are linked through DCs *(Diefenbach and Raulet, 2002)*. Initially, neutrophils, monocytes/macrophages, NK cells, and DCs are attracted to the site of a developing tumor by chemokines, pro-inflammatory cytokines, and "danger signals" such are heat-shock proteins (HSPs) or breakdown products of extracellular matrix. The latter ones are released as a consequence of the tissue disruption caused by neo-angiogenesis and/or invasive tumor growth. Tumor-infiltrating immune cells trigger/amplify

inflammatory responses (*Vicari and Caux, 2002*; *Gallucci and Matzinger, 2002; Coussens and Werb, 2002).*

NK cells recognize and kill tumor cells bearing "stress proteins" (ligands for NKG2D receptor), which are induced on their cell membrane either by the incipient inflammation or transformation process itself, and later those with down-regulated MHC class I expression or altered glycosylation of surface molecules *(reviewed in Smyth et al., 2002; Wu and Lanier, 2003; Raulet and Guerra, 2009).* In addition, they secrete numerous cytokines including IFN-γ, that is together with IFN-α and β referred to as the central coordinator of tumor – immune system interactions *(reviewed in Dunn et al., 2006).*

DCs activated by pro-inflammatory cytokines (e.g. IL-1β, TNF-α) and/or cross-talk with NK cells engulf and process tumor-derived apoptotic bodies as well as complexes formed by HSPs and tumor antigens. Subsequently, they migrate to lymphoid organs to complete their maturation process and (cross)-present antigenic peptides in a complex with MHC class I or II molecules to naive CD8+ and CD4+ T cells, respectively *(Huang et al., 1994; Banchereau and Steinman 1998; Gerosa et al., 2002; Li et al., 2002; Srivastava, 2002; Mocikat et al., 2003).* DCs also display tumor-derived glycolipids bound to CD1d membrane molecule, which are recognized by natural killer T (NKT) cells through their TCRs. Activated NKT cells secrete various cytokines (e.g. IFN-γ, IL-4, IL-13, IL-10, TNF-α, etc.) and induce apoptosis in cancer cells similarly as CTLs and NK cells, but independently of MHC expression *(Brutkiewicz and Sriram, 2002).* Recently, so called IFN-γ-producing killer dendritic cells (IKDCs), which can eliminate tumor cells in TRAIL and/or perforin-dependent manner, have been discovered *(Chan and Housseau, 2008).*

CD8+ cytotoxic T cells (CTLs) primed via the interaction with DCs, undergo clonal expansion and then, either exert the lytic activity against tumor targets or establish memory cells to be stimulated upon repeated encounter with the same antigen. On the other hand, CD4+ helper T cells differentiate into various subtypes depending on the local cytokine milieu (e.g. Th1, Th2, Th17, etc.), and act mainly as regulatory cells. The polarization of the immune system to Th1 responses was reported to be crucial for cancer immunosurveillance. Th1 cells characterized by IL-2 and IFN-γ production, among others, promote the macrophage activation, and help to maintain the viability and function of tumor-specific CTLs. Th2 cells secreting cytokines such as IL-4 and IL-13 provide co-stimulatory signals to B cells pre-activated with tumor antigens, and thus support the antibody formation. Tumor-specific antibodies (depending on their

isotype) can contribute to cancer cell elimination by mediating ADCC reaction or triggering the complement activation pathway *(Figure 13), (reviewed in Knutson and Disis, 2005; Smyth et al., 2006; Zitvogel et al., 2006; Johansson et al., 2008).*

As immune cell subpopulations are strongly influenced by special conditions within tumor microenvironment (e.g. hypoxia, low pH, chronic inflammation, mediators produced by tumor cells, etc.), they can paradoxically assist the tumor escape. An example is given by macrophages that, on one hand, kill cancer cells, present tumor antigens, produce T cell-stimulatory cytokines (M1 phenotype based on Th1/Th2 model), whereas, on the other hand, promote the tumor growth, invasion, angiogenesis, and metastasis (M2), *(Mantovani et al., 2004; Lewis and Pollard, 2006; Martinez et al., 2008).*

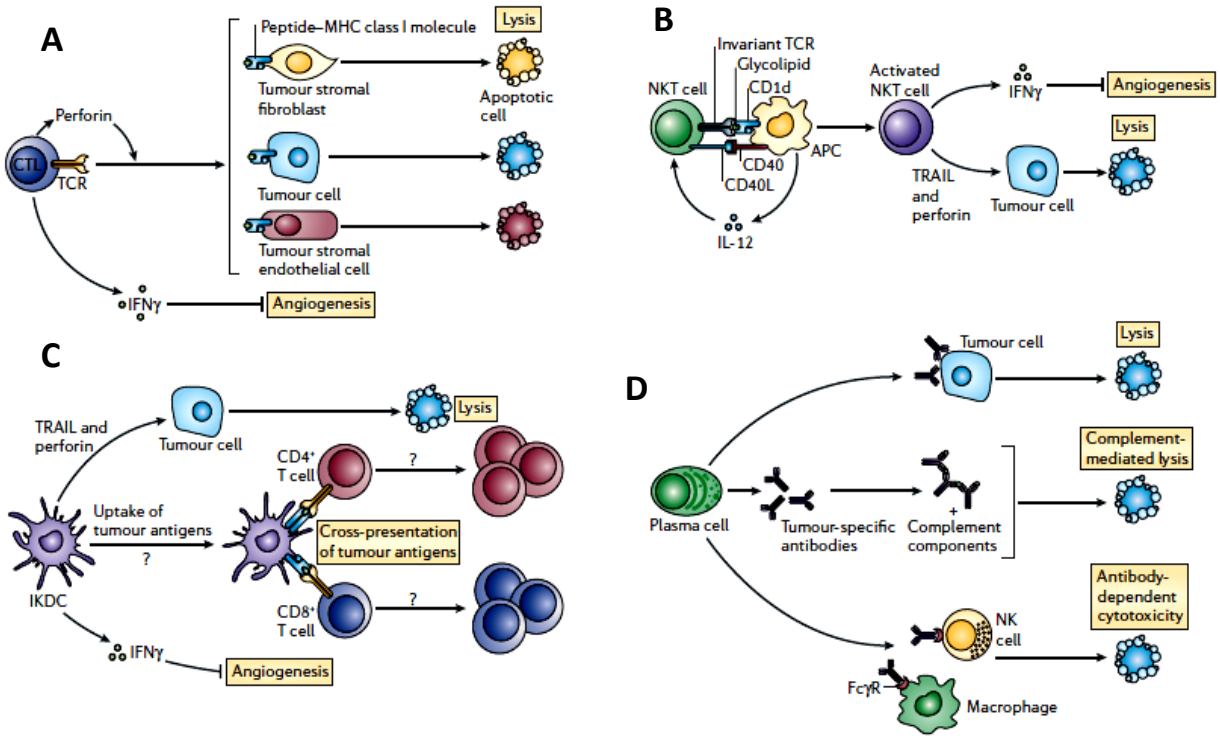



**Figure 13: Cancer immunosurveillance: the role of CTLs (A), NKT (B), dendritic (C), and plasma cells (D),** (see text; adapted from *Zitvogel et al., 2006*).

### *3.3.2.1.1. NK cells in cancer immunoediting*

Tumor infiltrating NK cells were associated with a favourable prognosis in patients with colorectal carcinoma *(Coca et al., 1997).* The participation of NK cells in cancer immunosurveillance was proved using two basic approaches: (i) NK cell depletion of experimental animals before tumor inoculation or chemically induced carcinogenesis that resulted in more aggressive tumor growth and/or metastasis than in NK cell-intact control group; (ii) systemic or local administration of cytokines known to boost NK cell functions (e.g. IL-2, IL-12, IL-15, IFN-α, β), which exerted antitumor effects *(Smyth et al., 2001; reviewed in Smyth et al., 2002; Wu and Lanier, 2003).*

NK cells might be directly and exclusively responsible for rejection of MHC class I-deficient tumors such as RMA-S lymphoma *(Karre et al., 1986),* otherwise cooperate with T cells *(Wu et al., 1995).* They were also found to be implicated in controlling liver metastasis *(Divino et al., 2000),* which is particularly relevant taking into account the abundance of NK cells in this organ. In addition, the liver NK cell subset (but not T cell one) was reported to constitutively express TRAIL molecule inducing apoptosis *(Takeda et al., 2001; Cretney et al., 2002).* Findings of *Ruggeri et al. (2002)* clearly indicated that allogeneic NK cells derived from the donor haematopoietic stem cells were able to mediate the GvL reaction in the recipient, and thus to prevent the relapse of myeloid leukaemia.

### *3.3.2.1.2. Interferons in cancer immunoediting*

The interferon family *(Table 4)* was originally recognized for its capacity to protect cells against viral infections, but currently it is known to have a crucial regulatory function in immune responses including cancer immunoediting *(reviewed in Stark et al., 1998; Dunn et al., 2006).* The antitumor activity of IFN-γ was demonstrated using IFN-$\gamma^{-/-}$, STAT1$^{-/-}$, or IFN-γR1$^{-/-}$ knockout mice, which were more susceptible to development of spontaneous, transplantable and/or chemically induced tumors than their wild-type counterparts, while introducing of IFN-γR1 into tumor-bearing IFN-γR1$^{-/-}$ animals leaded to the tumor rejection in a T cell-dependent manner *(Kaplan et al., 1998; Street et al., 2001).* This might be explained, in part, by IFN-γ ability to up-regulate the tumor cell expression of MHC class I and TAP1 molecules involved in antigen processing and presentation (*Shankaran et al., 2001).* Furthermore, IFN-γ inhibits angiogenesis and the cell proliferation, whereas promotes

apoptosis (via regulation of CD95, TRAIL, and caspase expression). The fact that several human tumor types showed defects in IFN-γ signalling as a result of genetic mutations, also supports the clinical relevance of this cytokine in malignant diseases *(reviewed in Dunn et al., 2006)*. Recently, it has been shown that increased level of SOCS-1, a negative regulator of IFN-mediated signalling, correlated with melanoma progression *(Li et al., 2004)*.

Importantly, early secretion of IFN-γ blocks the generation and/or immunosuppressive effects of CD4+/CD25+ regulatory T cells (Tregs), *(Nishikawa et al., 2005)*.

| Property | Type I IFNs | Type II IFN |
|---|---|---|
| | IFNα and IFNβ | IFNγ |
| Stimuli | • Viruses<br>• Other microorganisms | • Antigen–MHC complexes<br>• Activating NK-cell ligands<br>• IL-12 plus IL-18<br>• TLRs |
| Cells producing IFN | • All nucleated cells | • NK cells<br>• NKT cells<br>• T cells |
| Number of proteins | • IFNα: 12 (mice) and 12–13 (humans)<br>• IFNβ: 1 (mice and humans) | • 1 (mice and humans) |
| Cells expressing IFN receptors | • All nucleated cells | • All nucleated cells |
| Type of IFN receptor | • IFNAR: IFNAR1–IFNAR2 | • IFNGR: IFNGR1–IFNGR2 |
| Signalling molecules | • JAK1 and TYK2<br>• STAT1–STAT2–IRF9 complexes<br>• STAT1–STAT1 complexes | • JAK1 and JAK2<br>• STAT1–STAT1 complexes |

**Table 4: The key similarities and differences between type I and II interferons** (JAK = Janus kinase; TLRs = Toll-like receptors; TYK = tyrosine kinase; adapted from *Dunn et al., 2006*).

The primary role of type I interferons in anticancer response is to influence immune cells (e.g. activation of DCs, macrophages, NK, and T cells) rather than transformed ones (*reviewed in Dunn et al., 2006)*. However, *Takaoka et al. (2003)* found out that these cytokines also induced synthesis of tumor-suppressor protein p53.

### 3.3.2.2. Tumor escape

Tumors developed a series of strategies to evade immune surveillance that, according to *Smyth et al. (2006)* can be divided into two (rather overlapping) categories: (1) intrinsic escape mechanisms related to tumor cells themselves; (2) extrinsic ones associated with the host immune system. Examples of the former group include: (i) low levels of tumor antigens; (ii) heterogeneous phenotype of transformed cells within the tumor; (iii) absent or downregulated expression of molecules involved in antigen processing and presentation such are MHC class I, TAP, LMP, and β2-microglobulin; (iv) lack of MHC class II and co-stimulatory molecules (e.g. CD86, CD80); (v) the loss of response to IFNs; etc. Into the latter group fall: (i) immune ignorance; (ii) immune tolerance via induction of anergy in responding T cells or their deletion; (iii) suppression of the anticancer immune reaction by Tregs, CD11b+/Gr+ myeloid cells, tumor cell-derived factors, or conditions within tumor microenvironment (e.g. glucose deprivation, low pH, hypoxia); etc. *(Marincola et al., 2000; Seliger et al., 2000; Atkins et al., 2004; Spiotto et al., 2004; Dunn et al., 2006; Nagaraj and Gabrilovich, 2007; reviewed in Smyth et al., 2006).*

The immune ignorance/tolerance stems from the fact that tumor cells cannot elicit the adequate response because of anatomical barriers between them and effector cells or limited amounts and/or low immunogenicity of their antigens *(Cuenca et al., 2003; Ochsenbein, 2005)*. Moreover, tumor cells secrete soluble factors such as VEGF, iNOS, IDO, arginase-1, IL-10, and TGF-β inhibiting differentiation, maturation, and proper function of DCs (as well as NK and T cells). Thus, local DCs are usually of immature phenotype with defective ability to stimulate naive tumor-specific T cells. On the other hand, they promote generation of immunosuppressive Tregs *(Yang and Carbone, 2004; Ghiringhelli et al., 2005b; reviewed in Zou, 2005; Zitvogel et al., 2006)*. Qualitatively and/or quantitatively altered MHC class I expression on cancer cells represents further mechanism how tumors subvert the cytotoxic activity of CTLs. As discussed above, this can be overcome to some extent by NK cell involvement. However, several studies reported that chronic exposure of NK cells to membrane or soluble (shed from the tumor cell surface) NKG2D ligands led to down-regulation of their NKG2D receptors and subsequent impairment of NK cell function *(Oppenheim et al., 2005; Coudert et al., 2005; Holdenrieder et al., 2006; Zwirner, 2007)*. Cancer cells also possess ligands for NK and/or T cell inhibitory receptors enabling escape from NK/T cell-mediated killing (e.g. CEA – CEACAM-1, and PDL-1 (B7-H1) – PD-1, respectively), *(Dong et al.,*

*2002; Stern et al., 2005).* Finally, tumor cells can avoid T or NK cell-induced apoptosis via increased expression of c-FLIP (caspase-8 inhibitor) or conversely, down-modulation of death receptors. In contrast, tumor-derived FasL can trigger the programmed cell death in host lymphocytes bearing CD95 (Fas) molecules *(Irmler et al., 1997; Song et al., 2001; Whiteside, 2007).*

Cytokines and chemokines of cancer, stromal, or immune cell origin present in the tumor microenvironment (e.g. IL-4, IL-10, TGF- β, TARC, and MDC) drive a bias towards Th2-type immune response, generation and/or recruitment of Tregs, and Th17 cells. *In vivo* depletion of Tregs induced transient regression of experimental tumors, and increased efficacy of vaccine-mediated immunotherapy. Th17 cells were associated with cancer development due to synthesis of factors promoting/sustaining chronic inflammation such as IL-23 *(Danull et al., 2005; Bettelli et al., 2006; Gajewski et al., 2006; Zou, 2006; Wang and Wang, 2007; Johansson et al., 2008).*

The genesis of blood and lymphatic vessels is usually insufficient for progressively growing tumor mass, therefore hypoxia, low availability of glucose, and acidic pH occur within the tumor microenvironment. As a consequence, the level of hypoxia-inducible transcription factors HIF-1 and 2, which regulate expression of genes encoding pro-angiogenic proteins such as VEGF, but also those involved in apoptosis, glucose or extracellular matrix metabolism, is elevated in tumor and/or immune cells (e.g. tumor-infiltrating macrophages). In addition, increased frequency of genetic mutations, defects in DNA repair mechanisms, chromosomal fragile sites was observed under pressure of such conditions. All these events contribute to the selection, growth, and spread of tumor cell clones with better environmental adaptability, whereas suppress the host immune response (immune cells are more sensitive to the nutrient deprivation than cancer cells, low pH and hypoxia negatively influence their cytotoxic activity, migration, phagocytosis, receptor – ligand interactions, etc.), *(Fischer et al., 2000; Henning et al., 2004; Cham and Gajewski, 2005; Lewis and Pollard, 2006; reviewed in Gajewski et al., 2006).*

### 3.3.3. GLYCOSYLATION AND CANCER

It is generally accepted that aberrant glycosylation patterns on the cell surface are, among others, associated with cancer phenotype. This was originally reported by *Meezan et al. (1964)*, who observed that healthy fibroblasts possessed smaller membrane glycoproteins than their malignant transformed counterparts. Changes in glycosylation arise from altered levels of glycosyltransferases in tumor cells, and can include under/over-expression of naturally occurring glycans, neo-expression of those normally restricted to embryonic tissues, or appearance of entirely new structures (e.g. hyperbranched or truncated), *(reviewed in Dube and Bertozzi, 2005)*.

*N*-acetyl-glucosaminyl-transferase V (GlcNAc-TV, MGAT5) is responsible for β1,6 GlcNAc-branching of N-glycans, which in turn creates additional sites for linking the terminal sialic acid residues by sialyltransferases. The expression of gene for MGAT5 is up-regulated in various types of tumors, which correlates with the finding that it is regulated by RAS-RAF-MAPK signalling pathway, commonly showing the increased activity in cancer cells *(reviewed in Dennis et al., 1999; Dube and Bertozzi, 2005). Demetriou et al. (1995)* demonstrated that MGAT5-transfected lung epithelial cell line (Mv1Lu) formed solid tumors in experimental animals, exerted a loss of contact inhibition, and increased motility in the culture. Moreover, in MGAT5-deficient mice, regression in the tumor growth and metastasis was observed compared to intact littermates *(Granovsky et al., 2000)*. Thus, hyperbranched carbohydrate structures contribute to malignant transformation via influencing the intercellular interactions and the cell adhesion *(Guo et al., 2004).*

Cancer cells cultivated under hypoxic conditions also exhibited increased transcription of genes encoding sialyl- and fucosyltransferases that are involved in the synthesis of sialyl-Lewis$^x$ antigen (sLe$^x$), the ligand for endothelial or platelet selectins *(Koike et al., 2004)*, and therefore in promoting the metastatic spreading (*reviewed in McEver, 1997; Gorelik et al., 2001)*. In addition, patients whose tumor cells express sLe$^x$ (about 25-30% of patients with breast, colon, gastric, and thyroid cancer) have a much poorer prognosis for survival *(Alper, 2003)*. From this, it emerges again, that the tumor microenvironment plays a crucial role in directing the cancer evolution.

The tumor-associated carbohydrate antigens (TACAs) represent the majority of TAAs, and can be categorized into two major groups: (i) glycolipids such as GM2, GD2, GD3, and fucosyl

GM1 (gangliosides), or Lewis$^{y}$ and Globo H (neutral glycolipids); (ii) glycoproteins such as mucin-related epitopes Tn (GalNAcα-O-Ser/Thr), sTn (NeuAcα2→GalNAcα-O-Ser/Thr), and TF (Thomsen-Friedenreich antigen; Galβ1→GalNAcα -O-Ser/Thr), *(Vollmers and Brandlein, 2007).* No single modification in glycosylation seems to distinctly differentiate a normal cell from cancer one, instead each type of malignant tissue is characterized by a certain set of changes in glycan profile *(Table 5), (reviewed in Dube and Bertozzi, 2005).*

Increased serum levels of TACAs (e.g. PSA, CEA, CA125, etc.) are used for diagnostics of relevant cancer types *(reviewed in Dube and Bertozzi, 2005; Vollmers and Brandlein, 2007). Peracaula et al. (2003)* showed that characterization of *N*-glycans within PSA conferred the specifity upon diagnostic tests as it enabled to distinguish between PSA related to benign prostate hyperplasia and prostate cancer.

| Cancer glycan | Malignant tissue | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Ovary | Pancreas | Blood | Breast | Colon | Brain | Prostate | Skin | Lung |
| sLe$^{x}$ | | X | | X | X | | | | X |
| sLe$^{a}$ | | X | | X | X | | | | X |
| sTn | X | X | | X | X | | X | | X |
| TF | X | | | X | X | | X | | |
| Le$^{y}$ | X | X | | X | X | | X | | X |
| Globo H | X | X | | X | X | | X | | X |
| PSA | | X | X | X | | X | | | X |
| GD2 | | | X | | | X | | X | |
| GD3 | | | | | | X | | X | |
| Fucosyl GM1 | | | | | | | | | X |
| GM2 | X | X | X | X | X | X | X | X | X |

**Table 5: Common expression patterns of cancer glycans on malignant tissues** (adapted from *Dube and Bertozzi, 2005*).

*Van Gisbergen et al. (2005)* demonstrated that DCs adhered to colorectal cancer cells through interaction between their C-type lectin-like receptor DC-SIGN and Lewis$^{x/y}$ antigens present on tumor-associated antigen CEA, whereas they did not recognize normal colon tissue with low levels of Lewis structures. In addition, Tn antigens were identified as ligands for macrophage galactose-type lectin (MGL), *(van Vliet et al., 2005).* Thus, aberrant glycosylation patterns on

cancer cells, similarly as microbial glycans, might play a crucial role in the immune surveillance *(reviewed in Aarnoudse et al., 2006), (Figure 14)*. As described above, natural ligands for activating NK cell C-type lectin-like receptors remain unknown. However, *Ohayama et al. (2002)* observed that the extent of sialyl-Lewis$^{x}$ expression on tumor cells correlated with sensitivity to NK cell-mediated cytotoxicity probably triggered via CD94 receptor ligation.

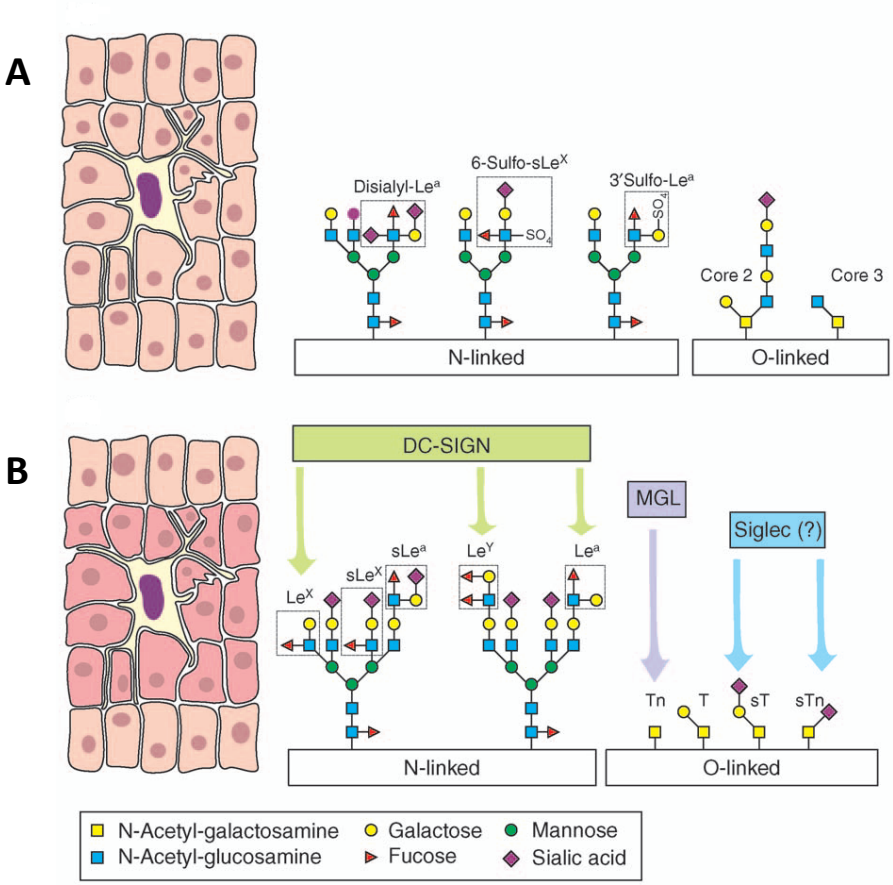



**Figure 14: Glycosylation in normal (A) and cancer (B) cells.** Normal breast and colon epithelial cells are rich in *N*-linked glycans carrying the epitopes such as disialyl-Le$^{a}$, 6-sulfo-sLe$^{x}$, and 3'sulfo-Le$^{a}$, whereas the O-glycans are of the core 3, or elongated core 2-type. Cancer cells possess different glycosylation patterns, rich in ligands for DC-SIGN (Le$^{x}$, Le$^{y}$, Le$^{a}$), MGL (Tn), and probably a yet unidentified Siglec (sT, sTn). (DC-SIGN = dendritic cell-specific ICAM-3 grabbing nonintegrin; Le = Lewis; MGL = macrophage galactose-type lectin; Siglec = sialic acid binding immunoglobulin-like lectin; adapted from *Aarnoudse et al., 2006*).

### 3.3.3.1. Carbohydrate vaccines in cancer immunotherapy

As many of TACAs represent altered "self" molecules, they lack inherent immunogenicity typical of viral or bacterial antigens. Thus, taking into account the necessity "to break" the immune tolerance, the development of anticancer vaccines is more challenging than those for treatment of infectious diseases. Several approaches were adopted to induce/enhance immune response against TACAs: vaccination with (i) whole or lysed tumor cells; (ii) purified or synthetic TACAs in the presence/absence of bacterial adjuvant such as *Salmonella minnesota* or Bacillus Calmette Guerin; (iii) TACAs conjugated to an immunogenic carrier such as bovine serum albumin (BSA), tetanus toxoid, or keyhole limpet hemocyanin (KLH) in the presence/absence of adjuvant e.g. QS-21 (purified saponin fraction obtained from the bark of the tree *Quillaja saponaria*). Carriers and/or adjuvants provide additional activating signals e.g. promote antigen processing and presentation by APCs, stimulate T cells, etc. *(reviewed in Slovin et al., 2005a; Niederhafner et al., 2008; Pashov et al., 2009).*

*Helling et al. (1994),* who dealt with evolution of ganglioside-based vaccines, demonstrated that GD3 covalently linked to KLH by reductive amination, and injected with QS-21 exerted the superior immunogenicity comparing with several combinations of carriers, adjuvants, and conjugation methods. Using this route of administration, high titres of anti-GD3 IgM and IgG, which triggered complement-dependent lysis of human melanoma cells expressing targeted antigen, were found in most experimental mice. GM2-KLH conjugate plus QS-21 exhibited similar effects in melanoma patients, moreover, induced anti-GM2 IgG antibodies mediating ADCC reaction *(Kitamura et al., 1995; Livingston et al., 1997).* Later, vaccination based on TACAs such are Globo H, TF, Tn, STn, $Le^y$, etc. was established and successfully tested in clinical trials *(Slovin et al., 1999; Sabbatini et al., 2000; Slovin et al., 2003; 2005b; Gilewski et al., 2007).* In about 60% of patients, conjugate vaccines against GD3, GD2, Globo H, $Le^y$, or TF elicited the formation of antibodies binding to antigen-positive cancer cells, which usually led to their lysis *(Slovin et al., 2005a).*

Monovalent vaccines (i.e. with one carbohydrate epitope), however, do not respect the degree of heterogeneity of TACAs present on the cell surface as well as that between individual transformed cells within the tumor. By combining TACAs to create a polyvalent vaccine, the multifaceted immune response can be evoked, and antibodies against each involved antigen produced, resulting in targeting of a greater percentage of cancer cells. Strategies for polyvalent

vaccine preparation include: (i) mixing of different monovalent vaccines; (ii) conjugation of more distinct TACAs to the same carrier; (iii) displaying of various TACAs on a polypeptide backbone that is subsequently coupled to a suitable carrier *(Slovin et al., 2005a; Niederhafner et al., 2008).*

A heptavalent vaccine containing GM2, Globo H, TF, STn, Tn, $Le^y$, and glycosylated MUC1, which were bound separately to one KLH molecule, induced the formation of antibodies reactive with all seven antigens, but their titres were comparable to those elicited by individual monovalent KLH-conjugates *(Ragupathi et al., 2003).* On the other hand, the pentavalent vaccine, where TACAs (Globo H, TF, STn, Tn, and $Le^y$) formed a part of one glycopeptide linked to KLH, elicited higher antibody titres compared to the pentavalent unconjugated vaccine (either alone or mixed with KLH). This pentavalent vaccine also exerted a superior IgM response against four used antigens (except for Tn) than pooled monovalent KLH-conjugated vaccines. Thus, the latter of the above-mentioned strategies of polyvalent vaccine development (so called dendrimeric) is the most sophisticated and efficient *(Ragupathi et al., 2006; reviewed in Niederhafner et al., 2008).*

### 3.3.3.2. Keyhole limpet hemocyanin in cancer immunotherapy

Keyhole limpet hemocyanin (KLH) is a cooper-containing respiratory glycoprotein from the hemolymph of the marine mollusk *Megathura crenulata* that was shown to stimulate both the humoral and cell-mediated immunity *(reviewed in Harris and Markl, 1999).* KLH is used not only as an anticancer vaccine carrier, but it also represents a safe and highly effective immunotherapeutic agent for superficial bladder cancer *(Jurincic-Winkler et al., 1995a; 1995b; 2000; Lamm et al., 2000).* Clinical trials demonstrated that KLH was even superior to mitomycin C-based chemotherapy in preventing bladder tumor recurrence, while exerting no adverse local or systemic side effects *(Jurincic-Winkler et al. 2000).*

Furthermore, KLH inhibited proliferation of breast, prostate, pancreas, oesophagus, and melanoma cancer cell lines *in vitro (Riggs et al., 2002; McFadden et al., 2003; McFadden et al., 2007).* Recently, *Rizvi et al. (2007)* reported that KLH delayed incidence of HTB68 human melanoma in athymic mice. In addition, when administrated in a combination with IFN-α, one of the standard cancer immunotherapy, it augmented its potential to reduce the tumor growth.

## 3.4. *N*-ACETYL-D-GLUCOSAMINE-SUBSTITUTED GLYCOCONJUGATES

Monosaccharides of *N*-acetyl-hexosamine type were reported to be capable of binding to the recombinant rat NKR-P1A molecule with affinity decreasing in the order ManNAc > GalNAc > GlcNAc >> TalNAc. Structural studies of NK cell receptors and their carbohydrate recognition domains revealed that the binding to NKR-P1 molecule was enhanced by the following ligand characteristics: (i) multivalency; (ii) linearity of oligosaccharide chain; (iii) joining of carbohydrate units by β (1→4) glycosidic linkage; (iv) presence of an aromatic subunit; or (v) negatively charged groups. Further possible factors influencing NKR-P1 receptor – ligand interactions include rigidity, density, and spacing of carbohydrate components as well as environmental conditions such as local pH (e.g. NKR-P1 is tightly associated with $Ca^{2+}$ at neutral pH, whereas it can be completely decalcified at pH = 10 resulting in the loss of carbohydrate binding). These observations contribute to the synthesis of functionally more potent NKR-P1 ligands, chemically defined multivalent glycoconjugates such as glycodendrimers *(Bezouska et al., 1994a; 1994b; Pospisil et al., 1995; Bezouska et al., 1997; Krist et al., 2001; Bezouska 2002; Lindhorst 2002; Pavlicek et al., 2004).*

For our studies, we have chosen GlcNAc-substituted glycoconjugates with the polyamidoamine (GN8P) or calix[4]arene (GN4C) scaffold. GlcNAc represents a common component of biological structures (e.g. extracellular matrix) that is, among others, crucial for protein and lipid glycosylation (including aberrant one during carcinogenesis). Moreover, carbohydrate sequences based on GlcNAc were shown previously to induce NK cell-mediated cytotoxicity *(Pospisil et al., 2000).* In contrast, the binding affinity of GalNAc oligomers to NKR-P1 molecule is diminished by the presence of β (1→6) glycosidic linkage, although GalNAc by itself was found to be its better ligand than GlcNAc. Furthermore, GalNAc do not appear to be suitable for calixarene substitution probably due to steric barriers. As for ManNAc, the synthesis of β-ManNAc linkages is considered to be one of the most complicated tasks in the carbohydrate chemistry *(Bezouska et al., 1994b; Krist et al., 2001; Krenek et al., 2007).*

### 3.4.1. *N*-ACETYL-D-GLUCOSAMINE-SUBSTITUTED PAMAM DENDRIMERS

Dendrimers, in general, are multi-antennary molecules usually constructed from their core to the surface (divergent synthesis) by assembling of basic modules (generation zero – G0) in a

radial, branch-upon-branch motif (G1, G2, etc.), *(Figure 15)*. These synthetic structures are used as carriers in drug delivery or gene transfection for the following reasons: (i) high level of possible control over their architectural design (size, shape, branching length/density, surface functionality); (ii) close matching of the size or scaffold of many important proteins (e.g. haemoglobin, histone clusters), DNA, etc.; (iii) relative non-toxicity and non-immunogenicity. The bioactive agents can be either encapsulated into the interior of dendrimers or chemically attached/physically absorbed onto their surface *(reviewed in Svenson and Tomalia, 2005).*

PAMAM dendrimers belong to the first complete dendrimer family (G0 – G7) to be synthesized and characterized, followed by their therapeutic applications. For instance, encapsulation of cisplatin within PAMAM dendrimers gave conjugates that exhibited slower release, higher accumulation in solid tumors, and lower toxicity compared to the free form of this anticancer drug *(Malik et al., 1999).*

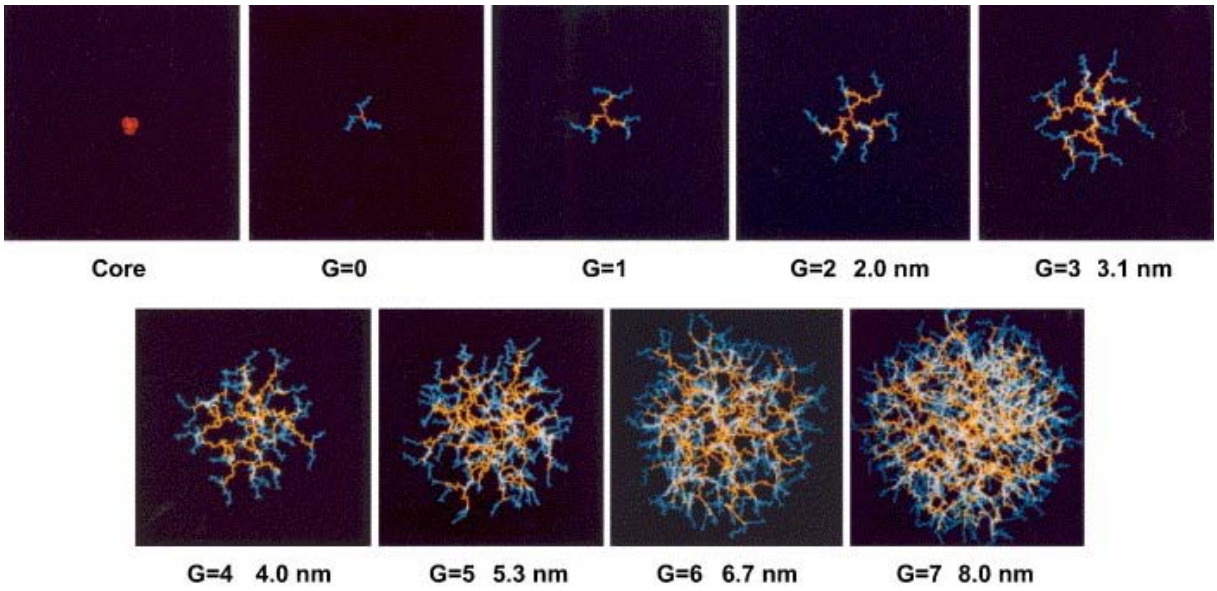



**Figure 15: The divergent synthesis of PAMAM dendrimers** (from the core to the generation 7 (G = 7) showing the linear increase in diameter and exponential growth of the surface group number; adapted from *Svenson and Tomalia, 2005*).

Dendrimers functionalized with a "glyco-coat" (glycodendrimers) were designed to mimic natural hyperbranched structures such as bacterial and tumor-associated glycoconjugates

*(Rockendorf and Lindhorst, 2001; Bertozzi and Kiessling, 2001)* or, as discussed above, to constitute potential ligands for NKR-P1 receptor. Plate inhibition assays confirmed that GlcNAc-substituted PAMAM dendrimers (tri-, tetra-, hexa-, and octavalent) exerted high binding affinity to the recombinant rat NKR-P1A molecule and identified the octavalent construct (GN8P) to be the best ligand probably as a consequence of spacing of GlcNAc units in its structure *(Bezouska et al., 1998)*. Later on, GN8P was also proved to bind to the recombinant mouse NKR-P1C$^{B6}$ protein *(Rozbesky, 2009)*.

**GN8P** is a symmetric molecule based on the first generation (G1) polyamidoamine scaffold with four branches per a side, each one terminated with thiourea-bridged GlcNAc *(Figure 16A)*. Presence of non-natural thiourea bridges in the structure confers upon this compound resistance to enzymatic cleavage by hydrolases, which is crucial for its stability when applied *in vivo (Lindhorst and Kieburg, 1996)*. GN8P was shown previously to increase natural killing of tumor cell targets *in vitro*. Furthermore, it delayed incidence of colorectal carcinoma in rats, and reduced the tumor growth and prolonged survival time of treated B16F10 melanoma-bearing mice. This was accompanied by the tumor infiltration with activated NK cells *(Pospisil et al., 2001; Vannucci et al., 2003)*.

### 3.4.2. *N*-ACETYL-D-GLUCOSAMINE-SUBSTITUTED CALIX[4]ARENES

Calix[4]arenes represent a versatile class of compounds, often being used as scaffolds in various supramolecular structures mainly due to the well-defined geometrical conformation of their functional groups, easy availability and work-up, even in large-scale reactions *(Vicens and Bohmer, 1991; Asfari et al., 2001)*.

The introduction of calix[4]arenes in glycoconjugates as a novel scaffold bearing sugar units enabled better control of three-dimensional structure, which can be achieved either by changing their conformation or by variation of calix[4]arene-sugar spacer *(Krenek et al., 2007)*. Besides this, they contain polyaromatic system, which often increases the binding affinity of carbohydrate ligands to their respective receptors via nonspecific interactions *(Krist et al., 2001)*.

A series of calix[4]arenes substituted with GlcNAc linked by a thiourea bridge were synthesized and subsequently tested for binding activity to the recombinant NKR-P1 molecule.

The best ligand, tetravalent GlcNAc-substituted calix[4]arene (GN4C), *(Figure 16B)* was used in further experiments to investigate its immunomodulatory effects.

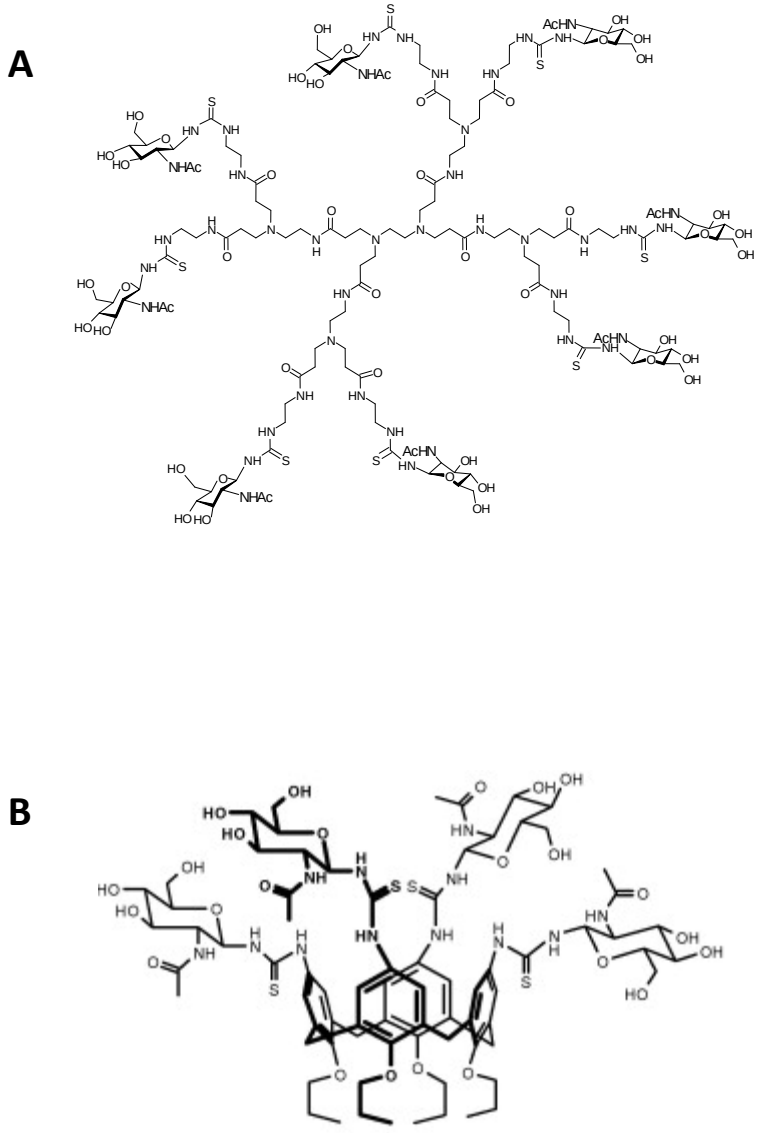



**Figure 16: Octavalent *N*-acetyl-D-glucosamine-substituted polyamidoamine dendrimer (A) and tetravalent *N*-acetyl-D-glucosamine-substituted calix[4]arene (B)** (formula drawn by Lindhorst TK, adapted from *Krenek et al., 2007*, respectively).

# 4. MATERIALS AND METHODS

## 4.1. STIMULI

### 4.1.1. GLYCOCONJUGATES

*N*-Acetyl-D-glucosamine-substituted polyamidoamine dendrimer (GN8P) kindly provided by Prof. T.K. Lindhorst (Christiana Albertina University in Kiel, Germany) was synthesized as described previously *(Lindhorst and Kieburg, 1996).* Briefly, the reaction between PAMAM-dendrimer of generation 1 bearing eight peripheral amino groups and 2,3,4,6-tetra-*O*-acetyl-β-*N*-acetyl-glucosaminyl isothiocyanate resulted in *O*-acetylated isothiourea-bridged glycodendrimer formation. After deacetylation, using sodium methoxide in methanol, and subsequent purification by gel permeation chromatography, GN8P was obtained as a white water-soluble lyophilisate.

*N*-Acetyl-D-glucosamine-substituted calix[4]arene (GN4C) was prepared as described in detail in *Krenek et al. (2007*) and kindly provided by Prof. V. Kren (Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague). The structure and purity of glycoconjugates were confirmed by mass spectrometry as well as nuclear magnetic resonance (*Krenek et al., 2007; Hulikova et al., 2009*).

### 4.1.2. OTHER STIMULI

Sheep red blood cells (SRBCs) were purchased from Biotest (Prague, Czech Republic), KLH and poly (I:C) from Sigma-Aldrich (St. Luis, MO, USA), and DNP-LPS from Biosearch Technologies (Novato, CA, USA).

## 4.2. PLATE INHIBITION ASSAYS

Plate inhibition assays were performed as described in *Bezouska et al. (1994b)*. Briefly, microtiter plates Immulon 4 (Dynatech Labs Inc., Chantilly, VA, USA) were coated with $GlcNAc_{23}BSA$ (1 µg/well; Sigma-Aldrich). Wells were blocked with 2% BSA-PBS for 2 hours at 4 °C. Then, $^{125}I$-labelled rat NKR-P1A or human CD69 receptor (specific activity: 1 MBq/µg of the protein) was added and incubated in the presence of indicated concentrations of tested inhibitors (GN4C, other GlcNAc-substituted calix[4]arenes, calix[4]arene scafold, GlcNAc) for 2 hours at 4 °C. Plates were washed four times with cold (4 °C) PBS, drained and dried. Radioactivity in each well was counted in the presence of 100 µL of Biodegradable Counting Scintillant (GE Healthcare, Fairfield, CT, USA) using Microbeta Trilux counter (Wallac, Turku, Finland). Nonspecific counts in wells not coated with $GlcNAc_{23}BSA$ were subtracted as a background. The experimental counts were related to maximal counts in wells without inhibitors to calculate % binding inhibition in individual wells. $IC_{50}$ concentrations (compound concentration required for 50% binding inhibition) were extrapolated on the complete inhibition curve containing experimental points from 3 duplicate measurements.

## 4.3. EXPERIMENTAL ANIMALS

Eight-week-old inbred C57BL/6, DBA/2, BALB/c mice (AnLab, Prague, Czech Republic), and F1 hybrids (C57BL/6 x BALB/c, C57BL/6 x DBA/2; Division of Immunology and Gnotobiology, Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague) were housed under natural day/night conditions (22 °C, 55% relative humidity), and fed on a commercial ST1 diet (Velaz, Prague, Czech Republic) *ad libitum*. All procedures were conducted in accordance with the European Convention for the Care and Use of Laboratory Animals as approved by the Czech Animal Care and Use Committee.

## 4.4. TUMOR CELL LINES

Established cell lines: B16F10 (mouse melanoma), CT26 (mouse colorectal carcinoma), P815 (mouse mastocytoma), YAC-1 (NK cell-sensitive mouse lymphoma), IC-21 (mouse macrophages transformed by Simian virus 40), and K562 (NK cell-sensitive human chronic

myeloid leukaemia), purchased from American Type Culture Collection (via Teddington, UK), were cultivated in RPMI-1640 medium (Sigma-Aldrich) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.05 mM 2-sulfanylethanol (2-mercaptoethanol), antibiotics (0.05 mg/mL gentamycin, 25 mg/mL amphotericin B) and 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY, USA). The incubation was performed in humidified atmosphere containing 5% $CO_2$ ($CO_2$ incubator; Jouan, St. Herblain, France).

## 4.5. TUMOR CELL INOCULATION

C57BL/6, BALB/c, or DBA/2 mice were injected s.c. with $10^6$ B16F10, CT26, and P815 cells/mouse in 0.1 mL phosphate buffered saline (PBS), respectively, into the lower back on day 0.

## 4.6. SURVIVAL EXPERIMENTS

C57BL/6 or BALB/c mice (10 per group) were injected with B16F10 melanoma and CT26 colorectal carcinoma cells as described above. GN8P (3 µg/mouse), GN4C (1.5 µg/mouse), and KLH (150 µg/mouse) were administrated i.p. either alone or as a combination of the glycoconjugate with KLH (i.e. GN8P+KLH, GN4C+KLH) every 3 days starting on day 11 after tumor inoculation (six doses of total). Control animals were treated with PBS by the same route. The tumor dimensions (length and width) were measured with a caliper every 2 – 3 days until all controls died. The tumor growth rate (the mean daily increase of the melanoma largest dimension) was calculated for each animal as the difference between the last and the first measurement divided by the time interval. At the end of the experiment (death of all experimental animals), curves of survival were drawn. During survival experiments, the peripheral blood was withdrawn from mouse tails (on day 27) to perform cytotoxicity assay, and evaluate tumor-specific antibody response.

## 4.7. EXPERIMENTAL DESIGN OF IMMUNOLOGICAL ASSAYS

If not specified otherwise, healthy and tumor-bearing mice were injected i.p. with 3 doses of GN8P (3 µg/mouse) or GN4C (1.5 µg/mouse) every 3 days starting on day 1 or 11, respectively. Immunization with KLH (100 µg/mouse) or DNP-LPS (50 µg/mouse) was performed on day of the first treatment with GN8P. PBS was given to control animals in the same time intervals. Poly (I:C) was administrated i.p. on day 1 (120 µg/mouse). Healthy and tumor-bearing animals were bled on day 8 and 18, respectively.

## 4.8. ISOLATION OF MONONUCLEAR CELLS

Spleens removed from experimental animals into tubes with H-MEMd medium (Sebac, Aidenbach, Germany) were squeezed through a nylon mesh and layered onto Ficoll-Hypaque density gradient (1.086), (Sigma-Aldrich). After 30 minute-long centrifugation, spleen mononuclear cells (SMCs) forming “a ring” on the border between Ficoll-Hypaque and medium were pulled out by a pipette, washed three times, counted, diluted to the desired concentration in RPMI-1640 medium, and used immediately for immunological assays or the cell sorting.

EDTA or citrate anti-coagulated human peripheral blood samples were collected from healthy donors from the Blood Transfusion Service (Prague, the Czech Republic). Peripheral blood mononuclear cells (PBMCs) were separated on Ficoll-Hypaque gradient similarly as mouse SMCs.

## 4.9. CELL SORTING OF NK AND CYTOTOXIC T CELLS

SMCs isolated from 3 mice of the identical experimental group, were collected (50 million cells/sample), and depleted of B cells using anti-CD19 (1D3) mAb conjugated with biotin, tetrameric antibody complex (anti-biotin, anti-dextran), and EasySep dextran coated magnetic particles (Stem Cell Technologies, Vancouver, Canada). The B cell depleted suspension was further stained with anti-CD49b-FITC (DX5) and anti-CD8a-PE (53-6.7) mAbs for positive selection of desired cell populations. Two-way sorting by FACS Vantage SE (Becton-

Dickinson) was applied to obtain purified NK cells (CD49b+/CD8a-) and CTLs (CD49b-/CD8a+) for cytotoxic assays. The purity of the corresponding populations was 97 – 99% after re-analysis.

## 4.10. CYTOTOXICITY ASSAYS

### 4.10.1. STANDARD CYTOTOXICITY ASSAY

The cytotoxic activity was estimated using the standard $^{51}$Cr-release assay. Effectors: mouse peripheral blood cells (PBCs), SMCs, sorted CTLs and NK cells or human PBMCs were seeded in pentaplicates into round-bottomed 96-well microtiter plates (NUNC, Roskilde, Denmark). Subsequently, $10^4$ tumor targets (B16F10, YAC-1, IC-21 cells for mouse model, or K562 cells for human system) labelled for 90 minutes with $Na_2{}^{51}CrO_4$ were added. After 3.5 and 18 hour-long incubation at 37 °C in humidified atmosphere containing 5% $CO_2$, the cell free supernatants were harvested (0.025 mL/sample), 0.1 mL of scintillation cocktail (SuperMix; Wallac) was added, and the radioactivity measured employing scintillation counter Microbeta Trilux (Wallac). The percentage of cytotoxicity was calculated according to the following formula: cytotoxicity [%] = 100 x (experimental cpm – spontaneous cpm) / (maximum cpm – spontaneous cpm). For spontaneous and maximal release controls, tumor targets were cultured alone or with 10% Triton X-100 (Sigma-Aldrich), respectively (*Fiserova et al. 2002*).

### 4.10.2. ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY

$Na_2{}^{51}CrO_4$-labelled B16F10 tumor targets were seeded in pentaplicates into round-bottomed 96-well microtiter plates ($10^4$ cell/well), and pre-incubated with 1:10 diluted sera from PBS, GN8P, GN4C, KLH, KLH+GN8P, or KLH+GN4C treated B16F10 melanoma-bearing C57BL/6 mice (4 °C for 20 min). Subsequently, SMCs isolated from spleens of intact animals were added as effectors. After 3.5 hour-long incubation, at 37 °C in humidified atmosphere

containing 5% $CO_2$, the cell free supernatants were harvested and the percentage of cytotoxicity was calculated, as described above.

## 4.11. FLOW CYTOMETRIC ANALYSIS

### 4.11.1. CELL SURFACE MARKER EXPRESSION

Cell suspensions prepared from spleens of individual mice (as described above) were re-suspended in PBS with 0.02% cold water fish skin gelatin and 0.01% sodium azide. The phenotype of cells was determined using the following monoclonal antibodies (mAbs) against surface markers of B cells: anti-CD45R/B220-Alexa405 (RA3-6B2); plasma cells: anti-CD138-biotin (281-2), T cells: anti-CD3-PECy5 (17A2), anti-CD8a-Alexa405, anti-CD4-biotin (H129.19); NK and NKT cells: anti-CD49b-FITC (DX5), anti-NK1.1-PECy7 (PK136). The expression of activation antigens on B cells was evaluated by means of anti-CD80-biotin (1G10), anti-CD86-APC (RMMP-2), and anti-I-A/I-E-PE (anti-MHC class II molecules; 2G9) mAbs. They were purchased either from Pharmingen (San Diego, CA, USA) or Caltag (San Francisco, CA, USA). The mixture of four to six mAbs was added to cells (30 min on ice). The cell surface markers labelled with biotinylated mAbs in the first step were detected with streptavidine-APC-Cy7 (Pharmingen). Samples were analyzed by LSRII cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA). The evaluation of measured data was performed using FlowJo software (Tree Star, Ashland, OR, USA). The percentage of positive cells was calculated from propidium iodide (Becton-Dickinson) negative cell population (live cells).

### 4.11.2. SERUM CYTOKINE LEVELS

For evaluation of serum cytokine levels, BD™ Cytometric Bead Array (Mouse Th1/Th2 cytokine kit; BD Bioscience, San Jose, CA, USA) was used. This assay is based on mixed bead populations with distinct fluorescence intensities, which are coated with capture antibodies specific for IL-2, IL-4, TNF-α, and IFN-γ. The samples were prepared according to standard manufacturer's protocol. Briefly, serum or standard sample was mixed with bead populations

and PE-conjugated detection antibody, and incubated in dark for 2 hours to form sandwich complexes. After washing, the samples were measured by LSRII cytometer. The evaluation of data was performed using FlowJo software. The cytokine concentration in pg/ml was calculated from calibration curves of standards.

### 4.11.3. INTRACELLULAR IFN-γ LEVELS

SMCs isolated as described above, were incubated with Golgi Stop (0.7 µL/$10^6$ cells; Pharmingen) for 6 hours in humidified atmosphere containing 5% $CO_2$. Then, the cells were stained with anti-CD3-PECy5 and anti-NK1.1-PECy7 mAbs (30 min on ice). After the cell membrane permeabilization with FACS$^{TM}$ permeabilizing solution (30 µl/well; 10 min in dark at room temperature; Becton-Dickinson), the cells were labelled with anti-IFN-γ mAb conjugated with biotin (30 min on ice). Subsequently, streptavidine-PE was added (30 min on ice). After each step, the cells were 2 times washed in PBS with 0.02% cold water fish skin gelatin and 0.01% sodium azide. Finally, the cells were fixed with 1% paraformaldehyde (Lachema, Brno, Czech Republic). The samples were measured by FACS LSRII. The evaluation of data was performed using FlowJo software.

### 4.11.4. TUMOR CELL-SPECIFIC ANTIBODIES

B16F10 cells were seeded in round-bottomed 96-well microtiter plates (2x$10^5$ cells/well), and washed in PBS with 0.02% cold water fish skin gelatin (Sigma-Aldrich). Then, 1:10 diluted sera from individual tumor-bearing mice (treated as described in *Chapter 4.6.*) were added (100 µl/well, 30 min on ice). The cells were blocked with 100 x diluted goat serum (10 µl/well, 20 min on ice) to avoid non-specific binding of secondary antibody, and stained with goat anti-mouse IgG-PE (100 x diluted; Immunotech, Marseille, France). After each step, the plates were extensively washed. B16F10 cells not incubated with serum and those incubated with sera from healthy animals were used as negative controls. Finally, the samples were analyzed by LSRII cytometer. The evaluation of measured data was performed using FlowJo software. The data were expressed as mean fluorescence intensity (MFI) of the live cells (Hoechst 33342-negative).

## 4.12. IMMUNOGLOBULIN ELISA

### 4.12.1. *EX VIVO* EXPERIMENTS

Sera obtained from mice immunized with KLH or DNP-LPS in the presence or absence of GN8 (as described in *Chapter 4.7.*) were collected and stored at -20 °C, until levels of anti-KLH or anti-DNP specific antibodies were measured by ELISA.

Flat-bottom, 96-well microtiter plates were coated with either KLH or DNP-BSA at the concentration of 10 μg/mL in 0.1 M $NaHCO_3$ (100 μL/well) and incubated overnight at 4 °C. Then, plates were washed (3x with Tween-PBS; 2x with PBS) and blocked with 5% BSA-PBS (22 °C, 2 h). Subsequently, three dilutions of each serum sample were added to the plate in duplicate (100 μl/well). After overnight incubation (4 °C) and extensive washing, Igs were detected with horseradish peroxidase-conjugated goat anti-mouse IgM, IgG or IgG2a (Jackson Immunoresearch Laboratories, West Grove, PA, USA). The reaction was stopped by adding 1M $H_3PO_4$. Plates were developed using TMB substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA), and the optical density (OD) read at 450 nm by automated ELISA reader (Rainbow Thermo; Tecan Spectra, Salzburg, Austria). The sera from non-immunized mice were used as negative controls.

### 4.12.2. *IN VITRO* EXPERIMENTS

SMCs from healthy C57BL/6, BALB/c, and DBA/2 mice were isolated as describe above. Those of C57BL/6 origin were left intact or depleted either of CD49b-positive or NK1.1-positive cell subpopulations by FACS Vantage SE (Becton-Dickinson) using anti-CD49b-FITC or anti-NK1.1-PECy7 mAb. SMCs, CD49b-negative, and NK1.1-negative cells were cultured in triplicate with DNP-LPS (1 μg/mL) in the presence or absence of 10 nM GN8P in RPMI-1640 medium supplemented with 2mM L-glutamine, gentamycin, 10% heat-inactivated fetal calf serum, and essential amminoacids (Gibco). Supernatants were harvested after a 5 day-long incubation at 37 °C in humidified atmosphere containing 5% $CO_2$. IgM levels were determined by ELISA as described above.

## 4.13. PLAQUE FORMING CELL ASSAY

Five days before the end of experiment, mice were immunized i.p. with 5% SRBCs in 0.5 ml PBS. The first dose of GN8P (3 µg/mouse) or PBS (in control animals) was administered on the day of immunization. The total treatment comprised two doses (on day 1 and 4).

The absolute number of antibody forming cells in the spleen was evaluated by modified Jerne's plaque forming cell (PFC) assay *(Sterzl and Mandel, 1964)*. Briefly, isolated SMCs (2.5 x $10^6$ cells/mL) re-suspended in RPMI-1640 medium were added to the mixture composed of melted agarose (Fluka Chemie AG, Buchs, Switzerland), 5% human serum albumin (Sigma-Aldrich), and 5% SRBC suspension, and pipetted onto Petri dishes, which were subsequently incubated at 37 °C for 2 hours. Hemolytic plaques were counted after incubation (37 °C, 30 min) with 1:9 diluted guinea pig complement (Sigma-Aldrich). To assess the number of IgG forming cells, anti-mouse IgG serum (Sigma-Aldrich) was added to Petri dishes prior to guinea pig complement. The results were expressed as the number of PFCs per spleen ($10^8$ splenocytes).

## 4.14. $^3$H-THYMIDINE PROLIFERATION ASSAY

Human PBMCs re-suspended in RPMI-1640 medium were seeded in tetraplicates into round-bottomed 96-well microtiter plates (2 x $10^5$ cells/well) and cultured in the presence or absence of the glycoconjugate (GN8P or GN4C) at the concentration of $10^{-4}$ – $10^{-9}$ mol/L at 37 °C in humidified atmosphere containing 5% $CO_2$. Two days later, $^3$H-thymidine (18.5 kBq/well; GE Health-Care Life Sciences, Amersham, UK) was pipetted to each well and allowed to incubate for additional 16 hours. The cells were harvested using 96-well harvester (Tomtec, Hamden, CT, USA), on glass fibre filters (Walac) and Melt-on scintillator sheets (Melti Lex$^{TM}$A; Walac) sealed in plastic sample bags, and analyzed on scintillation counter Microbeta Trilux. Results were expressed as stimulation index (SI = cpm experimental/cpm control).

## 4.15. PCR GENOTYPING

Genomic DNA for genotyping was obtained from mouse tails. Typical 3mm of mouse tails were incubated with lysis buffer containing 100 mM Tris-HCl, 5 mM EDTA, 0.5% SDS,

200 mM NaCl, and 40 µg ProteinaseK per tail (Qiagen, Hilden, Germany) in 55 °C for 4 hours. Homogenous samples were boiled at 95 °C for 10 minutes, diluted by water and used as a template for PCR. Amplification was carried out with 0.5 U of HotStarTaq$^{(R)}$ DNA polymerase (Qiagen), 400 nM primers, and an iCycler5 (Bio-Rad, Hercules, CA, USA). Gene for β-actin was used as a load control. Primers for amplification were following *β-actin* Forward: 5´*agagggaaatcgtgcgtgac* 3´, *β-actin* Reverse: 5´ *acggccaggtcatcactattg* 3´; *Nkr-p1a* F: 5´*acaagtaggggctgtgatgg* 3´, *Nkr-p1a* R: 5´*ctgaaaaccctgctgaaagc* 3´; *Nkr-p1b* and *d* F: 5´*agggagcaggaagagaggac* 3´, *Nkr-p1b* R: 5´*agtcttgtgggcactctaaa* 3´, *Nkr-p1d* R: 5´*agtcttgtgggcactctagc* 3´; *Nkr-p1f* F: 5´ *tctgaaatctggctgtgctg* 3´, and *Nkr-p1f* R: 5´ *tgggacttttgggttctttg* 3´. Two alleles of *Nkr-p1c* (allele *(A)* in BALB/c, and *(T)* in C57BL/6 mice) were distinguished by allele-specific PCR using common reverse primer: 5´*tgctttcagagtccatgtgc* 3´, forward primer for *Nkr-p1c(A)*: 5´ *gaaaatggcagctgtgcca* 3´, and forward primer for *Nkr-p1c(T)*: 5´*gaaaatggcagctgtgcct* 3´. Primers were designed in our laboratory using Primer3 Input software.

## 4.16. REVERSE TRANSCRIPTION (RT)-PCR AND REAL-TIME RT-PCR

Total RNA from SMCs was isolated using RNeasy Mini Kit (Qiagen). Five micrograms of RNA were transcribed into cDNA using cDNA Archive Kit (Applied Biosystem, Foster City, CA, USA). RT-PCR was performed with HotStarTaq DNA Polymerase (Qiagen) and iCycler5 (Bio-Rad). Real-time RT-PCR was carried out with FastStart SYBR Green Master (Roche, Mannheim, Germany) and iCycler5. PCR product specifity was checked by melt curve analysis. Primer sequences used for amplification were following *IFN-γ* F: 5´ *tcaagtggcatagatgtggaagaa* 3´, *IFN-γ* R: 5´ *catgaaaatcctgcagagcca* 3´; *IgG2a* F: 5´ *tgcaaggtcaacaacagagc* 3´*; IgG2a* R: 5´ *ggtccagtccacagcaattt* 3´. Primers were designed in our laboratory using Primer3 Input software. The gene of interest was normalized to the control gene β-2-microglobulin or 18S rRNA. Differences in gene expression between individual groups of mice were evaluated with Bio-Rad iQ5 2.0 software.

## 4.17. STATISTICAL ANALYSIS

The statistical significance of differences between two groups was calculated by Student's t-test and between more groups by one-way analysis of variance (ANOVA). P values ≤ 0.05 were considered as significant (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

# 5. RESULTS

## 5.1. THE ROLE OF *Nkr-p1c* GENE DIVERGENCE IN GN8P-MEDIATED ACTIVATION OF NK CELLS

GN8P was shown previously to bind to the rat recombinant NKR-P1A molecule *(Chapter 3.4.1)* that represents an ortholog to mouse NKR-P1C (NK1.1) isoform. However, C57BL/6 and BALB/c mouse strains differ from each other in NK1.1 expression, which is caused by *Nkr-p1c* gene divergence, leading to several differences in amino acid sequence of NKR-P1C receptor. A single amino acid substitution is responsible for the fact that anti-NK1.1 (PK136) mAb binds to NKR-P1C$^{B6}$, but does not to NKR-P1C$^{BALB/c}$ or NKR-P1C$^{DBA/2}$ *(Chapter 3.1.2.1.2.)*. In view of this, we investigated whether *Nkr-p1c* gene divergence between mouse strains (C57BL/6 and BALB/c were chosen as prototypes) can influence GN8P effects on NK cell functions.

### 5.1.1. *Nkr-p1* GENE FAMILY DISTRIBUTION AND NK1.1 EXPRESSION IN EXPERIMENTAL MICE

We verified distribution of *Nkr-p1* gene family (encoding individual NKR-P1 isoforms), *Nkr-p1c* gene divergence, and NK1.1 expression in mice used in our experiments by PCR genotyping and FACS analysis, respectively.

*Nkr-p1a* and *Nkr-p1f* genes were identified in all mouse strains. *Nkr-p1b* was of BALB/c and DBA/2, whereas *Nkr-p1d* of C57BL/6 origin. *Nkr-p1c* was present in two distinct gene forms indicated as allele A (*Nkr-p1c(A)*) in BALB/c as well as DBA/2 and as allele T (*Nkr-p1c(T)*) in C57BL/6 mice. F1 hybrids (C57BL/6 x BALB/c and C57BL/6 x DBA/2) inherited all *Nkr-p1* gene family members including *Nkr-p1c(A)* and *Nkr-p1c(T)* alleles *(Table 1)*.

FACS analysis showed that unlike C57BL/6 mice, DBA/2 and BALB/c ones were NK1.1-negative as they did not express the product of *Nkr-p1c(T)* gene form, which is recognized by

anti-NK1.1 mAb. F1 hybrids were NK1.1-positive, but with lower mean fluorescence intensity, when compared to C57BL/6 mice *(Table 1; Figure 1)*.

| Mouse strain | NK1.1 expression measured by FACS analysis | *Nkr-p1* gene family members | | | | | |
|---|---|---|---|---|---|---|---|
| | | *a* | *b* | *c*(A) | *c*(T) | *d* | *f* |
| C57BL/6 | high | + | - | - | + | + | + |
| BALB/c | negative | + | + | + | - | - | + |
| DBA/2 | negative | + | + | + | - | - | + |
| C57BL/6 x DBA/2 | low | + | + | + | + | + | + |
| C57BL/6 x BALB/c | low | + | + | + | + | + | + |

**Table 1: The relationship between distribution of *Nkr-p1* gene family members and NK1.1 expression in C57BL/6, BALB/c, DBA/2 mice, and F1 hybrids (C57BL/6 x DBA/2, C57BL/6 x BALB/c),** (presence or absence of *Nkr-p1* genes for individual NKR-P1 isoforms are indicated with + or -, respectively; adapted from *Hulikova et al., 2009*).

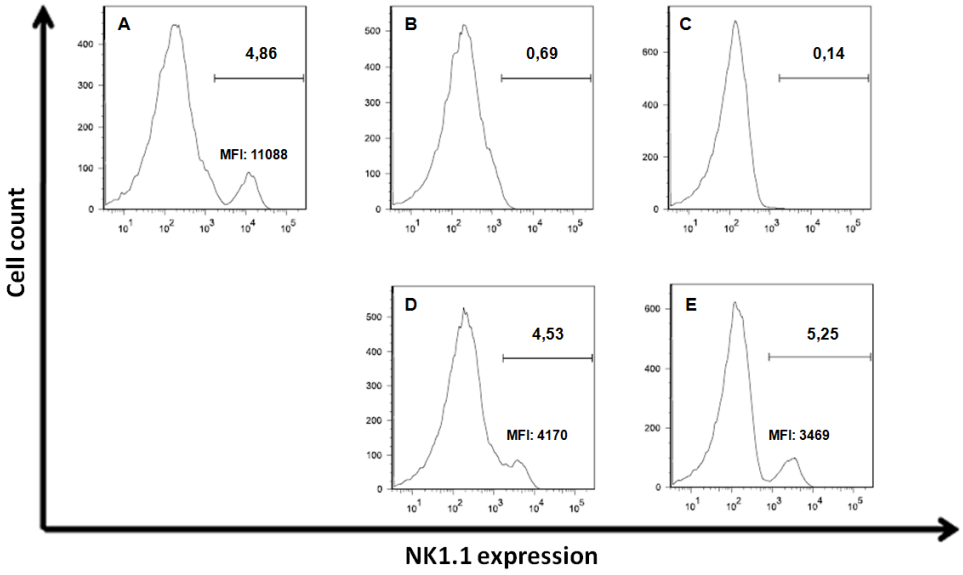



**Figure 1: NK1.1 expression in C57BL/6 (A), BALB/c (B), DBA/2 (C) mice, and C57BL/6 x BALB/c (D) and C57BL/6 x DBA/2 (E) F1 hybrids evaluated by FACS analysis.** Live, propidium iodide (PI)-negative cells with lymphocyte/monocyte morphology gated on the basis of FSC/SSC were analyzed for NK1.1 expression (adapted from *Hulikova et al., 2009*).

### 5.1.2. EFFECT OF GN8P ON NK CELL-MEDIATED CYTOTOXIC ACTIVITY

To determine the effect of GN8P on NK cell-mediated cytotoxicity, we performed the $^{51}$Cr-release assay using SMCs from healthy or syngeneic tumor-bearing C57BL/6, BALB/c, and DBA/2 mice as effectors against YAC-1 (NK cell-sensitive mouse lymphoma) tumor targets.

In general, C57BL/6 mice treated with GN8P showed significantly higher cytotoxic activity compared to controls (PBS group). However, healthy animals exerted greater increase than B16F10 melanoma-bearing ones ($p<0.01 – 0.001$ vs. $p<0.05 – 0.01$; *Figure 2A, B*). Instead, in BALB/c and DBA/2 mice, GN8P induced significant changes neither in healthy nor tumor-bearing animals *(Figure 2C – F)*.

### 5.1.3. EFFECT OF GN8P ON CYTOKINE SYNTHESIS

#### 5.1.3.1. Serum cytokine levels

To find out whether GN8P influences the cytokine secretion, we measured serum levels of major Th1 (IL-2, IFN-γ), Th2-type (IL-4), and pro-inflammatory (TNF-α) cytokines in C57BL/6 and BALB/c mice using FACS analysis.

In healthy C57BL/6 mice treated with GN8P, significant increase in serum levels of all tested cytokines ($p<0.01$, $p<0.001$, $p<0.001$, and $p<0.05$ for IL-2, IFN-γ, TNF-α, and IL-4, respectively) was detected, when compared to controls. IFN-γ and TNF-α, cytokines produced by NK cells, were influenced to the greatest extent, which indicates GN8P-induced NK cell activation. Tumor-bearing C57BL/6 mice, in response to GN8P administrations, showed significant enhancement only in IL-2 and IFN-γ levels ($p<0.05$ for IL-2 as well as IFN-γ), *(Figure 3A, B)*. On the other hand, we did not demonstrate significant effect of GN8P on the cytokine production in healthy or CT26 colorectal carcinoma-bearing BALB/c mice *(Figure 3C, D)*.

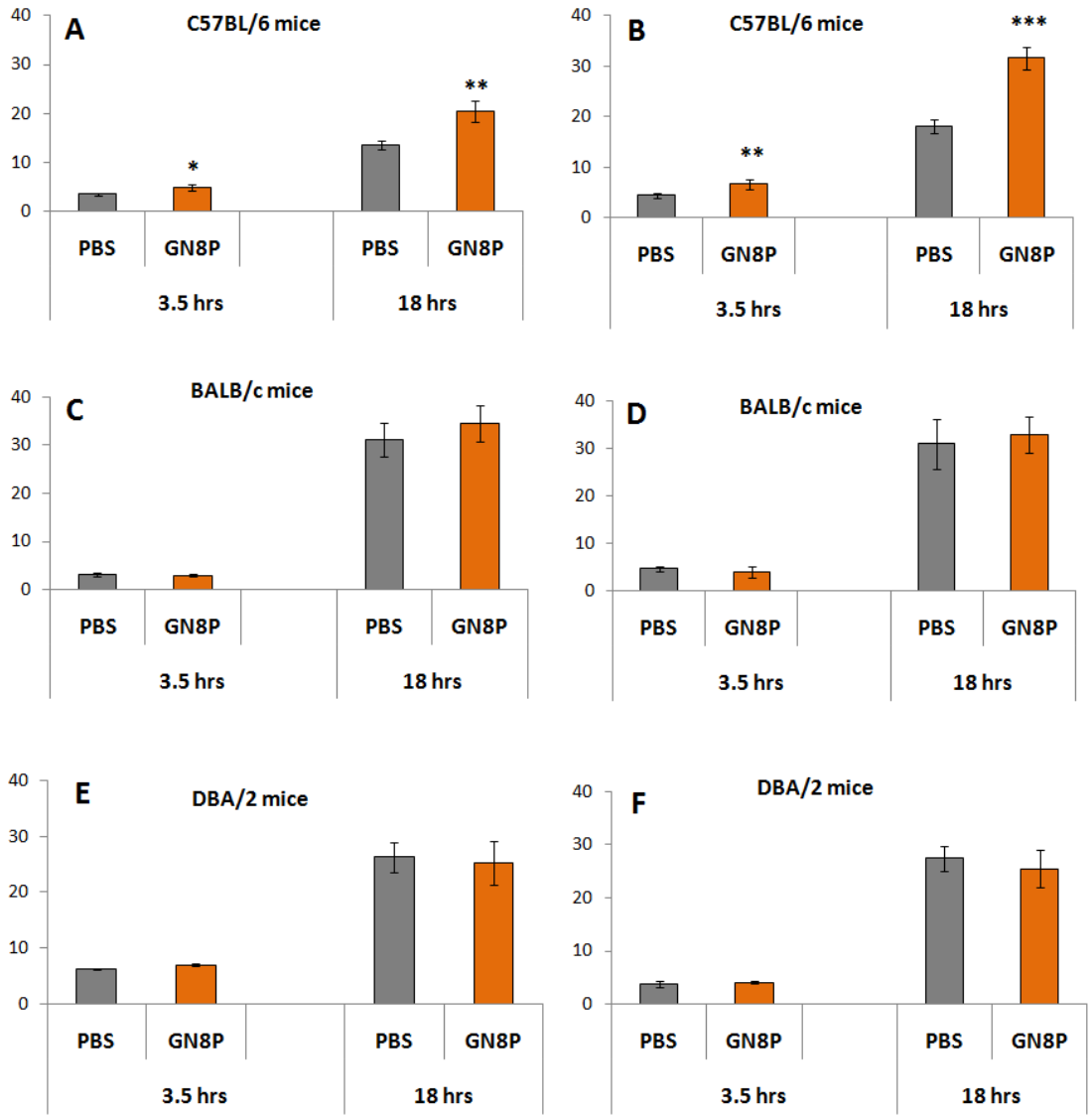



**Figure 2: Effect of GN8P on NK cell-mediated cytotoxic activity in tumor-bearing (A, C, E) and healthy (B, D, F) C57BL/6, BALB/c, and DBA/2 mice.** Healthy and syngeneic tumor-bearing animals were injected with 3 doses of GN8P or PBS and bled 24 hours after the last treatment. The cytotoxicity was determined after 3.5 and 18 hour-long incubation of SMCs with NK cell-sensitive YAC-1 tumor targets (effector to target ratio was 32:1). Data represent average ± standard deviation (SD) of values from 3 individual experiments (3 – 5 animals per group). Significant changes between GN8P treated mice and respective controls (PBS group) are marked by asterisk * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

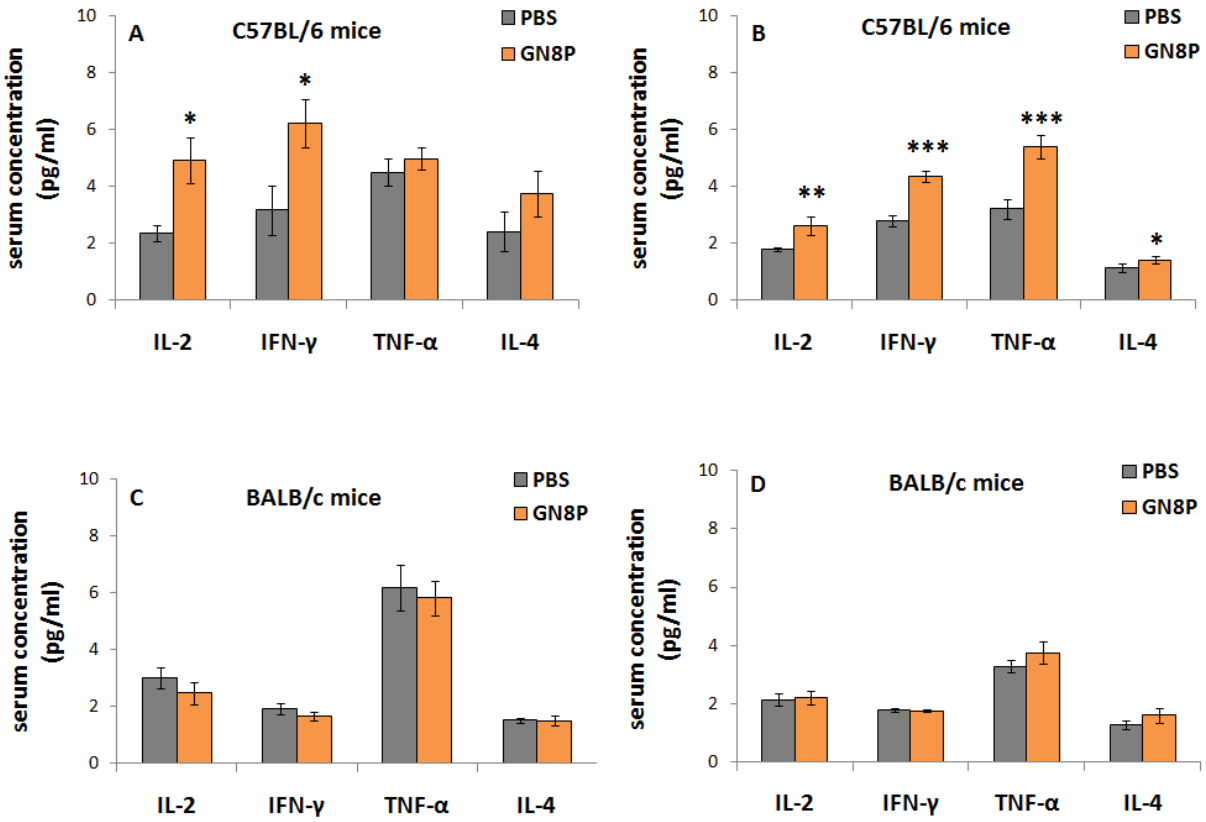



**Figure 3: Serum levels of IL-2, IFN-γ, TNF-α, and IL-4 in tumor-bearing (A, C) and healthy (B, D) C57BL/6 and BALB/c mice.** Healthy and syngeneic tumor-bearing animals were injected with 3 doses of GN8P or PBS and bled 24 hours after the last treatment. Serum levels of cytokines were determined using BD™ Cytometric Bead Array (Mouse Th1/Th2 Cytokine Kit). The cytokine concentration in pg/ml was calculated from calibration curves of standards. Results are expressed as average ± SD of values from 3 individual experiments (5 animals per group). Significant changes between GN8P treated mice and respective controls (PBS group) are marked by asterisk * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

### 5.1.3.2. IFN-γ mRNA expression

Real-time RT-PCR revealed that GN8P augmented mRNA expression for IFN-γ in both healthy and B16F10 melanoma-bearing C57BL/6 mice, whereas in BALB/c ones, mRNA level for IFN-γ remained unchanged *(Figure 4)*.

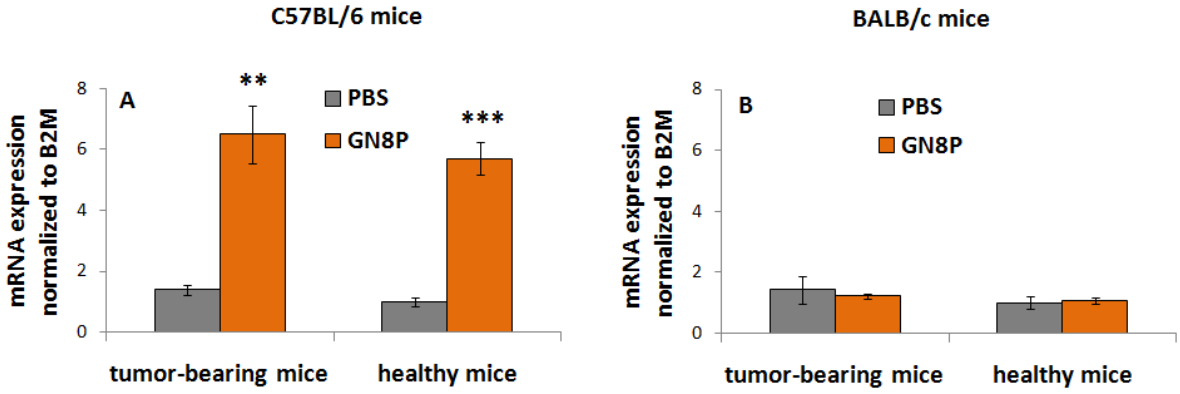



**Figure 4: Expression of mRNA for IFN-γ in tumor-bearing and healthy C57BL/6 (A) and BALB/c mice (B).** Healthy and syngeneic tumor-bearing animals were injected with 3 doses of GN8P or PBS and bled 24 hours after the last treatment. The total mRNA was isolated from SMCs of experimental mice. Level of mRNA for IFN-γ was determined by real-time RT-PCR and normalized to the expression of control gene β2-microglobulin (B2M). Results are expressed as average ± SD of values from individual mice (5 per group). Figure shows a representative example of three independent experiments with similar results. Significant changes between GN8P treated mice and controls (PBS group) are marked by asterisk * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

### 5.1.3.3. Cell subpopulations producing IFN-γ in C57BL/6 mice

To find out which cell subpopulations are involved in GN8P-induced enhancement of IFN-γ production in C57BL/6 mice, we measured intracellular levels of this cytokine using flow cytometry. Upon repeated GN8P administrations, significant increase of the percentage of IFN-γ-positive NK (CD3-/NK1.1+) and NKT (CD3+/NK1.1+) cells was observed in healthy ($p<0.001$ and $p<0.01$, respectively) as well as tumor-bearing ($p<0.01$ and $p<0.05$, respectively) animals *(Figure 5A, B)*. Counts of IFN-γ producing T cells (CD3+/NK1.1-) were elevated only in healthy mice, to a lesser extent comparing with NK and NKT cells *(Figure 5C)*. These results proved that NK cells represented potent producers of IFN-γ and that GN8P evoked the synthesis of this cytokine predominantly in NK1.1-positive cell subpopulations.

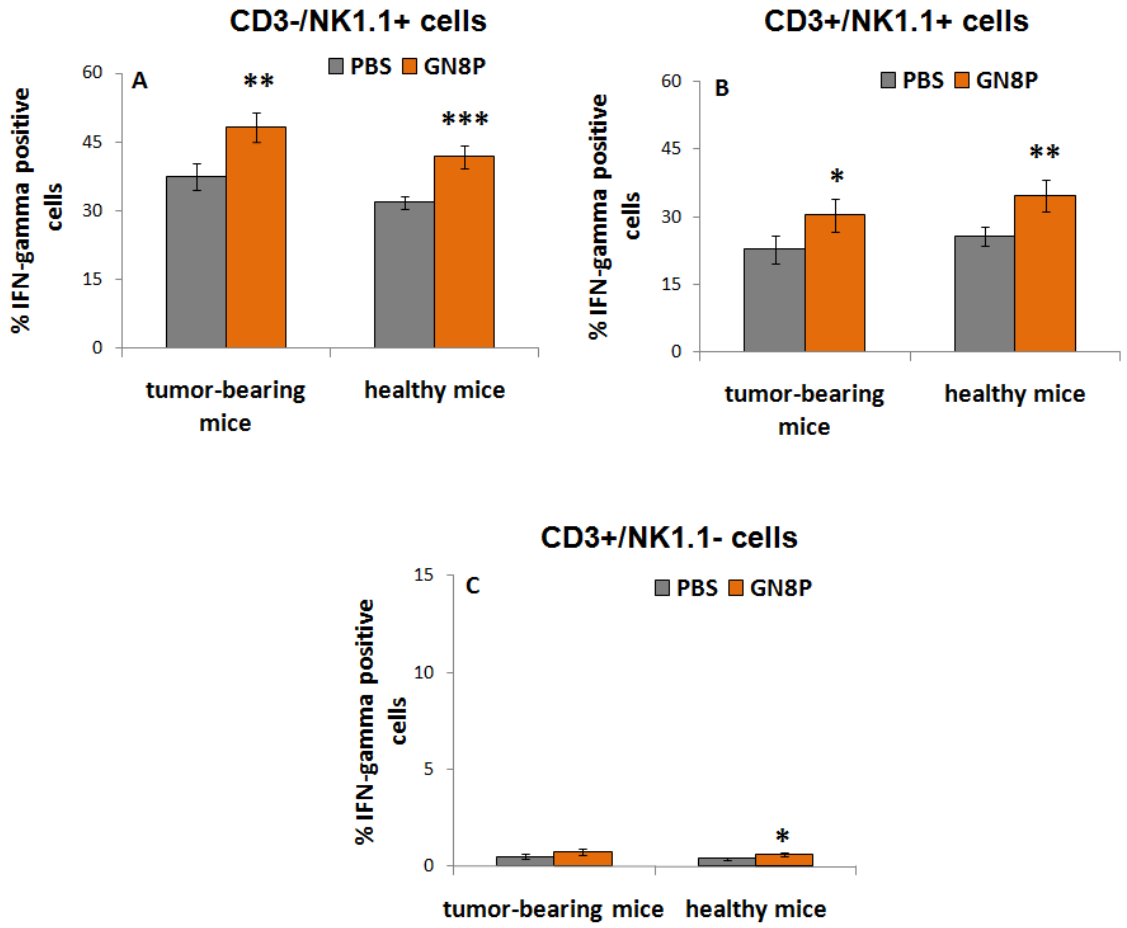



**Figure 5: Cell subpopulations producing IFN-γ in C57BL/6 mice.** Healthy and syngeneic tumor-bearing animals were injected with 3 doses of GN8P or PBS and bled 24 hours after the last treatment. SMCs with lymphocyte/monocyte morphology gated on the basis of FSC/SSC were at first analyzed for CD3 and NK1.1 expression. Then the percentage of IFN-γ-positive cells out of NK (CD3-/NK1.1+), NKT (CD3+/NK1.1+), and T cells (CD3+/NK1.1-) was determined. The data represent average ± SD of values from three performed experiments (5 mice per group). Significant changes between GN8P treated mice and controls (PBS group) are marked by asterisk * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

### 5.1.4. EFFECT OF GN8P ON SYNGENEIC TUMOR GROWTH

To find out whether GN8P anticancer properties are dependent on the mouse strain used as experimental model, we followed the tumor growth in C57BL/6 and BALB/c mice inoculated with B16F10 melanoma and CT26 carcinoma cells, respectively.

The tumor dimensions were measured every 2-3 days, starting on day 11 after tumor cell inoculation. Before the therapy initiation, the tumor area between the experimental groups of

individual mouse strains was comparable. On day 23, C56BL/6 mice treated with GN8P showed significantly smaller tumors than controls ($p<0.05$). This trend was sustained until all controls died (day 31). In contrast, GN8P did not produced significant differences in BALB/c mice *(Figure 6)*.

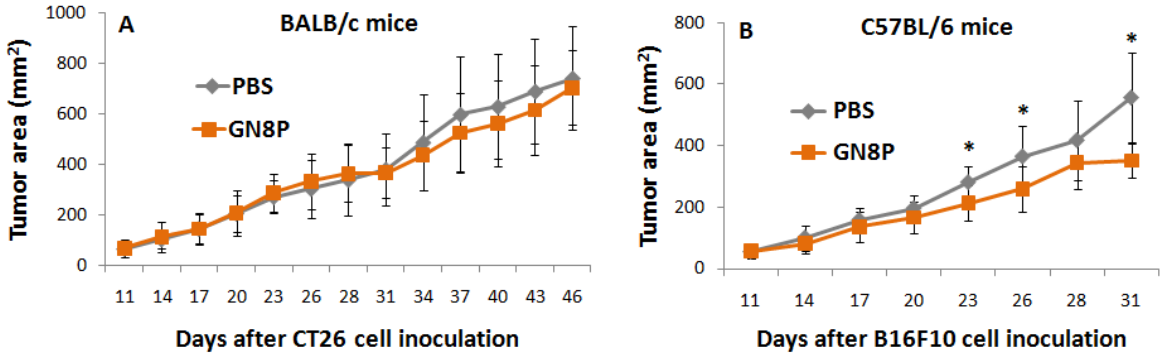



**Figure 6: Tumor growth in C57BL/6 and BALB/c mice treated with GN8P.** C57BL/6 and BALB/c mice (10 per group) were inoculated with B16F10 melanoma and CT26 colorectal carcinoma cells, respectively, and treated with 6 doses of GN8P. The tumor area represents the mean ± SD of values from individual mice. Figure shows a representative example of three performed experiments with similar results. The significant differences between GN8P treated mice and controls (PBS group) are marked by asterisk * $p\leq0.05$.

### 5.1.5. DISTRIBUTION OF SMC SUBPOPULATIONS AFTER GN8P TREATMENT

The expression of membrane markers on SMC subpopulations from tumor-bearing as well as healthy C57BL/6 and BALB/c mice was evaluated using FACS analysis to reveal the effect of GN8P on their proportion and activation state. We did not observe significant differences in the percentage of B cells (CD45R/B220+), cytotoxic (CD3+/CD8+CD4-) and helper (CD3+/CD4+CD8-) T cells comparing GN8P treated mice with controls (PBS group), *(Table 2)*.

| C57BL/6 mice | | healthy mice | | | tumor-bearing mice | | |
|---|---|---|---|---|---|---|---|
| | | PBS (%) | GN8P (%) | p value | PBS (%) | GN8P (%) | p value |
| T cells | CD3+ | 42.07 ± 3.37 | 40.88 ± 2.11 | 0.5519 | 43.83 ± 1.12 | 44.05 ± 2.53 | 0.9171 |
| Helper T cells | CD4+/CD8- | 54.47 ± 1.69 | 56.96 ± 1.56 | 0.0592 | 57.93 ± 3.03 | 61.32 ± 1.30 | 0.1074 |
| Cytotoxic T cells | CD8+/CD4- | 33.22 ± 1.12 | 31.96 ± 1.33 | 0.1581 | 35.65 ± 2.03 | 32.92 ± 0.90 | 0.0770 |
| B cells | CD45R/B220+ | 51.47 ± 1.36 | 55.32 ± 1.86 | 0.0772 | 54.5 ± 1.00 | 58.77 ± 1.61 | 0.0814 |

| BALB/c mice | | healthy mice | | | tumor-bearing mice | | |
|---|---|---|---|---|---|---|---|
| | | PBS (%) | GN8P (%) | p value | PBS (%) | GN8P (%) | p value |
| T cells | CD3+ | 34.83 ± 1.87 | 33.71 ± 1.76 | 0.4490 | 33.06 ± 3.50 | 27.38 ± 2.70 | 0.0674 |
| Helper T cells | CD4+/CD8- | 72.91 ± 1.32 | 73.81 ± 1.81 | 0.4740 | 74.62 ± 1.44 | 76.42 ± 0.67 | 0.0982 |
| Cytotoxic T cells | CD8+/CD4- | 22.24 ± 1.80 | 21.45 ± 1.01 | 0.5121 | 20.49 ± 2.10 | 17.98 ± 1.06 | 0.0788 |
| B cells | CD45R/B220+ | 50.14 ± 2.27 | 54.57 ± 2.25 | 0.0823 | 49.36 ± 1.39 | 52.04 ± 3.51 | 0.3152 |

**Table 2: Distribution of T and B cells in the spleen of tumor-bearing and healthy C57BL/6 and BALB/c mice.** Healthy and syngeneic tumor-bearing animals were injected with 3 doses of GN8P or PBS and bled 24 hours after the last treatment. The helper and cytotoxic T lymphocyte subpopulations were evaluated from gated CD3-positive cells.The data represent average ± SD of values from three performed experiments (5 mice per group), (adapted from *Hulikova et al., 2009*).

Although, GN8P did not influence the number of NK cells (CD3-/CD4-CD8-/CD49b+) and NKT cells (CD3+/CD4+CD8-/CD49b+) in C57BL/6 mice *(Figure 7A, B)*, it induced decrease in their NK1.1 expression *(Table 3)*.

The percentage of NK cells (control: 4.5 ± 0.32; 5.22 ± 0.49; GN8P: 4.85 ± 0.53; 5.05 ± 0.67 in healthy and tumor-bearing animals, respectively) and NKT cells (control: 1.17 ± 0.19; 1.09 ± 0.11; GN8P: 1.08 ± 0.12; 1.19 ± 0.2 in healthy and tumor-bearing animals, respectively) in spleens of BALB/c mice was comparable to that of C57BL/6 ones. The GN8P did not produce significant differences.

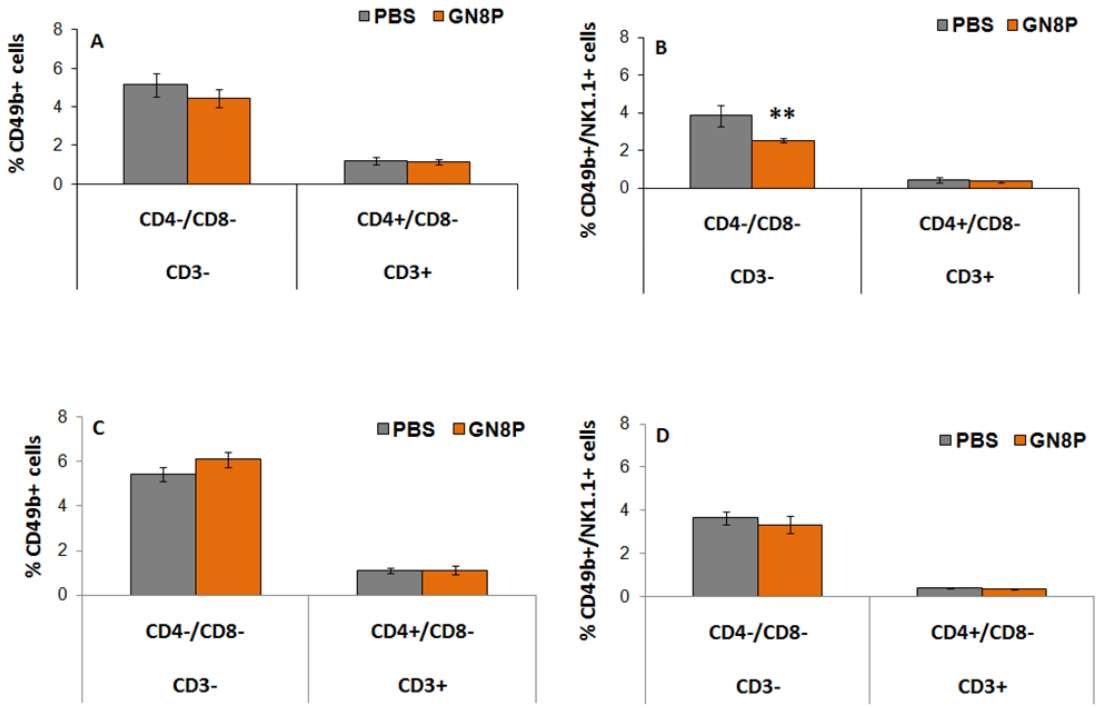



**Figure 7: Distribution of NK and NKT cells in the spleen of healthy (A, B) and tumor-bearing C57BL/6 mice (C, D).** Healthy and syngeneic tumor-bearing animals were injected with 3 doses of GN8P or PBS and bled 24 hours after the last treatment. Live, PI-negative SMCs with lymphocyte/monocyte morphology gated on the basis of FSC/SSC were at first analyzed for CD3 expression. CD3-negative or CD3-positive subpopulations were further analyzed for CD4 and CD8 expression. NK cells were defined as CD49b-positive cells out of CD3-/CD4-CD8- cell subpopulation, whereas NKT cells as CD49b-positive cells out of CD3+/CD4+CD8- cell subpopulation. The percentage of CD49b+/NK1.1+ NK cells was significantly decreased (** $p<0.01$) by GN8P treatment in healthy (B) but not tumor-bearing mice (D). The data represent average ± SD of values from three performed experiments (5 mice per group), (adapted from *Hulikova et al., 2009*).

| | healthy C57BL/6 mice | | | tumor-bearing C57BL/6 mice | | |
|---|---|---|---|---|---|---|
| | PBS | GN8P | p value | PBS | GN8P | p value |
| % NK1.1+ NK cells | 74.60 ± 10.46 | 57.00 ± 2.34 | 0.0227 | 67.00 ± 5.79 | 54.50 ± 6.87 | 0.0526 |
| % NK1.1+ NKT cells | 34.2 ± 11.34 | 29.72 ± 3.67 | 0.4777 | 35.4 ± 5.20 | 30.4 ± 5.43 | 0.2200 |

**Table 3: NK1.1 expression on gated NK (CD3-/CD4-CD8-/CD49b+) and NKT (CD3+/CD4+CD8-/CD49b+) cells**

### 5.1.6. GN8P-MEDIATED CHANGES IN mRNA EXPRESSION FOR NKR-P1C RECEPTOR

The GN8P significantly increased mRNA expression for NKR-P1C in healthy as well as tumor-bearing C57BL/6 mice, while did not induce any changes in BALB/c ones. These results indicate that the NKR-P1C receptor of C57BL/6, but not the divergent homolog of BALB/c mice, was engaged in the immune response evoked by the GN8P treatment.

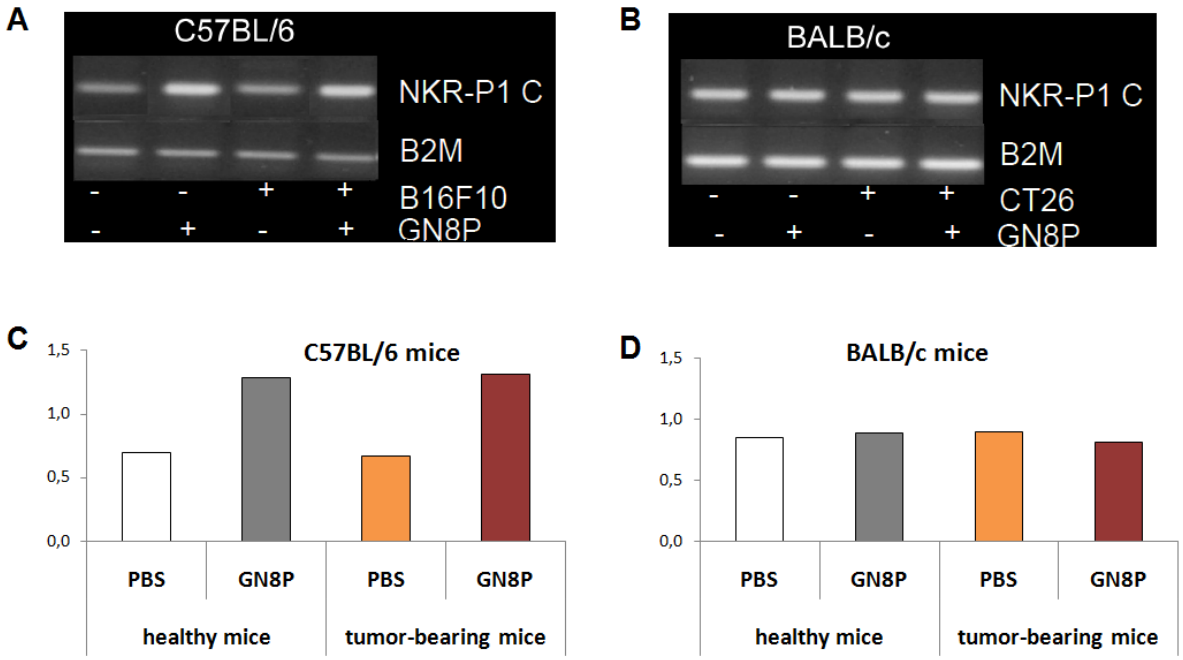



**Figure 8: Expression of mRNA for NKR-P1C receptor in C57BL/6 and BALB/c mice.** Healthy and syngeneic tumor-bearing animals were injected with 3 doses of GN8P or PBS. The total mRNA was isolated from SMCs of experimental mice. Level of mRNA for NKR-P1C was determined by semiquantitative RT-PCR and normalized to mRNA expression of the control gene B2M. The presence of the tumor burden or GN8P treatment is indicated by plus (+), whereas the absence by minus (-). Representative gel electrophoresis (A, B) of 3 experiments with similar results is shown. The gels were scanned and the densities evaluated as pixel intensity of NKR-P1C receptor bands normalized to those of B2M (densitometry analysis) for better comparison of experimental groups (C, D).

### 5.1.7. SERUM IgG2a LEVELS IN MICE TREATED WITH GN8P

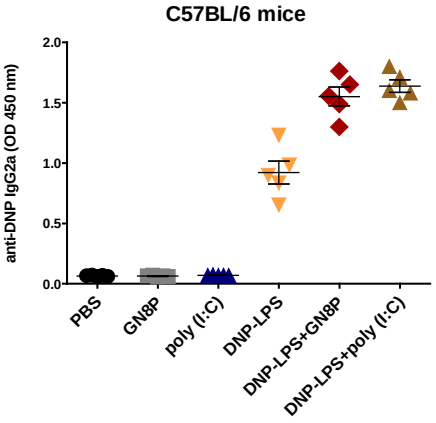
As activated NK cells were reported to regulate the antibody formation and to increase preferentially serum IgG2a levels *(Chapter 3.2.1.)*, we investigated whether GN8P is able to modulate IgG2a antibody response, specific for T-independent (DNP-LPS) as well as T-dependent (KLH) antigen, in NK1.1-positive (C57BL/6) and NK1.1-negative mouse strains (BALB/c and DBA/2) possessing different *Nkr-p1*c gene forms.

The both anti-DNP and anti-KLH IgG2a levels were significantly enhanced in GN8P-treated C57BL/6 mice compared with immunized controls (DNP-LPS and KLH group, respectively) and almost reached anti-DNP IgG2a response induced by poly (I:C), which was used as a positive control (DNP-LPS vs. DNP-LPS+poly (I:C) and KLH vs. KLH+poly(I:C) $p<0.001$). In contrast, neither anti-DNP nor anti-KLH IgG2a formation was influenced by GN8P administrations in BALB/c and DBA/2 mice. In NK1.1-positive F1 hybrids, which were tested to support the hypothesis that *Nkr-p1c* gene divergence could play a role in GN8P-mediated regulation of the antibody formation, IgG2a antibodies specific for the both used antigens were significantly mounted, but to a lesser extent than in C57BL/6 mice *(Figure 9)*.

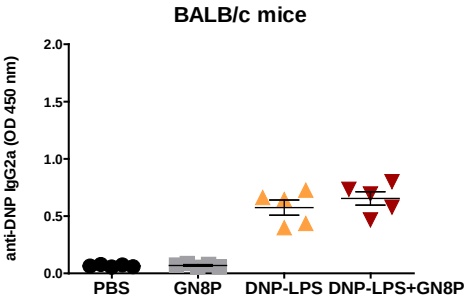
Taken together, elevated serum IgG2a levels induced by GN8P treatment were dependent on the presence of *Nkr-p1c*$^{B6}$ gene form (*Nkr-p1c(T)*), and thus on NK1.1 expression in experimental mice *(Table 1, Figure 1)*.

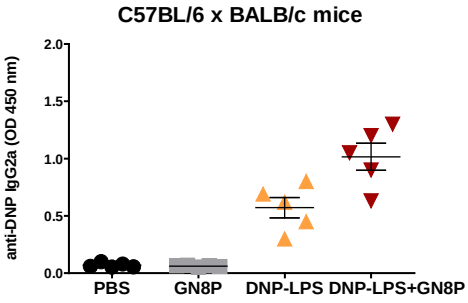
In addition, mounted IgG2a antibody secretion in C57BL/6 mice, but not in BALB/c ones correlated with increased serum as well as mRNA level for IFN-γ, which is known to support class switch to IgG2a isotype *(Figure 3, 4)*. As we proved by FACS analysis, GN8P potentiated IFN-γ production predominantly in NK cells *(Figure 5)*.

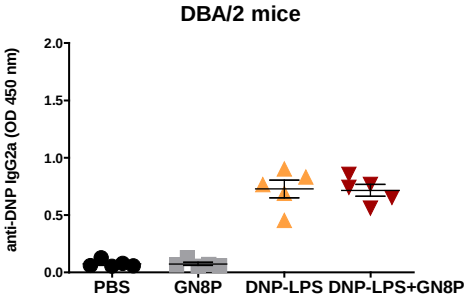
**A**

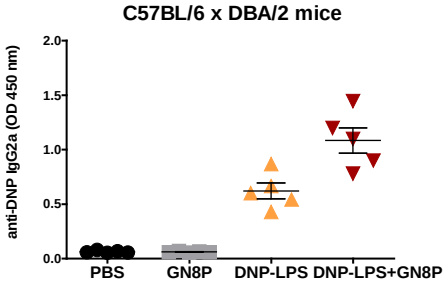


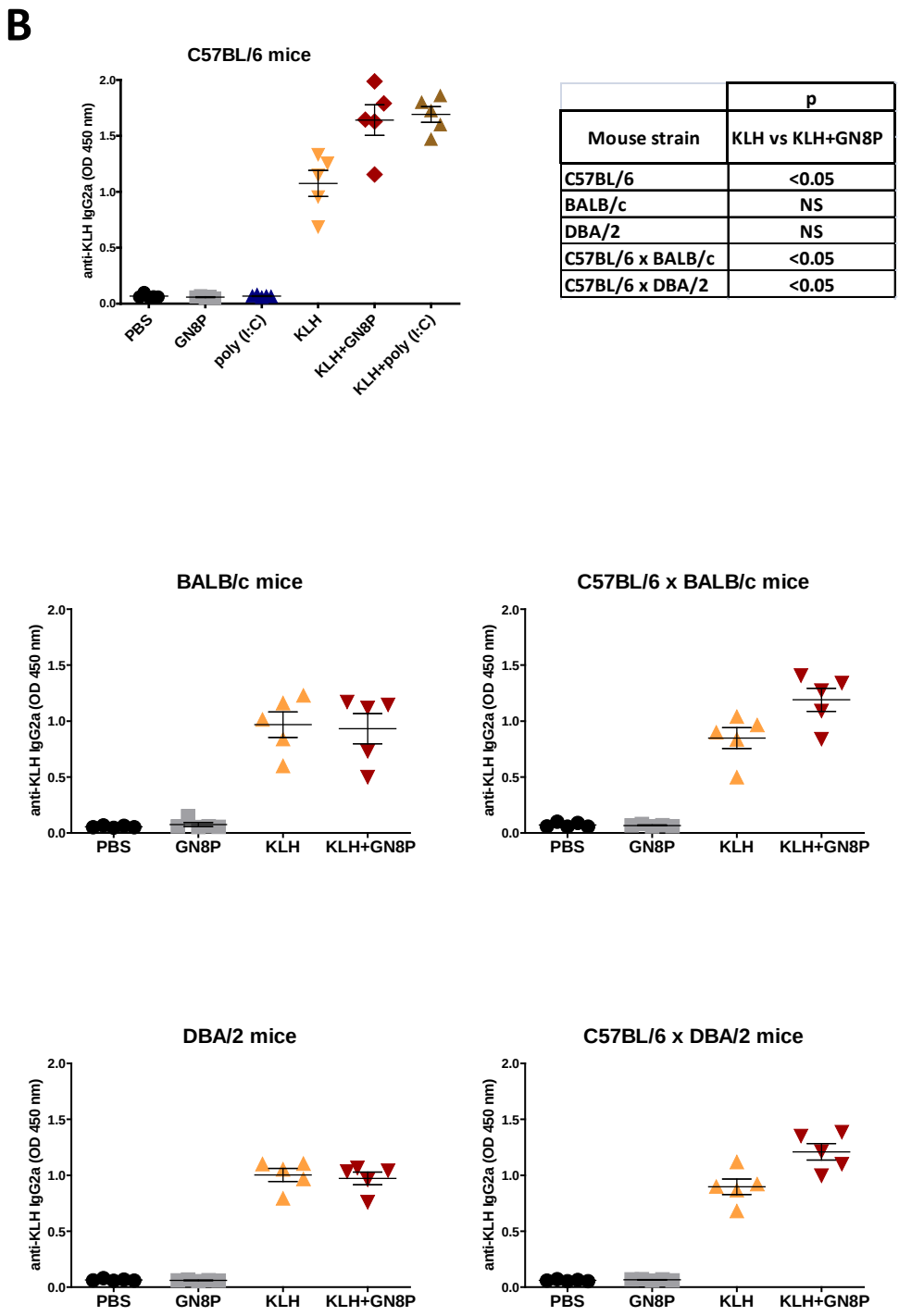

| Mouse strain | p |
|---|---|
| | DNP-LPS vs DNP-LPS+GN8P |
| C57BL/6 | <0.001 |
| BALB/c | NS |
| DBA/2 | NS |
| C57BL/6 x BALB/c | <0.05 |
| C57BL/6 x DBA/2 | <0.01 |






| Mouse strain | p<br>KLH vs KLH+GN8P |
|---|---|
| C57BL/6 | <0.05 |
| BALB/c | NS |
| DBA/2 | NS |
| C57BL/6 x BALB/c | <0.05 |
| C57BL/6 x DBA/2 | <0.05 |

**Figure 9: Antigen-specific serum IgG2a levels**. Animals were immunized with KLH or DNP-LPS and treated with either GN8P, poly (I:C), or PBS. Anti-DNP (A) and anti-KLH (B) IgG2a levels were measured by ELISA. Figure shows data for 1:10 serum dilution, which are representative of three independent experiments with similar results. The statistical analysis was performed by ANOVA for comparison between immunized mice treated with GN8P or poly (I:C) and immunized controls (KLH and DNP-LPS group). The significant changes are indicated in the tables (adapted from *Hulikova et al., 2009*).

### 5.1.8. EFFECT OF GN8P *ON IN VITRO* IgM FORMATION

The effect of GN8P on *in vitro* antibody formation was determined by ELISA using SMC supernatants harvested after a 5 day-long incubation with DNP-LPS in the presence or absence of GN8P.

We observed significant increase ($p<0.05$) in anti-DNP IgM levels comparing supernatants of SMCs from C57BL/6 mice incubated simultaneously with DNP-LPS and GN8P to those cultured with DNP-LPS alone. On the other hand, GN8P did not influence *in vitro* anti-DNP IgM levels in BALB/c and DBA/2 mice *(Figure 10).*

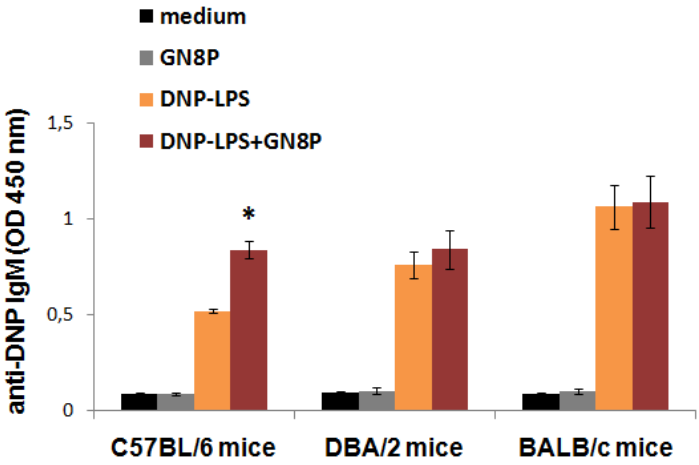



**Figure 10: *In vitro* IgM formation in C57BL/6, DBA/2, and BALB/c mice.** SMCs were cultured with DNP-LPS in the presence or absence of GN8P for 5 days. Subsequently, anti-DNP IgM levels in supernatants were measured by ELISA. SMCs incubated without antigen were used as controls. The data are expressed as average ± SD of triplicates. Figure shows one representative experiment of three with similar results.

In order to prove the involvement of NK cells or particularly NK1.1 (NKR-P1C$^{B6}$) receptor in GN8P-induced *in vitro* IgM formation in C57BL/6 mice, we performed this experiment after depletion of CD49b-positive or NK1.1-positive SMC subpopulations, which blocked the enhancement of anti-DNP IgM levels *(Figure 11)*. Anti-DNP IgG levels were not detectable in supernatants of SMCs after a 5 day-long incubation.

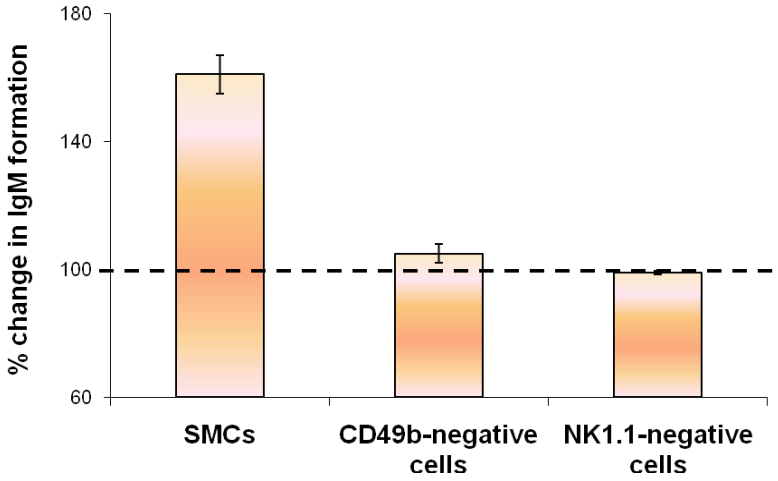



**Figure 11: *In vitro* IgM formation by CD49b or NK1.1-depleted SMCs in C57BL/6 mice.** SMCs, CD49b-negative or NK1.1-negative cells were cultured with DNP-LPS in the presence or absence of GN8P for 5 days. Subsequently, anti-DNP IgM levels in supernatants were measured by ELISA. The data are presented as percentage changes in IgM levels (OD 450 nm) comparing supernatants of cells cultured simultaneously with DNP-LPS and GN8P with those stimulated with DNP-LPS alone (stated as 100%; dashed line). Figure shows an illustrative example of three performed experiments with similar results. Significant difference ($p>0.05$) was observed only in case of SMCs (undepleted cells), (published in *Hulikova et al., 2009*).

## 5.2. B CELL RESPONSE TO GN8P IN C57BL/6 MICE

The results described above indicate that GN8P has a potential to modulate antibody formation via NK cell activation, in mice expressing NKR-P1C receptor encoded by *Nkr-p1c*$^{B6}$ gene form. The aim of the following experiments was to evaluate GN8P effect on the antibody response in C57BL/6 mice in more detail.

### 5.2.1. SERUM LEVELS OF ANTIGEN-SPECIFIC ANTIBODIES IN HEALTHY MICE

We determined IgM and IgG response eight days after immunization of mice with either KLH or DNP-LPS in the presence or absence of GN8P by Ig isotype-specific ELISA.

We did not observe significant differences either in anti-KLH nor anti-DNP IgM levels between GN8P-treated mice and controls primed with the antigen alone *(Figure 12A, B)*. However, anti-KLH as well as anti-DNP IgG formation was significantly increased after GN8P administrations *(Figure 12C, D)*.

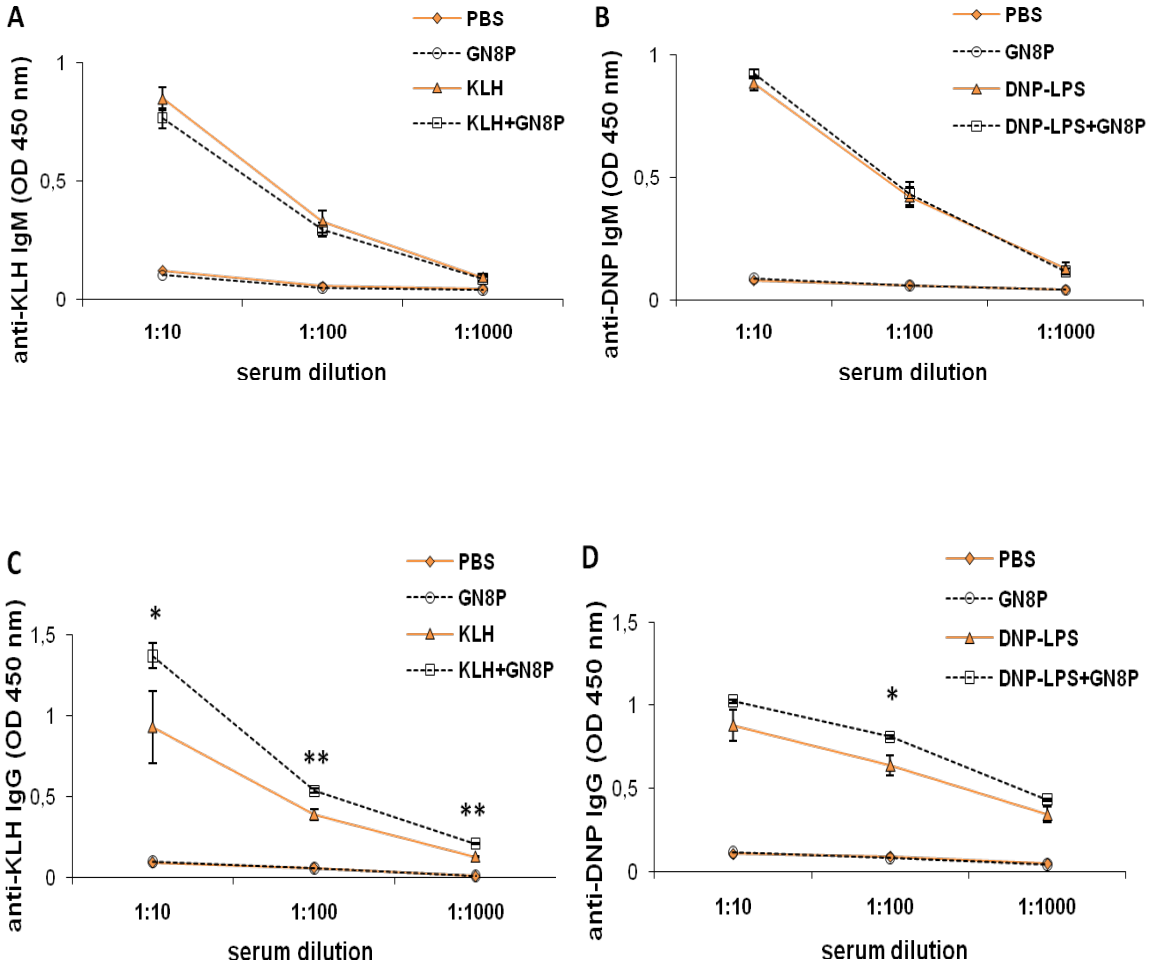



**Figure 12: Antigen-specific IgM and IgG levels in the serum of C57BL/6 mice**. Animals were immunized with KLH or DNP-LPS and treated with 3 doses of GN8P or PBS. Anti-KLH IgM (A) and IgG (C) or anti-DNP IgM (B) and IgG (D) levels were measured by ELISA. Non-immunized mice were bled to obtain control serum (dashed lines). Figure shows an illustrative example of three consecutive experiments with similar results. The data represent the average ± SD of values from individual mice (5 per group). The level of significance for immunized mice treated with GN8P (KLH+GN8P and DNP-LPS+GN8P group) vs. immunized controls (KLH and DNP-LPS group) are indicated as follows * $p \leq 0.05$, ** $p \leq 0.01$ (published in *Hulikova et al., 2009*).

We also evaluated IgG2a levels as the formation of this antibody subclass was reported to be regulated by activated NK cells. As shown above *(Figure 8)*, both anti-KLH ($p<0.05$) and anti-DNP ($p<0.001$) IgG2a levels were significantly enhanced in GN8P treated mice compared with immunized controls (KLH and DNP-LPS group, respectively).

### 5.2.2. SERUM LEVELS OF TUMOR CELL-SPECIFIC ANTIBODIES IN B16F10 MELANOMA-BEARING MICE

To investigate whether GN8P also influences tumor cell-specific antibody formation, we injected B16F10 melanoma-bearing mice with either PBS or GN8P, and measured their serum levels of IgG antibodies able of specific binding to B16F10 cells using FACS analysis. Intact B16F10 cells (not incubated with serum) and those incubated with sera from healthy animals were used as negative controls.

In tumor-bearing mice treated with PBS, we detected B16F10 melanoma-specific serum IgG levels (PBS group vs. healthy control $p<0.05$), which were further elevated by GN8P administrations (PBS vs. GN8P group $p<0.001$), *(Figure 13)*.

### 5.2.3. IgG2a mRNA EXPRESSION IN RESPONSE TO THE ANTIGEN

As GN8P treatment was found to augment serum IgG2a levels, we also tested its effect on mRNA expression for this antibody subclass in healthy as well as B16F10 melanoma-bearing mice.

Significant increase in mRNA levels for IgG2a was observed after GN8P administrations in both healthy and tumor-bearing animals. In case of healthy mice, we followed differences between experimental groups immunized with KLH (KLH vs. KLH+GN8P group), *(Figure 14A)*. As for mice inoculated with B16F10 melanoma, PBS and GN8P treated groups were compared *(Figure 13B)*. The results indicate that GN8P elevated mRNA for anti-KLH (healthy mice) as well as anti-B16F10 IgG2a antibodies (tumor-bearing mice).

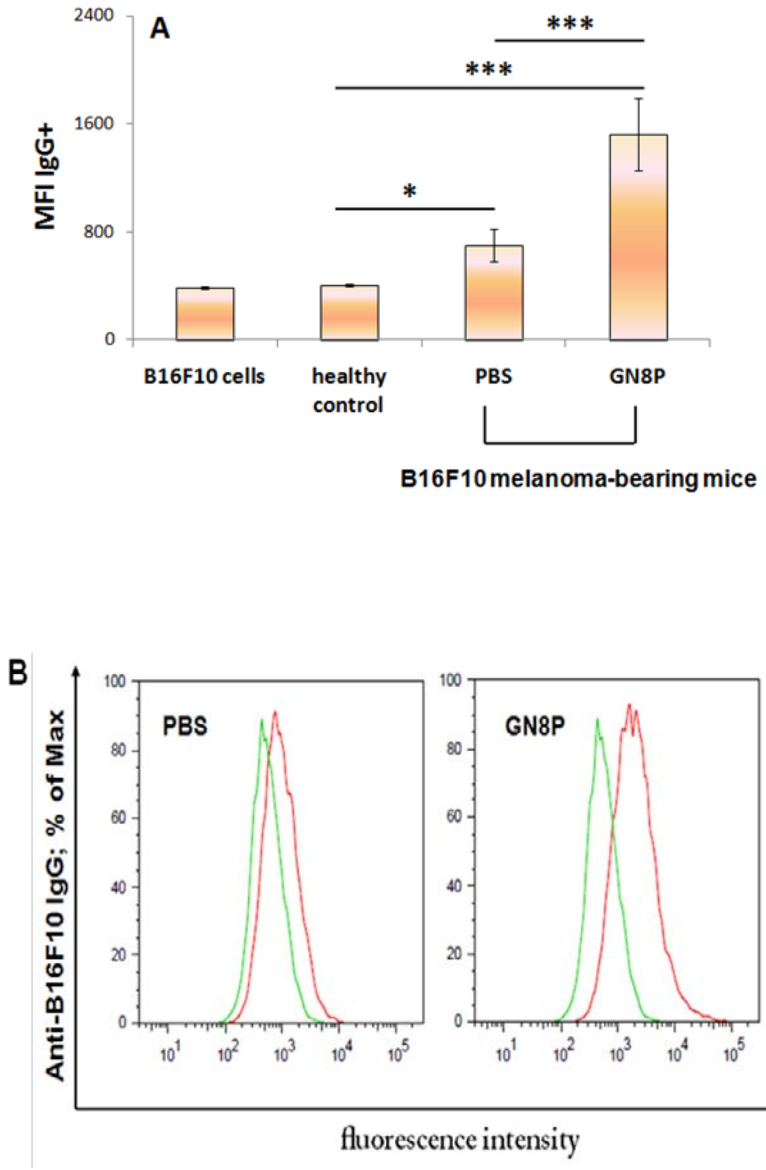



**Figure 13: Tumor cell-specific antibody formation.** Animals were inoculated with B16F10 tumor cells and treated with 3 doses of PBS or GN8P. Sera were collected 24 hours after the last treatment. To detect anti-B16F10 IgG levels, B16F10 cells were incubated with 1:10 diluted sera and anti-mouse IgG as described in Materials and Methods. Intact B16F10 cells (not incubated with serum) and those incubated with sera from healthy animals were used as negative controls. The results are expressed as (A) average ± SD of mean fluorescence intensity (MFI) of live B16F10 cells incubated with sera from individual mice (5 per group) or (B) histograms (B16F10 cells incubated with serum from healthy control are indicated in green, whereas those incubated with sera from PBS or GN8P treated B16F10 tumor-bearing mice in red). The statistical analysis was performed by ANOVA. The significant changes are marked by asterisk * $p \leq 0.05$, *** $p \leq 0.001$.

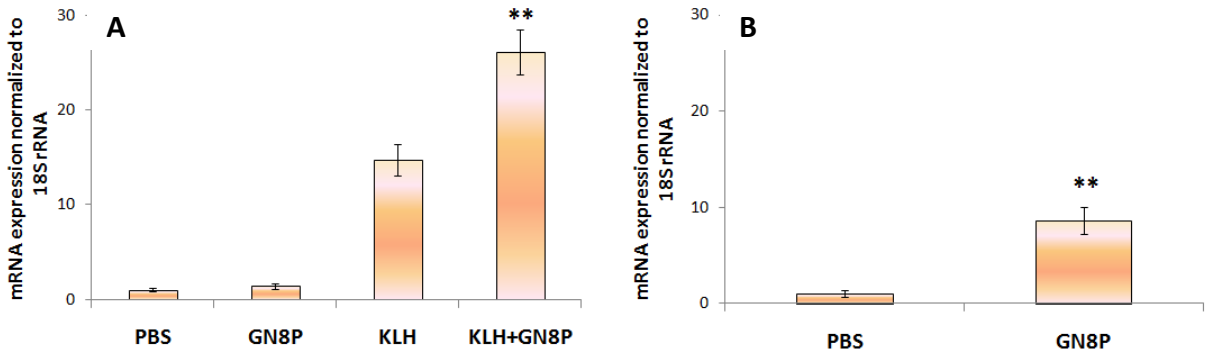



**Figure 14: Expression of mRNA for IgG2a.** Healthy animals (A) were immunized with KLH and treated with 3 doses of PBS or GN8P. B16F10 melanoma-bearing mice (B) were injected with GN8P or PBS by the same route as healthy ones. The total mRNA was isolated from SMCs of experimental mice. Level of mRNA for IgG2a was determined by real-time RT-PCR and normalized to the expression of control gene 18S rRNA. Results are expressed as average ± SD of values from individual mice (3 per group). Figure shows a representative example of three independent experiments with similar results.

### 5.2.4. NUMBER OF SPLENIC ANTIBODY FORMING CELLS IN RESPONSE TO SRBCs

To determine the absolute number of IgM and IgG forming cells in the spleen, we evaluated anti-SRBC antibody response six days after immunization by means of PFC assay.

We found significant increase in anti-SRBC IgG ($p<0.01$) as well as IgM ($p<0.05$) forming cell counts in spleens of GN8P treated mice, when compared with immunized controls (SRBC group), *(Figure 15).*

### 5.2.5. PROPORTION OF PLASMA CELLS AND ANTIGEN PRESENTING B CELLS

FACS analysis revealed that plasma cells identified as CD45R/B220+/CD138+ and CD45R/B220-/CD138+ subpopulations significantly enhanced their counts comparing healthy mice simultaneously treated with the antigen and GN8P (KLH+GN8P or DNP-LPS+GN8P group) to KLH or DNP-LPS immunized controls *(Figure 16A).* B16F10 melanoma-bearing animals injected with GN8P showed higher proportion of plasma cells than PBS treated group as well *(Figure 16B).*

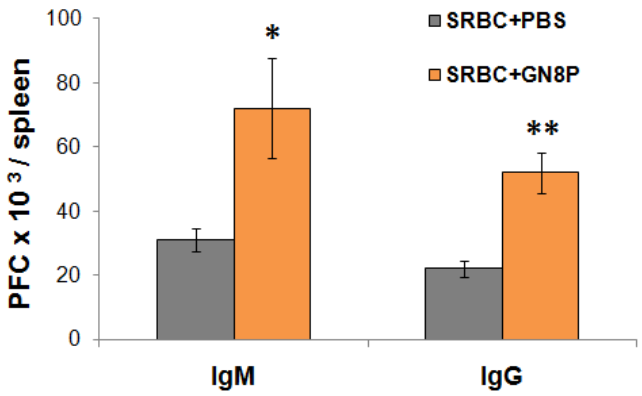



**Figure 15: Effect of GN8P on the number of plaque forming cells in the spleen.** Mice were immunized with SRBC and treated with either GN8P or PBS as described in Material and Methods. Results are expressed as the number of plaque forming cells (PFC) x $10^3$/spleen ($10^8$ cells). The data represent the average ± SD of values from three independent experiments (6 mice per group). Significant changes relative to controls (SRBC+PBS group) are marked by asterisk (* $p \leq 0.05$, ** $p \leq 0.01$), (published in *Hulikova et al., 2009*).

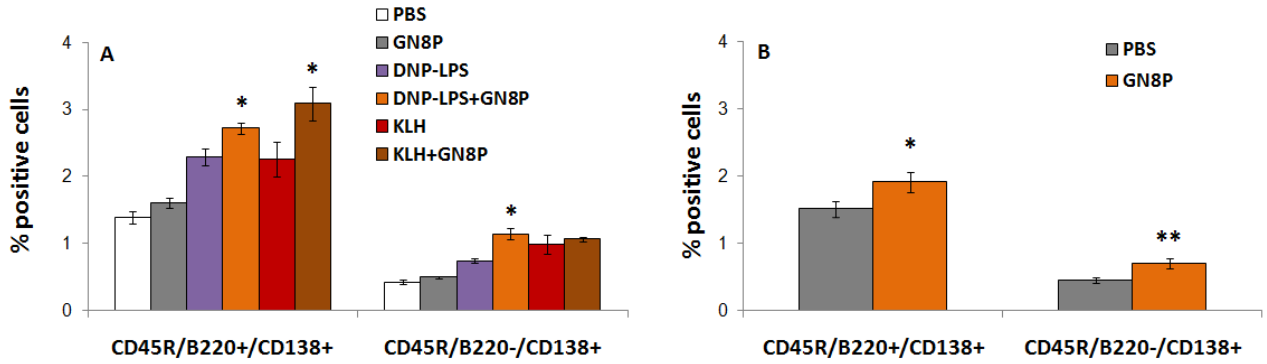



**Figure 16: Distribution of plasma cells in the spleen of healthy (A) and tumor-bearing C57BL/6 mice (B).** Healthy animals were immunized with KLH or DNP-LPS and treated with 3 doses of PBS or GN8P. B16F10 melanoma-bearing mice were treated with GN8P or PBS by the same route. Live, PI-negative SMCs with lymphocyte/monocyte morphology gated on the basis of FSC/SSC were analyzed for CD45R/B220 and CD138 expression. The significant changes in counts of CD45R/B220+/CD138+ and CD45R/B220-/CD138+ plasma cells between the following experimental groups: PBS vs. GN8P, DNP-LPS vs. DNP-LPS+GN8P, and KLH vs. KLH+GN8P are marked by asterix * $p \leq 0.05$, ** $p \leq 0.01$. The data are expressed as average ± SD of 3 performed experiments (5 mice per group).

Furthermore, the GN8P significantly elevated counts of I-A/I-E+/CD80+ and I-A/I-E+/CD86+/CD80+ antigen presenting B cells in healthy ($p<0.01$) as well as tumor-bearing ($p<0.001$) mice. The increase in the occurrence of I-A/I-E+/CD86+ B cells was observed only in tumor-bearing animals ($p<0.01$), *(Figure 17)*.

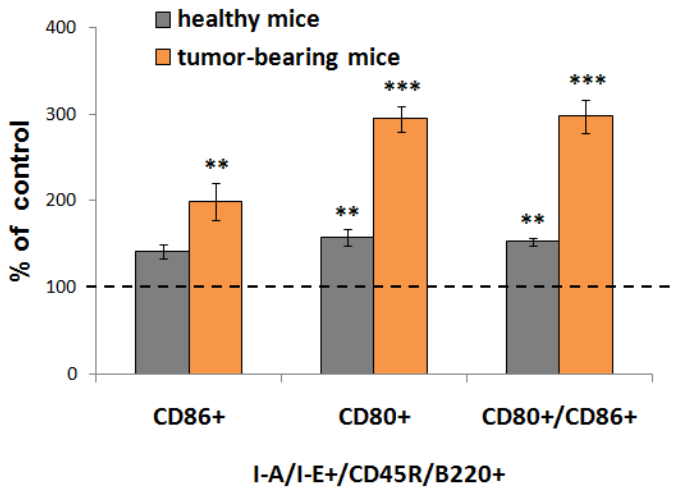
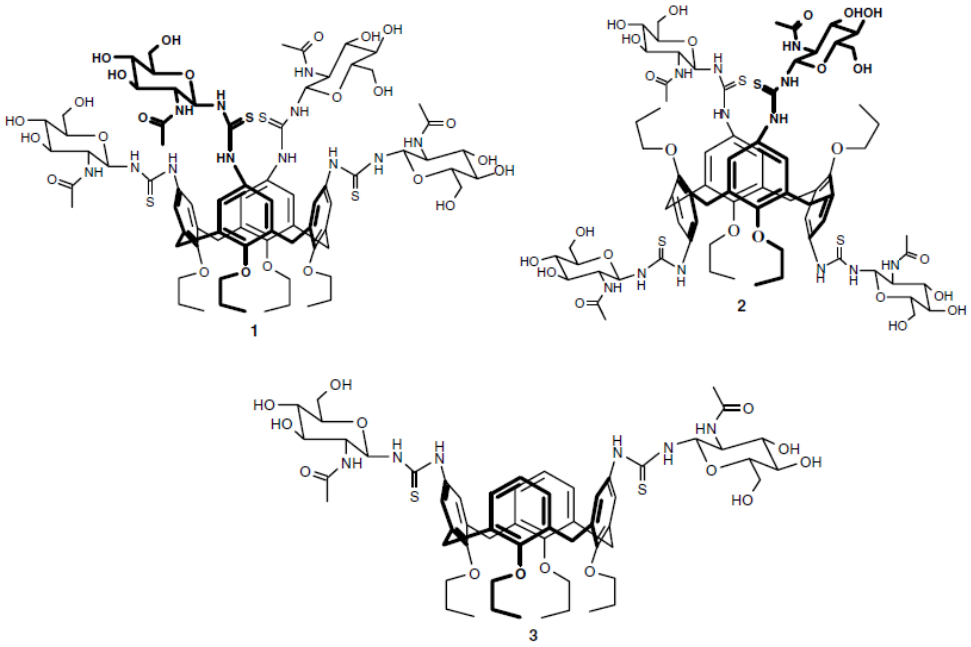



**Figure 17: Proportion of antigen presenting B cells in the spleen of healthy and tumor-bearing C57BL/6 mice.** Healthy and B16F10 melanoma-bearing animals were treated with 3 doses of GN8P or PBS. Live, PI-negative SMCs with lymphocyte/monocyte morphology gated on the basis of FSC/SSC were at first analyzed for I-A/I-E expression (MHC class II molecules). Then the percentage of B cells (CD45R/B220+) out of I-A/I-E-positive cells was determined. This B cell subpopulation was further analyzed for expression of costimulatory molecules CD80 and CD86. The data are presented as percentage of control (PBS group stated as 100%; dashed line). Significant changes relative to controls are marked by asterisk (** $p\leq0.01$, *** $p\leq0.001$).

## 5.3. GN4C: A NEW POTENTIAL LIGAND FOR NKR-P1 RECEPTOR

### 5.3.1. BINDING AFFINITY OF GN4C TO THE RECOMBINANT NKR-P1 PROTEIN

To evaluate the ability of newly synthesized GlcNAc-substituted cali[4]arenes, with varying geometry and number of GlcNAc subunits *(Scheme 1)*, to bind to NKR-P1 receptor, we determined the glycoconjugate concentration required for 50% inhibition in binding of $^{125}$I-labelled rat recombinant NKR-P1A molecule to its high affinity ligand $GlcNAc_{23}BSA$ ($IC_{50}$). The same plate inhhibition assay was performed using human recombinant CD69 molecule.



**Scheme 1: Newly synthesized GlcNAc-substituted calix[4]arenes** (1 = GN4C, cone structure with 4 GlcNAc subunits; 2 = 1, 3-alternate structure with 4 GlcNAc subunits; 3 = structure with 2 GlcNAc subunits; adapted from *Krenek et al., 2007*).

The GN4C displayed the highest inhibition of $GlcNAc_{23}BSA$ binding to the rat recombinant NKR-P1A molecule from all tested compounds ($IC_{50}$ = 2 x $10^{-10}$ M), and therefore was chosen for our further immunological studies, where we compared its effects to those of GN8P. Single

GlcNAc bound to NKR-P1A to a lesser extent ($10^{-7}$ M) than glycoconjugates, while calix[4]arene skeleton by itself exhibited a poor inhibition potential ($10^{-5}$ M), *(Figure 18A)*.

Surprisingly, GN4C was shown to be one of the best ligands for CD69 receptor described so far ($IC_{50}$ = 6 x $10^{-10}$ M). In adition, calix[4]arene skeleton by itself bound to CD69 molecule with higher affinity than other tested GlcNAc-substituted cali[4]arenes (compound 2, 3), *(Figure 18B)*.

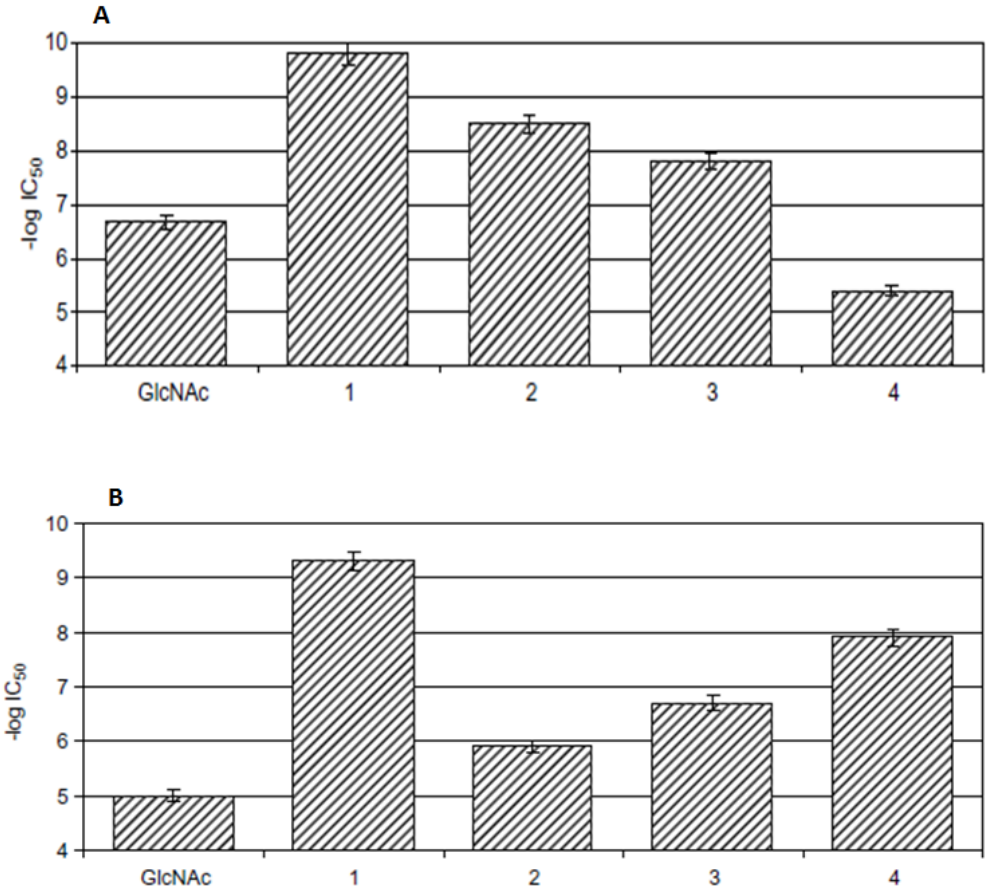



**Figure 18: Binding affinity of GlcNAc-substituted calix[4]arenes to the recombinant rat NKR-P1A (A) and human CD69 protein (B).** The results are expressed as average ± SD of values from 3 independent experiments. The GN4C is indicated as compound 1, other tested GlcNAc-substituted calix[4]arenes as compound 2, 3 *(see Scheme 1)* and calix[4]arene scaffold as compound 4. GlcNAc was used as reference structure ($IC_{50}$ = concentration required for 50% inhibition in binding of $^{125}$I-labelled rat recombinant NKR-P1A molecule to its high affinity ligand $GlcNAc_{23}BSA$; published in *Krenek et al., 2007*).

## 5.3.2 EFFECT OF GN4C ON FUNCTIONAL ACTIVITY OF HUMAN PBMCs

Initially, we tested effects of GN4C *in vitro*, on proliferation and cytotoxicity of human peripheral blood mononuclear cells.

### 5.3.2.1. Proliferation of human PBMCs

We observed that glycoconjugates modulated the proliferation of PBMCs in a dose-dependent manner. The GN4C exerted the greatest effect (at the optimal concentration of $10^{-8}$ mol/L) when compared to GN8P and calix[4]arene scaffold by itself *(Figure 19)*.

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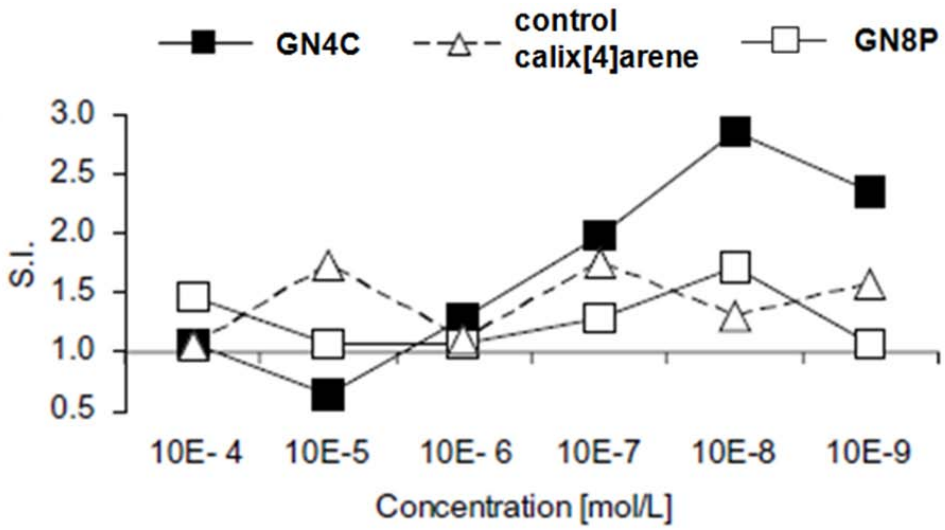



**Figure 19: Effect of glycoconjugates on the proliferation of human peripheral blood mononuclear cells.** The results are expressed as the stimulation index of control non-stimulated cells. The data represent average of 3 experiments (12 healthy donors, samples seeded in pentaplicates). SD does not exceed 10%. (S.I. = cpm experimental/cpm control; published in *Krenek et al., 2007*).

### 5.3.2.1. Cytotoxic activity of human PBMCs

$^{51}$Cr-release assay was performed using PMBCs from healthy donors as effectors and NK cell-sensitive K562 cell line (human chronic myeloid leukaemia) as targets. Before addition of tumor cells, PMBCs were incubated in the presence of GN4C, GN8P or calix[4]arene scaffold in the concentration range $10^{-4}$ – $10^{-9}$ mol/L for 18 hours.

When NK cell-mediated cytotoxicity was expressed as percentage of non-stimulated control (stated as 100%), GN4C increased it as follows: 158 ± 19% ($p<0.01$) and 170 ± 21% ($p<0.01$) at the concentration of $10^{-4}$ and $10^{-8}$ mol/L, respectively. GN8P induced significant enhancement in the lysis of K562 cells only at the concentration of $10^{-8}$ mol/L (115 ± 9%; $p<0.05$). Calix[4]arene scaffold by itself exerted similar effects as GN8P *(Figure 20)*.

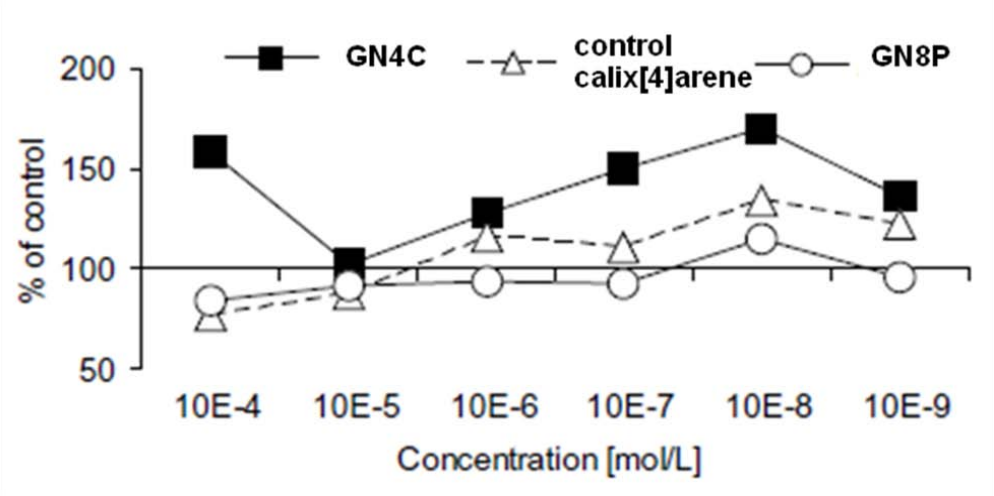



**Figure 20: Effect of glycoconjugates on cytotoxicity of human peripheral blood mononuclear cells.** Human PBMCs from healthy donors pre-incubated with GN4C, GN8P or calix[4]arene scaffold were used in standard 3.5 hour $^{51}$Cr-release assay as effectors and K562 cells as targets at E:T ratio 16:1. The results are expressed as percentage of non-stimulated control (stated as 100%). The data represent average of 3 experiments (12 healthy donors, samples seeded in tetraplicates). SD does not exceed 10% (published in *Krenek et al., 2007*).

## 5.3.3. EFFECT OF GLYCOCONJUGATES ON CYTOTOXIC CELLS IN C57BL/6 MICE

To prove that GN4C and GN8P influence predominantly the NK cell function, we performed the cytotoxic assay using SMCs, purified NK cells, or CTLs as effectors. SMCs were isolated from spleens of melanoma-bearing as well as healthy mice treated with PBS, GN8P or GN4C. NK cells (CD8-CD49b+) and CTLs (CD8+CD49b-) were separated from SMC suspension by two-way cell sorting. FACS re-analysis confirmed their high purity (more than 97% of the sorted cells displayed the required phenotype) and showed that 99.8% of CTLs were NK1.1-negative, while 94.1% of NK cells fell into CD49b+/NK1.1+ double positive compartment *(Figure 21)*.

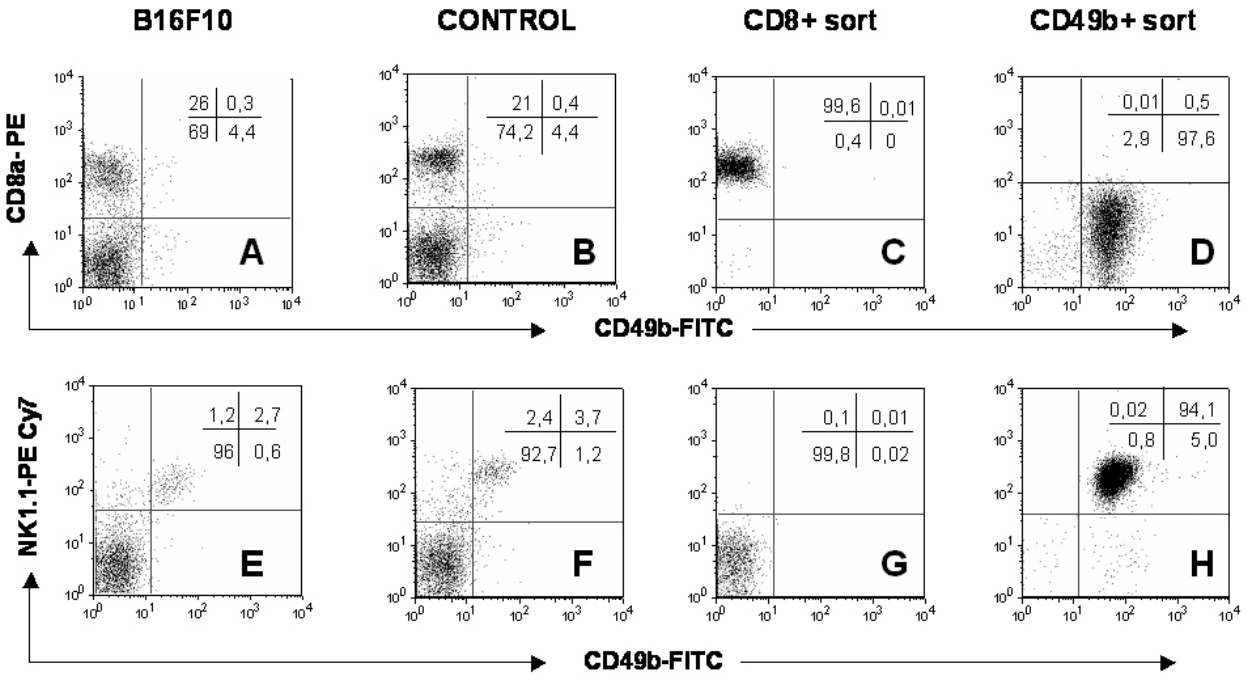



**Figure 21: Phenotype of spleen-derived cells used in the cytotoxicity assay.** We determined CTL and NK cell counts in B16F10 melanoma-bearing (A, E) and healthy (B, F) animals prior to the cell sorting. The sorted cells were re-analyzed to confirm the purity (C, D), and stained with anti-NK1.1 mAb to evaluate their NK1.1 expression (G, H). Numbers in the crosses indicate the percentage of cells in corresponding quadrants. The figure shows representative example of three performed experiments with similar results.

IC-21 (NKR-P1-dependent) or syngeneic B16F10 cell lines were used as targets for cytotoxic assay. In melanoma bearing mice, the GN4C significantly enhanced the overall (anti-B16F10) as well as NKR-P1-dependent (anti-IC-21) cytotoxicity of sorted NK cells *(Figure 22A, B).* In addition, this compound induced significant increase in SMC and CTL-mediated lysis of B16F10 targets *(Figure 22B).* On the other hand, the GN8P increased only the NKR-P1-dependent cytotoxic activity of NK cells *(Figure 22A).* In control mice, neither of tested glycoconjugates produced significant changes in the cytotoxicity against B16F10 cells *(Figure 22D),* whereas NKR-P1-dependent NK cell cytotoxicity against IC-21 cells was augmented by both the GN4C and GN8P (*Figure 22C).*

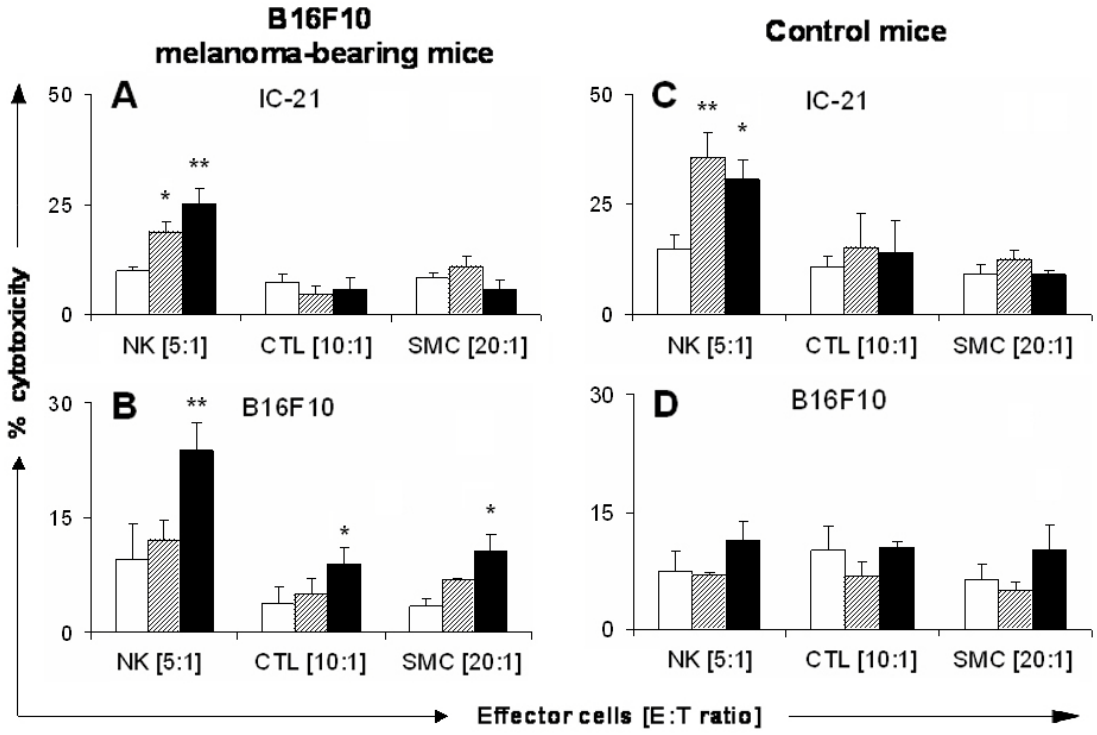



**Figure 22: Cytotoxic activity of SMCs, purified CTLs and NK cells.** B16F10 melanoma-bearing (A, B) and healthy (C, D) mice were treated with 3 doses of PBS, GN8P or GN4C. Sorted NK cells, CTLs and isolated SMCs were used in standard 3.5 hour $^{51}$Cr-release assay as effectors at E:T ratios indicated in brackets. IC-21 (A, C) and B16F10 (B, D) cell lines were used as targets. The data are expressed as mean ± SD of values from 3 individual experiments (3-5 animals per group). Significant changes between mice treated with the glycoconjugate (GN4C – closed, GN8P – shaded columns) and PBS (open columns) are marked as follows: * $p \leq 0.05$; ** $p \leq 0.01$.

## 5.3.4. ANTITUMOR ACTIVITY OF GLYCOCONJUGATES AND COMBINED THERAPY WITH KLH IN MOUSE B16F10 MELANOMA MODEL

### 5.3.4.1. The tumor growth and survival of experimental mice

The melanoma dimensions were measured every 2-3 days starting on day 11, before the therapy initiation, until all controls died. In the beginning, the tumor area was comparable in all experimental groups. From day 14 (after the first dose of therapeutic agents) up to the end of the observation period, the mice treated with GN4C ($p<0.01 – 0.001$) as well as KLH ($p<0.05 – 0.001$) showed significantly smaller tumors when compared to controls (PBS group). GN8P produced significant differences only after four doses (day 23; $p<0.05$), *(Figure 23A, B; Table 4)*. The combination of GN8P with KLH significantly decreased the tumor growth when compared to GN8P alone ($p<0.05$, $p<0.05$, and $p<0.01$ on day 17, 20, and 23, respectively). However, this was caused by action of KLH since the results from KLH and KLH+GN8P treated groups were similar *(Figure 23A)*. On the other hand, KLH exerted an inhibitory effect on GN4C *(Figure 23B)*. Thus, KLH did not act in synergism with glycoconjugates. *Table 4A* depicts results with the largest differences (day 23 and 26) in the tumor area between groups of treated animals and controls, and those obtained before the start (day 11) and after the end of therapy (day 31). Only on day 23 (12 days after the first dose of treatment), we found significantly reduced tumor size whichever the used therapeutic protocol (KLH+GN8P by 51.44%, GN4C by 50.9%, KLH by 37.44%, KLH+GN4C by 29.78%, and GN8P by 24%). The tumor growth rate was significantly decreased after GN4C ($p<0.01$) and, to a lesser extent, after KLH, and KLH+GN8P ($p<0.05$) treatment (*Figure 24)*.

The percentage of surviving mice and the survival time were evaluated on day 34 and until the death of all animals, respectively. On day 34, when all controls were dead, living animals were still present (at a different percentage) in all treated groups, and particularly after GN4C administration (90% of survivors), *(Figure 23C, D)*. The GN4C also induced the longest survival (63 days; mean 46.3±11.1 days; $p<0.01$), followed by KLH+GN4C (59; 36.4±12.1), KLH (48; 35.6±6.5), KLH+GN8P (48; 35.6±6.7), and GN8P (48; 32.4±7), compared to controls (34; 29.8±3.6), *(Figure 23E, F)*. The combination of KLH with glyconjugates again did not produce any synergistic action. In our experimental model, GN4C exhibited the most promising anticancer effects.

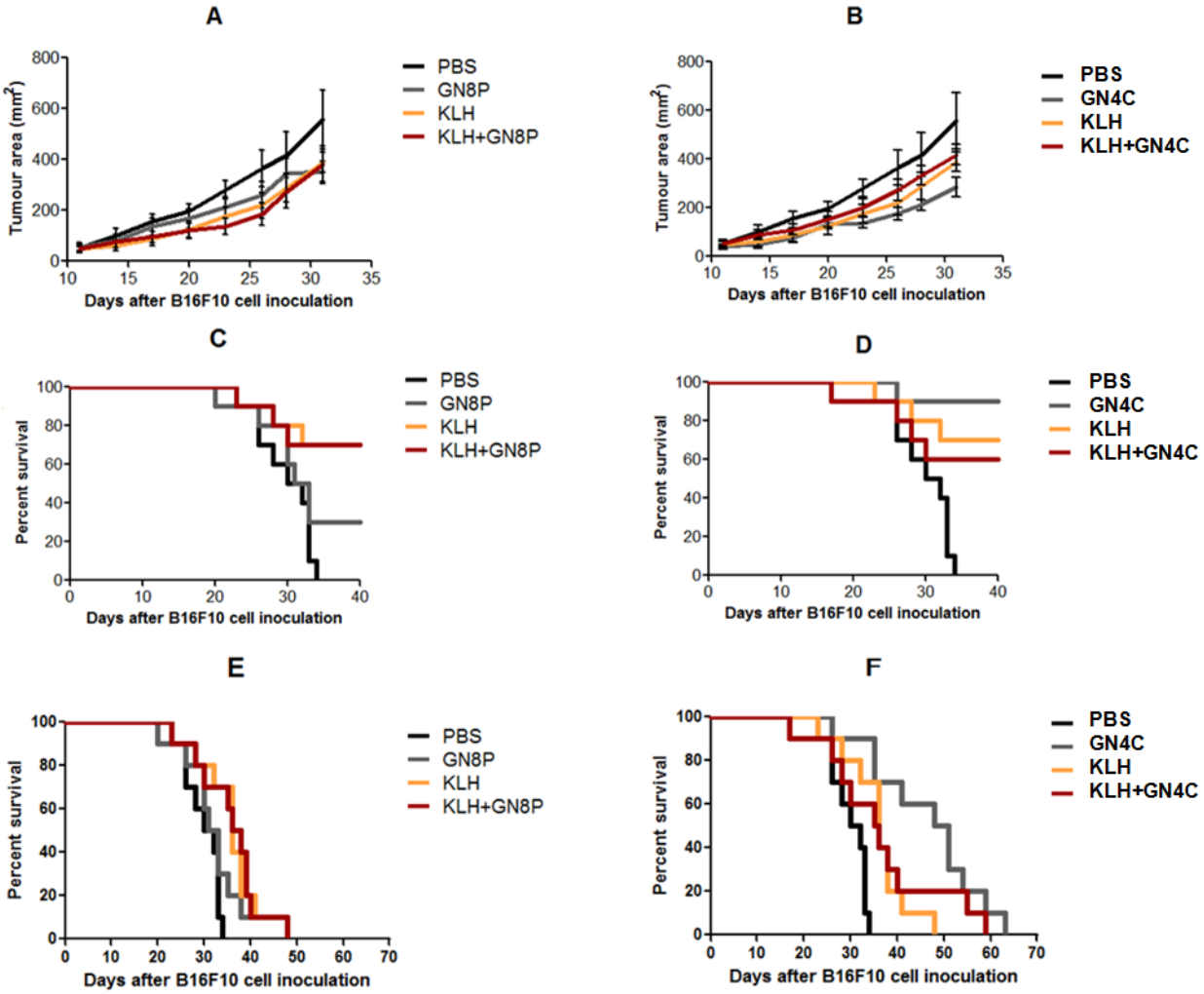



**Figure 23: Effect of glycoconjugates, KLH, and their combination on the tumor growth (A, B) and survival of B16F10 melanoma-bearing mice (C-F).** The mice were inoculated with B16F10 melanoma cells and treated with 6 doses of GN4C, GN8P, KLH, or combination of KLH with one of the glycoconjugates. Control animals were injected with PBS in the same time intervals. The tumor area represents the mean ± SD of values from individual mice. Figure shows a representative example of three performed experiments with similar results (*Hulikova et al.*, accepted for publication).

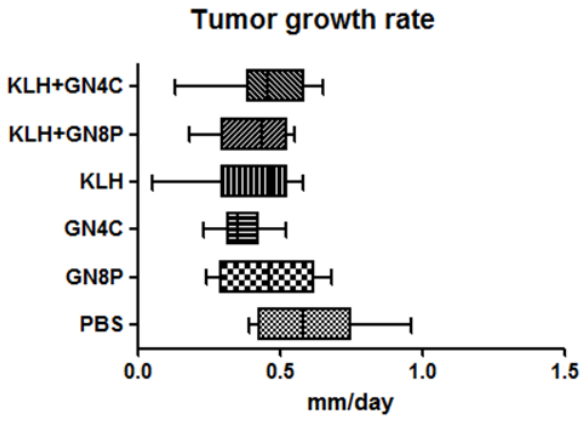
**A**

| Group of mice | Tumor area (mm2) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | on day 11 | | | on day 23 | | | on day 26 | | | on day 31 | | |
| | mean | SD | p | mean | SD | p | mean | SD | p | mean | SD | p |
| PBS | 54 | 22 | | 281 | 50 | | 365 | 101 | | 557 | 167 | |
| GN8P | 57 | 23 | NS | 213 | 57 | < 0.05 | 260 | 73 | < 0.05 | 351 | 57 | < 0.05 |
| GN4C | 47 | 19 | NS | 138 | 33 | < 0.001 | 175 | 35 | < 0.001 | 292 | 46 | < 0.01 |
| KLH | 48 | 21 | NS | 176 | 55 | < 0.001 | 219 | 71 | < 0.01 | 389 | 86 | < 0.05 |
| KLH+GN8P | 47 | 15 | NS | 136 | 46 | < 0.001 | 186 | 62 | < 0.01 | 379 | 107 | NS |
| KLH+GN4C | 51 | 12 | NS | 201 | 50 | < 0.01 | 273 | 61 | NS | 418 | 59 | NS |

**B**

| Group of mice | tumor area | | |
|---|---|---|---|
| | on day 23 | on day 26 | on day 31 |
| | p value | | |
| GN8P vs GN4C | < 0.01 | < 0.01 | NS |
| GN8P vs KLH | NS | NS | NS |
| GN8P vs KLH+GN8P | < 0.01 | NS | NS |
| GN8P vs KLH+GN4C | NS | NS | NS |
| GN4C vs KLH | NS | NS | < 0.05 |
| GN4C vs KLH+GN8P | NS | NS | NS |
| GN4C vs KLH+GN4C | < 0.01 | < 0.01 | < 0.001 |
| KLH vs KLH+GN8P | NS | NS | NS |
| KLH vs KLH+GN4C | NS | NS | NS |
| KLH+GN8P vs KLH+GN4C | < 0.05 | < 0.05 | NS |

**Table 4: Tumor area on day 11, 23, 26, and 31 after B16F10 melanoma cell inoculation in mice treated with GN4C, GN8P, KLH, and their combination.** The tumor area ($mm^2$) represents the mean ± SD of values from individual mice. Significant differences between treated groups and controls (A) as well as between groups with individual types of therapy (B) are depicted (NS = non-significant; *Hulikova et al.*, accepted for publication).

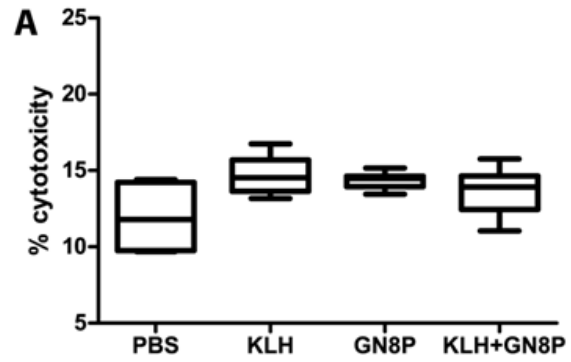



| Group of mice | Tumor growth rate (mm/day) | | |
|---|---|---|---|
| | mean | SD | p |
| PBS | 0.61 | 0.18 | |
| GN8P | 0.47 | 0.15 | NS |
| GN4C | 0.36 | 0.08 | < 0.01 |
| KLH | 0.41 | 0.16 | < 0.05 |
| KLH+GN8P | 0.4 | 0.12 | < 0.05 |
| KLH+GN4C | 0.45 | 0.15 | NS |

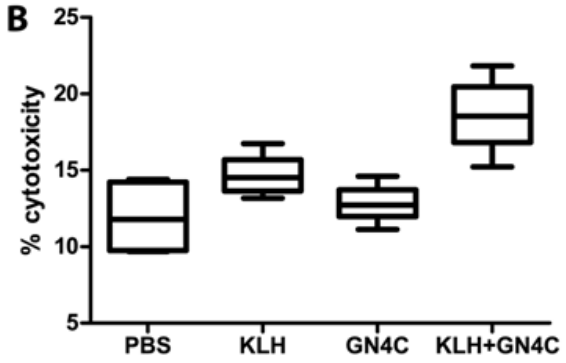
**Figure 24: Tumor growth rate (mm/day) in B16F10 melanoma-bearing mice treated with GN4C, GN8P, KLH, and their combination compared to controls (PBS group).** The boxes represent median and quartiles of values from individual mice. Statistical significance is indicated in the table (*Hulikova et al*., accepted for publication).

### 5.3.4.2. Cytotoxic activity of peripheral blood cells

To determine the effect of therapeutic agents on the cell-mediated cytotoxicity, we performed the $^{51}$Cr-release assay using peripheral blood cells (PBCs) from experimental mice as effectors and the syngeneic B16F10 melanoma cell line as targets. The tumor-bearing animals injected with PBS were used as controls. The B16F10 specificity of cytotoxic cells was proved by comparison with PBCs from healthy C57BL/6 mice. The tumor-bearing mice showed significantly higher lytic activity (11.93 ± 0.8) than healthy ones (9.99 ± 0.42; $p<0.05$). Significant increase of the cytotoxicity was observed in KLH+GN4C (18.61 ± 2.0, $p<0.001$), KLH (14.71 ± 1.11, $p<0.001$), GN8P (14.34 ± 0.52, $p<0.01$), and KLH+GN8P (13.66 ± 1.37, $p<0.05$) treated mice. The GN4C alone did not produce significant changes (12.85 ± 1.02) compared to controls *(Figure 25)*. We can conclude that KLH+GN8P combination did not exert synergistic action on the cytotoxic cell function. On the other hand, KLH potentiated the GN4C-induced lysis of B16F10 targets.




| | p |
|---|---|
| PBS vs KLH | < 0.001 |
| PBS vs GN8P | < 0.01 |
| PBS vs KLH+GN8P | < 0.05 |
| KLH vs GN8P | NS |
| KLH vs KLH+GN8P | NS |
| GN8P vs KLH+GN8P | NS |




| | p |
|---|---|
| PBS vs KLH | < 0.001 |
| PBS vs GN4C | NS |
| PBS vs KLH+GN4C | < 0.001 |
| KLH vs GN4C | < 0.01 |
| KLH vs KLH+GN4C | < 0.001 |
| GN4C vs KLH+GN4C | < 0.001 |

**Figure 25: Effect of glycoconjugates, KLH and their combination on cytotoxic activity of peripheral blood cells from B16F10 melanoma-bearing mice.** Eleven days after B16F10 melanoma cell inoculation, the mice were injected with GN8P and/or KLH (A) or GN4C and/or KLH (B) every three days (6 doses of total). PBS was administrated to controls in the same time intervals. The cytotoxicity was determined on day 27 (24 hours after the last treatment), after 18 hour-long incubation of PBCs with B16F10 tumor targets. The boxes represent median and quartiles of three independent experiments. Statistical significance is indicated in the tables.

### 5.3.4.3. Serum levels of B16F10 melanoma-specific antibodies

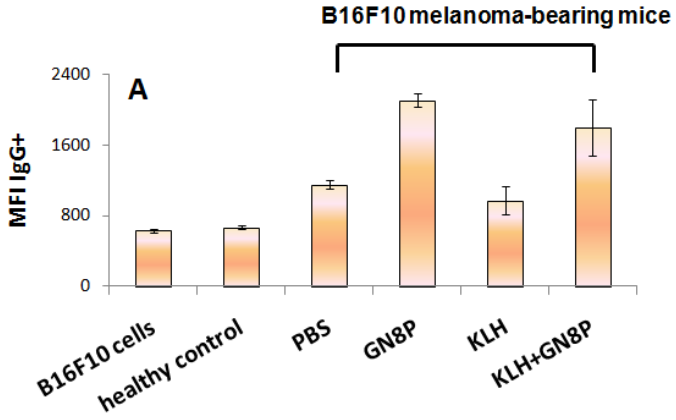
As described above, 3 doses of GN8P augmented the formation of antigen-specific IgG antibodies, including those directed against tumor cells, as well as mRNA expression for IgG2a (*Chapter 5.2.*). Therefore, using the different therapeutic protocol (6 doses of glycoconjugates or KLH administrated alone or as a combination), we also evaluated the serum levels of IgG able to bind to B16F10 melanoma cells by FACS analysis.

The mean fluorescence intensity of B16F10 cells incubated with serum from healthy mice was significantly lower compared to those incubated with sera from tumor-bearing animals, whichever the used type of treatment. However, only the therapy with GN8P ($p<0.001$) or GN4C ($p<0.05$) significantly elevated serum levels of B16F10 melanoma-specific IgG when compared to tumor-bearing controls (PBS group). The combination of KLH with glycoconjugates showed significant increase comparing to KLH alone ($p<0.01$ and $p<0.05$ for KLH+GN8P and KLH+GN4C, respectively), whereas we did not find significant differences between glycoconjugate and KLH+glycoconjugate groups. Thus, the mounted antibody formation was the result of glycoconjugate action, which was not potentiated by KLH.

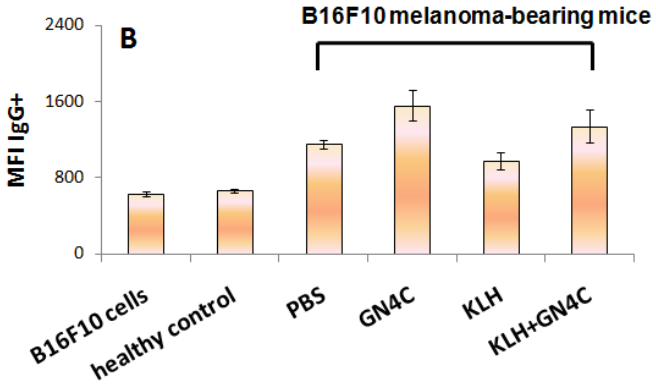
### 5.3.4.4. Antibody-dependent cell mediated cytotoxicity

To evaluate the clinical consequence of glycoconjugate-induced enhancement in serum levels of B16F10 melanoma-specific IgG antibodies, we performed $^{51}$Cr-release assay with SMCs from healthy C57BL/6 mice (effectors) and B16F10 melanoma cells (targets), which were pre-incubated with sera from tumor-bearing animals treated with PBS (controls), glycoconjugates, KLH or their combination (i.e. determination of ADCC reaction).

We observed increase in cytotoxicity of SMCs, when sera from GN8P ($p<0.01$), GN4C ($p<0.01$), KLH+GN4C ($p<0.01$), and KLH+GN8P ($p>0.05$) groups of mice were added to B16F10 tumor targets *(Figure 27)*, which correlated with the presence of anti-B16F10 IgG antibodies *(Figure 26)*.




| | p |
|---|---|
| healthy control vs PBS | <0.001 ↑ |
| healthy control vs GN8P | <0.001 ↑ |
| healthy control vs KLH | <0.05 ↑ |
| healthy control vs KLH+GN8P | <0.001 ↑ |
| PBS vs GN8P | <0.001 ↑ |
| PBS vs KLH | NS |
| PBS vs KLH+GN8P | NS |
| GN8P vs KLH | <0.001 ↓ |
| GN8P vs KLH+GN8P | NS |
| KLH vs KLH+GN8P | <0.01 ↑ |




| | p |
|---|---|
| healthy control vs PBS | <0.001 ↑ |
| healthy control vs GN4C | <0.001 ↑ |
| healthy control vs KLH | <0.05 ↑ |
| healthy control vs KLH+GN4C | <0.01 ↑ |
| PBS vs GN4C | <0.05 ↑ |
| PBS vs KLH | NS |
| PBS vs KLH+GN4C | NS |
| GN4C vs KLH | <0.01 ↓ |
| GN4C vs KLH+GN4C | NS |
| KLH vs KLH+GN4C | <0.05 ↑ |

**Figure 26: Effect of glycoconjugates, KLH and their combination on the formation of B16F10 melanoma-specific antibodies.** Eleven days after B16F10 melanoma cell inoculation, the mice were injected with GN8P and/or KLH (A) or GN4C and/or KLH (B) every three days (6 doses of total). PBS was administrated to controls in the same time intervals. Sera were collected 24 hours after the last treatment. To detect anti-B16F10 IgG levels, B16F10 cells were incubated with 1:10 diluted sera and anti-mouse IgG as described in Materials and Methods. Intact B16F10 cells (not incubated with serum) and those incubated with sera from healthy animals were used as negative controls. The results are expressed as average ± SD of MFI of live B16F10 cells incubated with sera from individual mice. Statistical significance evaluated by ANOVA is indicated in the tables.

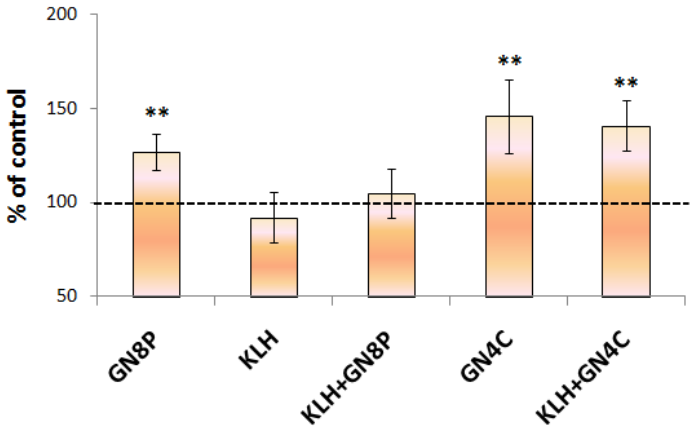



**Figure 27: Antibody-dependent cell mediated cytotoxicity.** Eleven days after B16F10 melanoma cell inoculation, the mice were injected with GN8P, GN4C, KLH or combination of KLH with one of the glycoconjugates every three days (6 doses of total). PBS was administrated to controls in the same time intervals. The ADCC reaction was determined on day 27 (24 hours after the last treatment) after 18 hour-long incubation of SMCs from healthy mice with B16F10 tumor targets pre-incubated with sera from treated tumor-bearing animals. The data are presented as a percentage of control (stated as 100%; dashed line). Figure shows an illustrative example of three performed experiments with similar results. Significant difference ($p>0.01$) was observed in case of GN8P, GN4C, and KLH+GN4C group.

# 6. DISCUSSION

The experimental work of this dissertation thesis is focused on evaluation of immunomodulatory properties of *N*-acetyl-D-glucosamine-substituted glycoconjugates with polyamidoamine (GN8P) or calix[4]arene (GN4C) scaffold, which were synthesized as mimics of physiological ligands for NK cell C-type lectin-like receptor NKR-P1 *(Bezouska et al., 1994a; 1994b; Lindhorst and Kieburg, 1996; Krenek et al., 2007).* We started from our previous results showing that GN8P bound with high affinity to the rat recombinant NKR-P1A molecule and triggered anticancer immune response in rat colorectal carcinoma as well as mouse melanoma model (i.e. tumor infiltration with activated NK cells, reduced tumor incidence and growth, prolonged survival time of experimental animals), *(Bezouska et al., 1998; Pospisil et al., 2001; Vannucci et al., 2003).*

Rat NKR-P1A, one of the first identified and studied NK cell lectin-like receptors *(Giorda et al., 1990)*, represents orthologous molecule to the mouse activating NKR-P1C isoform. In C57BL/6 mice, NKR-P1C receptor reacts with anti-NK1.1 mAb (PK136), whereas other mouse strains (e.g. BALB/c or DBA/2) are NK1.1-negative. Initially, this was attributed to the strain-specific deficiency in *Nkr-p1c* gene expression. However, currently it is known that mouse NKR-P1C receptor exists in two distinct forms NKR-P1C$^{B6}$ and NKR-P1$^{BALB/c}$ encoded by corresponding alleles (*Nkr-p1c*$^{B6}$ and *Nkr-p1c*$^{BALB/c}$, respectively). As a consequence of *Nkr-p1c* gene divergence, anti-NK1.1 mAb binds to NKR-P1C$^{B6}$, but does not to NKR-P1$^{BALB/c}$ protein (*Carlyle et al., 2006; reviewed in Mesci et al., 2006).* These findings put the question, whether there is a difference in response to GN8P treatment comparing NK1.1-negative and NK1.1-positive mouse strains.

We tested NK1.1 expression, *Nkr-p1* gene family distribution *(Nkr-p1 a, b, c, d, f* members), and *Nkr-p1c* gene divergence in mouse strains and F1 hybrids used in our experiments (C57BL/6 and BALB/c mice were chosen as prototypes) by FACS analysis and PCR genotyping, respectively *(Table 1; Figure 1).* Our results were in agreement with those obtained by *Carlyle et al. (2006) and Aust et al. (2009).*

As we considered GN8P to be a potential ligand for mouse NKR-P1C receptor, predominantly expressed on NK cells, we firstly evaluated its influence on NK cell functions (i.e. NK cell-

mediated cytotoxicity and cytokine secretion). The GN8P significantly increased the cytotoxic activity against NK cell-sensitive tumor targets YAC-1 *(Figure 2)* and production of IFN-γ, at the protein as well as mRNA level *(Figure 3, 4)*, in both healthy and syngeneic tumor-bearing C57BL/6 mice. FACS analysis confirmed that NK cells represented the major subpopulation involved in GN8P-induced enhancement of IFN-γ levels, followed by NKT cells *(Figure 5)*. In contrast, GN8P did not exert such effects in NK1.1-negative mouse strains *(Figure 2, 3, 4)*. These results indicate that GN8P is able to effectively engage NKR-P1C$^{B6}$, but not NKR-P1C$^{BALB/c}$ isoform, which is also supported by reports showing that cross-linking of NKR-P1C$^{B6}$ receptor with anti-NK1.1 mAb triggered NK cell-mediated cytotoxicity *(Karlhofer and Yokoyama, 1991)* as well as IFN-γ secretion by NK and NKT cells *(Arase et al., 1996)*. Increase in NK cell-mediated killing and release of IFN-γ, the cytokine playing a crucial role in anticancer immune response *(Chapter 3.3.2.1.; reviewed in Dunn et al., 2006)*, correlated with significant decrease in tumor growth observed in B16F10 melanoma-bearing C57BL/6 mice after GN8P administrations *(Figure 6)*. As we expected, significant differences in tumor area between CT26 carcinoma-bearing BALB/c mice injected with GN8P and controls (PBS group) were not found *(Figure 6)*. Serum levels of TNF-α, the pro-inflammatory cytokine, which was proved to promote the tumor growth and metastasis *(Chapter 3.3.1.; reviewed in Balkwill and Mantovani, 2001)*, were significantly elevated only in healthy, but not in melanoma-bearing C57BL/6 mice. The secretion of Th2-type cytokine IL-4, that can be produced by NKT cells, was stimulated by GN8P treatment to a lesser extent than Th1-type IFN-γ and IL-2 *(Figure 3)*. As described above *(Chapter 3.3.2.1.; reviewed in Johansson et al., 2008)*, polarization of the immune system to Th1 responses is crucial for cancer immunosurveillance.

We did not observe significant differences in the relative number of basic lymphocyte subpopulations (i.e. B, T, NK and NKT cells) in the spleen *(Table 2, Figure 7A, C)* comparing GN8P with PBS treated groups of animals, independently of mouse strain and health/tumor conditions. However, GN8P decreased NK1.1 expression on NK and NKT cells *(Table 3)*. This might imply that GN8P is predominantly up-taken by NK cells in a complex with NK1.1 receptor resulting in their functional activation (cytotoxic activity, IFN-γ synthesis). Similarly, *Aust et al. (2009)*, who prepared a new panel of mAbs specific for individual mouse NKR-P1 proteins, demonstrated that NKR-P1 expression on NK cells was down-regulated upon cross-linking with the relevant mAb or ligand. On the other hand, expression of mRNA for NKR-P1C$^{B6}$ receptor was significantly enhanced after GN8P administrations in both healthy and tumor-bearing mice suggesting a regulatory feed-back effect, whereas in BALB/c mice, in

general, GN8P did not induce any changes *(Figure 8)*. Furthermore, we showed that the GN8P competed with anti-NK1.1 mAb in phosphotyrosine protein signalling *(Pospisil et al., 2005)*, which provided another proof of GN8P-mediated stimulation of NK1.1-positive cells in C57BL/6 mice.

Taken together, all above discussed data confirm the hypothesis that *Nkr-p1c* gene divergence determines the susceptibility of mouse strains to GN8P treatment (unresponsiveness in BALB/c vs. activation of NK cell functions, including cancer surveillance in C57BL/6 mice). Although our immunological assays were performed before preparation of the mouse recombinant NKR-P1C$^{B6}$ molecule, they predicted (correlated with) the results of plate binding experiments showing that GN8P was its high affinity ligand *(Rozbesky, 2009)*. The biochemical studies with recombinant NKR-P1C$^{BALB/c}$ protein are currently in progress. We can only hypothesize that GN8P might bind to NKR-P1C$^{BALB/c}$ molecule with affinity too weak for triggering the signalling cascade, or might be not able to recognize it at all. Thus, variations in amino acid sequence, produced by *Nkr-p1c* gene divergence, could influence not only NKR-P1C – anti-NK1.1 mAb, but more importantly, carbohydrate – NKR-P1C receptor interactions. Similarly, the product of Ocil/Clr-like cytomegalovirus gene, which inhibits NK cell function, binds to NKR-P1B receptor dependently on the rat strain as a consequence of rat *Nkr-p1b* gene divergence *(Voigt et al., 2007)*.

Although NK cells are primarily known to serve as the first line of defence against malignant transformed and virus-infected cells, they are also able to regulate functions of other immune cell types, including B cells *(Chapter 3.2; reviewed in Yuan 2004)*, either via direct intercellular contact and/or cytokine secretion. We demonstrated that GN8P administrations in C57BL/6 mice significantly mounted serum levels of IgG2a antibodies specific for both T-independent (DNP-LPS) and T-dependent (KLH) antigen *(Figure 9)*, when compared with controls primed with the antigen alone. This finding is in agreement with previous reports showing that activated NK cells e.g. by poly (I:C), (in our experimental model by GN8P), preferentially up-regulated IgG2a formation *(Wilder et al., 1996; Koh and Yuan, 1997; 2000)*. Furthermore, the GN8P induced significant enhancement of anti-KLH *(Figure 12C)*, anti-DNP *(Figure 12D)* as well as anti-B16F10 melanoma (tumor-specific) IgG response *(Figure 13)*. Results of real-time RT PCR analysis indicate that the GN8P elevated mRNA transcripts for anti-KLH (healthy mice) as well as anti-B16F10 IgG2a antibodies (tumor-bearing mice), *(Figure 14)*.

It is generally accepted that specific antibodies participate in protection against certain viral and bacterial infections. However, their role in the control of tumor spreading is not single valued as elevated levels of antibodies against tumor antigens paradoxically correlated with decreased survival of patients with several cancer types. The possible involved mechanisms are following: (i) masking of tumor antigens against recognition by immune cells; (ii) promoting chronic inflammation via formation of immunocomplexes (link between cancer and inflammation is explained in *Chapter 3.3.1.*); etc. *(Gumus et al., 2004; reviewed in Johansson et al., 2008)*. On the other hand, anticancer vaccines, which elicit antibody response triggering ADCC reaction and/or complement-dependent lysis of tumor cells, were successfully tested in clinical trials *(Chapter 3.3.3.1; Slovin et al., 1999; Sabbatini et al., 2000; Slovin et al., 2003; 2005b; Gilewski et al., 2007).*

Antibody functions, in general, and in relation to cancer immunosurveillance, depend on Ig isotype/subclass. Namely, IgG2a antibodies represent the most efficient IgG subclass in mediating ADCC reaction under both *in vitro* and *in vivo* conditions *(Koh and Yuan, 2000; Nimmerjahn and Ravetch, 2005; 2007)*. In accordance with this knowledge, we demonstrated that SMCs (isolated from healthy C57BL/6 mice) showed increased cytotoxic activity against B16F10 tumor targets, which were pre-incubated with sera from B16F10 melanoma-bearing mice treated with GN8P *(Figure 27)*. Thus, GN8P-induced enhancement of anti-B16F10 IgG levels *(Figure 13)* resulted in augmented ADCC reaction. Not less importantly, IgG2a formation is indicated as "Th1-like" (antitumor) antibody response. NK cells were reported to programme B cells to switch to IgG2a via IFN-γ secretion (*Wilder et al., 1996; Koh and Yuan, 1997)*. In our experimental model, increased IFN-γ production by GN8P-activated NK cells is likely to be one of the events leading to up-regulation of serum and mRNA IgG2a levels as well.

Significant increase in the number of anti-SRBC IgG forming cells *(Figure 15)* and in percentage of CD138-positive plasma cells *(Figure 16)* revealed by PFC assay and FACS analysis, respectively, confirmed the modulatory effect of GN8P on antibody formation. Furthermore, GN8P promoted antigen presenting capacity of I-A/I-E-positive B cells by up-regulation of CD80 and CD86 co-stimulatory molecule expression *(Figure 17)*.

It was reported that *in vitro* IgM formation can be induced by activated NK cells *(Amirogena et al., 1990; Becker et al., 1990; Michael et al., 1991; Gray and Horwitz, 1995; Vos et al., 1998; reviewed in Yuan, 2004)*. In agreement, we found out that IgM levels in supernatants of SMCs

from C57BL/6 mice stimulated *in vitro* simultaneously with DNP-LPS and GN8P were significantly increased comparing to those primed with the antigen alone *(Figure 10)*. However, this enhancement was not observed after depletion of CD49b-positive or NK1.1-positive cell subpopulations *(Figure 11)*. These data proved that in C57BL/6 mice NK, eventually NKT cells (CD49b-positive cells with high expression of NK1.1 receptor) play a crucial role in modulation of antibody formation by GN8P.

On the other hand, GN8P did not change *in vitro* anti-DNP IgM levels in NK1.1-negative BALB/c and DBA/2 mice, measured under same conditions as in C57BL/6 ones *(Figure 10)*, which indicates that GN8P effects on antibody response are also dependent on NKR-P1C$^{B6}$ isoform. To support these results we evaluated anti-KLH and anti-DNP IgG2a levels in serum of BALB/c and DBA/2 mice as well as C57BL/6 x BALB/c and C57BL/6 x DBA/2 F1 hybrids. In BALB/c and DBA/2 mice, neither anti-DNP nor anti-KLH IgG2a formation was influenced by GN8P administrations *(Figure 9)*, while in NK1.1-positive F1 hybrids expressing both *Nkr-p1*$^{B6}$ and *Nkr-p1c* $^{BALB/c}$ gene forms *(Table 1*, *Figure 1)*, IgG2a antibodies specific for the used antigens were significantly mounted, but to a lesser extent than in C57BL/6 mice *(Figure 9)*.

From the above discussed data, we can conclude that: (i) GN8P participates in regulation of B cell response, at various levels, via NK cell activation; (ii) tumor-specific IgG antibodies induced by GN8P mediate ADCC reaction, and thus contribute to GN8P anticancer properties; (iii) inability of GN8P to increase *in vivo* IgG2a and *in vitro* IgM formation in NK1.1-negative mouse strains confirmed our hypothesis about the importance of *Nkr-p1c* divergence.

Despite the essential role of carbohydrates in a wide range of biological phenomena, including intercellular communications and immune recognition, there are relatively few glycodrugs (i.e. compounds, in which the sugar moiety carries the biological function) for clinical use *(reviewed in Williams and Davies, 2001)*. As described above *(Chapter 3.4.)*, the lectin binding potency and specifity of glycoclusters depend not only on the saccharide epitope density but also on the nature of the scaffold and the geometrical characteristics of multivalent assembly. There is a variety of core structures that can be constructed, which enables to organize and orient carbohydrate units in a multitude of topographic arrays. The introduction of calix[4]arenes as a novel scaffold of glycoconjugates brought the advantage of better control of the three-dimensional structure by changing their conformation or calix[4]arene – sugar spacer.

Moreover, an appropriate substitution of the calix[4]arene phenolic function ensures fixation of the desired conformation indefinitely in time *(Krenek et al., 2007).*

GlcNAc-substituted calix[4]arene (GN4C) used in our further studies was identified to be the best ligand for the rat recombinant NKR-P1A molecule *(Figure 18A).* Evaluating the immunostimulating properties of GN4C *in vitro*, we observed that this glycoconjugate enhanced the proliferation rate *(Figure 19)* and NK cell-mediated cytotoxicity of human PBMCs, to a greater extent than GN8P *(Figure 20).* Later, we proved the direct effect of GN4C on human sorted NK cells (CD56+/CD3-) showing that after GN4C treatment, they also displayed increased proliferation and cytolytic activity against both NK-sensitive K562 and NK-resistant HT-29 cell lines. Furthermore, fresh NK cells incubated with GN4C secreted higher levels of IL-2 than untreated controls. This cytokine might serve as the autocrine factor for proliferation and cytotoxicity enhancement *(Benson et al., 2010).*

Unlike rodents (mice and rats), in humans, only one non-polymorphic gene encoding NKR-P1A receptor (CD161) was described *(Chapter 3.1.2.1.2.; Lanier et al., 1994).* The engagement of NKR-P1A with its physiological ligand lectin-like transcript 1 (LLT-1) leads to inhibition of NK cell activity. However, signalling through this receptor, containing non-canonical ITIM-like motif in the structure, remains unclear *(Lanier et al., 1994; Aldemir et al., 2005; Rosen et al., 2005)*. The GN4C-induced enhancement of NK cell functions (cytotoxicity, proliferation, IL-2 secretion) might be explained by the following hypotheses: (i) involvement of other receptor(s) than NKR-P1A; (ii) dual function of NKR-P1A receptor depending on the type of ligand. We suggest CD69 early activation marker to be the candidate receptor, which could mediate GN4C effects, since GN4C was unexpectedly found to represent the highest affinity ligand for human recombinant CD69 molecule, identified so far *(Figure 18B).* In addition, the CD69 cross-linking on NK cells was reported to trigger the lysis of tumor targets *(Testi et al., 1994; Marzio et al., 1999; Sancho et al., 2005).* The second hypothesis is supported by observations of *Christiansen et al. (2006),* who identified carbohydrate moieties such as *N*-acetyl-lactosamine as potential ligands for human NKR-P1A receptor with probable activating function.

To evaluate *in vivo* effects of GN4C we used C57BL/6 mouse model. We proved again that both GN4C and GN8P influenced predominantly NK cell function as sorted NK cells, but not CTLs and SMCs, obtained from tumor-bearing or healthy animals treated with one of the glycoconjugates, showed significant increase in NKR-P1-dependent cytolysis of IC-21 targets

compared to controls (PBS group), *(Figure 22A, C)*. The GN4C also significantly enhanced CTL-mediated cytotoxicity against B16F10 targets in tumor-bearing mice, but to a lesser extent compared to its action on NK cells *(Figure 22B)*.

The above discussed findings on GN4C encouraged us to test the therapeutic anticancer properties of this glycoconjugate. We demonstrated for the first time that GN4C was able to significantly prolong the survival time of treated mice and slow the tumor growth in B16F10 melanoma model *(Figure 23, 24; Table 4)*. The GN4C exerted superior effects to the previously tested GN8P *(Vannucci et al. 2003)*, which was used in the current protocol in lower dose (comparable to that of GN4C). This correlated with results described above *(Figure 22B)* showing that the GN4C, but not the GN8P, significantly increased the cytotoxic activity of sorted NK cells as well as CTLs from melanoma-bearing mice against B16F10 targets. The nature of scaffold (calix[4]arene vs. polyamidoamine) could also play a role, which is supported by the recent study of *Bezouska et al. (2010)* reporting that carboxylated calix[4]arenes by themselves protected $CD69^{high}$ lymphocytes from apoptosis as a consequence of their binding to CD69 molecule. As the GN4C is the high affinity ligand for both NKR-P1 and CD69 molecule *(Figure 18)*, involvement of other cell subpopulations than NK1.1-positive ones can be expected (e.g. cytotoxic T cells). Moreover, we demonstrated that GN4C reduced the proliferation rate of transformed NK-92 cells (NK cell leukaemia), which was in agreement with down-regulated expression of genes for proteins promoting the cell growth (e.g. *EGFR1*, *c-MYC*). Simultaneously, GN4C decreased the expression of genes for pro-angiogenic factor VEGF, as well as glycosyltransferase MGAT5 in tumor cells, while increased their susceptibility to NK cell-mediated killing *(Benson et al., 2010)*. As described above *(Chapter 3.3., and particularly 3.3.3.)*, genes found to be affected by GN4C are crucial for the tumor growth, metastasis, and angiogenesis. Therefore, inhibition of their expression is of great interest. Based on these findings, we can suppose two principal mechanisms of GN4C antitumor activity: (i) triggering of NK (NKT and T) cell functions through NKR-P1 and/or CD69 receptor, and protecting them from apoptosis; (ii) modulation of the tumor growth, angiogenesis, and glycosylation pattern. NK cell activation as well as increased tumor immunogenicity could contribute to GN4C-induced enhancement of B16F10 melanoma-specific IgG levels mediating ADCC reaction *(Figure 26, 27)*.

As discussed in *Chapter 3.3.3.2.*, KLH was shown, among others, to decrease the tumor incidence and augment anticancer effects of IFN-α in athymic mice bearing HTB68 human

melanoma (*Rizvi et al., 2007)*. We demonstrated for the first time that KLH significantly reduced the tumor growth in syngeneic B16F10 melanoma mouse model, but to a lesser extent than GN4C. However, in our experimental schedule, KLH did not work in synergism with glycoconjugates. This might be caused by the fact that KLH and glycoconjugates, containing the multiple GlcNAc moieties *(Harris and Markl 1999; Krenek et al. 2007)*, could compete for shared carbohydrate-binding C-type lectin-like receptors important for tumor cell recognition. Discrepancies between the results of cytotoxic assay and survival parameters observed in GN4C treated mice *(Figure 25)* might be explained by the glycoconjugate-mediated recruitment of cytotoxic cells into the tumor microenvironment, reducing their number in the peripheral blood. As we described earlier, the glycoconjugate induced increase of activated NK cells inside the melanoma (*Vannucci et al. 2003*; *Hulikova et al*., accepted for publication). The promising antitumor properties of GN4C, involving both the carbohydrate structure as well calix[4]arene scaffold, make it perspective for further research in cancer immunotherapy.

# 7. CONCLUSIONS

In healthy as well as B16F10 melanoma-bearing C57BL/6 mice (NK1.1-positive, expressing NKR-P1C$^{B6}$ receptor), GN8P increased: (i) the lysis of NK cell-sensitive tumor targets; (ii) serum and mRNA levels of IFN-γ, which was synthesized by NK and NKT cells; (iii) expression of mRNA for NKR-P1C receptor. In contrast, GN8P did not exert these effects in BALB/c mice (NK1.1-negative, expressing NKR-P1C$^{BALB/c}$ receptor).

**The GN8P-mediated NK cell activation requires NKR-P1C$^{B6}$ expression.**

In C57BL/6 mice, GN8P (i) augmented antigen-specific (anti-KLH, anti-DNP, and anti-B16F10 melanoma) IgG and particularly IgG2a serum levels, and *in vitro* anti-DNP IgM formation; (ii) increased the number of CD138-positive plasma cells, anti-SRBC plaque forming cells, and antigen-presenting B lymphocytes in the spleen. In DBA/2 and BALB/c mice (expressing NKR-P1C$^{BALB/c}$), GN8P influenced neither *in vitro* IgM nor *in vivo* IgG2a response. The GN8P-mediated enhancement in IgG2a levels was restored in C57BL/6 x BALB/c and C57BL/6 x DBA/2 F1 hybrids (NK1.1-positive, expressing the both *Nkr-p1c* gene forms). After depletion of NK cells (CD49b+ or NK1.1+) in C57BL/6 mice, GN8P-induced increase of *in vitro* antibody formation was blocked. Up-regulated synthesis of IFN-γ, which is known to support class switch to IgG2a, was in correlation with secretion of this immunoglobulin subclass.

**The GN8P is involved in regulation of antibody formation via NK cell activation.**

In B16F10 melanoma model, the newly synthesized GN4C (i) stimulated not only NK cell but also CTL-mediated cytoxicity against B16F10 targets; (ii) elevated serum levels of B16F10 melanoma-specific IgG levels triggering ADCC reaction; (iii) significantly slowed the tumor growth and prolonged the survival time of experimental mice.

**The GN4C exerted *in vivo* anticancer effects, superior to those observed after therapy with GN8P.**

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# 9. PUBLICATIONS OF THE AUTHOR

## PUBLICATIONS RELATED TO THE DISSERTATION THESIS

### PAPERS PUBLISHED IN JOURNALS WITH IF

1. Krenek K, Kuldova M, **Hulikova K**, Stibor I, Lhotak P, Dudic M, Budka J, Pelantova H, Bezouska K, Fiserova A, Kren V: N-acetyl-D-glucosamine substituted calix[4]arenes as stimulators of NK cell-mediated antitumor immune response. *Carbohydr Res* 342, 1781-92, 2007

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1. Pospisil M, Vannucci L, Luptovcova M, Svoboda J, **Hulikova K**, Kuldova M, Bezouska K, Krist P, Kren V, Pesta M, Fiserova A: The interaction of glycodendrimers with tumor microenvironment. Book of Proceedings of the 3rd International Conference (Prague, Czech Republic, October 12-16, 2004) on Tumor Microenvironment: Progression, Therapy and Prevention. Medimond srl, Bologna, Italy; ISBN: 978-88-7587-135-2; 43-49, 2005

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1. Osmancik P, Jirmar R, **Hulikova K**, Peroutka Z, Pompachova A, Motovska Z, Widimsky P: A comparison of the VASP index between patients with hemodynamically complicated and uncomplicated acute myocardial infarction. *Catheter Cardiovasc Interv* 75(2):158-66, 2010

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