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NK buňky a jejich receptory v imunitní regulaci – možné cíle pro imunomodulaci

NK cells and their receptors in immune regulation – possible targets for immunomodulation

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Contents

Abstrakt (česky)

Přirození zabíječi - buňky NK hrají důležitou roli v imunitním dohledu a regulaci jednak přímým cytotoxickým působením na infikované, transformované či jinak poškozené buňky, ale také produkcí cytokínů a chemokínů. Výsledná odpověď je dána převahou stimulačních nebo inhibičních signálů, přenášených širokou paletou membránových receptorů. Zabíječské s Ig–příbuzné molekuly KIR2DL4 a LILRB1, které rozpoznávají vlastní HLA–G molekuly během těhotenství stejně jako NKR–P1 receptory lišící se ve funkci a počtu izotypů jsou druhově závislé a redukované v průběhu fylogeneze, zatímco NKG2D, reagující na stresem indukované proteiny a adenozínové receptory (AR) potlačující zánětovou reakci, zůstávají evolučně konzervované.

Cílem této práce bylo studium zapojení NK buněk a jejich receptorů v několika modelech imunitních poruch a u různých savčích druhů, za účelem získání nového náhledu na jejich funkci a možnost imunitní modulace.

Ukázali jsme zde, že výběr druhu ve studiu ovlivnění NK buněčné funkce může být v některých případech kritický. Reakce na glykany, s využitím syntetického GlcNAc terminovaného glykomimetika GN8P, měla protichůdné účinky na NK buněčnou funkci u lidí a C57Bl/6 myší. U lidí byla snížena cytotoxická aktivita NK buněk s vysokou expresí NKR–P1A, zatímco u myší podání GN8P aktivalo NK buňky a B16F10 specifickou tvorbu protilátek IgG2a izotypu, která následně zvýšila na protilátkách závislou cytotoxicitu (ADCC). Tento účinek byl pozorován pouze u myší nesoucích *Nkr-p1(T)* gen (kódující NK1.1 receptor). Endogenní hormon lidský chorionický gonadotropin (hCG), projevil jako většina hormonů, míru pleiotropie ve svém účinku. Pozorovali jsme posílení CD8 T buněk na úkor Th lymfocytů a zvýšenou expresi KIR2DL4 receptoru na NK buňkách, která podporuje produkci cytokinů. Navíc je tento účinek protichůdný k zamýšlenému užití hCG, tj. zlepšení výsledku postupů asistované reprodukce, protože takový profil byl pozorován u neúspěšných implantací. Evoluční konzervovanost adenozínových receptorů byla ověřena pomocí agonisty A_{2A}AR – CPCA, který měl srovnatelné tlumící účinky na NK buněčnou cytotoxicitu u všech sledovaných savčích druhů (člověk, prase, koza, potkan, myš) u zdravých i nádorových jedinců. Lokální mikrovlnná hypertermie (HT) *in vivo* do místa nádoru vykázala pozitivní účinek nejen na redukci nádorových buněk, ale také na NK buněčnou cytotoxicitu, přesto že celkové zastoupení NK buněk ve slezině nebylo ovlivněno. Tento postup je však omezen pouze na lokalizované, primární nádory. Pro další optimalizaci HT byly vyvinuty multifunkční nanočástice založené na lidském feritinu a nádorově specifických cílících struktur. Tato nanoplatforma může zvýšit efektivitu HT terapie i na cirkulující nádorové buňky nebo metastatická ložiska.

Naše výsledky prokázaly klíčové zapojení NK buněk v rozvoji a regulaci imunitní odpovědi v průběhu autoimunitních a reprodukčních poruch, nádorové transformaci nebo teplotního šoku. Tato práce přináší nové možnosti imunitní modulace prostřednictvím NK buněk, ale další výzkum je nutný k jejich plnému využití.

Zde poskytujeme základy k těmto výzkumům a následně možným budoucím klinickým aplikacím. V dalším studiu bude perspektivní sledovat vliv hormonálních hladin nebo autoimunitních změn u myších kmenů v závislosti na NKR–P1 a Ly49 fenotypu.

Abstract (english)

Natural Killers – NK cells play an important role in immune surveilance and regulation either by direct cytotoxicity towards infected, transformed or otherwise damaged cells, or by production of cytokines and chemokines. The resulting response of NK cells is given by the sum of stimulating and inhibiting signals, tranduced by a wide array of receptors. Killer Ig–like receptors KIR2DL4 and LILRB1, which recognize self HLA–G molecules in pregnancy, as well as NKR–P1 receptors, which differ in the number of isotypes, are species–dependent and reduced during phylogenesis. NKG2D, reacting to stress–inducible proteins, and adenosine receptors (AR), which supress the inflamatory reaction, remain evolutionary conserved.

The aim of this work was to study the involvement of NK cells and their receptors in several immune disorders and in various species, to provide new insights into their function and posisible immune modulation.

We have shown here, that the choice of species in the study of NK cell effector functions may be crucial in some cases. The reaction to glycans, using synthetic GlcNAc–terminated glycomimetics GN8P, exerted opposing effects on NK cell function in humans and C57Bl/6 mice. In humans, the glycomimetic decreased cytotoxic activity of high NKR–P1A expressing NK cells, while in mice it mounted an NK cell–mediated antibody formation and tumor–specific IgG2a production with subsequent increase in antibody dependent cellular cytotoxicity (ADCC). This effect was observed only in C57BL/6 mice expressing *Nkr–p1c(T)* gene (coding NK1.1 receptor). Endogenous hormone human chorionic gonadotropin (hCG), exerted as most other hormones a degree of pleiotropy in its effect. We observed a preference of cytotoxic over helper T cells and increased KIR2DL4 expression on NK cells, which renders them more prone toward cytokine production. Moreover, this effect proved to be antagonistic to the original intent of the hCG use – that is to improve the outcome of assisted reproduction courses, since such profile was observed during failed embryotransfers. A_{2A} adenosine receptor agonist CPCA on the other hand, was used to prove the evolutionary conserved mechanisms in its function, by thwarting NK cell cytotoxicity in healthy and immunocompromised subjects equally (human, pig, goat, rat, mouse). *In vivo* tumor–localized hyperthermia (LHT) proved to have beneficial effect on NK cell–mediated lytic activity, despite the NK cell distribution remained unchanged. This procedure is however limited to localized, primary tumors. For further optimization of LHT, multifunctional ferritin–based nanoparticles with tumor targeting structures were developed. This nanoplatform may increase the efficacy of LHT therapy onto circulating cancer cells or metastatic foci.

Our results proved the key involvement of NK cells in the development and regulation of immune response in autoimmune and reproductive disorders, tumor transformation or heat–induced stress. This work brings new options for NK cell–mediated immune modulation, but further research is needed to achieve their full potential. We here provide the basis for this research and its possible clinical applications in the future. It would be perspective in future studies to observe the hormonal levels or autoimmune changes in murine strains with varying NKR–P1 and Ly49 phenotypes.

1. Introduction

In humans, there are two major NK cell subpopulations present in the peripheral blood, based on the presence and the expression level of two specific surface markers – CD56 and CD16. Low CD56 expressing NK cells, termed $CD56^{dim}$ (CD16+) have high cytotoxic capability and are prevalent in peripheral blood, while the high–density CD56 ($CD56^{bright}$ – comprising approximately 1% of peripheral blood lymphocytes (CD16-)) have only marginal cytotoxic activity, and produce high amounts of IFNγ and TNFα (reviewed in (1)). These two subpopulations differ in the expression of over 400 gene transcripts (2).

Murine NK cells lack the expression of CD56 or other ortholog, but based on the expression of CD27, it is believed to distinguish the cytokine producer (CD27+) and cytotoxic NK cell subpopulations in mice (3, 4) as well, despite this division is not as clear–cut as in humans. Murine NK cells express CD49b, which could also be used to define NK cell populations in mice (1), and more recently a more suitable marker, NKp46, was described (5). The prototype murine NK cell marker NK1.1, which was frequently used in the past to define NK cells, is not even detectable on many murine strains due to the distinct allele expression in these strains (6). Nevertheless, two distinct NK cell populations are not enough to describe all the various effector functions exerted by NK. Thus the wide array of responses to outer stimuli must be established by the numerous NK cell receptors signaling pathways.

Unlike T or B cells, NK cells do not express clonally distributed receptor for antigen (7, 8). Instead, they are believed to act upon weighing the balance of many different inputs before they decide whether to kill a target (9, 10). To understand this moment of decision, one must realize that there are many different receptors on the surface of NK cells – some deliver activating and others inhibiting signals, while some may deliver both, depending on the circumstances and on the activation state of the cell (11). Inhibitory receptors are usually determined by the presence of immunoreceptor tyrosine–based inhibition motif (ITIM) sequence in their cytoplasmic tail, which recruits protein tyrosine phosphatase SHP–1 (12). It was proposed by Ljunggren and Kärre that the expression of self MHC molecules protects healthy cells from NK cell lysis and its absence or aberrant expression promotes the NK cell–mediated cytotoxicity (13). Thus malignant or virus–infected cells, which try to evade the T–cell recognition via MHC are attacked by NK cells (14). This would imply, that there has to be an "on" signal, whenever NK cells interact with a potential target (15). Activating receptors lack the ITIM sequence and rather associate via charged residue in the cytoplasmic tail with adaptor proteins (10). These proteins contain activation motifs (ITAMs) and are able to recruit protein tyrosine kinases and subsequently activate transcription (11).

NK cell receptors are able to respond to great numbers of target structures which are either directly associated with given pathogen infected or transformed cell, or indirectly by co–stimulation by cell population recognizing and presenting the pathogen–associated structure. Since NK cells are primarily designed to destroy malignant, infected, transformed or stress–damaged cells, they seldom recognize the exogenous pathogens themselves. While they can be directly activated by virus–derived proteins via their NKp (16) or Ly49H receptors (17), it only occurs when these proteins are expressed on the surface of the infected cell. As of yet, little conclusive evidence was presented to suggest direct NK cell activation by bacteria, or protozoan parasites (reviewed in (18)). Despite their inability to

recognize such pathogens directly, NK cells still play critical role in their clearance. Indirect activation of NK cells by bacteria is well–documented in numerous bacterial models and generally requires TLR–mediated activation of mDCs and monocytes or macrophages to secrete IL–12, IL–18 and type–1 interferons (19). More recent studies also pointed to the necessity of direct DC–NK cell contact for activation of effector function (20), suggesting DC–mediated antigen presentation and recognition by NK cells.

NK cells are involved in almost all aspects of immune response – aside from viral infection and malignant transformation in autoimmunity (21, 22), pregnancy (23) and transplantation immunology (24) not only by their cytotoxic effector function, but also as powerful cytokine producers. This makes NK cells excellent candidates for immune modulation and vaccine development (25). Among such development belong the recently emerging synthetic carbohydrate–based vaccines (26), which use multivalent glycans as mimetics of common antigens in a diverse set of pathologies (reviewed in (27)). Aberrant glycosylation with branched *N*–Acetyl–glucosamine (GlcNAc) subunits, among others, was also associated with ongoing oncogenesis and metastatic potential (reviewed in (28)). Tumor associated carbohydrate antigens (TACAs) are overexpressed on a variety of cancer cell surfaces, which present tempting targets for anticancer vaccine development. The local density of antigen rather than the total amount of antigen administered was found to be crucial for induction of high Tn antigen (*N*–Acetyl–galactosamine–serin–threonine)–specific IgG titers (29). Globo H (GH), for instance, is a hexasaccharide specifically overexpressed on a variety of cancer cells and therefore, a good candidate for cancer vaccine development (30). Glucosamine oligosaccharides corresponding to fragments of the bacterial surface poly–*N*–Acetyl–glucosamine were also previously synthesized to mimic the bacterial antigen (31) as well as other, dendrimeric GlcNAc structures (32). Saccharide structures are also emerging to be useful targeting tools to affect only selected tissues or cell subpopulations (33), or as anti–pathogenic structures (34). Multivalency plays a major role in biological processes and particularly in the relationship between pathogenic microorganisms and their host that involves protein–glycan recognition. These interactions occur during the first steps of infection, for specific recognition between host and bacteria, but also at different stages of the immune response. The search for high–affinity ligands for studying such interactions involves the combination of carbohydrate head groups with different scaffolds and linkers, generating multivalent glycocompounds with controlled spatial and topology parameters. By interfering with pathogen adhesion, such glycocompounds, including glycopolymers, glycoclusters, glyconanoparticles and glycodendrimers have the potential to improve or replace antibiotic treatments that are now being subverted by resistance. Bacteria present on their surfaces natural multivalent glycoconjugates such as lipopolysaccharides and S-layers that can also be exploited or targeted in anti–infectious strategies. Multivalent glycoconjugates have also been used for stimulating the innate and adaptive immune systems, for example with carbohydrate–based vaccines. As the understanding of the molecular mechanisms involved in NK cell activation and effector function grows, the expectations concerning NK cell–based immunotherapeutic approaches (*in vivo* modulation of NK cell activity, purification/expansion, adoptive transfer) increase (35). As shown earlier, determination of the NK cell numbers in the periphery or in the affected tissue is not as reliable a marker for disease progression or immune response, as when assessing the NK cell activation and differentiation state markers (36). Thus, the levels of expression for different activating and inhibitory receptors from NKR–P1, NKG2, KIR families, and others are being determined in order to ascertain

the effect of diverse mediators (neurotransmitter, purine nucleotides, and hormones) on NK cells function (36).

2. Aims

The goal of this work was to provide new insights and applications for NK cell–mediated immune modulation and on the receptors involved in these processes. Such data can provide the basis for more precise and balanced strategies of immune response alteration toward the desired effects. In order to provide such tool, it was first needed to study the receptors and their isoforms involved in both activation and inhibition of NK cell effector function upon stimulation – which are involved in direct cytotoxicity triggering and which tend to polarize the target cells toward cytokine production. It was also necessary to study the possible mediators and receptor ligands and whether they exert the same effect in different species, since many human immune disorders are studied in animal models. Thus, we concentrated at confirm the following points:

- Analyze the involvement of NKR-P1 receptor recognition systems in human autoimmune disorders and mouse tumor models employing synthetic glycan mimetics.
- Study the involvement of KIR2DL4 receptor in hormonal ovarian hyperstimulation protocol in assisted reproduction and its effect on the implantation rate.
- Determine the role of adenosine receptors in various species in antitumor immune response.
- Evaluation of therapeutic implications of local hyperthermia on NK cell effector function in cancer models and the establishing of versatile targeting platform using ferritin nanoparticles.

3. Materials and methods

3.1 Synthetic mediators

Octavalent *N*–Acetyl–β–D–glucosamine on polyamidoamine dendrimer scaffold kindly provided by Prof. T. K. Lindhorst (Christiana Albertina University in Kiel, Germany) and Prof. V. Kren (Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic) was synthesized and tested as described previously (32). Selective A_2 adenosine receptor agonist CPCA (5'–(*N*–cyclopropyl)carboxamidoadenosine) was purchased from Sigma–Aldrich, St. Louis, MO, USA)

3.2 Endogennous mediators

Mutated citrulinated vimentin (MCV, rheumatoid arthritis–specific autoantigen) was purchased from Orgentec, Mainz, Germany. Recombinant human vimentin (VIM) was purchased from Progen Biotechnik, Heidelberg, Germany. GnRH (gonadotropin-releasing hormone) antagonist (Cetrotide) and hCG (human chorionic gonadotropin, Ovitrelle) were purchased from , Merck Serono, Darmstadt, Germany.

3.3 Experimental animals.

Eight–week old inbred C57Bl/6, BALB/c, DBA2 mice (AnLab, Prague, Czech Republic) or three–month old Wistar rats (Charles River Labs, Wilmington, MA, USA) were housed under natural day/night conditions and fed *ad libitum* on a commercial ST1 diet (Velaz, Prague, Czech Republic). Tumor-bearing C57Bl/6 mice were inoculated with 10^6 syngeneic B16F10 melanoma cells in 0,1ml PBS subcutaneously into the lower back on day zero. Wistar rats were inoculated by the same route with $2x10^6$syngeneic C6 glioma cells and the MeLim pigs were used after developing spontaneous hereditary melanoma.

3.4 Patients and healthy donors

In autoimmune models, 86 patients were enlisted from the institute of Rheumatology (Prague, Czech Republic) with the following diagnoses: Rheumatoid Arthritis (RA, n=50), Osteoarthritis (OA, n=19), Dermatomyositis (DM, n=12) and Polymyositis (PM, n=5). In fertility disorder (FD) models, 66 women undergoing assisted reproduction protocol in the Institute for the Care of Mother and Child (Prague, Czech Republic) were enlisted and diagnosed with male (male FD, n=37) or female (female FD, n=29) cause of fertility disorder. Healthy donors peripheral blood from Blood transfusion department of Thomayer university hospital in Prague, Czech Republic was used as control (autoimmune, n=60; FD, n=37). Peripheral blood of patients with non–small–cell lung cancer (stage I-II, WHO PS 0–1) from the Department of Thoracic Surgery at the Lower Silesian Center in Wroclaw, Poland(37) was used in adenosine modulation study.

3.5 Isolation of peripheral blood, spleen, tumor–infiltrating and follicular–fluid cells

Peripheral blood samples were either lysed with ACK (ammonium chloride buffer with EDTA and $KHCO_3$) buffer for 10 minutes on ice and then washed twice (300xg, 4˚C, 5min) with cold PBS or as in case of spleen cells, layered on Ficoll–based density gradient and centrifuged (400x, 22˚C, 45min). Separated leucocytes were then collected and washed twice with either H–MEMd media (Institute of Molecular Genetics, Prague, Czech Republic) for use in cytotoxic assays or with cold PBS for use in flow cytometry assays.

Tumor-infiltrating leucocytes were prepared from individual melanomas minced by scissors, eluted through nylon mesh by repeated pipetting and separated on Ficoll-based density gradient. Follicular fluid samples were only lysed with ACK and then twice washed and used for flow cytometry.

3.6 Cell lines and cultures

Established cell lines K562 (human, NK cell–sensitive), YAC-1 (murine, NK–sensitive), B16F10 (murine, syngeneic melanoma) and C6 (rat glioma) used as target cells in cytotoxicity assays were maintained in RPMI–1640 medium (Sigma-Aldritch, USA), supplemented with 2mM L–glutamine, 1mM sodium pyruvate, 0.05 mM 2–mercaptoethanol, antibiotics (0.05mg/ml gentamycin,

25mg/ml amphotericin B) and 10% heat–inactivated fetal calf serum (FCS, Gibco, USA). All incubations were performed in 5% CO_2 and humidified atmosphere in 37˚C unless stated otherwise.

3.7 Experimental treatment procedures

For synthetic mediators of glycoconjugate basis, three consecutive doses of were administered every 3 days starting on day 10 after tumor inoculation, when palpable tumors developed. Mice were treated by intraperitoneal injection of 60μg/kg of mouse of polyamidoamine (GN8P) glycoconjugates in 0.1ml PBS or only by PBS in control animals. At the end of therapy, 2 hours after the last injection, the animals were sacrificed and spleens were removed for immunological studies. Adenosine agonist CPCA was administered *in vitro* to effector cells in 10 or 0.1μM concentrations for 18 hours in CO_2 incubator.

In fertility disorder studies, GnRH antagonist (0.25mg) was administered at day 7 of the menstrual cycle (fixed regimen) and given daily until the day of hCG administration (6500 IUs, *sc*, 34–36 hours before peripheral blood and oocyte collection in order to coincide with oocyte maturation).

MCV, VIM and GN8 were incubated with purified effector NK cells (CD56+CD3- one–way cell sorting) at 37˚C for 24 hours in autoimmune disorder diagnosed patients.

Purified NK cells (CD56+CD3- one–way cell sorting) of RA diagnosed patients before initiation of therapy were incubated with MCV, VIM and GN8 for 24 hours at 37˚C in CO_2 incubator and used for mRNA isolation and further evaluation.

Local hyperthermia (HT) of melanoma-bearing C57BL/6 mice was performed under anesthesia (ketamine: 1.9 mg/mouse and xylazine: 0.25 mg/mouse weighing 25-30 g, *i.p.*). The superficial, intra-tumor and rectal temperature of mice has been monitored during the whole period of HT application by the fluoro-optic thermometer (LUXTRON, Luxtron Corporation, CA, USA). Target temperature (42ºC) in the tumor was reached during one minute and was maintained using pulses of microwaves for 7 min. Rectal temperature did not surpass 38ºC. We performed three consecutive heatings in the hyperthermia group at days 10, 14 and 17 after tumor cells inoculation. Twelve hours after the third session of hyperthermia all mice were euthanized and tumors and spleens were removed for further analysis. All animal experiments and 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*. All patients and healthy donor samples were collected and processed with the informed consent of all donors.

3.8 Cytotoxicity assays

The *in vitro* cell-mediated cytotoxicity was estimated using the standard ^{51}Cr-release assay with SMC or PBMC as effectors. Effector SMC or PBMC (3.2×10^5/100 μL per well) were seeded in tetraplicates and in case of adenosine receptor studies cultivated in the presence of A2 AR agonist CPCA at a concentration of 10 or 0.1 μM for 18 hours. 10^4 target cells labeled for 90min. with $Na_2{}^{51}CrO_4$ were added to the effector cells in round-bottomed 96-well microtiter plates (*Nunc*, Denmark). Evaluation of cell lytic activity was performed after 4 or 18h incubation in the presence of target cells at 37°C. The cell-free supernatants were harvested (50μL per sample), 0.1 mL of

scintillation cocktail (SuperMix; *Wallac*, Finland) was added, and the radioactivity measured employing scintillation counter Microbeta Trilux (*Wallac*). The percentage of cytotoxicity (ctx) was calculated according to the formula ctx[%]=100×(experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm).

3.9 Flow cytometry.

Phenotype analysis of cells using specific surface markers was following: Monoclonal antibodies labeling for living cells – Propidium iodide or Hoechst 33258, B lymphocytes – CD45R/B220, CD19, anti–IgM, Plasma cells: CD138, CD80, CD86, I–A/I–E, and IgG mAbs. T lymphocytes – CD3, CD4, CD8, NK cells – CD49b, CD56, CD16, and activation antigens NK1.1, CD161, NKG-2D, CD69, KIR2DL4 and LILRB1 were purchased from BD Pharmingen (Becton Dickinson, NJ, USA), Caltag (San Francisco, CA, USA) or eBioscience (San Diego, CA, USA) and analyzed by FACS LSR II (BD). Five to eight-color stainings were performed, according to the manufacturer's standard protocol. Evaluation of cytometry data was performed using FlowJo version 7.6.5 software (Tree Star Inc., Ashland, OR, USA).

3.10 Statistical analyses.

Statistical significance of differences between groups was calculated by two tailed Student's T–test for comparison between two groups (control vs. treatment). D´Agostino and Pearson omnibus normality test was employed to test for Gaussian distribution of values and if not passed, Mann–Whitney U test was employed to check for significance instead of Student´s T–test. One–way ANOVA was used to test for differences between three or more groups. T–test, ANOVA and Mann–Whitney p values lower than 0.05 were considered as significant ($p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***).

4. Results

4.1 CD161 receptor participates in both impairing NK cell cytotoxicity and the response to glycans and vimentin in patients with rheumatoid arthritis.

J. Richter, V. Benson, V. Grobarova, J. Svoboda, J. Vencovsky, R. Svobodova, A. Fiserova.
Clinical Immunology (2010) **136,** 139-147.

In this study, we investigated the mechanisms of NK cell functional impairment, which was described previously in systemic autoimmune diseases (38). We focused on the involvement of CD161 in this process, specifically on its response to synthetic saccharide mimetic, octavalent *N*–acetyl–β–D–glucosamine–terminated dendrimer (GN8P) and endogenous RA–specific marker, mutated citrullinated vimentin (MCV). Patients with RA, polymyositis (PM) and dermatomyositis (DM) were compared to patients with osteoarthritis (OA) and healthy donors.

We confirmed the highly significant impairment of NK–mediated cytotoxicity in patients with autoimmune disorders (RA, PM, DM) compared to healthy donors or patients with OA. This impairment was accompanied with only slight decrease in the number of cytotoxic ($CD56^{dim}$) NK cell

subpopulation. Thus, we tested the NK cell activity after 30–minute preincubation with the synthetic GN8P. We found, that NK cells were divided into two groups – either they were unaffected by the GN8P glycomimetic, or their activity was impaired by the preincubation. The GN8P–inhibited group exerted significantly higher expression of CD161 on $CD56^{dim}$ NK cells in both HD and RA patient groups. When we assessed the CD161 expression on the mRNA level, RA patients had significantly more CD161–mRNA, which further increased after incubation with MCV.

We can thus conclude that there is a subset of NK cells (~ 50%), which react to GN8P glycomimetic by impaired cytotoxic effector function and this subpopulation can be identified by the elevated level of CD161 expression. This expression can be further increased by MCV in both RA patients and in healthy donors, pointing to a more universal effect of MCV. In addition, these results make us believe, that the impaired NK cell cytotoxicity in autoimmune patients is caused by ligand binding to CD161, which is in accordance with the findings of Rosen et al. (39).

The effect of synthetic mediators (GN8P) on NK cell activity may not be the same in murine models, since rodents express various isoforms of NKR–P1 (A, B, C, E and F) when compared to single human *Nkr–p1a* gene (40). And since human NKR–P1A (CD161) was reported to have both inhibitory (41) and stimulatory (42, 43) properties it was needed to further confirm, whether the same GN8P–mediated inhibitory effect will be observed in mice with various isoforms of NKR–P1.

4.2 *N*–acetyl–β–D–glucosamine–coated polyamidoamine dendrimer modulates antibody formation via natural killer cell activation.

K. Hulikova, V. Benson, J. Svoboda, P. Sima, A. Fiserova.
International Immunopharmacology (2009) **9**, 792-799.

Since NK cells are known to mediate IgG production (44) , we investigated whether GN8P modulates antibody response specific for T–independent and T–dependent (soluble as well as corpuscular) antigen in C57BL/6 mice. To prove the involvement of NK and NKT cells and particularly NK1.1 receptor in the mechanism of GN8P effect, we determined Ig levels *in vitro* in supernatants of spleen mononuclear cells (SMCs) depleted of CD49b–positive or NK1.1–positive subpopulations. Finally, we examined whether GN8P increases antigen–specific IgG2a levels in DBA/2 and BALB/c mice, which are considered NK1.1–negative based on FACS analysis.

The GN8P mimetic significantly elevated the relative number of plasma cells and CD80/CD86/MHC–II expressing B cells in C57BL/6 mice, which is the basis of antigen presentation and antibody formation. In addition, we detected significantly higher serum levels of IgG2a specific for both T–independent and T–dependent antigen after GN8P administration, when compared with the response to the antigen alone.

In DBA/2 and BALB/c mice, unlike in C57BL/6, GN8P treatment did not influence serum antigen–specific IgG2a levels. Therefore, we suggest that GN8P effect on antibody formation in mice might be dependent on the presence of NKR-P1C isoform encoded by *Nkr–p1c(T)* gene allelic form present in C57BL/6 strain. We do not expect involvement of NKR-P1A and NKR-P1F isoforms (activating receptors) as their genes are present in DBA/2 and BALB/c as well as in C57BL/6 mice. As for NKR-P1B and NKR-P1D isoforms, *Nkr–p1b* gene in DBA/2 and BALB/c mice exerted 94.9%

homology with *Nkr–p1d* in C57BL/6 mice. In addition, they both have inhibitory function (6) and have a mutual ligand, Clr-b (45).

To further solidify the conclusion that NK cells are involved in the GN8P–induced antibody formation, IgM levels were measured in response to DNP–LPS antigen (2,4–dinitrophenylated lipopolysaccharide). SMC, CD49b–negative or NK1.1–negative cells were cultured with DNP–LPS in the presence or absence of the glycomimetic for 5 days. While in SMC, the IgM level was significantly increased in the presence of the glycomimetic, we observed no such change in either CD49b–negative or NK1.1–negative populations. Since most CD49b–positive NK cells are also NK1.1–positive, this does not come as a surprise.

In conclusion, GN8P has shown a potential to modulate specific antibody formation via NK cell stimulation. This stimulation seems to be dependent on the surface expression of NKR–P1C isoform encoded by C57BL/6 *Nkr–p1c(T)* gene allele.

However, this study is limited by its focus on the administration of synthetic, exogenous antigens against which GN8P glycomimetic mounts increased antibody response. The practical applications in tumor or immune therapy needed further investigation in the *in vivo* models.

4.3 *N*–acetyl–β–D–glucosamine–coated polyamidoamine dendrimer promotes tumor–specific B cell responses via natural killer cell activation.

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International Immunopharmacology (2011) **11**, 955-961.

To evaluate possible GN8P–mediated anti–tumor immune response, several different parameters needed to be investigated. For this purpose, we determined serum anti–B16F10 melanoma IgG levels, IgG2a mRNA expression, and antibody dependent cell–mediated cytotoxicity (ADCC) in tumor–bearing C57BL/6 mice. Changes in counts of basic lymphocyte populations in the spleen evoked by GN8P were followed, with focus on B cells and their differentiation stages as well. Finally, we estimated the synthesis of interferon–gamma (IFNγ) and IL–4, cytokines involved in the regulation of immunoglobulin class switch (44, 46), to reveal the mechanism of NK–mediated GN8P effects.

GN8P administration to tumor–bearing animals did not significantly change the percentual distribution of basic immune cell populations as T helper, T cytotoxic, B cells or NK cells. However, there was a significant increase in CD80/CD86/MHC–II expressing B cell population levels, as well as increased counts of CD138 positive plasma cells, which was previously observed in healthy animals (47).

Serum tumor–specific IgG levels were naturally significantly higher in melanoma–bearing mice when compared to healthy animals, but this increase was further augmented by GN8P administration. This increase was represented not only by the number of IgG positive B16F10 melanoma cells, but also by the level of coverage by the IgG on these cells. To determine, whether these IgG antibodies had any impact on tumor clearance, we performed the antibody–dependent cell cytotoxicity assay, using B16F10 melanomas as targets – preincubated with sera of GN8P or PBS treated melanoma–bearing mice. Significant increase in cytotoxicity was achieved with sera of GN8P–treated animals. IgG2a antibodies were reported previously to represent the most efficient IgG subclass in mediating ADCC reaction (48) and when tested here, their mRNA expression levels were increased

several–fold after the glycomimetic administration, indicating this subclass to be the major one of the IgG detected before.

Since IgG2a–oriented class switch does not usually occur in B cells by itself, but under the effect of IFN–γ or IL–4 (46, 48), we investigated the fate of these two chemokines as well. Both exerted significant increases in expression on the mRNA level and IFNγ was almost exclusively produced by NK cells after GN8P administration, which points to a positive feedback loop established by NK cells. Natural killers, upon interaction with GN8P, increased the production of IFNγ, thus induced the IgG2a class switch in B cells and their differentiation into plasma cells and then used the provided tumor–specific IgG2a for enhanced tumor clearing via ADCC, releasing more tumor–associated antigenic material in the process.

In conclusion, GN8P–activated NK cells potentiate tumor–specific IgG formation, triggering ADCC reaction as well as antigen presentation by B cells. These results illustrate the importance of carbohydrate recognition in NK cell–mediated regulation of adaptive immunity, with benefit for anticancer immune responses.

4.4 Ovulation stimulation protocols utilizing GnRH–antagonist/hCG, promote cytotoxic cell populations, predominant in patients with embryo implantation complications.

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While hCG is known to have immunomodulatory properties, we aimed to assess its effect on immunological changes, with respect to HLA-G binding receptors and embryo implantation success. This study involved 103 subjects, including patients undergoing COH protocols (n=66), divided on the basis of the pair's fertility disorder (FD) causes (female FD – represents immune compromised system, n=29; male FD – represents healthy immune system, n=37), and age matched healthy women (HD – represents uninfluenced healthy immune system, n=37). The relative distribution of T cell (CD3+/CD4+, CD3+/CD8+) and NK cell ($CD56^{bright}$/CD16-, $CD56^{dim}$/CD16+) populations was evaluated together with HLA–G ligands KIR2DL4 and LILRB1 expression by flow cytometry in the peripheral blood of all subjects, as well as in patient follicular fluids. CD161 and NKG–2D receptor levels were also observed, but remained unpublished for the prevailing lack of significant changes in their expression. This allowed us to sufficiently describe the effect of antagonist COH protocol on the immune system and correlate it to the implantation success rate.

First, we demonstrated that while age is a known detrimental factor for the outcome of ARTs, it has no significant effect on the composition of the observed immune parameters. Next, we compared the healthy donor samples with samples of male and female FD and revealed the massive changes in the immune system composition, mediated by the COH protocol. The CD4/CD8 ratio (or Th:CTL index) was severely pushed toward cytotoxic T–lymphocyte (CTL) preference and furthermore, those CD3/CD8 positive T cells had also significantly decreased levels of the inhibitory LILRB1 receptor. While the counts of NK cell populations were influenced only in the case of female FD ($CD56^{dim}$ NK cells upregulated), which points to COH–independent cause, the KIR2DL4 levels were significantly higher in ART patients.

These data show that hCG promotes cytotoxic T cell populations with lower inhibition potential (lack of inhibitory receptors) and NK–mediated cytokine production, since KIR2DL4 was described to activate potent cytokine production but only weak cytotoxicity (49-51). LILRB1, as an inhibitory receptor among T cells, causes impaired signaling through TCR and decreased IL–2 and IFNγ production (52, 53), further increasing the immune potential for IFNγ production. This may have a detrimental effect on embryo implantation, since $CD56^{bright}$ NK cells (54) and $CD56^{dim}$ NK cells (55) also produce IFNγ, which in high doses has detrimental effect on embryo implantation (56). We have further shown in this study, that patients with increased counts of CD8+ T cells or with higher KIR2DL4+ $CD56^{bright}$ NK cell levels have notably decreased implantation rates, further confirming the detrimental effect of hCG on ART outcome.

The CD161 receptor on either subset of NK cells did not exert any significant change upon hCG treatment and appears to be unaffected by the COH protocol. NKG2D was only slightly downmodulated on $CD56^{dim}$ cells (HD=88.8±7.9; Patients=82.2±15.6; p=0.0036) and only in the peripheral blood.

Here, follicular fluid T and NK cells, which were described previously in patients with idiopathic infertility or endometriosis (57, 58) as key players, revealed no significant differences in their relative distribution or in the expression of HLA–G ligands, when successful and failed embryotransfer groups were compared.

In conclusion, the commonly used COH protocol influences the CD4/CD8 index, promoting cytotoxic T cells and increases the expression of KIR2DL4 on NK cells, rendering them more prone for cytokine production.

These results provide another valuable modulator of the immune response, but as in previous cases, remain entirely focused on one NK cell receptor in a specific pathological situation. While being useful in specific cases (promoting certain cell populations over others), a more universal approach to the immune system modulation remains elusive and a different mediator is required for these purposes.

4.5 NK cell–mediated cyototoxicity modulation by A2 adenosine receptor agonist in different mammalian species.

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Folia Microbiologica (2009) **54**, 364-368.

In this study, our goal was to provide evidence whether adenosine–mediated inhibition of NK cell function is universal among most common mammal species or whether it is specialized for humans and rodents. To achieve this goal, we used the synthetic A_2AR agonist 5'–(*N*–cyclopropyl)carboxamidoadenosine (CPCA) to influence the cytotoxic activity of mononuclear cells isolated from human donors, C57BL/6 mice, Wistar rats, Ladrace–Duroc pigs and White Shorthaired goats. Both healthy and tumor bearing hosts were used (except goats) to observe any tumor–mediated precoditioning of effector cells (tumor escape strategies include adenosine release) and standard ^{51}Cr–release cytotoxicity assay was performed against sepcies–specific NK–sensitive target cells (K562: human, pig, goat; C6: pig, goat rat; YAC–1: mouse, rat; B16F10: mouse)

The *in vitro* addition of $A_{2A}AR$ agonist, 10–micromolar CPCA, downmodulated the NK cell cytotoxic activity in both healthy and tumor–compromised subjects of all tested species. In several

cases, even 100nM concentration was sufficient to exert significant decrease in lytic activity. A slight effector cell conditioning was visible in tumor–bearing rats, which exerted lower degree of lysis inhibition by CPCA, when compared to healthy counterparts, but overall, the effect was comparable.

In conclusion, these data, obtained by an *in vitro* stimulation of effector cells derived from different mammalian species, indicate that CPCA can be considered a universal NK cell attenuator.

A universal attenuator as CPCA could exert undesirable effects if applied systemically. Two approaches can be applied in order to circumvent systemic or pleiotropic effect: either to rely on other than chemical means of immune modulation or to use a specific–targeting platform for the delivery of chemical and biological compounds.

4.6 Immunological response in the mouse melanoma model after local hyperthermia.

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Physiological Research (2008) **57**, 459-465.

Here, we investigated the effect of LHT on *in vivo* and *in vitro* induction of NK and CTL–mediated cytotoxicity and on the changes in effector cell subpopulation proportions and activation state in LHT–treated B16F10 melanoma–bearing C57BL/6 mice. These mice were first inoculated with B16F10 melanoma cells, and after palpable tumors were developed, underwent LHT under anesthesia. Furthermore, to distinguish the effect of HT on effector cells and tumors, we incubated either effectors or targets in 42°C prior to a standard ^{51}Cr–release assay.

As for spleen immune cell subpopulations, neither CTL nor NK cells exerted significant changes in their occurrence induced by the local hyperthermia *in vivo*. Their activation state, indicated by the presence of the very early activation antigen CD69, was also unaltered. The only change observed was in the higher counts of CD69 positive CD11b cell in the tumor site – other changes were ascribed to the anesthesia itself.

The functional assay pointed to increased effector lytic activity against NK–sensitive YAC–1 target cells after *in vivo* applied LHT. When *in vitro* HT of 42°C was applied to target cells only, healthy mice–derived effectors exerted significantly decreased lytic activity (probably due to the tumor cell–mediated release of adenosines). Tumor–compromised effectors exerted a slight increase in cytotoxicity against *in vitro* hyperthermed targets, pointing to an unknown melanoma–based preconditioning of the effector cells. Effector cells after *in vitro* HT exerted slight, but significant decreases of lytic activity, probably due to the temperature reaching the limit of beneficial febrile response.

In conclusion, *in vivo* LHT proved to have beneficial effect on NK cell–mediated lytic activity, despite NK cell numbers or receptors repertoire remained unchanged. This, together with *in vitro* HT data suggests indirect effect on NK cells and the linking elements identification would require further study in more precisely controlled conditions.

Despite the simplicity and benefits of hyperthermia, its effects are systemic, despite its localized application. Most immune–related pathologies and immune–modulating applications require specific targeting to achieve even measurable effectiveness. An answer may be in the recent development of targeted nanoparticles discussed below.

4.7 Selective targeting of melanoma by PEG–masked protein–based multifunctional nanoparticles.

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International Journal of Nanomedicine (2012) **7**, 1489-1509.

This study aims at the *in vitro* stability, affinity and specificity of melanoma–targeted nanoparticles, on their *in vivo* circulation and organ accumulation in the mouse C57BL/6 melanoma (B16F10) model. It was our goal to prove these particles stable, specific for the melanoma cells and having low accumulation in irrelevant organs with retained circulation. The particles were cloned, purified, PEGylated, fluorescently labeled and after structure and stability was confirmed, were introduced into *in vitro* cultures and living animals.

The results shown a good stability and availability of the nanoplatform, with unique spectral properties, allowing fluorescent imaging (other molecules or magnetic/radioactive particles could be used to describe the fate of the particle). We further demonstrated its specificity for the targeted B16F10 melanomas both *in vitro* and *in vivo*. In addition, the PEG molecule masking increased its circulation in the system to several hours, when compared to minutes for the unmasked particle.

During *in vivo* imaging, we observed specific binding to melanocytes in the affected skin with intact (non–fluorescent) surrounding healthy tissue. Even single infiltrating melanoma–cells were well discerned against the dark background. Increased but comparably lower accumulation of nanoparticles was observed in the liver Kupffer cells and in the kidney glomeruli. This accumulation was however much lower, than previously reported for unmasked construct with different targeting moiety (59).

In conclusion, we developed a rationally designed modified human ferritin–based multifunctional nanoplatform, with well–defined parameters and modularity in the targeting moiety, present on the surface of each of the 24 subunits. Thus, this platform can be equipped with specific ligands or monoclonal antibodies and loaded with mediators or heat–inducing particles, achieving specifically targeted immune modulation in almost any system.

5. Discussion

One of the goals of this thesis was to describe the NK cell reaction to glycans. The role of glycosylation and protein–glycan recognition in autoimmune diseases had been proven in the past and is being intensively studied in the present (60, 61). Aberrant glycosylation was also repeatedly reported during malignant tumor growth and was associated with metastatic potential (28), while other, bacterial LPS–derived glycans are long known to act in cellular activation and immune response enhancement (62, 63). Octavalent GlcNAc mimetic, GN8P, was used in the studies herein to simulate branched glycan structures, appearing during autoimmune disorders or malignant growth. According to the last findings, there is no clear evidence about the direct binding of glycan (GlcNAc–terminated) structures to NKR–P1 receptor; nevertheless, it is important for NK, and subsequently other type of immune cells activation. CD161, a member of the C–type lectin–like receptor family, was reported

previously to bind LacNAc (*N*–acetyl–Lactosamine) epitopes and could thus be involved in the GN8P mimetic recognition either alone or more probably in cooperation with other receptors. As shown in this thesis, RA patients with increased CD161 surface and mRNA expression on/in NK cells responded to the glycomimetic by attenuated NK cell–mediated cytotoxicity. Since similar effect can be achieved with CD161 natural ligand LLT1 (39), we can hypothesize a similar mechanism of binding. Further study with LLT1 as a binding competitor in GN8P–CD161 interaction investigation is needed to test this hypothesis as well as to ascertain the involvement of other, CD161–associated co–receptors. In mice, there are several orthologs of CD161, NKR–P1A, B, C, D and F (64). While B and D isoforms contain an ITIM motif and are considered inhibitory, A, C and F lack it and were reported to associate with FcεRIγ and to have activation potential (64). In this thesis, it was presented that serum levels of IgG2a specific for both T–independent and T–dependent antigens were elevated after GN8P administration in C57Bl/6 mice. Since resting NK cells fail to modulate antibody responses, but if activated, preferentially upregulate IgG2a formation (65, 66), we can attribute this elevation to NK cells. In addition, we presented results that only NK1.1–positive C57Bl/6 mice responded to GN8P administration, while NK1.1–negative strains (Balb/c and DBA/2) were unresponsive. Therefore, we suggest that GN8P effect on antibody formation in mice might be dependent on the presence of NKR–P1C isoform encoded by *Nkr–p1c(T)* gene form (present in C57Bl/6 strain). We do not expect involvement of NKR–P1A and NKR–P1F isoforms (activating receptors) as their genes are present in DBA/2 and BALB/c as well as in C57BL/6 mice. As for NKR–P1B and NKR–P1D isoforms, *Nkr–p1b* gene in DBA/2 and BALB/c mice exerted 94.9% homology with *Nkr–p1d* in C57BL/6 mice. In addition, they both have inhibitory function (6) and have a mutual ligand, Clr–b (45). The involvement of NK cells in the GN8P–mediated antibody formation was further studied in the melanoma–bearing C57Bl/6 mice, where the glycomimetic administration evoked tumor–specific IgG2a upregulation, NK cell–mediated IFNγ production and increased ADCC effector function (67). Since IFNγ promotes Ig class switch towards IgG2a (46) and IgG2a is one of the most potent IgG subclass in mediating ADCC (48), the involvement of NK cells was further validated. Yet again, the exact mechanism of glycomimetic–NK cell interaction needs to be further studied with NKR–P1C(T) natural ligands as binding–competitors, but these are as of yet still unknown. The glycomimetic effect on NK cell function was well described in humans and mice and in both cases, NKR–P1 receptor was involved. In recent studies, more and more attention is being given to the tumor–associated carbohydrate antigens (TACA) and their use in the preparation of carbohydrate–based vaccines (29, 30). Our data on NK cell reaction to GlcNAc based glycomimetic may prove quite useful in the future design of such vaccines as well as other applications.

We have shown here, that the choice of species in the study of receptor–ligand interactions may be crucial in some cases. For instance, the synthetic immune glycomimetic, GN8P, proved to have quite the opposite effect on the function of NK cells when human and C57BL/6 murine systems were compared. This may be due to the fact, that there is only one NKR–P1A isoform in humans with both activation and inhibitory potency (39, 41, 43), while in mice, these functions are divided between individual activating/inhibitory isoforms (68). Moreover, some murine strains (DBA/2, BALB/c) were utterly unaffected, despite having only one amino acid substitution in their NKR–P1C receptor and expressing NKR–P1A, which is considered an ortholog of the human CD161.

Among widely used, endogenously present and well–described immune modulators belong the hormones. In further study presented here (Publication 4) we have shown, that the widely used controlled ovarian hyperstimulation protocol implementing GnRH antagonist/hCG treatment significantly increases KIR2DL4 expression on the surface of NK cells. This receptor is known to activate IFNγ cytokine production, but only weak cytotoxicity (50, 51) in NK cells and can thus be used to further modulate the NK cell function. As most hormones, hCG also shows a degree of pleiotropy in its effect, influencing CD4 and CD8 T cells and their receptors as well as NK cells. Thus, this effect needs to be targeted to NK cells to achieve an exact and accurate modulation of NK cell function. That is where the protein–based multifunctional nanoparticles presented here come into view and could be used for selective NK cell targeting with hCG load for KIR2DL4 upregulation. Further studies involving these nanoparticles need to be performed to make the use of hCG as NK cell modulator specific.

Another endogenous and well–described immunomodulator is the purine nucleoside adenosine, often released by transformed cells under the hypoxic conditions of the tumor microenvironment. It influences many physiological functions (*e.g.*, vasodilatation, lipolysis, *etc*.) (69) and its levels are dramatically increased during hypoxic conditions, tissue damage and inflammation. Adenosine inhibits inflammatory activity of neutrophils, macrophages and lymphocytes, protecting the ischemic tissues from massive damage (70). Adenosine receptors (AR) are also involved in the inhibition of cytotoxic activity and cytokine production by activated NK cells (71, 72). Here we have proven the synthetic A_{2A} adenosine receptor agonist CPCA to be a universal NK cell cytotoxicity attenuator in several mammalian species in both healthy and immunocompromised subjects. These findings could be applied in transplantation immunology to prevent the host versus graft reaction in the early phases, but without effective targeting, the entire host immune system could become compromised. Thus, further studies with CPCA specific targeting to target tissues/populations need to be performed to achieve practical applications for NK cell–mediated lysis attenuation. Such specificity can be achieved either in conjunction with tissue–targeting saccharide structures (33) or with the nanoplatform presented in this thesis.

Stress inducible proteins, ligands of the activating NKG2D (73), provide another possibility how to activate NK cells. Hyperthermia represents an elegant way of contact–less attenuation of tumor growth, while inducing the responsiveness of the immune system, by increased presentation of these proteins (74). Higher temperatures (≥42°C) have directly cytotoxic and immunogenic effect on malignant tissue (75), and if properly focused only on the tumor itself, can provide tumoricidal temperature in the affected site, while maintaining febrile temperature range in the surrounding. This could prove very beneficial, since such range was reported previously to activate spleen cells (76), NK cells (77), cytotoxic T cells and T helper cells (78, 79). On the other hand, implementation of hyperthermia can be used only seldom on barely detectable metastatic cells, early neoplasia or leukemia. Again, if properly targeted to these cells by the abovementioned nanoplatform, the ferritin core can be loaded with microwave–inducible heat generating particles (80) and could thus ensure the targeted heating of circulating metastatic or leukemic cells or in the case of inaccessible tumors. Further research as to the specific particle load, temperature ranges and targeting moieties need to be performed in order to achieve future useful clinical applications in this field as well.

Among recent trends in tumor–immunotherapy is the specific or passive targeting of tumor–suppressants or immune mediators into the tumor site (81, 82). This proves especially important and useful in the case of metastatic cells, leukemia or in the case of inaccessible tumors. The nanoparticle platform presented here stands out by its simple versatility – it could be applied not only in one specific, controlled system, but with proper targeting structure and immune mediator load can be used in almost any pathology. Its employment for specifically targeted hyperthermia, when loaded with heat–generating particles (80), is also notable but further studies, using this versatile system need to be performed, before practical applications are achieved.

6. Result summary, conclusions and remarks.

In this work, NK cells and their receptors were intensively studied in several mammalian species in order to acquire new insights on their function, response to glycan structures (GN8P), hormones (hCG), tumor evasion strategies (adenosines) and stress inducible proteins (hyperthermia). This allowed us to provide new tools for NK cell–mediated immune modulation. Together with immune modulation via localized hyperthermia and modular targeting nanoplatform, these findings provide new tools for immune modulation via the regulation of NK cell functions.

Based on the work provided here, we came to the following conclusions:

- In humans, there is a subset of NK cells (~ 50%), which react to the synthetic glycomimetic GN8P by impaired cytotoxic effector function and this subpopulation can be identified by their higher levels of CD161 expression. This expression is increased by mutated citrullinated vimentin (MCV) in both rheumatoid arthritis patients and in healthy donors on the mRNA level. In addition, these results make us believe, that the impaired NK cell cytotoxicity in autoimmune patients could involve the ligand interaction with CD161.
- The synthetic glycomimetic – GN8P has shown a potential to modulate specific antibody formation via NK cell stimulation in the C57BL/6 murine model. This stimulation was shown to be dependent on the surface expression of NKR–P1C isoform encoded by C57BL/6 *Nkr–p1c(T)* gene allele. Glycomimetic–induced NK cells, via IFNγ production, potentiate tumor–specific Ig formation, triggering ADCC reaction as well as antigen presentation by B cells. These results illustrate the importance of carbohydrates recognition in NK cell–mediated regulation of adaptive immunity, with benefit for specific anticancer immune responses.
- The commonly used GnRH antagonist/hCG ovulation stimulation protocol increases the expression of KIR2DL4 on NK cells, influences the CD4/CD8 index, promoting cytotoxic T cells. This immunological profile was associated with decreased implantation rates.
- A_{2A} adenosine receptor agonist, CPCA, used in our *in vitro* evaluation of cytotoxic effector cells derived from different mammalian species, indicates for a universal NK cell cytotoxicity attenuator.
- *In vivo* tumor–localized hyperthermia proved to have beneficial effect on NK cell–mediated lytic activity, despite the NK cell numbers or receptors repertoire remained unchanged.
- We have developed a melanoma–specific tailor made ferritin–based multifunctional nanoplatform, with well–defined parameters and modularity in the targeting moiety. This platform can

be equipped with specific ligands on heat–generating nanoparticles singly or combined with the abovementioned mediators, achieving immunotherapeutic procedures in various systems.

This work brought further knowledge in the possibilities of NK cell modulation, nevertheless, further research needs to be performed for practical applications. More studies with the combination of the herein studied modulators targeted to specific cell populations by the established nanoplatform are required.

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8. Publications

8.1 Publications in extenso *related to the presented dissertation thesis:*

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8.2 Publications in extenso *not related to the presented dissertation thesis.*

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9. Abbreviation index

ADCC	Antibody Dependent Cellular Cytotoxicity
AR	Adenosine Receptor
CD	Cluster of Differentiation
COH	Controlled Ovarian Hyperstimulation
CPCA	5'–(*N*–cyclopropyl)carboxamidoadenosine
CTL	Cytotoxic T Lymphocyte
DC	Dendritic Cell
DM	Dermatomyositis
EDTA	Ethylene Diamine Tetraacetic Acid
FCS	Fetal Calf Serum
FD	Fertility Disorder
GlcNAc	*N*–Acetyl–glucosamine

GN8P	Octavalent *N*–Acetyl–β–D–glucosamine on polyamidoamine scaffold
GnRH	Gonadotropin–Releasing Hormone
hCG	human Chorionic Gonadotropin
HD	Healthy Donor
HLA	Human Leukocyte Antigen
HT	Hyperthermia
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ITAM	Immunoreceptor Tyrosine–based Activation Motif
ITIM	Immunoreceptor Tyrosine–based Inhibition Motif
KIR	Killer Immunoglobulin–like Receptor
LacNAc	*N*–Acetyl–Lactosamine
LHT	Local Hyperthermia
LILR	Leukocyte Immunoglobulin–Like Receptor
LLT1	Lectin–Like Transcript 1
MCV	Mutated Citrullinated Vimentin
MHC	Main Histocompatibility Complex
NK	Natural Killer
NKR–P1	Natural Killer Receptor Protein 1
OA	Osteoarthritis
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PEG	Poly–Ethylene Glycol
PM	Polymyositis
RA	Rheumatoid Arthritis
SHP	Src Homology Phosphatase
SMC	Spleen Mononuclear Cells
TACAs	Tumor–Associated Carbohydrate Antigens
Th	T helper cell
TLR	Toll–Like Receptor
Tn antigen	*N*–Acetyl–galactosamine–serin–threonine
TNFα	Tumor Necrosis Factor alpha