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Hämatologie und Onkologie
Direktor: Universitätsprofessor Dr. med. M. Hallek

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Inaugural-Dissertation zur Erlangung der Doktorwürde
der Medizinischen Fakultät
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vorgelegt von:
Ayşe Çetintaş
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2. Berichterstatterin: Universitätsprofessorin Dr. med. Dr. rer. nat. M.-R. Schweiger

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Die dieser Arbeit zugrundeliegenden Daten wurden mit meiner Mitarbeit im Labor der Innate Immunity Group und in den Räumlichkeiten der Universität zu Köln erhoben:

Den Umgang und die Arbeit mit den Versuchstieren habe ich nach der Teilnahme am FELASA-Kurs in Köln vom 31.08. – 04.09.2015 und nach Anleitung durch Maximiliane Schuldner erlernt. Meine Aufgaben bestanden darin, die Tiere im Tierstall in der Pathologie der Universität zu Köln regelmäßig auf Anzeichen einer Erkrankung zu untersuchen. Bei Erkrankung erfolgte der Abtransport der betroffenen Mäuse in das Labor, wo ich zunächst circa 25µl Blut aus der Schwanzspitze sowie ein Stück der Schwanzspitze zur Genotypisierung entnahm.
Im Anschluss resezierte ich die betroffenen Lymphknoten und die Milz und führte eine Bilddokumentation durch. Aus den Organen stellte ich Zellsuspensionen her, die dann für die weiteren Versuche genutzt wurden. Einen Teil der Zellsuspensionen wurde für die Zellkultur bereitgestellt.

Die Genotypisierung der Mäuse unterlag meiner Verantwortung. Nach Aufbereiten der Schwanzspitze erfolgten die PCR-Vorbereitungen und die anschließende Auftragung auf die Gele, welche dann von mir ausgelesen und beschriftet wurden.

Die Zellen in der Kultur wurden von mir gesplittet, um eine Überwachsung in den Petrischalen zu verhindern. Für die ex vivo Behandlung entnahm ich eine vor definierten Anzahl an Zellen, denen ich unterschiedliche Dosierungen von zytostatischen Medikamenten zugab. Am darauffolgenden Tag wurden die Zellen dann von mir untersucht.

Für die Durchführung der Flow Cytometry färbte ich mit Unterstützung durch Maximiliane Schuldner die Zellen mit Antikörpern. Diese wurden dann im Anschluss untersucht.

Für die Durchführung der Real Time PCR isolierte ich RNA aus den Zellen, die von den Mäusen entnommen wurden, und überschrieb sie mithilfe eines Kits in DNA um. Die DNA wurde nach Zugabe von Primern untersucht.

Danksagung

Ein großes Dankeschön möchte ich als erstes an Prof. Elke Pogge von Strandmann richten. Vielen Dank, dass Du es mir ermöglicht hast meine Arbeit im Labor der Innate Immunity Group durchzuführen. Danke für die Betreuung, die Gespräche und Ideen. Es war eine unglaubliche und sehr anspruchsvolle Erfahrung, die ich immer in Erinnerung halten werde.

Weiterhin möchte ich mich bei Maximiliane Schuldner bedanken. Es war nicht immer leicht für Dich mich einzuarbeiten und mir alles von null auf zu erklären, aber Du warst immer sehr geduldig und hast mir sehr geholfen. Vielen Dank auch für das Korrekturlesen der ersten Seiten meiner Doktorarbeit und die Verbesserungsvorschläge!

Ich möchte mich noch bei dem gesamten Laborteam für die schöne Zeit, die interessanten Diskussionen und gemeinsamen Essen bedanken. Auch wenn die Zeit im Labor anstrengend und manchmal auch frustrierend war, war es trotzdem immer lustig und motivierend!

Zu guter Letzt, möchte ich mich bei meiner Familie bedanken, die mich im gesamten Zeitraum unterstützt hat. Danke an Franziska Schmitz, die mit mir die langen Bibliothekstage zum Verfassen der Doktorarbeit geteilt und die schönen und erst einmal letzten Kaffeepausen in der Uniklinik Köln verbracht hat.

Ich widme diese Arbeit meinem Vater Fehmi Çetintaş

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1. Glossary

7-AAD	7-aminoactinomycin D
Ac-	Acetyl-
ACK	Ammonium-Chloride-Potassium Buffer
ANAC	Anacardic acid
Ara-C	Cytarabin
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related
B cell	Bone marrow derived lymphocytes
C646	Histone acetyltransferase (HAT) inhibitor
CBP	CREB-binding protein
CD	Cluster of differentiation
CGK733	ATM & ATR kinase inhibitor
ChIP	Chromatin immunoprecipitation
Chk 1	Checkpoint kinase 1
CMMC	Center For Molecular Medicine
Cre	An enzyme able to recognize specific DNA sequences
CREB	cAMP response element-binding protein
CRISPR	Clustered regularly interspaced short palindromic repeats
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EyMyc	Transgenic mouse, expressing Myc under the Eμ Ig heavy chain enhancer
FACS	Fluorescence-activated cell sorting
fl	floxed
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPI	Glycosylphosphatidylinositol
H60	Histone 60
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDACi	HDAC inhibitor
HEK-293	Human embryonic kidney
HUVEC	Human umbilical vein endothelial cells
IAP	Inhibitor of apoptosis protein
IgM	Immunglobulin M
IL	Interleucin
IFN-γ	Interferon-gamma
KLRK-1	Killer cell lectin like receptor K1
KU55933	ATM inhibitor
LBH	Panobinostat – HDAC inhibitor
loxP	Locus of Crossover in P1
mAB	Monoclonal Antibody
MCF-7	Michigan-Cancer-Foundation-7 (breast cancer cell line)
MHC	Major histocompatibility complex
MICA/B	MHC class I polypeptide-related sequence A/B
mRNA	Messanger Ribonucleic acid
MULT-1	Murine UL16-binding protein-like transcript 1
Nf-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NK cell	Natural killer cell

NKG2D	Natural killer group 2, member D
NKp46	Natural killer cell protein 30
p300	A Histone acetyl transferase
p53	Transcritpion factor regulating gen expression after DNA damage
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RAE-1	Retinoic acid early inducible 1
RNA	Ribonucleic acid
RPMI	Rosewell Park Memorial Institute
T cell	Thymus derived lymphocyte
TAE	Tris-acetate-EDTA buffer
TER	Monoclonal antibody
tg	transgene
TSA	Trichostatin A
ULBP1-3	UL16 binding protein 1-3
wt	wildtype

2. Introduction

2.1 The immune system

The immune system protects the body against pathogens such as bacteria, viruses, fungi, parasites. The body's own defence also plays a crucial role in fighting malignant cells. It can be divided in the innate immune system and the adaptive immune system. Both systems are linked together but work in different manners.

The innate immune system belongs to the first line defence and is present since birth. It works unspecific and the immune reaction takes place immediately after recognizing foreign microorganisms. The cells that are involved are macrophages, granulocytes and Natural Killer cells [1].
Major histocompatibility complexes I (MHC-I) which are located on the cell-surface are able to present intracellular proteins. In this way foreign proteins, that seem to be harmful for the host, can be recognized by cytotoxic T-cells (CD8+) and Natural killer cells which can directly eliminate the affected cells by cell lysis [2].
Antigen-presenting cells such as dendritic cells, macrophages and B-Lymphozytes carry MHC-II on the cell-surface. Through phagocytosis they can take up extracellular material/ pathogens and present them through MHC-II-receptors on the cell surface. This process activates CD4+ T-Lymphozytes which can initiate the activation of the adaptive immune system [1, 2].

The fact that the activation of the adaptive immune system takes longer causes a delayed but more specific reaction. However, the reinfection with the same pathogen leads to an immediate and effective immune reaction. A crucial fact is that B-cells form an immunological memory which leads to a lower activation threshold so that a second contact with the same antigen will cause a faster and more specific Ig-release than the first contact [1].
The innate immune system is able to directly interfere with pathogens and crucial in fighting changed cells. Additionally, it is now also described that NK cells are able to form an antigenic-specific immunologic memory defining NK cells as high specific cells of the immune defence [2].

2.2 The immune system in fight against cancer

The first idea of tumor immunosurveillance and suppressed tumor formation thanks to the immune system was described by Paul Ehrlich in 1909. He suggested that tumor proliferation is usually suppressed depending on the resistance of the host [3].
In 1997, Engel et al. showed that immunodeficient mice tend to have a significantly higher rejection after transplantation of tumor than the immunocompetent mice. They concluded that there must be an immunoselection which leads to a better elimination of tumor cells in organism with a functioning immune system [4].
In order to achieve a better understanding of tumor immunosurveillance, an epidemiological study could show that a low peripheral blood NK cell activity is linked to an increased cancer risk [5]. Thus, NK cells play a crucial role in fighting cancer cells.
But tumor cells do also appear in immunocompetent hosts, despite immune surveillance. According to Malladi et al. cancer cells can enter a slow-cycling state and remain latent for a period of time. In this time cancer cells can develop different mutations which lead to an increased resistance towards immunity [6, 7]. Accordingly, Hanahan and Weinberg updated the Hallmarks of cancer by adding (inter alia) the ability of immune evasion of tumor cells [7,8].

Hitherto, it is known that the environment of different tumor entities have an immunosuppressive function like downregulating ligands for NK cells or shedding ligands from the cell surface in order to protect themselves from the antitumor response of the immune system [9, 10].
However, for many years the mechanism of the NK cell reaction toward changed cells seemed mostly unclear [11].

2.3 Natural killer cells as a major component of the innate immune system

Natural killer cells are cytotoxic lymphocytes and play an essential role in the innate immune system and the early defence against foreign cells [2]. They were discovered in 1975 in early experiments that showed the capacity of these cells to lyse virally infected and tumorous cells without having previously been sensitized to them [12].
Through specific receptors NK cells can distinguish foreign cells and initiate apoptosis, a programmed and controlled cell death, to eliminate these cells. NK cells are major producer of different cytokines, like Interferon-y, Il-3, Il-10, and chemokines. These molecules can in turn activate other immune cells like macrophages or dendritic cells [13].

By releasing perforins, pores are formed within the target cell membrane and cytokines can enter the cell through these pores which in turn can activate caspases and thus lead to rapid cell death [14, 15].

2.3.1 The mechanisms of how the balance between activated and not-activated NK cells is maintained.

NK cells have to distinguish between healthy cells and harmful cells. In order to control the cytotoxic potential towards healthy host cells, the activation of NK cells is therefore tightly regulated by positive and negative signals [16].

Infected or genetically modified cells such as tumor cells can lose the expression of MHC-I which makes it impossible for T- and B-cells to recognize and distinguish them from the body's own cells. This theory is called as the "missing-self" and was defined by Klas Klärre in the 1980ies. NK cells are able to detect changed or stressed cells such as infected or tumorous cells which lack the expression of MHC I. Usually, NK cells express inhibitory receptors for MHC-I molecules. The loss of MHC I leads to an activation of NK cells as there is no inhibitory signal anymore [17].

Besides inhibitory receptors, NK cell also feature activating receptors. Changed cells upregulate ligands for the NK cell receptors which leads to a stress-induced self-recognition [18].
The best-known activating receptor is NKG2D (Natural Killer group 2 D). This receptor is a lectin-like, type 2 transmembrane receptor encoded by the KLRK1 gene which is located on chromosome 12 in humans and chromosome 6 in mice [18,19].
NKG2D is expressed by all NK cells and also found on T cells. All CD8+ T cells in humans and all activated CD8+ T cells in mice feature this type of receptor. One could also detect expression on some CD4+ T cells [19].

Both ways cause a tumour cell elimination either directly through the NK cell-mediated cytotoxity or through production of cytokines [10].

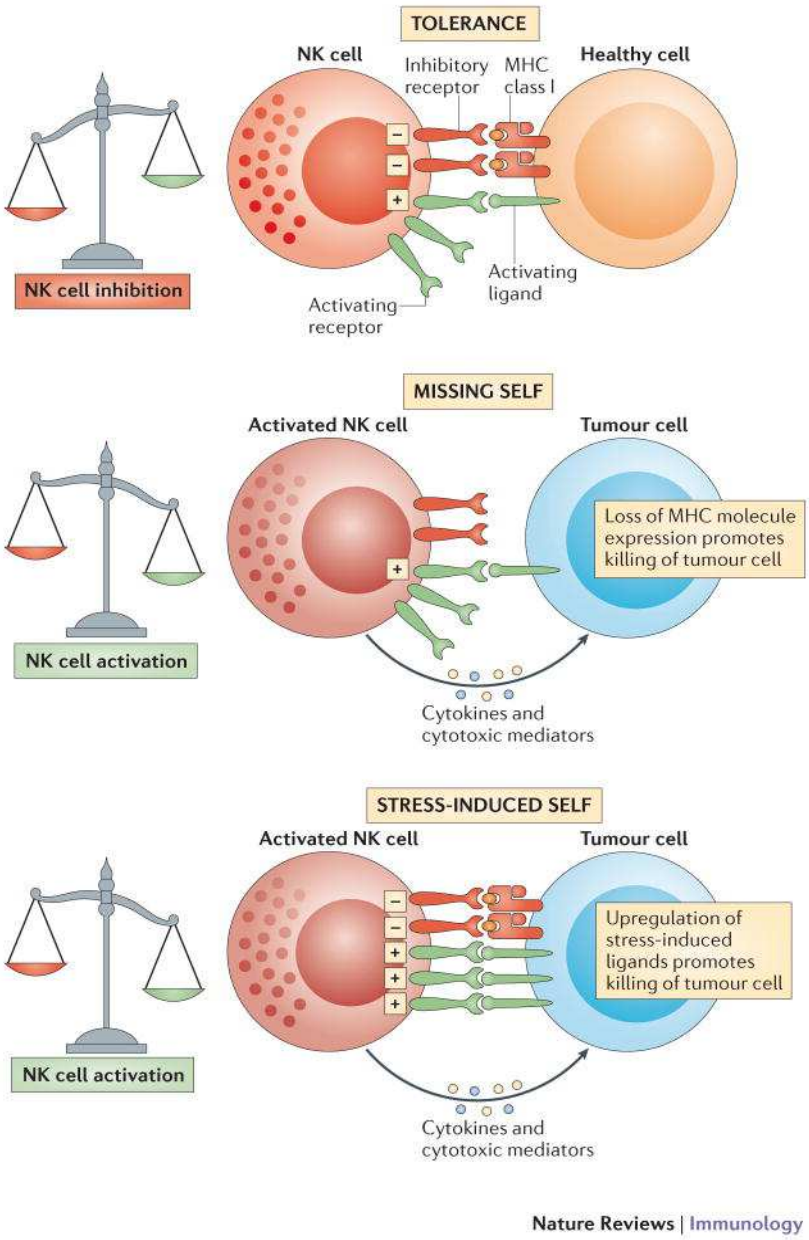

Figure 1: Vivier et al., 2012. NK cell recognition of tumour cells. a | NK cells are tolerant to normal self cells as the 'strength' of activating signals is dampened by engagement of inhibitory receptors. b | NK cells are selectively activated by stressed cells as they express a density of cell surface ligands for activating receptors which overcomes signalling via inhibitory receptors. c | this NK cell activation leads to tumour elimination directly (cytotoxicity) or indirectly (production of cytokines such as IFN-γ) [10].

2.3.2 NKG2D-ligands in human and mice

Ligands that bind to the NKG2D receptor are induced-self proteins, found on infected and abnormal cells. On the surface of normal cells, they are absent or present only at low levels [20, 21].

Different ligands for NKG2D are known for human and mice: the major histocompatibility

complex (MHC) class I-like molecules MICA and MICB and up to six different isoforms of ULBP are found to bind the NKG2D-receptor in human. Whereas mice show five isoforms of RAE-1 proteins, one MULT-1 protein and three different H60 proteins that are able to bind to the NKG2D-receptors. It is worthwhile noting that not all mice seem to express all isoforms [21 - 24].

It is still not clear why there are so many different ligands for NKG2D in human and mice. It is assumed that the ligands are regulated in a different manner, especially in different stress situations. The binding of various ligands to the same receptor might stimulate the NK cells in a different context and may differ in various cell types [19].
To sum up, the upregulation of NKG2D ligands is a complex process which is not yet completely understood but plays a crucial role in the defence against changed cells [19].

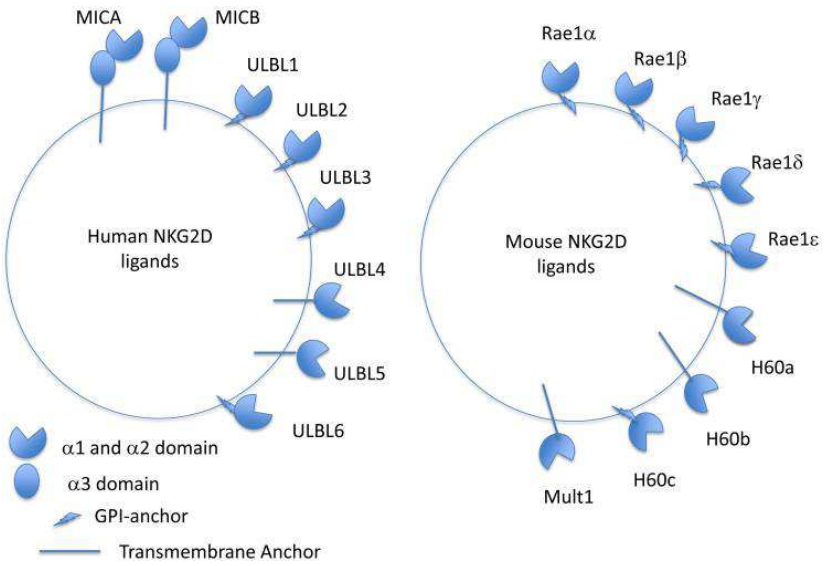

Figure 2: Lanier LL, 2015. Schematic diagram showing all human and mouse NKG2D ligands identified to date [18].

2.4 DNA damage pathway: a potent inducer of NKG2D ligand expression

To understand the defence mechanism of NK cells against cancer cells, it is crucial to know how the upregulation of ligands for NKG2D takes place on changed cells and how it can be enhanced.
There are several ways known so far that are responsible for regulating the expression of NKG2D ligands including transcription, mRNA and protein stabilization and cleavage from the

cell surface. The Cellular stress pathways are a main contributor in expressing ligands [19]. Transformation of cells, tumorigenesis and rapid cell proliferation lead to deregulated DNA replication which causes DNA breaks. This genomic instability is linked to the DNA damage response. By activation of Ataxia Telangiectasia Mutated kinase (ATM) and the DNA damage sensor kinase also known as Ataxia Telangiectasia and Rad3-related protein (ATR) a cascade including several mediators like Chk1, Chk2 and p53 is set in motion. This process results in a cell cycle arrest, apoptosis or DNA Repair [25 - 27].

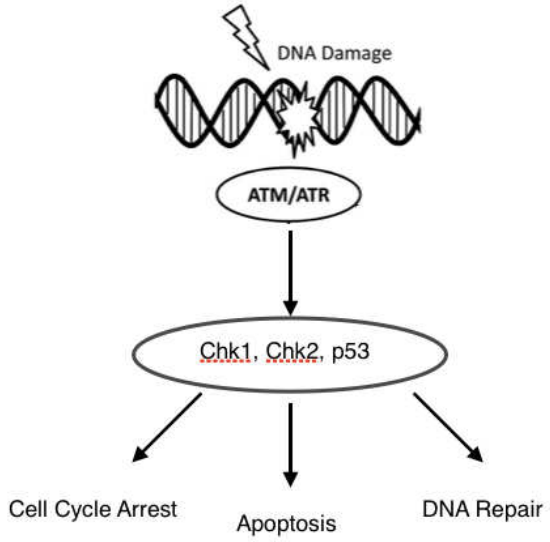

Figure 3: Simplified scheme of the DNA damage response. DNA irregularities are detected by ATM/ATR. They activate Chk1, Chk2 and p53 leading to the response including growth arrest, DNA repair and/or apoptosis.

Therefore, DNA damage inducing chemotherapeutics also lead to an upregulation of NKG2D ligands. Previous studies showed that IAP inhibitors, cytostatic drugs, were able to activate the DNA damage response by showing increased phosphorylation levels of Chk1 and γH2A histones in Hodgkin Lymphoma cell lines [28].

2.5 Ligand induction through HDAC inhibition is more potent and not dependant on the DNA damage response

For a long time, it was assumed that the function of Histone deacetylases (HDAC) inhibitors is linked to the DNA damage pathway. Previous data showed that HDAC inhibitors (HDACi) lead to the phosphorylation of ATM and are therefore inducers of the ATM-dependant DNA damage signal [29, 30].

The enzymes Histone deacetylases (HDAC) remove acetyl groups from histone and non-histone proteins which leads to condensed chromatin and thus transcriptional repression. Non-histone proteins such as the tumor suppressor genes p53 and Nf-κB become inactive. HDACs are often upregulated in cancer cells leading to growth and spread of malignant cells [27].
To repress the function of HDACs, the analogue inhibitor became more and more interesting in anti-cancer therapies. HDAC inhibitors (HDACi) reverse the function of HDACs and activate the transcription of regulatory genes and the non-histone proteins. In some patients, a benefit was shown in clinical studies by adding HDACi to DNA damage inducing chemotherapies [29].

In order to investigate which mechanism leads to a stronger upregulation of NKG2D ligands several experiments were done prior to the work mentioned in this thesis. First, different DNA damage inducing agents (Gemcitabin, Ara-C, Aphidicolin, Bleomycin and Cisplatin) and the HDACi Trichostatin A (TSA) were incubated with different cell lines. Interestingly, TSA was more potent in inducing MICA/B production than the used DNA damage inducing agents.
A FACS-based NK cell killing assay also revealed that NK cells were significantly more potent in lysing HDACi-treated cell lines than cell lines without a treatment.
Assuming that HDACi is dependent on ATM and ATR, the next step was to find out whether this hypothesis is true. By inhibiting the DNA damage kinases ATM and ATS through CGK733 and ATM through KU55933, a significant ligand induction through HDACi was still possible indicating that ligand upregulation through HDACi does not depend on the DNA damage pathway and that DNA damage-independent factors are also involved [32].

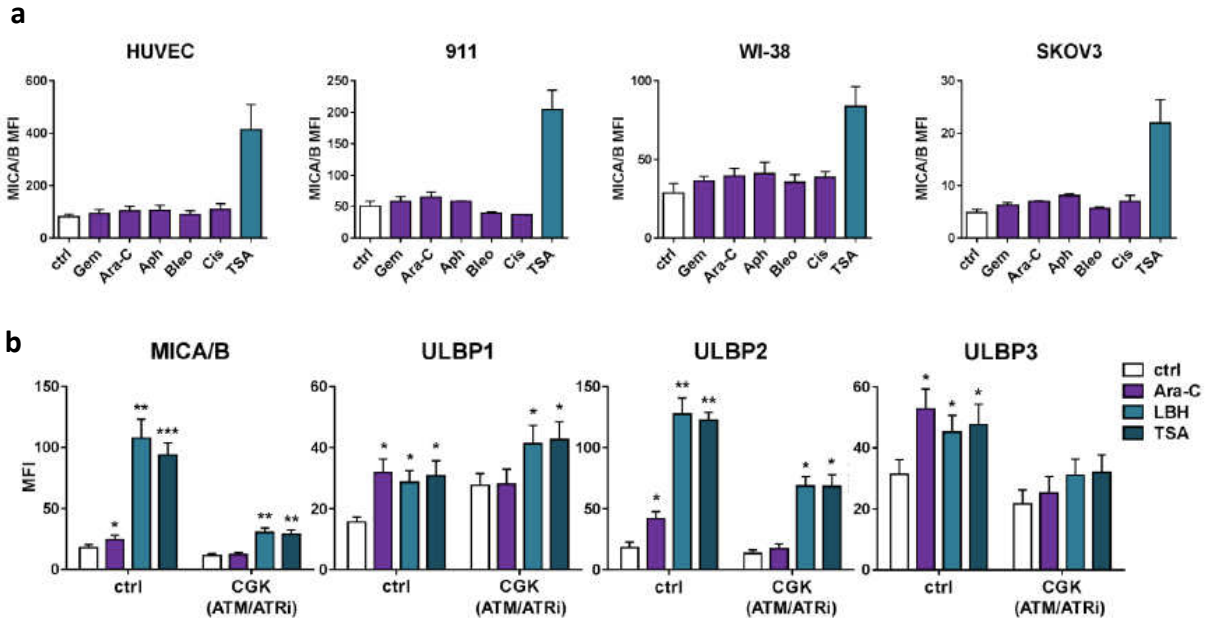

Figure 4: Sauer M., 2015. (a) Cell lines were incubated with DNA-damage agents (Gemcitabin, Ara-C, Aphidicolin, Bleomycin, Cispatin) and TSA for 16 hours. Surface expression of MICA/B were measured via Flow cytometry. (b) HEK-293 cells with ATM/ATR inhibitor-treatment (CGK733) or no treatment followed by a treatment with either Ara-C, LBH589, TSA or no further treatment for 16 hours. Then, surface expression of MICA/B, ULBP1, ULBP2 and ULBP3 were measured via Flow cytometry.

2.6 HDAC inhibitors depend on the acetyltransferase CBP/ p300

The antagonist effect of HDACs is done by Histone acetyltransferases (HAT). The CREB-binding protein (CBP) and p300 are HATs and acetylate up to over 70 proteins like histones, NF-κB and p53. They also function as adapter proteins and establish a connection between the transcription factors and the transcription machinery which enhances gene transcriptions.
A whole genome knockout of CBP and p300 is lethal in mice, worms and flies, underlining the importance of these proteins [33-35]. They are often mutated in different cancers and been described as tumour suppressor genes [36].

Having found out that the DNA damage pathway is not the main contributor in upregulating NKG2D ligands through HDACi, it was assumed that the acetylation of proteins plays a role. By inhibiting CBP and p300 through anacardic acid (ANAC), which is a general HAT inhibitor, and C646, an inhibitor of p300, an upregulation MICA/B was not observed. Furthermore, under this condition, only a reduced expression of MICA/B, after the use of HDACi, was seen. This reveals that ligand induction though HDACi depends on the acetyltransferases CBP and p300. This was again proven by genetically knocking out CBP and p300 in different cell lines. The

loss of CBP and p300 induced a decreased induction of NKG2D ligands after treatment with the HDACi LBH589 compared to wild type cells and led also to a decreased basal cell surface expression of MICA/B [32].

The tested mouse cell lines revealed that HDACi also induced an enhanced Rae-1 expression, however not in the same extend as in human cell lines. Furthermore, the inhibition of CBP/ p300 through Anacardic acid and C646 in these mouse cells prevented NKG2D ligand induction revealing that CBP and p300 also play an important role in mouse NKG2D ligand regulation [32].

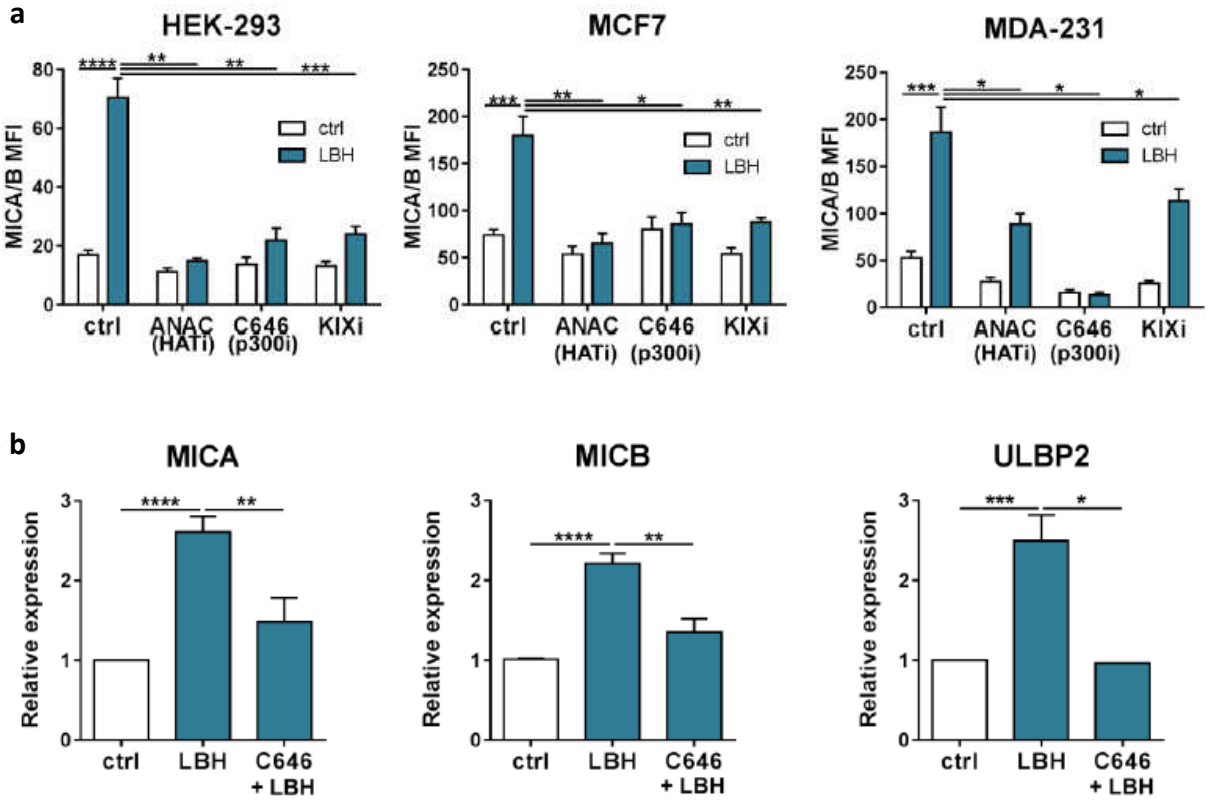

Figure 5: Sauer M., 2015. (Modified) (a) Cell lines were incubated with ANAC or C646 and treated with LBH589. (b) HEK-293 cells were preincubated with or without C646 for 3 hours followed by a treatment with LBH-589 for 1 hour. mRNA expression labels of MICA/B and ULBP2 were measured via Real Time PCR.

2.7 The use of the EμMyc lymphoma mouse model to confirm the importance of CBP/ p300

In order to verify the previous data in vivo, showing that CBP and p300 are major regulators of NKG2D ligands, an EμMyc lymphoma mouse model with the use of the Cre/loxP system was established for this thesis.

The EμMyc transgenic mouse is a model for high incidence spontaneous lymphoma. After introducing a DNA sequence isolated from a B-cell tumor into the mouse genome, the next steps are followed by breeding. Usually, affected mice develop lymphomas in the first 5 months of life [37].

The EμMyc lymphoma mice were crossed with CD19Cre mice which there obtained through the use of the Cre/loxP System. The Cre/loxP is a recombination system which enables the deletion of certain DNA-sequences in organism. The beginning and the end of a target gene is marked through a loxP which are detected and cut through the recombinase Cre. The both loxP ends are linked together and the target gene is deleted. In this project the genes coding for CBP and p300 were deleted and the B cells expressed the Cre recombinase [38 - 40].

The aim of crossing these mice was to obtain the genotype CD19Cre tg/wt, EμMyc tg/wt, CBP fl/fl, p300 fl/fl. The control mice were wildtype for CBP/p300. Both groups were observed in tumor growth and age of disease. Further experiments were planned to show the expression of NKG2D ligands in each group.

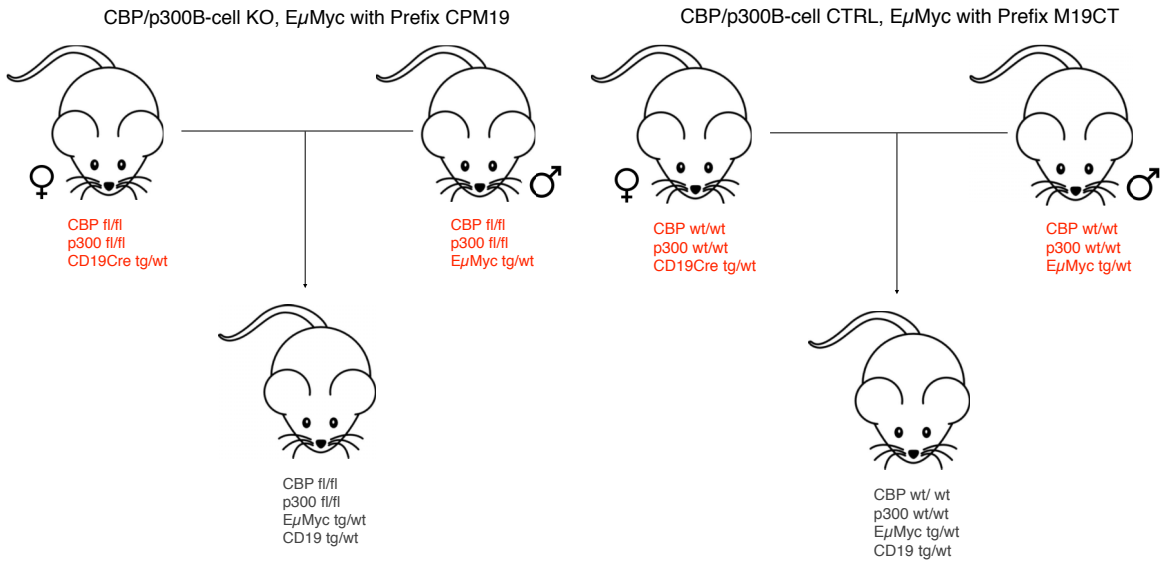

Figure 6: An EμMyc-lymphoma-mouse model was established by using the CRISPR/Cas9 method. All tested mice were positive for EμMyc and CD19. For the test-group, mice with floxed CBP/p300 were crossed with each other. While the control-mice were wildtype for CBP/p300.

2.8 Aims of this thesis

The importance of NK cells in fighting cancer cells and tumor immunosurveillance is described in multiple works. But it is still not clear how the ligand induction for NKG2D is regulated and especially how the ligand induction is enhanced for a better defence against tumor cells in cancer therapies.
For a better understanding of the regulatory mechanism of NK cells and the NKG2D ligand expression, the content of this thesis includes the following aims:

1. Monitoring the tumor formation, growth and phenotype of the mice generated with the use of the Eμ-Myc lymphoma mouse model and the Cre/loxP system.
2. Revealing the importance of the acetyltransferases CBP/p300 for NKG2D ligand induction in vivo of a mouse model with a genetic knockout of CBP/p300 compared to a mouse-model without knockout of CBP/p300.
3. Ex vivo treatment of CBP/p300-deficient cells with DNA damage inducers and HDAC inhibitors in order to observe ligand induction compared to cells with functioning CBP/p300.

3. Material and methods

3.1 Materials

Animals:

Animals were held in the CMMC of the University of Cologne. For breeding, mice aged over 8 weeks were used. For mating, generally female mice with genotype CD19Cre tg/wt, EμMyc wt/wt, CBP fl/fl, p300 fl/fl and male mice with genotype CD19Cre wt/wt, EμMyc tg/wt, CBP fl/fl, p300 fl/fl were applied.

Cell lines:

The Cell lines listed below were kept in RPMI 1640 GlutaMAX (for added ingredients see section *Chemicals*). They were used as comparison to the cells isolated from mice.

Name	Origin
EμMyc	Lymphoma cell line isolated from an EμMyc mouse (Massachusetts, Institute of Technology)
Myc-303	Lymphoma cell line isolated from an EμMyc mouse (Laboratory of the University of Cologne)

Antibodies/ Marker:

Antigen	Isotype	Application and dilution/concentration
Acetyl-Histone H3 (Lys18)	Rabbit IgG mAb	iFLOW1:50
Ac-H3 (K9)	Rabbit IgG mAb	ChIP 1:200
Ac-Lysine	Polyclonal rabbit IgG	FACS 1:200
Ac-p65 (K310)	Polyclonal rabbit IgG	FACS 1:200
B220	Anti-mouse/human CD45	FACS 1:200
b-Actin	Mouse IgG1	WB 1:2500
CBP	Polyclonal rabbit IgG	WB 1:1000, ChIP 5μg/ml
CD3	T-cell antigen	FACS 1:200
CD69		FACS 1:50

CREB	Rabbit IgG mAb	ChIP 1:200
GAPDH	Rabbit IgG mAb	WB 1:4000
IgM		FACS 1:10
MICA	Mouse IgG1	FACS 5µg/ml
MICA/B	Mouse IgG2a	FACS 1:200
MICB	Mouse IgG2	FACS 2,5µg/ml
MULT-1	Rat IgG2a	FACS 1:10
NKG2D	Mouse IgG1	FACS 1:100
NKp46	Anti Mouse CD335 antibody	FACS 1: 50
Rae-1	Rat IgG2a	FACS 1:10
TER-119	Rat IgG2b	FACS 1:100
ULBP1	Mouse IgG2a	FACS 2,5µg/ml

Inhibitors/Cytostatic agents:

Name	Function	Concentration used
Actinomycin D	Transcription inhibitor	2 µM
Ara-C	Deoxynucleoside analog	10-500 nM
Cyclophosphamid	Double strand breaks in DNA	200mg/kg BW
LBH (Panobinostad)	HDAC inhibitor	0,1-5 nM

Buffers:

Name	Ingredients	Function
ACK Buffer	1.5 M NH4Cl, 100 mM KHCO3, 10 mM EDTA-2Na	Erythrocytes Lysing Buffer
Alkaline Lysis Buffer	25 mM NaOH, 0.2 mM EDTA	DNA extraction from ear tags/ tail tips
FACS Buffer	PBS with 0.2 % BSA, 0.2% sodium acide	Substance dilution
Neutralisation Buffer	40 mM Tris-HCL	Neutralisation of Alkaline Lysis Buffer
PBS	137 mM NaCl, 8.1 mM Na2HPO4, 2.7 mM KCL, 1.5	Substance dilution

	mM KH2PO4, pH 7.4	

Primer:

Name	Sequence
Eμ-Myc Forward Primer	cag ctg gcg taa tag cga aga g
Eμ-Myc Reverse Primer	ctg tga ctg gtg agt act caa cc
CD19 Forward Primer	aca gag gga ggc aat gtt gt
CD19 Reverse Primer	ccc aga aat gcc aga tta cg
CD19-Cre Reverse Primer	tgc cag acc aaa gaa ctt cc
CBP LoxP Forward Primer	cct ggt tgc cta tgc taa gaa ag
CBP LoxP Reverse Primer	ggt atg ctg gta tca ctg gga
CBP647 + 219AS	ctg ctc tac cta aat tcc cag
CBP 607 + 324AS	ggg gaa att ttg gtc tgg taa g
P300 Primer 1	ctc agt tta tgt agc acc cc
P300 Primer 2	cag tag atg cta gag aaa gcc
P300 Primer 3	ctc tac atc cta agt gct agg

Chemicals:

Name	Application
4% Formaldehyde	iFLOW
Agarose-Powder	Genotyping
DNA-Stain	Genotyping
Methanol	iFLOW
RPMI (Glutamin, 500 µl b-Mercaptoethanol, 50 ml FBS 10%)	Medium for cell culture
TAE	Genotyping

3.2 Methods

Genotyping

To determine the genotype of the mice the ear tags or alternatively the tail tip of young mice (usually 8 weeks old) were cut and lysed by using Alkaline Lysis Buffer and incubated at 95°C for 60 minutes. Afterwards Neutralisation Buffer was added.
For PCR, 2µl isolated DNA per mouse was used. A PCR for EµMyc and CD19-Cre took separately place. The primermix for EµMyc and CD19-Cre, H2O and the Mastermix were added to the DNA. For Primer details see section *Materials*.
The PCR was performed in a C1000 Touch Thermal Cycler (BioRad, München, Germany) by an initial heating at 95°C minutes for 5 minutes, followed by 40 cycles of 60 seconds at 95°C, 60 seconds at 60°C and 90 seconds at 72°C. In a last step, samples were kept at 72°C for 5 minutes.

After PCR, the DNA samples were applied on Agarose-gel for electrophoresis in order to determine the bands which were located at 830 bp for EµMyc and 600 bp for CD19-Cre transgene.

For validation of deleted p300 and CBP, DNA from tail tips of dead mice were extracted by using DNA Easy Kit. The PCR was performed with the primers which are mentioned above.

Flow cytometry

In a biweekly cycle approximately 50 µl blood was drained from the tail of the mice. The blood was prepared with ACK Buffer and washed with PBS. The same procedure was done with the blood of terminally ill mice. Furthermore, after detection of primary tumor signs like growing lymph nodes and reduced general condition, single cell suspensions from the spleen and lymph nodes were prepared by passing the tissue through a cell strainer. To isolate the tissue cells and lymphocytes from the erythrocytes, the cell suspension was layered on mouse ficoll and spinned for 20 minutes. Afterwards the upper layer was separated and washed with PBS.
The cells were stained with antibodies either directly or through labelled secondary antibodies. The process was followed by an incubation for 30 minutes on ice in the dark. In a last step, 7-AAD was added to mark dead cells in order to exclude them from the measurement.
The analyses were performed with FACSCalibur (Becton Dickinson) or Gallios (Beckman Coulter).

For intracellular flow, 4% Formaldehyde in PBS was used to fix the cells. Afterwards the mixture was incubated at 37°C for 12 minutes followed by incubation on ice for 60 seconds. After spinning the cells down, the supernatant was removed, the cells were resuspended in ice-cold Methanol and incubated again on ice for 30 minutes. This procedure was followed by resuspension of the cells in a staining mix containing Acetyl-Histone H3 Rabbit mAb and (after incubation) with Dylight 659 Donkey-anti rabbit IgG as secondary antibody. An isotype control was done with unlabelled Rabbit IgG.

Real-time PCR

The M&N NucleoSpin kit was applied to extract RNA from cells. For measuring the RNA concentration SpectraMax M4 was used. The Revert Aid First Strand cDNA kit from Thermo scientific was used for synthesis of cDNA from 1µg RNA. Diluted cDNA was mixed with forward and reverse primer for Rae-1 and HPRT. SYBR Green PCR jump start was added and the Real-time PCR was performed. With an initial heat activation for 15 minutes at 95°C followed by 40 cycles of 15 seconds at 94°C, 30 seconds at 55°C and 30 seconds at 72°C.

Ex Vivo Treatment

Priorly isolated cells from mice were kept in culture with RPMI. Depending on growth rate the cells were split two to three times a week.
For ex vivo treatment, 1x10^5 cells/ml per well were used and treated for a total of 1 hour and 16 hours. LBH and Ara-C were applied in different concentration. LBH was used from 0,1 nM to 5 nM. Ara-C was titrated from 10 nM to 500 nM. An untreated control with equivalent volume of DMSO was present in every treatment procedure.

Software

For analysing the flow cytometry data FlowJo X was used. The bar charts and figures were created with GraphPad Prism 6 and Pages version 5.6.2.

4. Results

4.1 Tumor onset and progression do not seem to depend on the loss of CBP/p300

Before analysing the ligand expression in vivo and ex vivo we compared the two mice group in regard to tumor appearance and degree of severity.
Generally, mice developed tumor in an age between 86 and 159 days, independently from gender and genotype. Therefore, tumor onset was not linked to the age of the mice which is consistent with the present spontaneous tumor model. Other symptoms that occurred with tumor onset, as far as it was observed, were reduced general condition, fatigue and loss of appetite.
As soon as mice showed the first signs of a tumor, which were detectable from the outside, the tumor were extracted, counted and measured.
No differences in tumor onset and tumor progression were observed upon the two mice groups. Lymph node swelling occurred particularly in the cervical and axillar region, the inguinal and abdominal region were also affected. The mice showed generally up to 8 enlarged lymph nodes. The tumor size differed not only between the two mice groups but also within each group. Furthermore, we compared the size of the spleen which is usually affected when suffering of lymphoma. The spleen measured an average size of 20 mm. Again, we could not detect any differences.

To summarize, the loss of CBP/p300 does not seem to influence the tumor progression and the survival time. However, it is worthwhile noting that the differences might be masked by the heterogeneity of tumor onset and age between the two mice group.

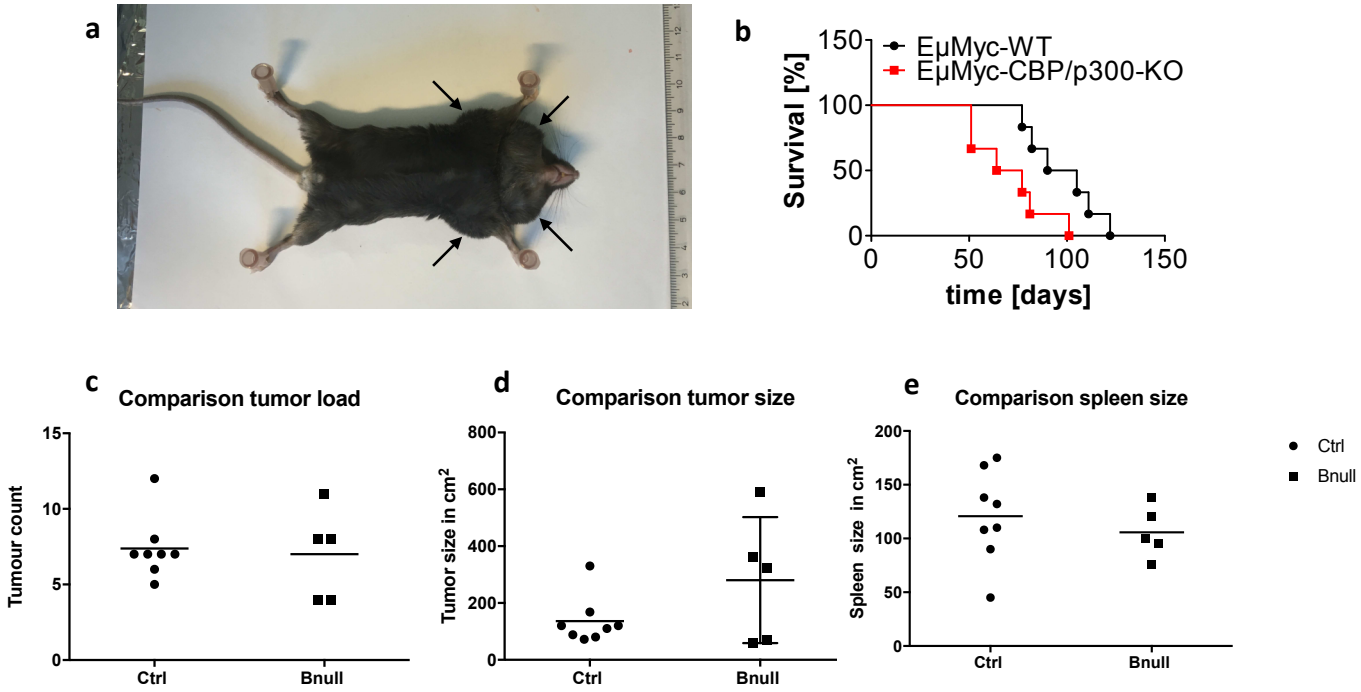

Figure 7: Tumor onset and proression does not seem to depend on CBP/p300. (a) Terminally ill mice with visible and palpable tumor cervical left and right as well as axillar left and right. Enlarged lymphnodes were also found inguinal (not shown). (b) The survival time did not vary. In general, mice died after 120 days. (c) Tumor load in Bnull mice and control mice did not differ. Generally, mice showed up to 7 enlarged lymphnodes, but the count varied greatly. (d) Tumor size differed in Bnull- and Control-mice. The Bnull-mice partly tend to have extrem enlarged lymphnodes but this only applied to indiviudal cases. (e) The spleen is generally affected in lymphoma. Spleen size was measured in both groups showing that there is no significant difference.

4.2 The loss of both acetyltransferases, CBP and p300, might not be compatible with life

First, it was crucial to detect whether a loss of CBP and p300 is present in the KO group in order to form the two mice groups. A PCR from the DNA, isolated from the tail tip, was performed using specific primers to verify the genotype. The PCR results showed either a deletion of CBP or p300, but never both genes. All mice, either with a deletion of CBP or p300, were included in the KO group.

The absence of a complete deletion of both acetyltransferases could be an indication for the importance of them in an organism and that at least one of them needs to be functioning for the development and/ or survival of B-cells.

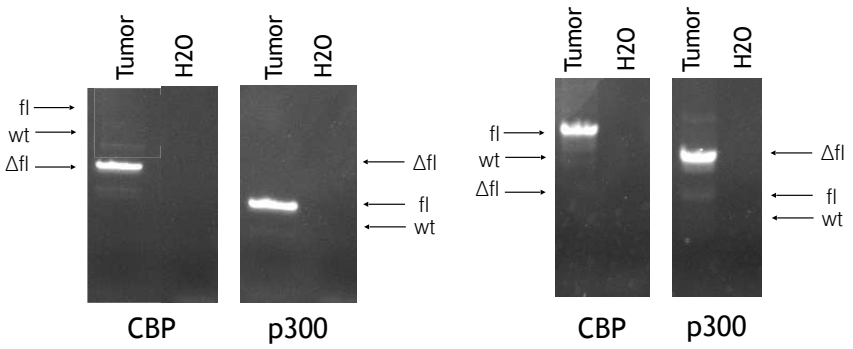

Figure 8: The loss of both acetyltransferases, CBP and p300, might not be compatible with life. CBP/p300 deficient EμMyc mice show deletion of either CBP or p300, but never both. PCR was performed using genomic DNA extracted from tumor cells of terminally ill Bnull mice. Using specific primers, recombined or non-recombined genes were detected. The left data shows a Bnull Mice with deletion of CBP. The right data shows a Bnull mice with deletion of p300.

4.3 CBP/p300 are major regulator of the mouse NKG2D-L RAE-1

In order to analyse the NKG2D-L expression, tumor cells were isolated from peripheral blood, lymph nodes and spleen.

First, the surface expression of RAE-1 and MULT-1 on peripheral blood cells were analysed by flow cytometry showing that only the ligand RAE-1 was significantly reduced in the CBP/p300 deficient cells, while the surface expression of MULT-1 remained unaffected. A possible explanation for this could be that the ligands RAE-1 and MULT-1 are regulated independently.

Furthermore, we concentrated on RAE-1 and measured the RAE-1 expression on tumor cells isolated from lymph node (tumor), spleen and blood cells from CBP/p300-deficient EμMyc mice and EμMyc control-mice without deficiency via Real Time PCR. Again, RAE-1 was significantly diminished on the cell surface. Via Real Time PCR, the transcript levels of RAE-1 were analysed to reveal that the expression of RAE-1 is also reduced on molecular level in the Bnull-mice compared to the control-mice. Therefore, the data correlates with the reduced surface expression of RAE-1.

In vitro experiments with the cell line MCA-205 could also show that upon inhibition of CBP/p300 with C646, the RAE-1 expression was blocked while MULT-1 expression was not reduced. The cells were preincubated with C646 in order to inhibit p300, followed by a treatment with ANAC and LBH. The Rae-1 and MULT-1 surface expression was measured via Flow cytometry.

To sum up, the acetyltransferases CBP/p300 were identified as major regulator of the mouse NKG2D-L RAE-1 in vitro and in vivo.

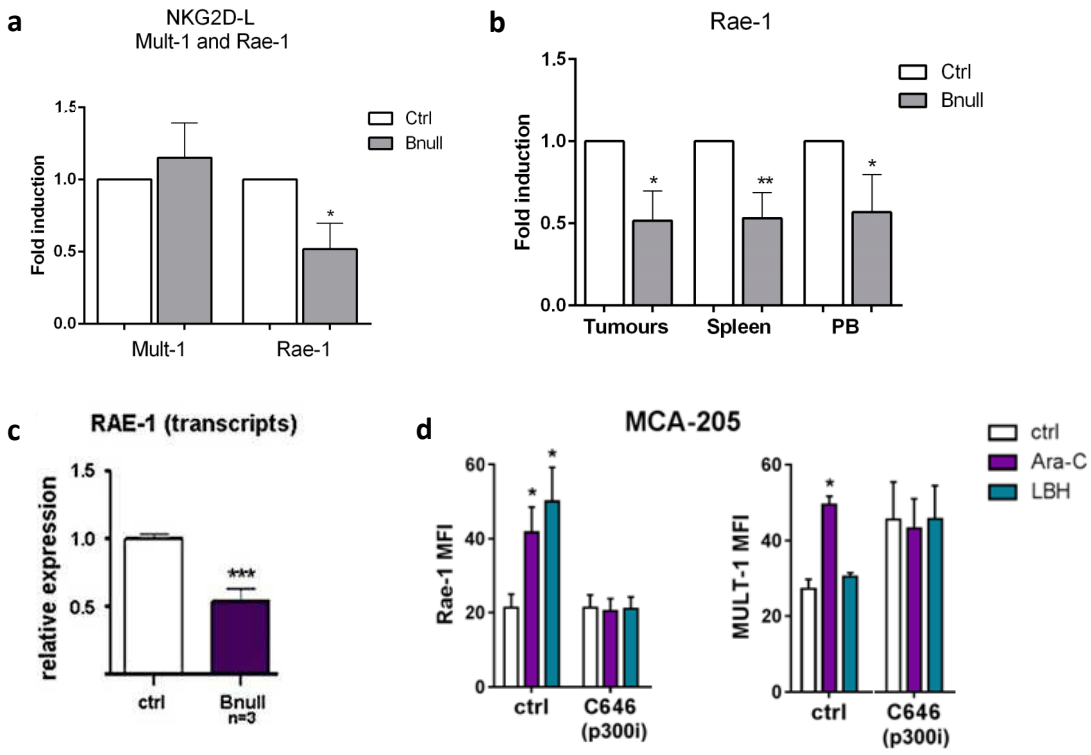

Figure 9: CBP/p300 are major regulator of the mouse NKG2D-L RAE-1. (a) The NKG2D ligand expression on peripheral blood tumor cells show that only RAE-1 is significantly reduced in the Bnull mice, while MULT-1 is not affected by the deficiency of CBP/p300. (b) RAE-1 expression on tumor cells isolated from lymph nodes, spleen and peripheral blood of EμMyc-control mice or EμMyc with CBP/p300 deficiency. Significant reduction of RAE-1 on all cells from the mice with CBP/p300-deficient B cells. (c) RAE-1 transcript levels detect by Real Time PCR. (d) MCA-205 cells were preincubated with 8μM C646 followed by a treatment with 5nM LBH589 for 16 hours. After treatment surface expression of MULT-1 and RAE-1 were detected by flow cytometric analyses.

5. Discussion

5.1 The importance of NKG2D and its ligands for tumor surveillance

Natural killer cells are crucial for the innate immunity as they form the first barrier when it comes to tumor surveillance. In the last decades, the NKG2D receptor and the NKG2D ligands were declared to play a significant role in cancer immune surveillance [41, 42]. But the function of the immune system and especially the function of NK cells is usually impaired in organism with various tumors [43,44]. This is attributed to the ability of tumor cells to evade the immune system through the expression of MHC I molecules to inhibit NK cell cytotoxicity or through reducing the expression of NKG2D ligands on the cell surface to hinder a recognition by NK cells [44,45]. Unfortunately, the detailed molecular mechanisms still remain unclear which is why it is necessary to carry out further analysis to understand the working manner of NK cells and how their activation can be induced in order to use the derived information for developing immunotherapeutic approaches.

The main aim of this work and its underlying paper was to find out how ligand induction for NKG2D receptor is regulated and which mechanism is the main regulator. For a long time, it was assumed that the DNA damage response is the main mechanism to upregulate ligand expression [46,47]. We could show that the ligand induction for NKG2D is not only dependent on the DNA damage response but the inhibition of histone deacetylases and thus the enhanced acetylation of proteins play an important role in the transcriptional regulation of them. In several human and murine cell lines, the upregulation of NKG2D-L was stronger upon HDACi-treatment than upon the treatment with DNA-damaging agents. Several agents of the HDACi class were able to induce MICA/B on the cell-surface.
It is known that HDACi regulate ATM which is a major regulator of the DNA damage response and sensitize cancer cells towards therapies containing DNA damaging agents [48]. Therefore, it was assumed that the upregulation of NKG2D ligands caused by HDACi depend on ATM/ATR. But even the inhibition of the DNA damage response through blocking ATM/ATR with CGK733 or Ku55933 resulted in a ligand upregulation indicating that HDACi do not depend on the DNA-damage response and lead to the strongest upregulation of NKG2D ligands compared to DNA-damage inducing agents.

5.2 The acetyltransferases CBP/p300 are crucial for the regulation of NKG2D ligands

After HDACi seemed to be the main regulator of ligand induction, it was assumed that the enhanced acetylation after inhibiting HDAC might be responsible. The acetyltransferases CBP/p300 are important for the acetylation of various proteins and enable gene transcription by regulating chromatin accessibility [49]. They play a key role in tumors, as they are often mutated in various cancer forms and are described as tumor suppressor genes [50,51,52].
We wanted to find out whether a loss of CBP/p300 affected the ligand upregulation through HDACi. Therefore, CBP/p300 were blocked by using HAT-inhibiting agents or deleted CBP/p300 with the help of genetic modification to proof the previous findings in vitro and in vivo experiments.

CBP/p300 were blocked witch ANAC or C646 in various cell lines derived from men and mice. The following treatment with HDACi did not lead to a ligand induction. Ligand induction was also diminished after knocking out CBP/p300 in HEK-293 cells by using the CRISPR/Cas9 method.
For further investigation in vivo, we started with the breeding of conditional B cell CBP/p300 knockout EμMyc-lymphoma mice. After ligand induction through HDACi was also possible in murine cell lines, we assumed that there must be similarities with men in view of the regulation of NKG2D ligands and it would be a good possibility to verify the findings.

We expected that the mice with the CBP/p300 knockout would present themselves with an accelerated tumor growth due to the fact that CBP/p300 are described as tumorsuppressor genes and are often correlated with the appearance of various cancer types [54,55].
The loss of CBP/p300 did not affect the tumor development and progression in mice although it is difficult to determine this information as the used lymphoma-mouse-model is a model with spontaneous tumor onset and differences could be masked by the heterogeneity of this.
Nevertheless, we found out that the mice never had a deletion of both acetyltransferases CBP and p300. It may be well assumed that mice with a deletion of both die premature in early gestation and an organism needs at least one of these acetyltransferases functioning to survive and develop without any malformation of organs [53].

The main results of this thesis were that the loss of CBP/p300 resulted in a diminished surface expression and reduced transcript levels of RAE-1 upon treatment with HDACi indicating that CBP/p300 are major regulator of the mouse NKG2D ligand RAE-1. This data correlated with the previous findings. The NKG2D ligand MULT-1 was not affected by a knockout of CBP/p300. We hypothesize that this ligand is regulated in a different way and might have a special function for the regulation NK cells.

5.3 Difficulties with ex vivo treatments of the cells isolated from the EμMyc mice

To investigate the collected data, we tried to grow the cells isolated from the peripheral blood, the tumor (lymph nodes) and spleen of the mice in the cell culture. The aim was to treat the cells with HDAC inhibiting agents as well as DNA damage inducing agents and reproduce and secure the data collected previously. Approximately 1x10^5 cells/ ml were incubated in RPMI per well. Then, various dosages of the HDACi LBH starting from 0,1 nM up to 5 nM, TSA 10 – 400 nM and the DNA damage inducer Ara-C 10 – 500 nM were added. The treatment was followed by an incubation for 16 hours.

After treatment, we wanted to measure the ligand expression on the cell surface via Flow Cytometry. Unfortunately, most of the cells were dead, even in the lowest used dosage of HDACi. It was not possible to collect the data as it was not analyzable.

We retried the experiment with various cells from the mice we used for the other experiments and titrated extreme low dosages of the mentioned agents. We also tried to reduce the treatment duration to 1 hour. But we always obtained the same results: mostly all cells were not alive.

We suggest, that before treatment with HDACi or DNA damage inducers, it is necessary to maintain cell lines that grow well and have proper cell division. In our case, the used cells had a good growth rate before treatment. It was necessary to split them regularly, so they did not out grow. The used medium was also not affected. But it is conceivable that it takes much more cell cycle of division to establish a stable cell line.

5.4 Can the results help to build new therapeutic options?

The cells of the innate immunity, particularly NK cells, are directly involved in cancer immunosurveillance. Various studies could show that a low activity of NK cells is correlated with an increased risk for cancer in men [54]. The absence or dysfunction of NK cells is associated with spontaneously developing tumors and tumors induced by chemical carcinogens in mice [55].

All these results provide evidence that NK cells are essential when it comes to cancer defence. This is why it is so important to obtain more information in this field in order to apply it to refine approaches in developing cancer therapies.

Here, we could proof that the ligands for NKG2D are regulated by the acetyltransferases CBP/p300 and an upregulation is enhanced through HDACi. But it seems that the ligands are regulated in different ways in human as well as in mice. An induction through HDACi was seen in various cell lines while the effect differed from cell line to cell line. Even the two ligands known in mice seem to be regulated separately. While RAE-1 was inducible, MULT-1 remained unaffected.

It is questionable to what extent it is possible to transfer this data collected from mice to men. It is definitely important to extend the research in this field for a better understanding of how the ligands are regulated and especially how the NK cell function can be enhanced. Further consideration would be to use a humanized mouse model in order to improve the transferability of the data.

Another idea is to directly test tumor cells derived by human who have been treated with HDACi.

For sure, we can say that triggering the NKG2D activation could possibly enable to overcome the ability of tumor cells to evade the innate immunity and enhance the body's own defense system against malignant cells and to achieve a better outcome in cancer therapies.

5.5 Conclusion

Ligand induction for the NKG2D receptor is a complex and yet not completely understood mechanism. The underlying data presented in this work provides the evidence that CPB/p300 are crucial for the regulation of the ligand RAE-1 for the murine NKG2D receptor.

CBP/p300 are acetyltransferases which facilitate gene transcription by establishing a connection between the transcription factors and the transcription machinery. They enable the expression of ligands which in turn can activate NK cells. The activation of NK cells is indispensable for the innate immune system in order to eliminate changed cells such as tumor cells.

The findings help to understand the function of NK cells and how they are regulated. Further analysis in this field is important to expand the knowledge about NK cells and tumor surveillance and to make use of it in order enhance cancer therapies for men.

6. Summary

Natural killer cells (NK cells) play an important role in the innate immune system as they are crucial for the defence against cancer cells. In order to avoid an autoreactive activation, NK cells are strictly regulated and need to get activated first. The best known and investigated activating receptor is the Natural-killer group 2 member D (NKG2D). Several ligands are known for binding this receptor and thus activating NK cells. These ligands are generally not expressed on the cell surface of normal cells, but on changed cells they are upregulated.

Previous studies could show that the ligand-induction is maintained by the DNA-damage-pathway, whereas the molecular mechanism remains unknown. The DNA-damage-pathway can be activated through several mechanisms, amongst others through Histone-deacetylase-Inhibition (HDACi).

The pre-project of this project revealed that a ligand induction is still possible while inhibiting the DNA-damage kinases Ataxia Telangiectasia Mutated (ATM) and Ataxia Telangiectasia and Rad3-related (ATR) using HDACi, indicating that there must be other mechanisms, which are independent from the DNA-damage-pathway, that regulate the ligand expression for NKG2D. Furthermore, the ligand induction through HDACi could be blocked by inhibiting the acetyltransferases CREB-binding protein (CBP) und p300.

This projects aim was to investigate the role of CBP and p300 for the NKG2D-ligand induction using an EμMyc-lymphoma-mouse-model with a genetic knockout of CBP and p300. We could show that, compared to the EμMyc-lymphoma-mouse-model without a knockout, the loss of CBP/p300 led to a diminished expression of ligands on the surface of tumor cells. Additionally, we identified CBP/p300 as major regulator for the expression of the Retinoic acid early inducible 1 (RAE-1), which is a NKG2D-ligand in mice.

This work gives crucial evidences for the importance of CBP/p300 as NKG2D-ligand-inductor, in men and mice.

These findings could lead to the development of immunotherapies that can optimize the immune system of patients suffering from malignancies and improve their overall survival.

7. Zusammenfassung

Natürliche Killerzellen (NK-Zellen) nehmen eine wichtige Stellung im angeborenen Immunsystem ein, da sie unter anderem eine große Bedeutung für die Tumorabwehr haben. Zur Vermeidung einer autoreaktiven Funktion sind NK-Zellen streng reguliert und bedürfen einer gezielten Aktivierung über Rezeptoren. Der bislang am besten beschriebene und untersuchte aktivierende Rezeptor heißt Natural-killer group 2 member D (NKG2D). Diverse Liganden können durch Andocken an diesen Rezeptor zu einer Aktivierung von NK-Zellen führen und so eine primäre Immunantwort gewährleisten. Diese Liganden sind auf der Zelloberfläche gesunder Zellen kaum exprimiert, wohingegen sie auf veränderten Zellen hochreguliert aufzuweisen sind.

Voruntersuchungen konnten zeigen, dass eine Induktion der Liganden-Exprimierung über die DNA-Damage-Antwort erfolgt, welche unter anderem durch die Inhibierung von Histon-Deacetylasen (HDAC) aktiviert wird. HDAC-Inhibitoren (HDACi) führen zu einer Hemmung der Transkriptionsunterdrückung und Phosphorylierung von DNA-Damage-Kinasen.
Die Vorarbeit zu diesem Projekt machte deutlich, dass HDACi auch unter Inhibierung der DNA-Damage-Kinasen Ataxia Telangiectasia Mutated (ATM) und Ataxia Telangiectasia and Rad3-related (ATR) zu einer NKG2D-Liganden-Expression führen, sodass davon auszugehen ist, dass auch DNA-Damage-unabhängige Faktoren eine Rolle spielen. Weiterhin konnte gezeigt werden, dass eine Liganden-Hochregulierung durch HDACi durch Inhibieren der Acetyltransferasen CREB-binding protein (CBP) und p300 gehemmt werden kann.
Für dieses Projekt erfolgte zur weiteren Untersuchung des Einflusses von CBP und p300 auf die NKG2D-Liganden, die Etablierung eines EμMyc-Lymphom-Maus-Modells mit einem genetischen Knock-Out von CBP und p300. Als Vergleichsgruppe diente ein EμMyc-Maus-Modell ohne genetischen Knock-Out von CBP/p300. Die Ergebnisse der Untersuchungen zeigten das ein Fehlen von CBP/p300 zu einer verminderten Liganden-Exprimierung auf Tumorzellen führt. Außerdem konnte in vivo und in vitro deutlich gemacht werden, dass CBP/p300 ein wesentlicher Regulator für die Expression von Retinoic acid early inducible 1 (RAE-1), einem NKG2D-Liganden in Mäusen, ist.

Diese Arbeit legt bedeutende experimentelle Nachweise für die Wichtigkeit von CBP/p300 als NKG2D-Liganden-Induktoren dar, sowohl in Menschen, als auch in Mäusen.

Die gewonnenen Erkenntnisse können einen wesentlichen Beitrag zur Entwicklung immuntherapeutischer Ansätze leisten, um das Immunsystem onkologischer Patienten gegenüber dem Tumorleiden zu optimieren und damit ein besseres Gesamtüberleben zu ermöglichen.

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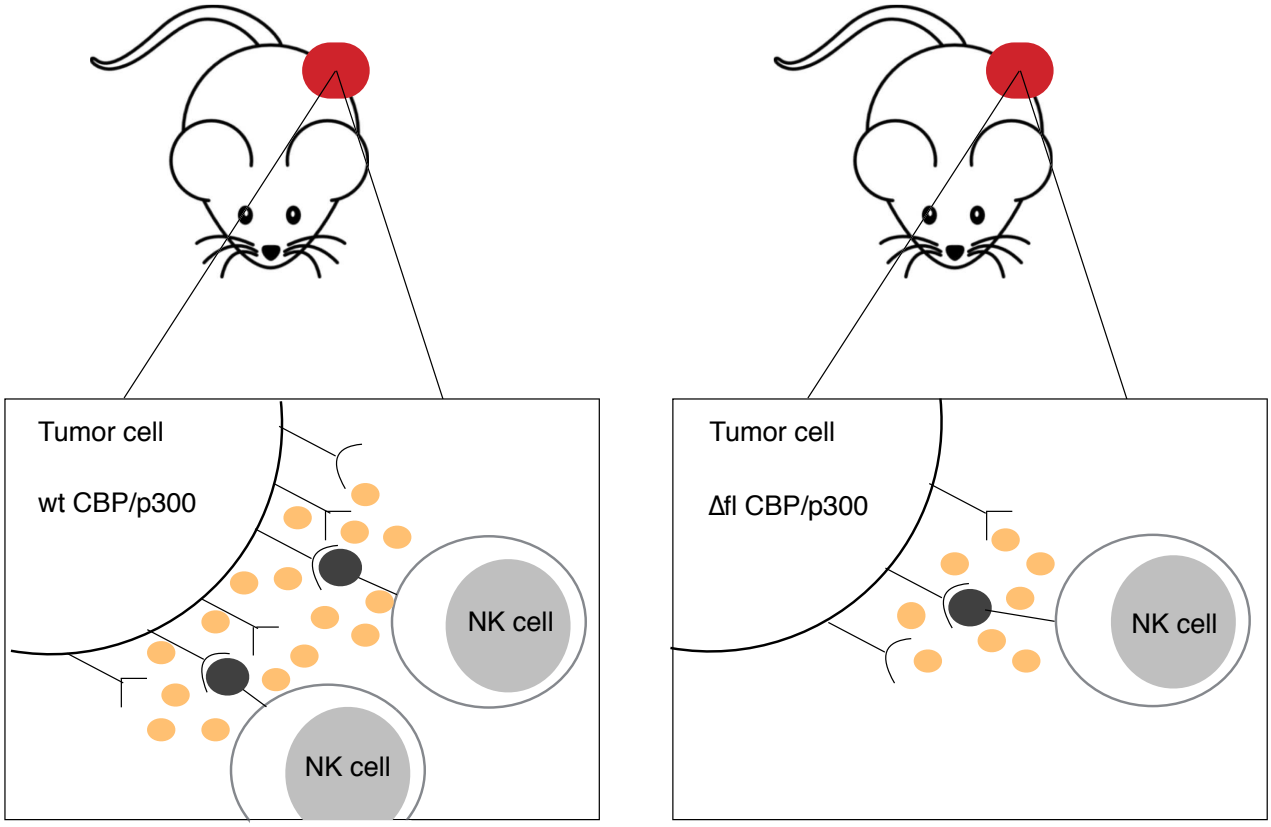
9. Preliminary Publication

Sauer M, Schuldner M, Hoffmann N, Cetintas A, Reiners KS, Shatnyeva O, Hallek M, Hansen HP, Gasser S, Pogge von Strandmann E (2017). *CBP/p300 acetyltransferases regulate the expression of NKG2D ligands on tumor cells.* Oncogene. 36(7): 933-941.

10. Appendix

Graphical abstract



Model showing the role of CBP/p300 for NKG2D ligand upregulation for NK cells in mice. Tumor cell with loss of the acetyltransferases CBP/p300 shows a diminished ligand count on the cell surface leading to less binding and thus less activation of NK cells. After activation, NK cell releases cytokines and chemokines which stimulate apoptosis of the tumor cell.