Immunomodulatory Effects of Adiponectin

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Berlin, den 01.10.2012

Sabrina Wilk

"Nur wer nicht sucht, ist vor Irrtum sicher."

Albert Einstein, 1945

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TABLE OF CONTENTS

LIST OF ILLUSTRATIONS **I**
TABLE OF DIRECTORY **III**
ABBREVIATIONS **IV**
SUMMARY **VI**
ZUSAMMENFASSUNG **VIII**

1 INTRODUCTION 1

1.1 ADIPONECTIN 1
1.1.1 GENE AND EXPRESSION 1
1.1.2 PROTEIN AND RECEPTORS 2
1.1.3 ROLE OF ADIPONECTIN 4
1.1.4 SIGNALING PATHWAYS 7
1.2 IMMUNOMODULATORY EFFECTS OF ADIPONECTIN 10
1.2.1 ANTI-INFLAMMATORY ACTIVITIES OF ADIPONECTIN IN *IN VIVO* MODELS 11
1.3 T CELLS 12
1.3.1 REGULATION OF T CELLS 12
1.3.2 ADIPONECTIN AND T CELLS 15
1.4 NATURAL KILLER CELLS 15
1.4.1 ADIPONECTIN AND NATURAL KILLER CELLS 18
1.5 AIM OF THE STUDY 19

2 MATERIAL 20

2.1 SUBJECTS 20
2.1.1 HUMAN 20
2.1.2 ANIMAL MODEL 20
2.1.3 CELL LINES 20
2.2 REAGENTS 22
2.2.1 CELL ISOLATION AND SEPARATION REAGENTS 22
2.2.2 KITS 22
2.2.3 CELL CULTURE MEDIA 22
2.2.4 PRIMERS FOR QRT-PCR 23

2.2.5 ANTIBODIES 23
2.2.6 FLUORESCENCE DYES 25
2.2.7 STIMULATING AND ACTIVATING AGENTS 26
2.2.8 CHEMICALS, SOLUTION AND BUFFERS 26
2.3 EQUIPMENT 27
2.4 SOFTWARE 28

3 METHODS 29

3.1 CELL CULTURE AND PREPARATION 29
3.1.1 ISOLATION OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCS) 29
3.1.2 ISOLATIONS OF HUMAN CD3 T CELLS 29
3.1.3 ISOLATION OF HUMAN NK CELLS 29
3.1.4 ISOLATION OF MURINE SPLENOCYTES AND PERIPHERAL CELL 29
3.1.5 THAWING OF CELL LINES 30
3.1.6 FREEZING OF CELLS 30
3.2 STIMULATION 30
3.2.1 STIMULATION OF T CELLS 30
3.2.2 STIMULATION OF NK CELLS 31
3.3 FLOW CYTOMETRY 31
3.3.1 STAINING PROCEDURES 31
3.3.2 GATING STRATEGIES 31
3.4 CONFOCAL MICROSCOPY 32
3.5 QUANTITATIVE REAL-TIME-PCR 32
3.6 ASSAYS 33
3.6.1 ANALYSIS OF PROLIFERATION AND APOPTOSIS 33
3.6.2 CD107A DEGRANULATION ASSAY 33
3.6.3 *IN VITRO* KILLING ASSAY 34
3.7 STATISTICAL ANALYSIS 34

4 RESULTS 35

4.1 IMMUNOMODULATORY EFFECTS OF APN ON T CELLS 35
4.1.1 ADIPORS EXPRESSION AND LOCALIZATION IN HUMAN T CELLS 35
4.1.2 ADIPORS EXPRESSION ON MURINE T CELLS 41
4.1.3 ADIPORS REGULATION ON T CELLS 42
4.1.4 FUNCTIONAL EFFECTS OF APN ON T CELLS 47

4.2 IMMUNOMODULATORY EFFECTS OF APN ON NK CELLS 51
4.2.1 ADIPORS EXPRESSION ON HUMAN NK CELLS 51
4.2.2 ADIPORS EXPRESSION ON MURINE NK CELLS 52
4.2.3 ADIPORS REGULATION ON NK CELLS 54
4.2.4 FUNCTIONAL EFFECTS OF APN ON NK CELLS 54

5 DISCUSSION 62

5.1 APN AND T CELLS 62
5.2 APN AND NK CELLS 66

6 REFERENCES 70

7 ATTACHMENTS 78

7.1 PUBLICATIONS 78
7.2 POSTERS 78
7.3 CURRICULUM VITAE FEHLER! TEXTMARKE NICHT DEFINIERT.

LIST OF ILLUSTRATIONS

Figure **1**	Location and genomic structure of ADIPOQ	1
Figure **2**	Domains and configurations of Adiponectin	3
Figure **3**	Pleiotropic role of Adiponectin	5
Figure **4**	Involvement of Adiponectin in intracellular signaling pathways	8
Figure **5**	Interaction of TCR/CD3 complex with MHC molecule on APC	13
Figure **6**	Comparison of mouse and human NK cells	17
Figure **7**	AdipoRs expression on human $CD3^+$ T cells	35
Figure **8**	Intracellular and extracellular expression of AdipoR1 in peripheral blood $CD3^+$ T cells and $CD14^+$ monocytes	36
Figure **9**	Intracellular and extracellular expression of AdipoR1 and AdipoR2 on bone marrow cells	37
Figure **10**	Intra- and extracellular expression of AdipoRs on lymphatic leukemia cell line Jurkat and myeloid leukemia cell line K562	37
Figure **11**	Intracellular co-localizations of AdipoRs with TIRC 7 in human T cells	39
Figure **12**	Intracellular co-localization of AdipoR1 with clathrin-coated vesicles and CTLA-4	40
Figure **13**	Distribution of AdipoRs in murine $CD3e^+$ T cells	41
Figure **14**	Expression of AdipoRs on murine $CD3e^+$ T cells of WT and APN-KO mice	42
Figure **15**	Regulation of AdipoRs on SEB stimulated human $CD4^+$ and $CD8^+$ T cells	42
Figure **16**	Co-expression of AdipoR1 and CTLA-4 on human $CD137^+$ T cells	43
Figure **17**	AdipoR1 surface expression on influenza activated human T cells detected by tetramer staining	44
Figure **18**	AdipoR1 surface expression on influenza activated human T cells detected by IFNγ staining	45
Figure **19**	Time course of human AdipoR1 surface expression	46
Figure **20**	Regulation of AdipoRs on murine T cells	46
Figure **21**	Influence of APN on the generation of human antigen specific T cells	47
Figure **22**	Influence of APN on AdipoR1 expression of SEB activated T cells	48
Figure **23**	APN impact on cytokine producing human $CD8^+$ T cells and on *ex vivo* cytokine production	48

Figure **24**	APN influence on apoptosis and proliferation of human CD3/CD28 activated T cells	49
Figure **25**	APN influence on apoptosis and proliferation of human influenza activated T cells	50
Figure **26**	Influence of APN deficiency on murine antigen activated T cells	50
Figure **27**	AdipoRs expression on human $CD56^+$ NK cells	51
Figure **28**	AdipoRs expression on murine splenic $Nkp46^+$ NK cells	52
Figure **29**	Surface AdipoR1 distribution on murine NK cell subsets of WT and APN-KO mice	53
Figure **30**	Regulation of surface AdipoRs expression on murine $Nkp46^+$ NK cells	54
Figure **31**	Functional effects of APN on TLR-L induced IFNγ secretion of human NK cells	55
Figure **32**	APN influence on human NK cell degranulation and cytotoxicity	56
Figure **33**	Increased NK cell frequencies and numbers of APN-KO mice	57
Figure **34**	Murine NK cell subsets of uninfected and infected WT and APN-KO mice based on CD11b and CD27 expression	58
Figure **35**	Degranulation of murine NK cells of uninfected and infected WT and APN-KO animals	59
Figure **36**	NKG2D expression on NK cells of uninfected and infected WT and APN-KO animals	60
Figure **37**	$CD94^{high}$ and $CD94^{low}$ subsets of murine NK cells in uninfected and infected WT and APN-KO mice	61
Figure **38**	IFNγ production by NK cells of uninfected and infected WT and APN-KO mice	61

TABLE OF DIRECTORY

Table **1**	Cell lines	21
Table **2**	Antibodies with human specificity	24
Table **3**	Antibodies with murine specificity	25
Table **4**	Secondary antibodies	25
Table **5**	Fluorescence dyes	25
Table **6**	Recombinant proteins and cytokines	26
Table **7**	Toll-like receptor agonists and other stimuli	26
Table **8**	Acquired chemicals and solutions	26-27
Table **9**	Buffers	27
Table **10**	Equipment	27-28

ABBREVIATIONS

Not additionally listed are symbols for chemical elements and international abbreviations for SI units.

aa	amino acid
AdipoR1 / R2	adiponectin receptor 1 / 2
AdipoRs	adiponectin receptors
AMPK	adenosine monophosphat activated protein kinase
APC	allophycocyanin
APN	adiponectin
APN-KO	adiponectin-deficient [knock-out]
APPL-1	adaptor protein containing pleckstrin homology domain, phosphotyrosine-binding domain and a leucine zipper motif 1
BFA	brefeldin A
bp	base pair
BSA	bovine serum albumin
CD	cluster of differentiation
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen 4
Cy	cyanin
CVB3	coxsackievirus B3
DC	dendritic cell
DCMI	inflammatory dilated cardiomyopathy
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
IFNγ	interferon γ
IL	intereukin
i.p.	intraperitoneal
Iono	ionomycin
JNK	c-Jun N terminal kinase
kbp	kilo base pair
LPS	lipopolysaccharide
mAb	monoclonal antibody

MAPK	mitogen activated protein kinase
min	minutes
mTOR	mammalian target of rapamycin
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PHA-L	phytohemagglutinin L
PMA	phorbol myristate acetate
PPAR	peroxisome proliferator-activated receptors
qRT-PCR	quantitative real time polymerase chain reaction
rpm	revolutions per minute
R848	Resiquimod
SD	standard deviation
SEB	Staphylococcal enterotoxin B t
SEM	standard error of the mean
SNP	single nucleotide polymorphism
STAT	signal transducer and activator of transcription
TIRC7	T cell immune response cDNA 7
TLR-L	Toll-like receptor ligand
TLR4	Toll-like receptor 4
TLR8	Toll-like receptor 8
TNFα	tumor necrosis factor α
WT	wild type

SUMMARY

Adiponectin (APN), an adipokine constitutively produced by fat tissue is present abundantly in different oligomeric forms in human plasma and has been shown to exert anti-inflammatory effects in various disease models. While the influence of APN on monocytes, macrophages and epithelial cells has been extensively investigated, little has been known about its role in modulating lymphocytes and their function, especially T and natural killer (NK) cells so far. This study describes the distribution of adiponectin receptor 1 and 2 (AdipoR1 and 2) on human and murine T cells, their regulation upon antigen stimulation and the effects of APN on T cell responses. Further, it deals with the AdipoRs expression on human and murine NK cells and their subsets, and characterizes the APN influence on human NK cells as well as on murine NK cells *in vitro* and in a murine model.

In this thesis I show that only a minority of human and murine T cells expresses AdipoRs on their surface but most T cells store AdipoRs intracellular. Human AdipoRs co-localize with T cell immune modulatory molecules CTLA-4 and TIRC7 within clathrin coated vesicles. Antigen stimulation results in an upregulation of both receptors on the surface of human and murine T cells. The expression is mostly restricted to antigen activated T cells as determined by CD137 and tetramer staining and is co-expressed with human CTLA-4. Incubation of APN results in a diminished antigen induced T cell expansion. Analysis of the mechanism on the generation of antigen activated T cells displays an enhancement of apoptosis and an inhibition of proliferation by APN. In accordance, APN deficient [(knockout)-KO] mice exhibit reduced frequencies of $CD137^{+}$ T cells upon Coxsackie virus B3 infection. Additionally, APN diminishes human T cell cytokine production in response to antigen stimulation.

The majority of human $CD56^{dim}$ NK cells express surface AdipoRs while most $CD56^{bright}$ NK cells lack AdipoRs. In contrast, only a small subset of murine mature NK cells expresses surface AdipoRs but the majority stores them intracellular and exposes them on the cell surface upon CVB3 infection in a similar manner as human T cells. Cytotoxicity is not impaired by APN while Toll-like receptor (TLR) ligand (TLR-L) induced interferon γ (IFNγ) secretion is reduced in human NK cells. Uninfected APN-KO mice reveal elevated frequencies of NK cells with lower effector NK cells displaying a decreased cytotoxic degranulation. In line with decreased frequencies of $CD11b^{high}$ $CD27^{high}$ and $CD94^{high}$ effector NK cells NKG2D expression required for NKG2D-dependent cytolytic activation is lower in uninfected APN-KO mice. Upon CVB3 infection murine NK cell function including degranulation and IFNγ production was restored.

Taken together these data describes APN as an anti-inflammatory negative T and NK cell regulator. In future studies it is therefore of great interest to analyze the prognostic and therapeutic potential of APN in inflammatory diseases.

ZUSAMMENFASSUNG

Adiponektin (APN), ein vom Fettgewebe kontinuierlich sekretiertes Adipozytokin zirkuliert in hohen Konzentrationen im Plasma. Neben seinen vielseitigen metabolischen Funktionen, wird APN als ein anti-inflammatorischer Modulator beschrieben. Während der Einfluss von APN auf Monozyten, Makrophagen und epitheliale Zellen mehrfach untersucht wurde, existieren nur wenige Daten, die die Wirkung von APN auf Lymphozyten beschreiben.

In dieser Arbeit werden die Adiponektin Rezeptor 1 und 2 (AdipoR1 und 2) Expression auf humanen und murinen T Zellen, ihre Regulation während der Antigenaktivierung, sowie die Effekte des APNs auf T Zellen beschrieben. Des Weiteren wird die AdipoRs Expression auf humanen und murinen NK Zellen und deren Subpopulationen gezeigt und die Wirkung von APNs auf die Funktion humaner NK Zellen charakterisiert. Zusätzlich wird in dieser Arbeit der Effekt von APN auf murine NK Zellen in einem *in vitro* Model dargestellt.

Wir zeigen, dass lediglich ein geringer Prozentsatz an humanen und murinen T Zellen AdipoRs auf der Oberfläche exponiert, während beide Rezeptoren von der Mehrheit der T Zellen intrazellulär exprimiert wird. Humane AdipoRs ko-lokalisieren in T Zellen gemeinsam mit den Immunmodulatoren CTLA-4 und TIRC7 in Clathrin umhüllten Vesikeln. Die Stimulation von T Zellen mit Antigenen resultiert in einer Oberflächenexpression beider Rezeptoren auf humanen und murinen T Zellen. Die AdipoRs Oberlächenexposition beschränkt sich hauptsächlich auf antigenaktivierte T Zellen, welche durch die Expression von CD137 und durch Tetramerfärbungen charakterisiert wurden, und mit CTLA-4 ko-exprimiert werden. APN verringert die antigeninduzierte T Zell Expansion indem die Apoptose von $CD137^+$ T Zellen verstärkt und deren Proliferation inhibiert. Damit einhergehend weisen APN defiziente [(knockout)-KO] Mäuse nach Coxsakie Virus B3 (CVB3) Infektion erhöhte Frequenzen von $CD137^+$ T Zellen auf. Zusätzlich verringert APN die antigenstimulierte Zytokinsekretion von humanen T Zellen.

Im Gegensatz, exprimieren nahezu alle humanen $CD56^{dim}$ NK Zellen beide APN Rezeptoren auf ihrer Oberfläche, während den meisten $CD56^{bright}$ NK Zellen die Oberflächenrezeptoren fehlen. Demgegenüber exprimiert nur eine kleine Subpopulation muriner, reifer NK Zellen beide Rezeptoren auf ihrer Oberfläche. Die meisten murinen NK Zellen speichern die AdipoRs intrazellulär und exponieren diese nach CVB3 Infektion an der Oberfläche. APN beeinflusst nicht die Zytotoxizität, inhibiert jedoch die Toll-like Rezeptor (TLR) Ligand

(TLR-L) stimulierte IFNγ Sekretion humaner NK Zellen. APN-KO Mäuse weisen erhöhte NK Zell Frequenzen auf, wobei ihre zytotoxische Degranulation erniedrigt ist. Übereinstimmend mit verringerten $CD11b^{high}$ $CD27^{high}$ und $CD94^{high}$ Effektor NK Zellen, ist die NKG2D Expression in APN-KO Mäusen vermindert. Die CVB3 Infektion führt zur Wiederherstellung der NK Zell Funktionen, einschließlich der Zytotoxizität und IFNγ Produktion.

Insgesamt beschreiben diese Daten APN als einen anti-inflammatorischen, negativen T und NK Zell Regulator. Die Analyse des prognostischen und therapeutischen Potentials von APN im Zusammenhang mit entzündlichen Erkrankungen könnte somit von zentraler Bedeutung für zukünftige Studien sein.

1 INTRODUCTION

1.1 Adiponectin

1.1.1 Gene and expression

Adiponectin (APN) was discovered simultaneously by for groups in the mid-1990s. Scherer *et al.* as well as Hu *et al*. isolated adiponectin cDNA from murine adipocyte cell lines and named it Acrp30 *(adipocyte complement-related protein of 30kDa)* and AdipoQ respectively [1, 2]. Further it was found as the most abundant transcript in human adipose tissue (apM1, *adipose most abundant gene transcript 1*) [3] and purified from human plasma as a gelatin-binding protein, GBP28 [4].

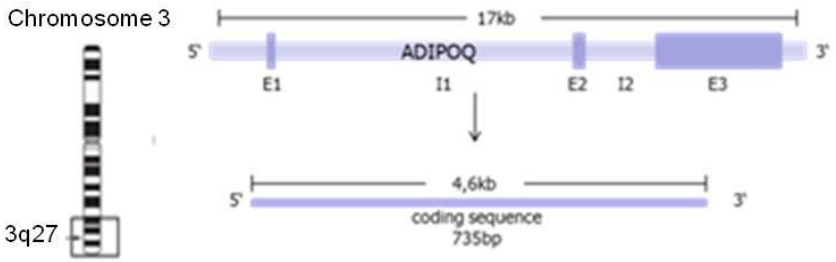

Figure 1: Location and genomic structure of ADIPOQ. Human ADIPOQ is located on chromosome 3q27 consisting of three exons (E1-E3) and two introns (I1-I2). The human ADIPOQ spans 17kb. Within the 4.6kb mRNA 735bp are coding for the 244aa protein. Modified [5].

The human adiponectin gene, *ADIPOQ* is located on chromosome 3q27 encoding for a 244-aa polypeptide and sharing 84% protein sequence identity with mouse APN (encoded on Chr.16 16.16.0cM). The coding sequence of 735bp is transcribed [5] from a 17kb spanning genomic region containing three exons and two introns (Fig.1) [6]. The translation starts at exon 2 and ends at exon 3 so that exon 1 and parts of exon 3 are left untranslated [6]. The special locus is associated with the susceptibility to type 2 diabetes and cardiovascular disease, two diseases highly related with the protective role of APN [7]. Within 16 identified polymorphisms of *ADIPOQ* [8] many are associated with clinical conditions.

APN is mainly synthesized by adipocytes and therefore ranks among the fat derived hormones named adipokines. Several studies also reported expression in skeletal muscle [9], liver [10] and cardiac tissue [11]. Plasma APN levels determined by various genetic, hormonal, inflammatory, dietary and pharmacological factors, range from 2 to 30µg/ml, thus

accounting for 0.01% of total plasma proteins in humans [12]. Plasma values are decreased in obese [13] and diabetes type 2 [14] patients as well as in individuals with cardiovascular diseases [15]. Values increase with weight loss thereby enhancing sensitivity to insulin [16]. Studies for circulating APN levels reported that five single nucleotide polymorphisms (SNPs) were genome-wide significant as determinants for APN plasma values, whereas SNPs in the *ADIPOQ* locus demonstrated the strongest association [17]. Surprisingly APN values inversely correlate with cardiovascular diseases [14], obesity-linked diseases like insulin resistance, the development of type 2 diabetes and obesity itself. The expression of APN decreases with obesity, considered as a chronic inflammation, and insulin resistance, determined by pro-inflammatory cytokines (TNFα, IL-6, IL-18) suppressing APN transcription [18]. Further studies have shown a relationship between APN levels and gender with higher values in women than men although women have a higher body fat content. This paradoxical relationship might be due to the involvement of sex steroids as shown by Nishizawa *et al*. [19]. Finally, human and mice *in vitro* and *in vivo* studies revealed that APN expression and secretion is regulated at both transcriptional and posttranscriptional levels. One of the key players is the nuclear receptor peroxisome proliferator activated receptor γ (PPARγ) which acts as a transcription factor. Its activation leads to increased APN expression and secretion, due to the binding of PPARγ to a putative PPARγ responsive element (PPRE) on the APN promotor [20]. PPARγ is activated by its natural ligands such as free fatty acids and eicosanoids and by its agonists such as thiazolidinediones (TZDs). These anti-diabetic drugs are used in the treatment of type 2 diabetes which results in enhanced insulin sensitivity [18, 19]. Posttranscriptional regulation of APN including hydroxylation, glycosylation and disulfide bound formation is determined among others by PPARγ induced genes coding for chaperons involved in the multimerization of APN [18, 20].

1.1.2 Protein and receptors

Structurally APN contains four differentiable domains: an amino-terminal signal sequence, followed by a species specific variable region, a collagenous domain and a carboxy-terminal globular domain that binds to the adiponectin receptor (AdipoR) [21]. The 30kDa monomeric form has not yet been detected in the circulation and is thought to appear only in the adipocyte. Synthesized as single subunits the full-length monomers trimerize through their globular domains and form low molecular weight (LMW) complexes, which themselves

associate through the collagenous domains via disulfide bonds to hexamers (middle molecular weight structures, MMW) and higher molecular weight (HMW) structures (Fig. 2). There exists a fourth configuration of APN, the globular one (gAPN), which results of a cleavage of the monomeric form.

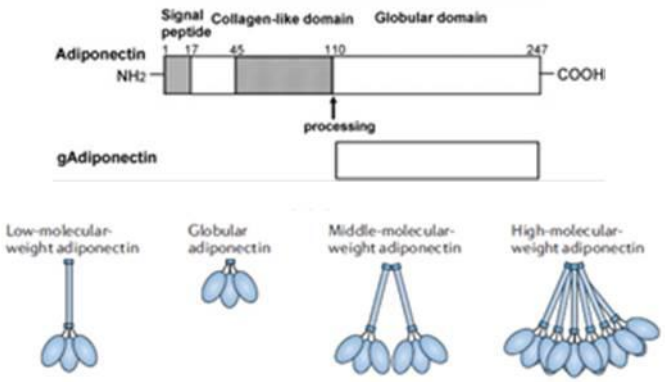

Figure 2: **Domains and configurations of APN**. APN contains four domains which form different structures of the protein at which the LMW and HMW are the most abundant in plasma [19, 25]

In plasma the protein is mainly found as a hexamer and HMW isoform. [8, 12]. It has been suggested that the various isoforms have different target tissues and/or different biological effects [5]. Whereas the HMW isoform is considered to be responsible for its pro-inflammatory actions, the LMW isoform exerts the anti-inflammatory effects [22], but this requires the measuring of APN isoforms when studying its action and function. On the other hand circulating APN is composed of all oligomers hence it might be the ratio between them, rather than their respective concentrations, which determines the physiological activity of APN [23]. Further posttranslational modifications of the adipokine like hydroxylation and glycosylation determine its activity and receptor binding [5].

APN exerts its function mainly through binding with its globular domain to two receptors, Adiponectin receptor 1 and 2 (AdipoR1/AdipoR2). Both receptors are structurally related and are with 95% identity highly conserved in humans and mice [21]. Protein sequence alignment shows that the internal N-terminal 69 aa of AdipoR1 and the 79 aa of AdipoR2 are specific for each receptor whereas the rest of these proteins is highly homologous with 80% identity. It is therefore not surprising that although both receptors have similar effects they also have individual signaling preferences (elaborated in section 1.1.4) [24] AdipoR1 and 2 are expressed at detectable levels in most tissues like skeletal muscle, endothel, brain and in the heart. Studies also report expression of both receptors in cells of the immune system like

monocytes, macrophages, B and NK cells but only a small percentage of T cells [25]. Whereas AdipoR1 is the major receptor in skeletal muscle and heart, AdipoR2 is most abundant in the liver [16]. Studies demonstrated that both receptors bind globular APN and full-length APN but with different affinities. AdipoR1 serves as a high-affinity receptor for globular APN and a low-affinity receptor for full-length APN. In contrast AdipoR2 is an intermediate affinity receptor for both isoforms. Each receptor consists of seven transmembrane domains, but they are structurally, topologically and functionally distinct from all other reported G-protein coupled receptors with an internal N-terminus and external C-terminus [21]. Hug *et al*. [26] identified a third receptor being capable of binding APN, T-cadherin. As a cell-surface receptor T-cadherin lacks an intracellular domain which is required for signal transduction. Consequently T-cadherin is only able to serve as a co-receptor by competing with AdipoR1 and AdipoR2 for APN binding or is believed to interfere with APN signal transduction [12, 27]. Effects of APN are also regulated by AdipoRs distribution and expression levels, which in turn are defined genetically, through mediators and by physiological state, like obesity and insulin resistance [16]. Insulin and obesity negatively regulates the levels of AdipoRs mRNA [28, 29]. PPARγ and α are also able to modulate AdipoRs [30].

1.1.3 Role of Adiponectin

Adiponectin has pleiotropic biological effects in different organs and tissues (Fig. 3). It is characterized as insulin-sensitizing, antiatherogenic, cardioprotective, tumor growth limiting, anit-angiogenic and immunomodulatory /anti-inflammatory adipokine whereas its main metabolic functions span the regulation of glucose and lipid metabolism. It increases fatty acid oxidation in skeletal muscle and liver and the translocation of the glucose transporter 4 (GLUT 4) in skeletal muscle [12, 31], which is important for the cellular glucose uptake. APN suppresses hepatic glucose output and reduces the synthesis of glucose in the liver leading to an improved insulin-signaling and –sensitivity [12]. The absence of the APN gene in APN deficient [knock out (KO)] mice has no appreciable effect when placed on a normal diet, but on a high fat/sucrose diet they develop severe insulin resistance [16]. In accordance administration of APN protects mice with diet induced obesity against insulin resistance [32], the major ingredient of type 2 diabetes. Tow SNPs related pathophysiological conditions are low APN plasma concentrations [6], high risk of type 2 diabetes [9], obesity and insulin resistance [10] and cancer [11]. Studies revealed that low initial plasma concentrations of

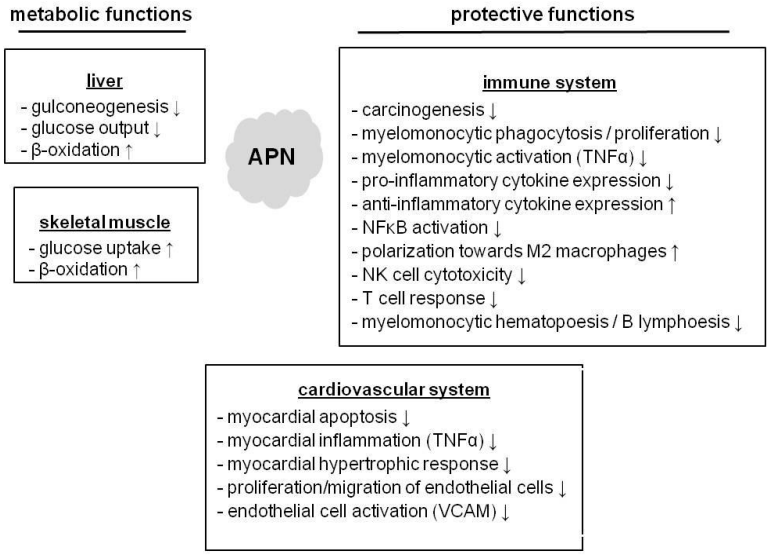

Figure 3: Pleiotropic role of APN. APN is involved in metabolic functions, plays a role in the cardiovascular system and negatively regulates the immune system. ↓ indicates inhibition, ↑ indicates activation. TNFα, tumor necrosis factor α; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; VCAM, vascular cell adhesion molecule 1

APN are strongly correlated with the development of type 2 diabetes [14, 33] and high levels of APN are associated with a reduced risk of type 2 diabetes [34]. Several studies have shown that expression levels of APN are diminished in obesity. The resulting decreased effect of APN possibly plays a causal role in the development of insulin resistance. The following hyperinsulinemia or an existing obesity leads to a decreased expression of both receptors which in turn results in a reduced sensitivity to APN and insulin resistance , a so called "vicious cycle"[16]. This suggestion is confirmed by studies which show diminished levels of APN and AdipoRs in obesity, insulin resistance and diabetes [28, 31]. Consequently, APN protects against insulin resistance and type 2 diabetes through its abilities to improve the systemic carbohydrate and lipid profiles [35].

The activation of endothelial cells and macrophages display an essential component in the development of atherosclerosis. APN was shown to decrease endothelial TNFα induced adhesion molecule expression thereby inhibiting the adhesion of macrophages [36]. Furthermore, APN reduces the ability of macrophages to transform into foamy cells [37] and suppresses the TNFα induced endothel activation through inhibition of NFκB [29]. *In vivo* experiments demonstrated that adenoviral mediated APN overexpression reduces the formation of atherosclerotic lesions in apolipoprotein E knockout (ApoE-KO) mice, a mouse model of atherosclerosis, via the inhibition of TNFα and vascular cell adhesion molecule -1

(VCAM-1) expression [38]. In agreement, APN overexpressing ApoE-KO mice are protected from developing atherosclerosis compared to wild type (WT) mice [39].

Low APN levels are associated with cardiovascular diseases. Hypoadiponectinemia is associated with coronary artery disease, acute coronary syndrome and hypertension. It directly affects signaling in cardiac myocytes and has advantageous effects on several pathological heart conditions, including cardiac hypertrophy and myocardial infarction [40]. Compared to WT mice APN-KO mice develop larger infarcts which are associated with increased myocardial apoptosis and TNFα expression. Adenoviral delivery of APN diminished infarct size, apoptosis and TNFα production [41]. Further pressure overload in APN-KO mice resulted in enhanced concentric cardiac hypertrophy, which was attenuated by adenoviral administration of APN in both APN-KO and WT mice [42]. The beneficial effects of APN are referred to an inhibited inflammatory response, decreased myocyte death and hypertrophic response [40]. APN plasma values also inversely correlate with classical cardiovascular risk factors such as body mass index (BMI), high blood pressure and C-reactive protein (CRP) levels [43].

Adiponectin has been found to exert anti-angiogenic effects, through the inhibition of endothelial cell proliferation and migration [44, 45]. However, many reports describe APN as a stimulator of angiogenesis in APN-KO mice [46] and in a model of overexpression [47].

A number of studies indicated an inverse relationship between APN levels *in vivo* and the risk of malignancies. Low levels of plasma APN were found in endometrial cancer, postmenopausal breast cancer, colon cancer, renal cancer, gastric cancer, prostate cancer leukemia and other hematological malignancies. Two mechanisms are thought to exert APN's anti neoplastic effects on tumor cells. First, APN can act directly via receptor mediated signaling pathways on cancer cells expressing AdipoRs and secondly, it may modulate insulin sensitivity at the target tissue resulting in an impaired inflammatory response and angiogenesis [12]. *In vitro* studies, dependent on the used APN isoform, indicated that involved receptor mediated signaling pathways lead to the induction of apoptosis [48-50], and the inhibition of migration [51, 52] and proliferation [53, 54] in cancer cells. Indirectly, among others, Adiponectin has an anti-proliferative effect via selectively binding to several mitogenic growth factors [45]. This interaction constricts the involved growth factors from their binding to the membrane receptors and prevents the induction of proliferation of tumor cells.

Finally APN has been found to be an important regulator of the immune response affecting carcinogenesis by inhibiting the growth and inducing apoptosis of myelomonocytic progenitor cells [55].

1.1.4 Signaling pathways

Adiponectin stimulates several intracellular signaling pathways when binding to its receptors including **p**eroxisome **p**roliferator **a**ctivated **r**eceptors (PPARs), **n**uclear **f**actor -κB (NFκB), **m**ammalian **t**arget **o**f **r**apamycin (mTOR), MAPK (**m**itogen **a**ctivated **p**rotein **k**inase) cascade, **s**ignal **t**ransducer and **a**ctivator of **t**ranscription (STAT3) and **a**denosine **m**ono**p**hosphat (AMP) activated **p**rotein **k**inase (AMPK) [12] (Fig. 4). Recent studies identified among others [24] the **a**daptor protein containing **p**leckstrin homology domain, **p**hosphotyrosine-binding domain and a **l**eucine zipper motif (APPL-1) interacting N-terminal and intracellular with AdipoRs thereby participating in APN signaling by mediating and enhancing its effects [56, 57]. Overexpression of APPL-1 enhances the effects of APN whereas the suppression by small interfering RNA (siRNA) abrogates APN function including the phosphorylation of AMPK and the associated glucose uptake and fatty-acid oxidation [56]. APPL-1 further functions as mediator of other signaling pathways through intracellular binding to receptors and proteins. These signaling pathways mainly impair cell proliferation, survival and apoptosis, endosomal trafficking and chromatin remodeling [58, 59].

AMPK plays a central role in APN signaling. It is a master regulator of cellular energy homeostasis responding to low intracellular levels of ATP. As an energy sensor it positively regulates signaling pathways that replenish ATP supplies like transcription and translocation of glucose transporter 4 (GLUT4) resulting in increased glucose uptake and fatty-acid oxidation. On the other hand it negatively regulates several proteins central to ATP consuming processes leading to a downregulation and/or inhibition of gluconeogenesis, lipid and protein synthesis. Metabolic functions and the insulin sensitizing effects of APN are among others (see PPRAα) mainly exerted through the phosphorylation of AMPK via APPL-1 promoting glucose utilization. This results in increased fatty-acid oxidation, increased glucose uptake and reduced gluconeogenesis. Through AMPK, APN is involved in the regulation of AMPK downstream targets, like mTOR and p53. Whereas the activated,

phosphorylated AMPK inhibits mTOR resulting in the suppression of cell proliferation, stimulation of p53 results in cell cycle arrest and the induction of apoptosis [56].

The peroxisome proliferator-activated receptors (PPARs) are nuclear, ligand dependent, transcription factors involved in lipid metabolism, glucose homeostasis, cellular differentiation, apoptosis and inflammation control. They are activated endogenously by fatty-acid derivatives and eicosanoids, additionally by leukotrienes (PPARα) and prostaglandins (PPARγ).

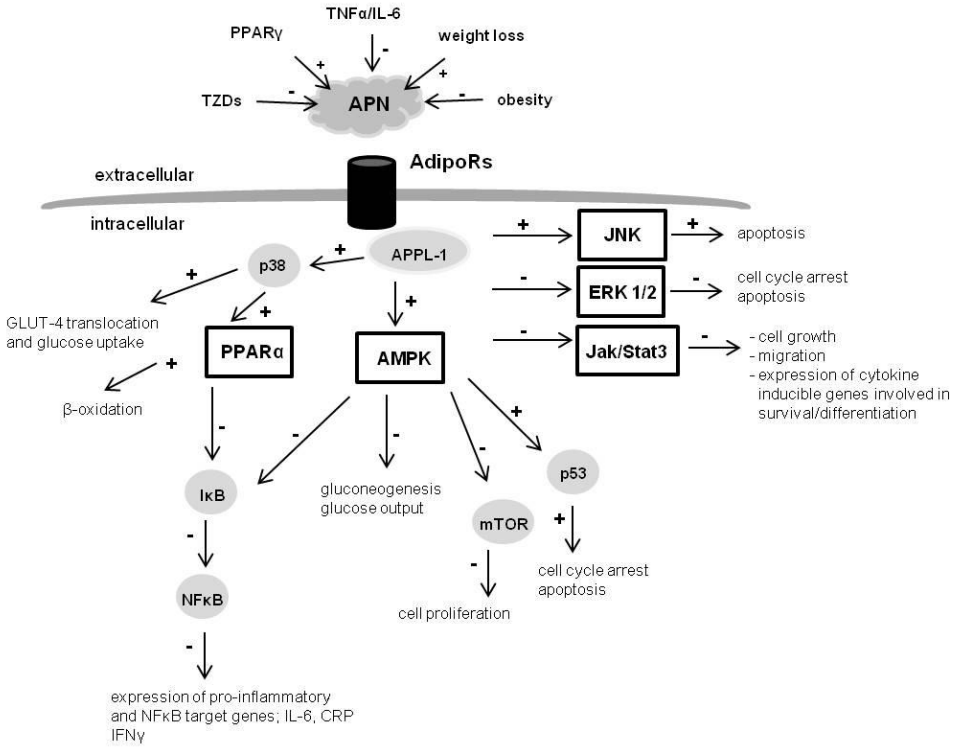

Figure 4: **Involvement of APN in intracellular signaling pathways.** APN exerts its functions by modulating several signaling pathways and their mediators. Depicted are important through APN impaired cell signals and factors regulating APN. If JNK, ERK 1/2 and Jak/Stat3 are activated or inhibited via APPL-1 remains to be investigated. - indicates inhibition, + indicates activation. TNFα, tumor necrosis factor α; IL-6, interleukin 6; TZD, thiazolidinediones; PPARα (γ), peroxisome proliferator activated receptor α (γ); APPL-1, adaptor protein containing pleckstrin homology domain, phosphotyrosine-binding domain and a leucine zipper motif; AMPK, adenosine monophosphat activated protein kinase; JNK, c-Jun-N-terminal kinase; ERK 1/2, extracellular signal-related kinases 1and 2; Jak/Stat3, Janus kinase/signal transducer and activator of transcription; mTOR, mammalian target of rapamycin; IκB, inhibitor of kappa-light-chain-enhancer of activated B cells; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; p38,mitogen activated protein kinase p38; p53, tumor protein 53

Whereas PPARα is highly expressed in liver, skeletal muscle, kidney, heart and the vascular wall, PPARγ is predominantly detected in adipose tissue, intestine and macrophages [60]. Studies revealed that APN metabolic functions can be further explained by the APN activation of PPARα resulting in enhanced fatty-acid combustion, energy consumption and improved insulin sensitivity *in vivo* [12, 32]. Interestingly, studies indicated that AMPK is mainly activated by AdipoR1 whereas stimulation of PPARα could be attributed to binding to AdipoR2 [61]. However, it remains an active field of research which isoforms of APN primarily triggers the activation of AMPK [12].

APN exhibits tumor growth limiting effects by interfering with Jak (Janus kinase)/STAT3 and several MAPK cascades. Cytokines like IFNα and γ, as well as IL-6 signals through the Jak/STAT pathway and ensure a rapid signal translation. The cytokine induced phosphorylation of receptor associated Jaks is followed by the phosphorylation of STATs, which dimerize, translocate to the nucleus and activate the transcription of cytokine inducible genes involved in regulation of cell survival and differentiation. APN decreases STAT3 phosphorylation and reduces cell growth and migration. Moreover, it was found to suppress constitutively active STAT3 signaling [62]. MAPK cascades are composed of MAPKKK (MAP-kinase-kinase-kinase), a MAPKK (MAP-kinase-kinase) and MAPK connected in series. MAPK are divided into three groups following three signaling pathways the stress activated kinase c-Jun-N-terminal kinase (JNK), the stress and tumor necrosis factor activated kinase p38 and the mitogen activated, extracellular signal-related kinases 1and 2 (ERK). Studies demonstrated an involvement of APN in all three signal transductions. APN increased JNK activity resulting in apoptosis [62, 63] and p38 activity, which is associated with metabolic effects of APN [57]. In contrast Erk1/2 signaling is inhibited by APN leading to cell cycle arrest and apoptosis [50, 64]

The anti-inflammatory effects of APN could be explained by activation of PPARs which inhibit the expression of pro-inflammatory genes in various cell types, like cytokines and C reactive protein (CRP) [60, 65]. Further, anti-inflammatory effects are partially based on the APPL-1 dependent AMPK mediated activation of phosphatidylinositol 3-kinase (PI3K) pathway and the suppression of NFκB pathway through suppression of inhibitor NFκB phosphorylation [29, 66] which results in decreased expression of pro-inflammatory NFκB target genes like a variety of chemokine receptors and ligands, IL-6, CRP and IFNγ. A variety of mediators of the above mentioned pathways are cross linked among each other and to

further cell signals. Consequently, APN is indirectly involved in a number of signal translations.

1.2 Immunomodulatory effects of Adiponectin

Among its metabolic, antiatherogenic, cardioprotective and anti-oncogenic properties several studies indicate APN to be involved in the regulation of the immune system. The majority of these findings refer to the effects of APN on cells of the innate immune system and inflammation, only little is known about the influence of APN on the adaptive immune system and/or response.

APN was shown to influence the function of myelomonocytic cells by reducing the phagocytic activity of human macrophages, inhibiting the proliferation of myelomonocytic cells and the production of tumor necrosis factor (TNF) in response to stimulation with lipopolysaccharide (LPS) [55]. It impairs the leukocyte adhesion by inhibiting the adhesion molecule expression on the endothel [36]. APN also reduces secretion of pro-inflammatory cytokines TNFα [55], IL-6 [67] and IFNγ by LPS activated human macrophages and induces the production of anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist (IL-1RA) in dendritic cells (DCs), monocytes and macrophages [68]. Further APN negatively regulates the response of macrophages to Toll-like receptor (TLR) stimulation through suppression of TLR induced NFκB activation [69]. Moreover, APN *in vitro* and *in vivo* promotes the macrophage polarization towards the anti-inflammatory M2 phenotype and supports the resolution of inflammation [70]. Recently APN was also identified as a negative regulator of NK cell cytotoxicity [71].

Other data illustrate pro-inflammatory effects of APN depending on isoform and environment. gAPN and the HMW isoform was shown to induce the inflammation mediators TNFα and IL-6 in monocytes, macrophages and the endothel through the activation of NFκB [72, 73]. Saijo *et al*. described APN dependent enhancement of IL-8 secretion in the presence of LPS whereas in the absence of LPS the IL-8 secretion was suppressed. Further LPS presence results in an increased phagocytosis of apoptotic cells by macrophages when culturing with APN [74].

Concerning the effects of APN on mediators of the adaptive immune system little is known. Several published effects of APN on the adaptive immune system are indirect. Wolf *et al*.

revealed that APN incubation of macrophages leads to a reduced ability to evoke an allogenic T cell response [68]. Further APN modulates the DC activity which results in a decreased T cell proliferation and an increased percentage of $CD4^+CD25^+Foxp3^+$ regulatory T cells [75] and reduces T cell recruitment in atherogenesis through downregulating T cell chemoattractants in macrophages [76]. Finally, APN was shown to act *in vivo* and *in vitro* not only as a hematopoietic stem cell factor stimulating proliferation while retaining the cells in a functionally immature state [77] but also as a stimulating factor of adult hippocampal neural stem cells [78]. Contrary, APN was also revealed as a negative regulator of hematopoiesis inhibiting directly proliferation of myelomonocytic progenitors [55] or indirectly through inducing changes in stromal cells and hence suppressing B cell lymphopoesis [79] . Through its immunomodulatory effects APN has a considerable influence on carcinogenesis.

1.2.1 Anti-inflammatory activities of Adiponectin in *in vivo* models

APN has been shown to possess profound immunoinhibitory and anti-inflammatory effects in various disease models. In accordance APN-KO mice are more susceptible to inflammatory diseases.

In APN-KO mice APN administration had beneficial effects and alleviated disease severity by suppressing the TNF expression in the liver in alcoholic and non-alcoholic fatty liver disease [80] as well as in carbon tetrachloride induced liver fibrosis where APN attenuates the induced fibrosis [81]. In addition, APN protects from endotoxin induced liver injury in another model of fatty liver, the $KK\text{-}A^y$ obese mouse model through modulation of TNFα [82]. Moreover APN-KO mice are more prone to inflammatory diseases like colitis [83] and sepsis [84]. Compared to WT, APN-KO mice displayed higher mortality and significant increased inflammatory cytokine levels [84]. Adenoviral supplementation of APN has been shown to have protective effects due to the inhibition of chemokine production in intestinal endothelial cells, infiltration of macrophages and the synthesis of pro-inflammatory cytokines [83]. APN was also found to exert anti-inflammatory effects in viral myocarditis mouse models. Leptin-deficient ob/ob (OB) mice showed impaired expression of cardiac adiponectin that may contribute to the progression of viral myocarditis through enhanced expression of TNFα**.** [85]. Consistently, APN replacement and induction of cardiac APN expression attenuates myocardial damage provoked by viral myocarditis and survival [86, 87]. Finally, cardiac allografts transplanted into APN-KO mice showed severe acute rejection relative to

transplants in WT mice accompanied by increased accumulation of $CD4^+$ and $CD8^+$ T lymphocytes, significantly higher levels of TNFα and IFNγ [88]. Interestingly, the disruption of AdipoRs results in systemic inflammation [61].

1.3 T Cells

T cells are highly specialized lymphocytes belonging to the adaptive immune system and account for 70% of the lymphocytes in human blood. They originate from the bone marrow, develop in the thymus and are responsible for the regulation of immune responses, specific elimination of pathogens and play a central role in cell-mediated immunity. T cells can be distinguished from other lymphocytes like B and natural killer (NK) cells by bearing a T cell receptor (TCR) which enables the specific recognition of pathogens, infected and transformed cells. To realize these functions precisely T cells pass various steps of selection preventing them to recognize "self-antigens" via TCR during their development in the thymus and later in peripheral lymphoid organs.

The TCR is a transmembrane, heterodimeric receptor with a cytosolic C terminus and a extracellular N terminus. The exposed part is classified into a constant and variable region aligned and stabilized by disulfide bonds. The variable domains determine the specificity of a given TCR which is the result of somatic gene rearrangements and alternative splice processes. The TCR is associated with the CD3 protein complex The CD3 molecule is responsible for the signal transduction of the TCR and its translocation to the T cell surface. Other co-receptors associated with the TCR are CD4 and CD8 molecules. The expression of either CD4 or CD8 divides T cells into the major classes: $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells. These co-receptors mediate the interaction of the TCR with the constant region of MHC class I or II molecules during the antigen recognition of T cells [89, 90]

1.3.1 Regulation of T cells

T cells are activated in peripheral lymphoid organs by APCs presenting processed antigens via MHC molecules. In addition to the interaction between MHC molecules and TCRs associated with CD4 or CD8, T cells require further signals to be activated and to proliferate and differentiate into effector cells (Fig. 5). The co-stimulatory signal needed is the binding of constitutive expressed CD28 molecule/receptor on the T cell surface with B7 proteins (CD80

and CD86) on APCs. B7 protein expression on APCs is induced and upregulated during an innate immune response. With assistance of CD3 involved in the signal transduction the T cell starts to produce IL-2 and to express the IL-2 receptor and enhances auto- and paracrine the differentiation and proliferation signals [90]. Activation of human and mice T cells is characterized among others through their surface expression of CD137 interacting with 4-1BBL on activated APCs and can be detected by mAb against CD137 [91]. Cross-linking of CD137 is required for T cell expansion and survival, as well as for cytokine production [92, 93].

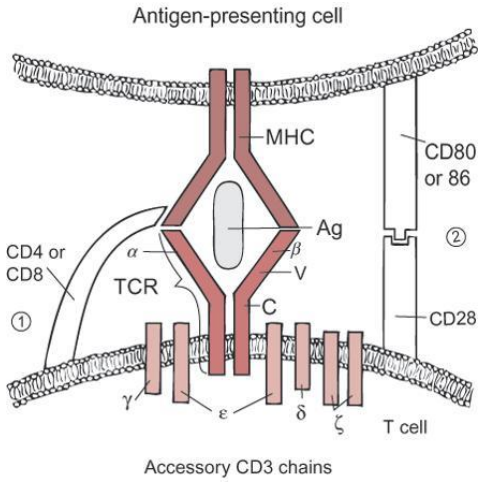

Fig. 5: Interaction of TCR/CD3 complex with MHC molecule on APC. The α and β chains of the (TCR) bind to antigen (Ag) loaded MHC complex on an APC. Additionally CD4 or CD8 interacts with the MHC. Both actions stimulate the T cell (1st signal) through the accessory CD3 chains. However, without a 2nd (co-activation) signal, the T cell is anergic or tolerant. The TCR have constant (C) α and β (or γ and δ) chains and variable (V) regions. (1) = 1st signal; (2) = 2nd signal. (modified merckmanuals.com)

In the course of activation T cells expose an inhibitory receptor being similar to CD28 and binding B7 proteins; cytotoxic T lymphocyte antigen 4 (CTLA-4). CTLA-4 has a comparatively higher binding affinity to B7 proteins and ensures through a negative feedback loop the downregulation of a continuous immune response. Another inhibitory protein induced after T cell activation is T cell immune response cDNA 7 (TIRC7) which inhibits further T cell proliferation and cytokine response [94, 95]. Regulatory proteins accumulate predominantly in cytoplasmatic compartments and are transported after activation to the cell surface via cellular traffic systems. CTLA-4 and TIRC7 were shown to co-localize and are stored in clathrin-coated vesicles [94] built in order to transport molecules within and between the cells. Additional to TCR and co-stimulatory signals provided during the activation T cells

upregulate and activate adhesion molecules like Lymphocyte-function-associated protein 1 (LFA-1) to increase the adhesion to the APC. LFA-1 ligation to intracellular adhesion molecule 1 (ICAM-1) on APCs prolongs cell contact and allows the proper activation of T cells.

$CD8^+$ cytotoxic T cells protect the body by killing cells which are transformed and infected with intracellular pathogens. These cells recognize their targets by binding to MHC class I molecules loaded with peptide antigens. MHC class I molecules are expressed on every nucleated cell of the body. After activation $CD8^+$ T cells release their cytotoxic granules including perforin and granzyme B. Perforin forms pores in the target cell membrane and allows granzyme B to enter, activate the caspase cascade and induce apoptosis. During this exocytose process CD107a is transiently located on the T cell surface and can be used as a marker for degranulation. Another way to induce apoptosis in target cells is via Fas-ligand (FasL) expressed on activated $CD8^+$ T cells. FasLs bind to the transmembrane Fas receptor on target cells and induces apoptosis in a caspase 8 dependent way. Activated cytotoxic T cells are also able to secrete IFNγ and provoke a non-lytic antiviral immune response through e.g. induction of MHC molecules. Most effector function of $CD8^+$ T cells depend on the presence of IL-2 and IFNγ released by $CD4^+$ T cells or IL-1, TNFα or IFNα provided by activated cells of the innate immune system [90].

$CD4^+$ helper cells activate and direct other immune cells. They are essential in antibody production by B cells, activate and help $CD8^+$ cytotoxic T cells and maximize the effector function of macrophages. Activation of $CD4^+$ cells by loaded MHC class II molecules and co-stimulatory signals induce the release of cytokines and the differentiation into subclass of $CD4^+$ T cells, among them T_H1 or T_H2 cells. T_H1 cells produce IFNγ, IL-2 and TNFα and promote macrophages to kill phagocytosed pathogens. Furthermore, T_H1 cells stimulate the lytic activity of $CD8^+$ T cells. T_H2 cells secrete IL-4, IL-5 IL-6, IL-10 and IL-13 and stimulate the differentiation of B cells into plasma cells as well as the production of antibodies. The differentiation of $CD4^+$ cells into T_H1 or T_H2 cells promote the immune system into a cell mediated or humoral immune response and is driven by the released cytokines of APCs. IL-12 induces the development of T_H1 and suppresses T_H2 growth whereas IL-4 is required for the T_H2 differentiation and suppresses T_H1 development [90].

1.3.2 Adiponectin and T cells

To date little is known about the direct modulatory effects of APN on T cells and their function. All described effects of APN on T cells are determined and caused by cells of the innate immune system. The APN incubation of macrophages resulted in reduced T cell recruitment and decreased allogenic T cell response [76] whereas the modulation of DC activity led to a decreased T cell proliferation and higher frequencies of T regulatory cells [75]. Further studies reported the expression of AdipoRs on the surface of a small subpopulation of T cells [25, 96]

1.4 Natural Killer Cells

Natural killer cells (NK cells) are key players and the first line of defense of the innate immune system and are considered to be involved in the regulation of the immune response. Proliferation, NK cell-mediated cytotoxicity against tumor and virus infected cells and IFNgamma (IFNγ) production display the important defense mechanisms of NK cells which are regulated by a wide range of receptors, cytokines and hormones.

Originally NK cells were identified as large granular lymphocytes having the ability to lyse tumor cells without prior sensitization. Later these cells were recognized as a separate lymphocyte lineage developing in the bone marrow through discrete stages of maturation [97, 98] and are present throughout lymphoid and non-lymphoid tissues. Consistent with data in the mouse the human NK cell turnover in blood is around two weeks [99, 100]. Human NK cells comprise 2-18% of total lymphocytes in peripheral blood and spleen whereas murine NK cells represent around 2% of splenocytes [101].

The mechanism by which NK cells recognize and discriminate target cells from healthy cells depends on a dynamic equilibrium of inhibitory and activation receptor signaling through corresponding NK cell receptors. Inhibitory NK cell receptors detect the presence of self-molecules, MHC class I molecules on potential target cells through MHC class I-specific receptors. The absence of MHC class I molecules on target cells, downregulated by viruses, in transformed cells and upon stress, results in a loss of the inhibitory signal in the NK cell and leads to NK cell mediated cytotoxicity [102] via induction of apoptosis. Inhibitory MHC class I specific NK cell receptors include most killer cell immunoglobulin-like receptors (KIRs) in

humans, lectin-like dimers of the Ly49 family in mice, CD94-NKG2A heterodimers in both species and receptors recognizing non-MHC self-molecules [103, 104]

Activating receptors on NK cells, like NKG2D, NKp46, CD94-NKG2C/E, low affinity Fc receptor CD16 (only in human) through which NK cells exert antibody-dependent cell cytotoxicity (ADCC), and natural cytotoxicity receptors (NCR) recognize ligands on target cells expressed upon stress or bind infectious non-self-ligands, like Toll-like receptors. TLR are receptors belonging to the pattern recognition receptors (PRR) of the innate immune system and recognize structures which are broadly shared by pathogens. Bearing several Toll-like receptors (TLR), *in vitro* exposure of NK cells to TLR ligands (TLR-L) such as lipopolysaccharide (LPS) results in enhanced IFNγ production as well as in increased cytotoxicity [104]

Distinct NK cell subsets have been defined and characterized in human as well as in mice based on phenotypic, functional and anatomic features (Fig. 6).

Human NK cells are identified by the absence of the surface molecule CD3 and the expression of CD56. These lymphocytes further can be divided into $CD56^{dim}$ and $CD56^{bright}$ subsets differing in their function and phenotypic properties. 90% of peripheral NK cell are $CD56^{dim}$ and express high levels of KIRs and CD16. $CD56^{dim}$ NK cells display enhanced cytotoxicity and ADCC against target cells via perforin and granzyme than the $CD56^{bright}$ subset [105]. $CD56^{bright}$ NK cells are $CD16^{-}$, have high levels of CD94-NKG2C/E receptors and produce large amounts of cytokines such as IFNγ in response to stimulation with interleukin (IL)-12, IL-15, IL-18 [105] and TLR-L [106]

Besides the fact that all NK cells express the heterodimeric IL-2 receptor (IL-2Rβγ) with intermediate affinity for IL-2, this subset appears to express the heterotrimeric high affinity IL-2Rαβγ and is therefore able to proliferate in response to low dose of IL-2. Both subsets express constitutively receptors for monocyte-derived cytokines such as IL-1, IL-10, IL-12, IL-15 and IL-18. Thus their function and activation can be mediated by monocytes [105]. Several groups proposed that $CD56^{bright}$ cells are precursors of the CD56dim NK subset displaying longer telomere length and the ability to differentiate in contact with fibroblasts into $CD56^{dim}$ NK cells [78, 79].

Mouse NK cells mainly characterized by the expression of NKp46 and DX5 and the absence of CD3 can be subdivided into four subsets, based on their developmental expression of CD11b and CD27. The maturation process starts at a $CD11b^{low}CD27^{low}$ state and leads the

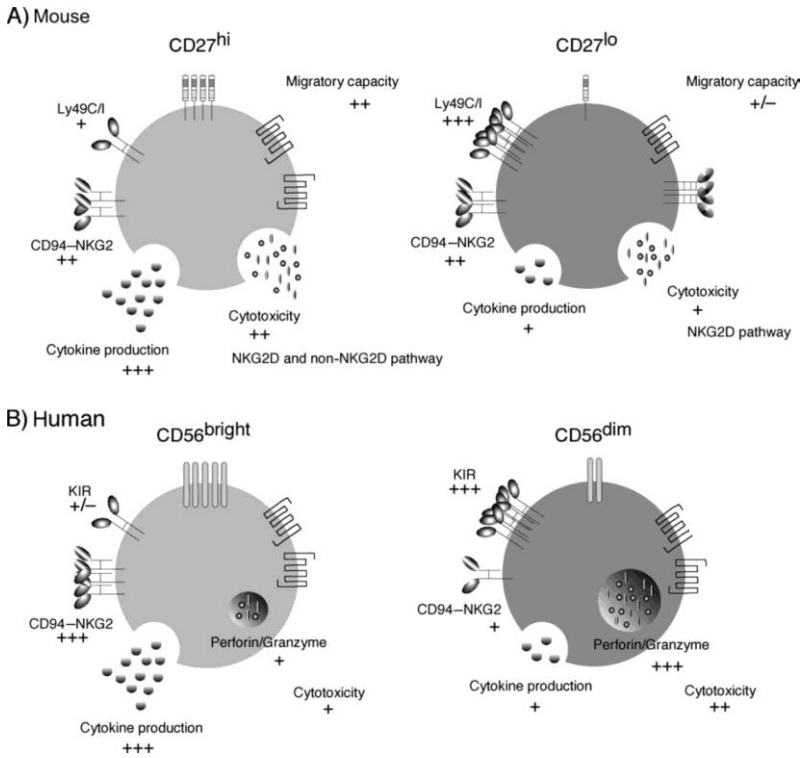

Fig.6: Comparison of mouse and human NK cells. (A) $CD27^{hi}$ NK cells produce larger amount of cytokines upon monokine (e.g. IL-12/IL-18) stimulation and show higher migration capacity than $CD27^{low}$ ($CD27^{lo}$) NK cells. The major proportion of the $CD27^{low}$ subset expresses inhibitory Ly49 receptors and therefore, their cytotoxic activity is more tightly regulated than that of the $CD27^{hi}$ subset. $CD56^{bright}$ NK cells produce high levels of cytokines in response to monokines compared with $CD56^{dim}$ NK cells. The human $CD56^{dim}$ subset has a high expression of KIRs and exhibits higher cytotoxic activity than the human $CD56^{bright}$ subset. Adapted from [107].

NK cells through $CD11b^{low}CD27^{high}$, $CD11b^{high}CD27^{high}$ and $CD11b^{high}CD27^{low}$ stages, each of them having distinct phenotypic features and gene profiling. The $CD11b^{low}$ subset is mostly found in bone marrow, lymph nodes and liver whereas $CD11b^{high}$ NK cells display the majority in spleen, peripheral blood and lung. Transcriptome analysis revealed the largest differences between these two subsets. Further the $CD11b^{low}$ subset can be divided into $CD27^{high}$ and $CD27^{low}$ NK cells. Both subsets, $CD11b^{low}CD27^{low}$ [double negative (DN)] and $CD11b^{low}CD27^{high}$, are closely related to each other but differ in their proliferative potential with $CD27^{low}$ having the highest potential to proliferate and giving rise to all other subsets. Hence, the DN NK cells are suggested to be the immature NK cells with low levels of Ly49 receptor and DX5. The $CD11b^{high}$ subset, with high levels of Ly49 receptor, can also be further subdivided into $CD27^{low}$ and $CD27^{high}$ NK cells with the $CD11b^{high}CD27^{high}$ [double positive (DP)] subset displaying the most potent effector cells. Both subsets show comparable

cytotoxic capacity against class I MHC mismatched NK cell sensitive (NKG2D ligand expressing) target cells, however, only the DP NK cells are able to kill MHC class I and NKG2D ligand expressing cells. Thereby, the DP NK cell subset displays a non-NKG2D-dependent cytotoxicity suggesting activation by a pathway distinct from that of NKG2D. Moreover, the $CD11b^{high}CD27^{high}$ subset produce higher amounts of IFNγ compared to $CD11b^{high}CD27^{low}$ NK cells and possesses a lower threshold to be activated [107]. The $CD11b^{high}CD27^{low}$ subset is shown to be most susceptible to spontaneous apoptosis and appears to show the end stage of NK cells before cell death. [107, 108].

1.4.1 Adiponectin and Natural Killer cells

In various studies APN was shown to display anti-inflammatory activity. Concerning its role in NK cell function and activity there exist few and conflicting data whereas the expression of AdipoRs on NK cells was approved [25, 96].

Kim *et al.* [71] described APN as a negative regulator of IL-2 induced cytotoxicity in murine NK cells. APN was found to suppress IL-2 enhanced but not basal cytotoxicity by induction of AMPK resulting in an AMPK mediated inhibition of NFκB activation. IFNγ, one of NFκB target genes, also enhances NK cell cytotoxicity and can be induced by IL-2. In this study APN was shown to down regulate IFNγ production leading to an inhibition of FasL (Fas ligand) and TRAIL (tumor necrosis factor related apoptosis inducing ligand) expression. FasL and TRAIL are important ligands for the induction of apoptosis when binding to FasR and death cell receptors on target cells.

On the other hand APN was described as inducer of cytotoxicity in lean human subjects and significantly increased smoke reduced IFNγ production. Further, it was shown to restore smoke reduced NK cell activation and cytotoxicity through enhancing the release of cytotoxic granules [109].

1.5 Aim of the Study

Various studies with APN-KO mice revealed the anti-inflammatory role of APN in several disease models moderating the course of disease. The effect of APN on monocytes and macrophages, members of the innate immune system, as well as on epithelial cells has been extensively analyzed but little has been known so far about the modulating actions of APN on lymphocytes.

The aim of the study was to assess the function and molecular mechanism of APN on T and NK cells.

The specific objectives were as follows:

- The analysis of AdipoRs expression on human and murine T and NK cells including their regulation

- The study of the effects of APN on activated T cells and their functions with regard to the generation of antigen activated T cells, apoptosis, proliferation and cytokine responses

- The study of the effects of APN on stimulated NK cells including cytotoxicity and cytokine response

- The investigation of *in vivo* T and NK cell responses in APN deficient mice upon Coxsackie virus B3 (CVB3) infection

2 MATERIAL

2.1 Subjects

2.1.1 Human

Human peripheral blood was collected from healthy volunteers. Bone marrow cells were collected from femoral head of donors who underwent operation. Studies were approved by the Institutional Ethics Committee and informed consents were obtained from the subjects.

2.1.2 Animal Model

APN-KO mice, which carry a deletion of the *adiponectin* gene, on a C57BL/6 background and C57BL/6 wild-type (WT) mice were purchased from Jackson laboratories. All mouse strains were bred in the Forschungsinstitut für Experimentelle Medizin (Berlin, Germany). WT and APN-KO mice, aged 8 to 10 weeks, were inoculated with Coxsackie virus B3 (CVB3) (Nancy strain, 5- 10^5 plaque-forming units ip) diluted in 0.2 ml saline as previously described [110]. Three and seven days after viral infection, mice were sacrificed and splenocytes and blood were aseptically removed.

2.1.3 Cell lines

Following cell lines were used to identify AdipoRs expression and for NK cell degranulation assays:

Tab. 1: Cell lines

Cell line:	**Jurkat**
Organism:	Homo sapiens
Gender:	male
Tissue	lymphoma
Celltype	lymphoblast, Leukemia, acute T cell; T lymphocyte
Growth properties	suspension
Description	Immortalized line of T lymphocytes that are used to study acute T cell leukemia. Their primary use, however, is to determine the mechanism of differential susceptibility of cancers to drugs and radiation.
Cell line:	**K562**
Organism:	Homo sapiens
Age:	53 years
Gender:	female
Tissue:	bone marrow
Celltype:	lymphoblast, chronic myelogenous leukemia
Growth properties:	suspension
Description:	The cells spontaneously differentiate into precursors of the erythroid, granulocytic and monocytic series. The line is EBNA negative.
Cell line:	**YAC-1**
Organism:	Mus musculus (mouse)
Strain:	A/Sn
Tissue:	lymphoma
Celltype:	lymphoblast
Growth properties:	suspension
Description:	Moloney murine leukemia virus (Mo-MuLV) induced lymphoma. The cells are sensitive to the action of NK cells and are useful in assays of NK cell activity.

2.2 Reagents

2.2.1 Cell isolation and separation reagents

Mononuclear cells from human and murine blood or spleen were separated with Biocoll (Ficoll) separating solution (density 1.077g/ml) (Biochrom AG, Germany) or Histopaque 1083 separating solution (density 1.083g/ml) (Sigma Aldrich Inc., USA). To obtain single cell suspension spleens were filtered through 70µm nylon meshes (Becton Dickinson GmbH, USA). Murine blood was treated with red blood cell lysis buffer (ACK buffer). Cells were washed consistently with human or murine FACS buffer.

2.2.2 Kits

Human T cells were depleted by CD3 MicroBeads (positive selection) or isolated by Human Pan T cell isolation kit II (negative selection) (Miltenyi Biotec, Germany). Human NK cells were isolated by NK cell isolation kit (Miltenyi Biotec). If not differently described human T cells were activated with T cell Activation/Expansion kit containing CD3/CD28 beads (Miltenyi Biotec). RNA was extracted using RNeasy Mini Kit (Qiagen, Germany) and reverse transcribed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Germany). Real-time quantitative PCR was performed using TaqMan® Universal PCR Master Mix (Applied Biosystems) and corresponding primer sets (s. 2.2.4 Assays).
Apoptosis was detected by using FITC Annexin V Apoptosis Detection Kit I (Becton Dickinson) and proliferation by BrdU Flow Kit® (Becton Dickinson GmbH). Cytotoxicity was determined with LIVE/DEAD® Cell-mediated Cytotoxicity Kit (L7010, Invitrogen Corporation, USA). Assays were performed according to the manufactures instructions.

2.2.3 Cell culture media

Isolated human PBMCs, sorted T and NK cells were incubated and cultured in Iscove's Modified Dulbecco's Medium, (IMDM, PAA Laboratories, Austria), containing 10% heat-inactivated human AB serum (Valley Biomedical Inc., USA), 2mM glutamine and 1% Penicillin/Streptomycin (PAA Laboratories). Isolated splenocytes, murine cells and cell lines

were held in Roswell Park Memorial Institute medium (RPMI 1640, PAA Laboratories), supplemented with 10% heat-inactivated FCS (PAA Laboratories), 2mM glutamine, 1% penicillin/streptomycin. The culture medium of murine cells was supplemented with 500U/ml IL-2. RPMI 1640 medium was used as freezing medium containing 10% DSMO and 40% serum.

2.2.4 Primers for qRT-PCR

The following primer sets from Applied Biosystems were used for qRT-PCR:

TaqMan® Gene Expression Assays for:

human AdipoR1 (Hs00360422_m1),
human AdipoR2 (Hs00226105_m1),
human 18s rRNA (Hs99999901_s1)
murine Nkp46 (Mm00456776_g1)
murine IFN-γ (Mm01168134_m1)

2.2.5 Antibodies

Antibodies were used for flow cytometry or immunofluorescence staining for successive confocal microscopy. All antibodies, accept AdipoR1 and AdipoR2, against human antigens were raised in mice. AdipoR1 and AdipoR2 were raised in rabbit.
Following molecular antibodies were used to identify human cell surface or intracellular antigens:

Tab. 2: Antibodies with human specificity

Antigen	Clone	Dilution	Company
AdipoR1	polyclonal	1:300	Phoenix Pharmaceuticals, USA
AdipoR2	polyclonal	1:300	Phoenix Pharmaceuticals
CD3	SK7	1:50	BioLegend, USA
CD4	SK3	1:20	Becton Dickinson GmbH
CD14	MφP9	1:10	Becton Dickinson GmbH
CD8	SK1	1:10	Becton Dickinson GmbH
CD19	HIB 19	1:10	Becton Dickinson GmbH
CD137	4B4-1	1:5	Becton Dickinson GmbH
CTLA-4	BNI3	1:7	Becton Dickinson GmbH
TIRC 7			kindly provided by Nalan Utku,
Clathrin heavy chain	23/clathrin heavy chain	1:200	Becton Dickinson GmbH
TNFα	MAB11	1:500	Becton Dickinson GmbH
IFNγ	B27	1:500	Becton Dickinson GmbH
IL-2	MQ1-17H12	1:10	Becton Dickinson GmbH
CD107a	H4A	1:5	Becton Dickinson GmbH
Influenza tetramer A2	/	1:12.5	Beckman Coulter, France
HIV tetramer A2	/	1:12.5	Beckman Coulter

Tab. 3: Antibodies with murine specificity

Antigen	Clone	Dilution	Company
CD45	30-F11l	1:100	eBioscience Inc., USA
CD3e	145.2C11	1:100	eBioscience Inc.
CD4	GK1.5	1:200	eBioscience Inc.
CD8b	eBioH35-17.2	1:500	eBioscience Inc.
CD11b	M1/70	1:1000	eBioscience Inc.
CD27	LG 7F8´9	1:125	eBioscience Inc.
Nkp46	29A1.4	1:100	BioLegend
DX5	DX5	1:50	BioLegend
CD94	18d3	1:200	eBioscience Inc.
CD107a	eBio1D4B	1:40	eBioscience Inc.
CD137	1AH2	1:50	Becton Dickinson GmbH
IFNγ	XMG.1	1:40	Becton Dickinson GmbH
NKG2D	CX5	1:200	BioLegend

Tab. 4: Secondary antibodies

Antibody	Fluorochrome	Dilution	Company
Goat anti rabbit mAb (GAR)	Alexa Fluor® 488	1:400	Invitrogen Corporation
Goat anti mouse mAb (GAM)	Cy™ 3	1:100	Jackson ImmunoResearch, USA

2.2.6 Fluorescence dyes

Tab. 5: Fluorescence dyes

Dye	Company
Live Dead-Pacific Blue	Invitrogen Corporation
DAPI	Sigma Aldrich Inc

2.2.7 Stimulating and activating agents

2.2.7.1 Recombinant proteins and cytokines

Tab. 6: Recombinant proteins and cytokines

Protein/Cytokine	Final concentration	Company
Human Adiponectin	3µg/ml	R&D Systems Inc., USA
Human IL-2	50U/ml	Chiron, Great Britain
Human IL-7	10ng/ml	R&D Systems Inc.
Human IL-12	1ng/ml	ImmunoTools, USA
Murine IL-2	500U/ml	eBioscience Inc.

2.2.7.2 Toll-like receptor agonists and other stimuli

Tab. 7: Toll-like receptor agonists and other stimuli

Toll-like receptor agonist/stimuli	Final concentration	Company
LPS from E. coli S form for TLR 4	100ng/ml	ALEXIS Biochemicals, Swiss
R848 for TLR-8	2µg/ml	ALEXIS Biochemicals, Swiss
SEB	3µg/ml	Sigma Aldrich Inc.
PHA-L	1µg/ml	Sigma Aldrich Inc.
Influenza matrix protein 58-66aa	10µg/m	ThermoHybaid GmbH, Germany
HIVA2 peptide	10µg/ml	ThermoHybaid GmbH

2.2.8 Chemicals, Solution and Buffers

Tab. 8: Acquired chemicals and solutions

Name	Company
Isoflurane	Baxter GmbH, Germany
BFA	Sigma Aldrich Inc.
BSA	Sigma Aldrich Inc.
BD CompBeads, anti-mouse Ig	Becton Dickinson GmbH
BD Lyse	Becton Dickinson GmbH
BD Perm	Becton Dickinson GmbH

Continuation Tab. 8: Acquired chemicals and solutions

EDTA	Sigma Aldrich Inc.
FleboGamma	BioTest AG, Germany
FCS	PAA Laboratories
Formaldehyde	Sigma Aldrich Inc.
Monensin	Merck KGaA,
Fluoromount-G™	Southern Biotech, USA
PBS without Ca and Mg	PAA Laboratories
Trypanblue	Merck KGaA;

Tab. 9: Buffers

Name	**Ingredients**
ACK buffer	0.15 M NH_4Cl,
	0.01M $KHCO_3$,
	0.07 mM EDTA in H_20
FACS buffer human	PBS with 2% FleboGamma
FACS buffer murine	PBS with 2% FCS
MACS buffer	PBS
	0.5% BSA
	2mM EDTA

2.3 Equipment

Among the current laboratory equipment the following hardware was used.

Tab. 10: Equipment

Type	**Company**
Cell strainer 70µm	Becton Dickinson GmbH
Centrifuge 5415D	Eppendorf AG, Germany
FACS Canto II analyzer	Beckton Dickinson GmbH
FACS LSR II analyzer	Beckton Dickinson GmbH
FACS tubes	Beckton Dickinson GmbH
GeneAmp PCR System 9700	Applied Biosystems

Continuation Tab. 10: Equipment

Heraeus Multifuge 3SR+	Thermo Fisher Scientific Inc., USA
Leica TCS SP5 confocal microscope	Leica Microsystems GmbH, Germany
NanoDrop ND 1000	Thermo Fisher Scientific Inc.
Neubauer counting chamber	Lo Laboroptik, Germany
Microscope BX 300	Will Wetzler, Germany
Superfrost ultra plus slides	Thermo Electron LED GmbH, Germany
Vacutainer blood collection system	Beckton Dickinson GmbH

2.4 Software

Flow cytometry data were generated by BD FACSDiva software v6.0 and analyzed with Flow.Jo v9.4.1.0 (Treestar). Leica LAS AF Lite Software was used by Jordi Magrane and Diana Elligsen-Merkel to evaluate and quantify confocal fluorescence microscopy images. RNA quality and quantity was estimated using NanoDrop software. Real-Time PCR data were analyzed by 7500 sequence detection software (SDS) v1.4. Besides Microsoft Office 2010 (Microsoft), statistical analysis and calculations were performed by Graphpad Prism.

3 METHODS

3.1 Cell culture and preparation

3.1.1 Isolation of human peripheral blood mononuclear cells (PBMCs)

Blood was collected using the BD Vacutainer blood collection system. Blood was diluted 1:1 with PBS and separated under sterile conditions by gradient centrifugation using Ficoll with 850 g for 20 minutes at room temperature (RT) without brake. The PBMC fraction was collected, diluted 1:1 with PBS, centrifuged at 400 g for 10 minutes at RT and resuspended in IMDM medium containing 40% AB serum. Cells were counted using the Neubauer counting chamber and trypan blue for discriminating alive cells.

3.1.2 Isolations of human CD3 T cells

Isolation of human T cells was performed using MACS® Pan T cell isolation kit II (negative selection) or CD3 MicroBeads (positive selection) according to the manufactures instructions from PBMCs. In summary, negative selection was performed by depletion of non-T cells whereas during positive selection T cells were labeled with CD3 MicroBeads.

3.1.3 Isolation of human NK cells

Human NK cells were isolated from PBMCs using the MACS® NK cell isolation Kit. Separation was performed as described by the manufactures' instruction. Briefly, all non-NK cells were magnetically labeled and isolated from the PBMCs over a MACS® Column placed in a magnetic field. Non-NK cells were retained on the column while the untouched NK cells pass through..

3.1.4 Isolation of murine splenocytes and peripheral cell

Mice were sacrificed by isoflurane inhalation at indicated time points. Spleens and blood were aseptically removed. Heparinized blood was treated three times with ACK buffer to remove red blood cells. Single cell suspensions were made by splashing the spleen through a 70 µm

cell strainer and diluting the suspension in cold PBS. All obtained cells were counted and cultured in RPMI culture medium containing L-glutamine, supplemented with 10 % FCS, 1% each of penicillin/ streptomycin and 500U IL-2 at 37°C with 5% CO2, or used immediately after washing and resuspending in FACS buffer. Absolute murine NK cell numbers were calculated from total isolated cell numbers using percentages of $Nkp46^{+}$, $CD3^{-}$ lymphocytes

3.1.5 Thawing of cell lines

Frozen cells were stored in cyroconservation tubes in liquid nitrogen. 20 mL of 37°C warm Iscoves medium with 4% AB serum were prepared and 1 mL of frozen cell suspension was diluted stepwise with the warm culture medium. The 20 mL solution containing the thawed cells was washed and finally resuspended in PBMC culture medium (Iscoves with 10% AB serum).

3.1.6 Freezing of cells

For long term storage in liquid nitrogen cells were frozen in freezing medium. 1ml of freezing medium was used to resuspend 10^7 cells in ice-cold cyroconservation tubes. Freezing container was used to store cells in -80°C before transferring to the nitrogen tank.

3.2 Stimulation

3.2.1 Stimulation of T cells

Functional analyses of T cells were performed on PBMCs rested overnight. Antigen activated T cells were generated from PBMCs following stimulation with SEB or influenza peptide in 96 well plates in the presence of rhIL-2 and rhIL-7. PBMCs were seeded in 200µl of medium at concentrations of 2 x 10^5 per well and analyzed at various time points after stimulation. APN was added 24h before stimulation. Activated and antigen specific T cells were determined by CD137 surface expression, cytokine production and by tetramer staining. Expansion of influenza specific T cells determined by cytokine response was analyzed at day 9 of culture upon 6h restimulation with influenza peptide. BFA was added at the last 4h of restimulation. Antigen specific T cell cytokine production was measured in freshly isolated PBMCs following 24h stimulation with influenza peptide. BFA was added at the last 22h.

3.2.2 Stimulation of NK cells

For NK cell stimulation PBMCs and sorted NK cells from healthy donors were primed with rhIL-12 and preincubated with APN for 24h. Sorted NK cells were seeded in 200µl of medium at concentrations of 2.5 x 10^5 per well in 96 well plates. Stimulation was performed with the Toll-like receptor (TLR) ligands LPS and R848 for additional 24h. BFA was added at the last 6h of stimulation. Intracellular IFN-γ production of NK cells was analyzed via flow cytometry.

3.3 Flow cytometry

3.3.1 Staining procedures

Cells were harvested and washed with FACS buffer. 1mM EDTA/PBS was added for 10min at room temperature. Before the addition of antibodies the cells were washed. Extracellular cell markers were stained for 20min at 4°C, washed and resuspended in FACS buffer.

Intracellular molecules were stained after the extracellular staining procedure. Therefore cells were washed in 1x BD Lyse to remove red blood cells and permeabilized with 1x BD Perm. Intracellular staining was performed 30min at 4°C. Finally, cells were washed and resuspended in FACS buffer.

AdipoRs staining was performed using fluorescence labeled goat anti rabbit secondary antibody. AdipoRs staining was performed prior to CD antigen staining except in case of intracellular staining of the receptors.

3.3.2 Gating strategies

For the expression analysis of AdipoR1 and AdipoR2 dead cells and "cell doublets" were excluded. Human lymphocytes were gated on CD3 expression for identifying T cells, further subdivided in $CD4^+$ and $CD8^+$ T cell subsets, on $CD3^-$ $CD56^+$ NK cells, CD14 monocytes and CD19 B cells. NK cells were further divided in $CD56^{high}$ and $CD56^{dim}$ populations. AdipoR1 and AdipoR2 expression was studied on indicated PBMCs subsets. Activated and antigen specific T cells were identified by CD137 and cytokine expression as well as by influenza matrix protein 58-66 tetramers.

Murine T cells were identified as CD3e positive cells and subdivided into $CD4^+$ and $CD8a^+$ populations whereas NK cells were defined by the absence of CD3e and the expression of Nkp46 and DX5. CD45 was used to identify leukocytes in isolated murine blood. NK cells were further divided using the maturation markers CD11b and CD27. CD94 and NKG2D were used to characterize effector NK cells. Intracellular IFNγ staining was used to analyze cytokine response of $CD3e^-$ $Nkp46^+$ splenocytes. AdipoRs expression was analyzed on different murine cell subsets.

3.4 Confocal microscopy

Confocal microscopy was used to analyze the localization of AdipoRs with cytoplasmic compartments and molecules. After PHA-L stimulation, CD3 cells, isolated with Pan T Cell Isolation Kit II (Miltenyi Biotech) were intracellular stained for AdipoRs visualized by Alexa Fluor® 488 conjugated goat-anti rabbit antibody and co-stained against clathrin heavy chain, CTLA-4 and TIRC7. Clathrin, CTLA-4 and TIRC7 were visualized by Cy3-conjugated anti mouse antibody. Cells were fixed with 2% formalin in PBS, adhered to glass slides by centrifugation via cytospin and mounted.

Confocal analysis of clathrin-coated vesicles, CTLA-4 and AdipoRs was performed by Jordi Magrane from Weil Medical Collage of Cornell University. Images were collected in a Leica Confocal Microscope using a 63 oil immersion lens (NA1.4), standard pinhole of 1 AU, and optical intervals of 0.4 mm; a 2.5 zoom was applied. Standard 488 and 543nm excitation wavelengths were used for the detection of Alexa488 and Cy3.

Confocal fluorescence microscopy of AdipoRs and TIRC7 localization was done by Diana Elligsen-Merkel from CellAct Pharma GmbH used. Leica fluorescence microscope DMI 4000 B. Confocal microscopy was performed as described in [94].

3.5 Quantitative Real-Time-PCR

Quantitative Real-Time-PCR was used to determine AdipoR mRNA levels in human T cells and CD3 depleted cells, as well as Nkp46 and IFNγ mRNA expression levels in murine splenocytes. CD3 T cells were separated from PBMCs by pan T-cell isolation kit II (negative selection) or by CD3 MicroBeads (positive selection). Total RNA from murine splenocytes,

human CD3 cells or CD3-depleted PBMCs was extracted and reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit according to the manufactures description. Quality and quantity were checked by Agilent 2100 Bioanalyzer. Real-time quantitative PCR was performed using TaqMan® Universal PCR Master Mix. Evaluation was performed by DDCt method.

3.6 Assays

3.6.1 Analysis of proliferation and apoptosis

Human antigen specific T cells were generated from PBMCs by CD3/CD28 activation using T cell Activation/Expansion kit or by stimulation with influenza peptide. 72h after stimulation, APN was added to the culture for additional 24h and the frequencies of proliferating and apoptotic antigen specific cells were assayed by flow cytometry using BrdU Flow Kit® and FITC Annexin V Apoptosis Detection Kit I. Antigen-specific T cells were defined as $CD3^{+}CD137^{+}$.

3.6.2 CD107a degranulation assay

NK cell degranulation of human and murine NK cells was measured by CD107a secretion [111]. Human PBMCs were stimulated with rhIL-2 (100U/ml), APN (3µg/ml) or simultaneously with IL-2 and APN for 20hours and incubated with K562 target cells at an effector to target ratio of 1:1 and 1:2. Sterile mAb against CD107a was added to cells, cultured for one hour at 37°C and incubated with 2mM monensin to prevent intracellular protein transport. Five hours later cells were stained with mAb against CD3, CD8 and CD56 and analyzed by flow cytometry. Dead cells were excluded from analysis.

Murine splenocytes from uninfected and CVB3 infected mice were incubated in the presence of 500U/ml rmIL-2 with YAC-1 target cells at an effector to target ratio of 50:1 and 100:1 and incubated with CD107a antibody. One hour later 7.5µg/ml brefeldin A was added to the culture. Cells were cultured for a total time of four hours, harvested, stained for CD3e, CD8a and Nkp46 and analyzed by flow cytometry. Dead cells were excluded from analysis.

3.6.3 *In vitro* killing assay

Cytotoxicity of human NK cells was measured by the LIVE/DEAD® Cell Mediated Cytotoxicity Kit (Invitrogen). K562 target cells were labeled in a dilution of 1:100 with the 3,3'dioctadecylloxacaebocyanin fluorescence dye for 30min, washed twice with PBS and incubated with sorted NK cells at effector to target ratio of 1:1 and 1:4. Cells were treated with 3µg/ml APN and/or 100U/ml IL-2 and cultured with the provided propidium iodid (PI) solution for three hours at a dilution of 1:400. Cells were analyzed using flow cytometry.

3.7 Statistical analysis

Analysis of human data was performed using one-tailed Wilcoxon signed rank test. Significance of mice data were calculated by one-tailed Mann-Whitney –U test. All graphs are shown as mean ± SD or SEM.

4 RESULTS

4.1 Immunomodulatory effects of APN on T cells

4.1.1 AdipoRs expression and localization in human T cells

To study the effects of APN on T cells we first analyzed the expression of both AdipoRs on human peripheral blood T cells of healthy donors by flow cytometry. Consistent with published data, *ex vivo* surface receptor staining revealed that only a small fraction of $CD3^+$ T cells expose AdipoR1 and AdipoR2 on their surface. Whereas almost all human $CD14^+$ monocytes expressed AdipoRs extracellular, <10% of both receptors were detected on the $CD3^+CD4^+$ (mean 6,6% for AdipoR1 and 7,6% for AdipoR2) and $CD3^+CD8^+$ (mean 7,5% for AdipoR1 and 7,3% for AdipoR2) T cell subpopulations (Fig.7A).

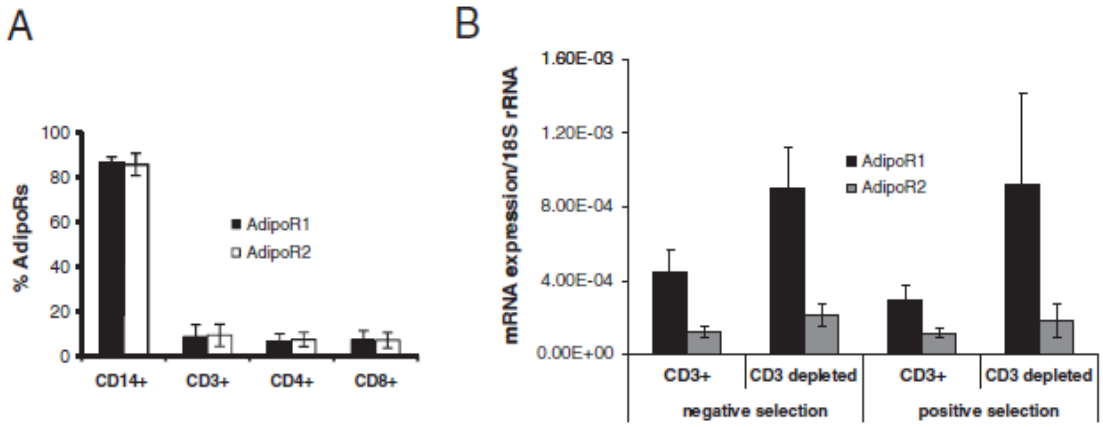

Figure 7: **AdipoRs expression on human $CD3^+$ T cells. (A)** Surface expression on $CD14^+$ monocytes and T cell subpopulations assessed by flow cytometry. Represented are the frequencies of AdipoR1 and AdipoR2 expression on the respective PBMC subpopulation (n=6; mean±SD). **(B)** AdipoRs mRNA expression levels in $CD3^+$ T cells and CD3 depleted PBMCs. mRNA levels of AdipoRs of cell fractions received by negative and positive magnetic selection are depicted relative to 18S rRNA transcription (negative selection n=4, positive selection n=3; mean±SEM). Published in [112].

In contrast, the measurement of AdipoR1 and AdipoR2 mRNA levels by quantitative Real-Time PCR (RT-PCR) illustrated that $CD3^+$ T cells express almost half the amount of both receptor transcripts when compared with CD3 depleted PBMCs (Fig.7B). To exclude changes in the AdipoRs mRNA transcription dependent on the CD3 selection method we performed quantitative assessment of both receptors by using negatively and positively separated $CD3^+$ T cells. In both cases results were similar independent of the isolation method and showed

high receptor's transcript levels in $CD3^+$ T cells conflicting with the flow cytometry data. According to this, intracellular (i.c.) flow cytometry staining of AdipoRs were performed on peripheral blood $CD3^+$ T cells. Figure 8 depicts the comparison between the surface and intracellular expression of AdipoR1 in $CD3^+$ T cells and $CD14^+$ monocytes. While AdipoR1 was expressed on the surface (e.c.) of most monocytes, $CD3^+$ T cells showed mainly an intracellular expression of AdipoR1 and AdipoR2 (data not shown). The same results were obtained for $CD3^+$ $CD4^+$ and $CD4^+$ $CD8^+$ T cell subsets (data not shown).

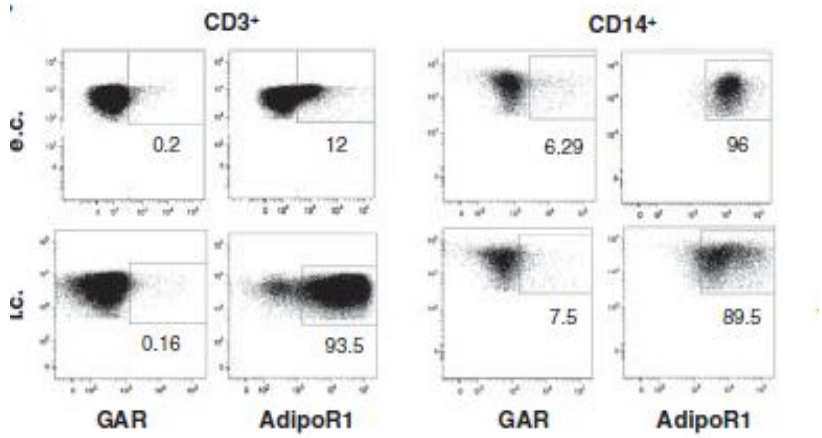

Figure 8: Intracellular (i.c.) and extracellular (e.c.) expression of AdipoR1 in peripheral blood $CD3^+$ T cells (left) and $CD14^+$ monocytes (right). One representative staining with AdipoR1 specific mAb is shown. GAR indicates negative control stained with goat-anti rabbit secondary Ab for visualizing AdipoR1 expression. Numbers indicate frequencies of positive cells. Published in [112].

As $CD3^+$ T cells originate from lymphoid precursors in the bone marrow and are able to re-enter it during their life spam we analyzed the AdipoRs distribution on bone marrow T cells being exposed to a different microenvironment than peripheral blood T cells. Similar to T cells in the blood stream bone marrow T cells displayed only low frequencies of extracellular AdipoRs expression, as shown for $CD3^+$ $CD4^+$ T cells whereas intracellular expression was detected on almost all $CD3^+$ $CD4^+$ T cells (Fig. 9A). In contrast to $CD19^+$ B cells (Fig. 9B) and $CD14^+$ monocytes (Fig. 9C) found in bone marrow which all exposed the receptors on the surface.

In line with the expression pattern of AdipoRs on T cells the expression of both receptors on the T lymphatic leukemia cell line Jurkat was limited to the cytoplasm whereas the myeloid leukemia cell line K562 demonstrated high extracellularly AdipoR1 and Adipor2 expression (Fig. 10).

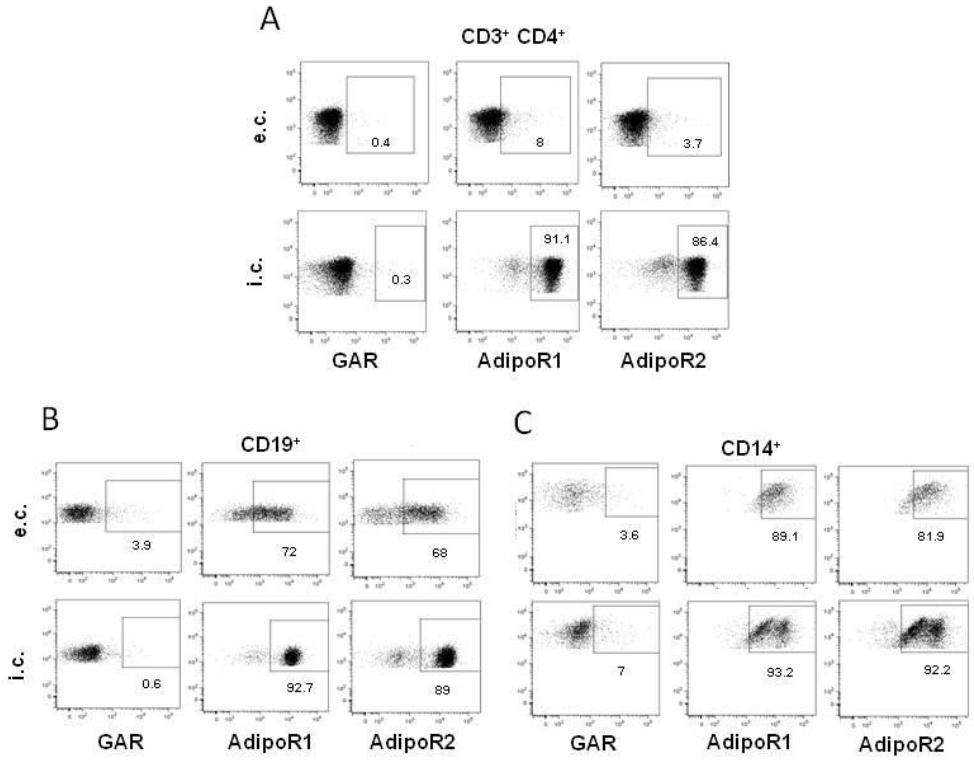

Figure 9: Intracellular (i.c.) and extracellular (e.c.) expression of AdipoR1 and AdipoR2 on bone marrow cells. Representative dot plots of receptor expression in (A) in bone marrow $CD3^+$ $CD4^+$ T cells, (B) bone marrow $CD19^+$ B cells and (C) in bone marrow $CD14^+$ monocytes. GAR indicates negative control stained with goat-anti rabbit secondary Ab. Numbers indicate frequencies of positive cells.

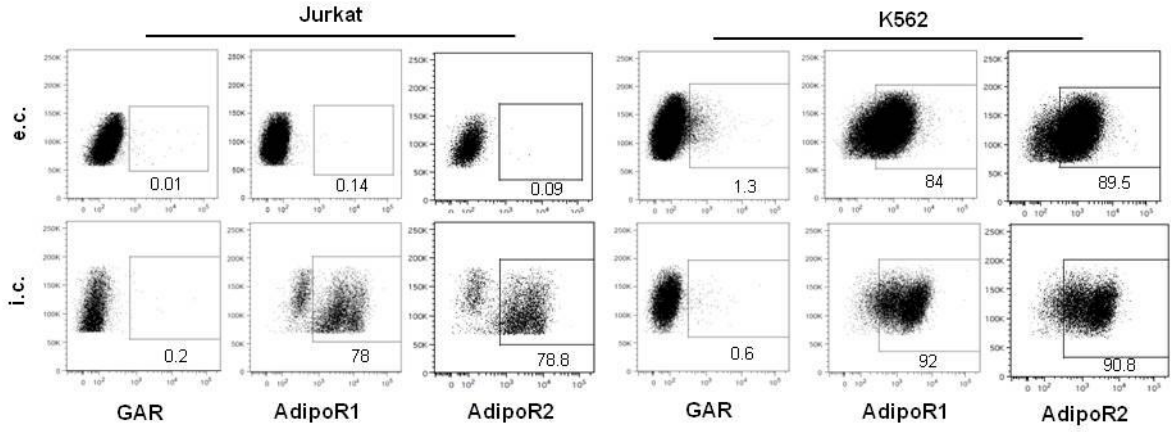

Figure 10: Intra- and extracellular expression of AdipoRs on lymphatic leukemia cell line Jurkat and myeloid leukemia cell line K562. Representative staining of AdipoRs is shown. GAR indicates negative control, numbers frequencies of positive cells. Published in [112].

Given that the majority of $CD3^+$ T cells express AdipoRs intracellularly and APN was shown as an inhibitory protein in cells of the innate immune system we hypothesized that AdipoRs might be stored like other T cell regulatory molecules in cytoplasmatic compartments. After isolation of PHA stimulated peripheral blood T cells and intracellular staining of AdipoRs, $CD3^+$ cells were co-stained against TIRC7, CTLA-4 and clathrin, a marker for clathrin-coated vesicles. Fluorescence labeled cells were counterstained with DAPI to exclude cell debris and adhered to glass slides. Localization was visualized by confocal fluorescence microscopy. As shown in Figure 11 staining of AdipoRs and TIRC7 defined by green and red fluorescence, respectively, revealed a co-localization of both structures near the cell surface of T cells resulting in a yellow fluorescence after overlay of both pictures. However, AdipoR2 illustrated a weaker co-localization with TIRC7 compared with AdipoR1 and TIRC7. Further similar analyses were performed to study the localization of AdipoR1 in clathrin-coated vesicles and with CTLA-4 within $CD3^+$ T cells. Staining with specific anti-AdipoR1, anti-clathrin and anti-CTLA-4 mAb demonstrated a co-localization of AdipoR1 and CTLA-4 (Fig. 12D), as well as clathrin. (Fig. 12A, B). Orthogonal sections across the z-image stack from the cells shown in Fig12A (dotted area) represent strong cellular association of AdipoR1 and clathrin near the plasma membrane. The quantitative analysis of co-localization using single optical plane taken at the mid-section of a cell revealed a Pearson's correlation coefficient $R(r) = 0.69 \pm 0.11$. The Pearson's correlation coefficient between AdipoR1 and CTLA4 was $R(r) = 0.43 \pm 0.11$. These results depicted that the majority of T cells express the AdipoRs intracellularly, store them in clathrin-coated vesicles where they co-localize with CTLA-4 and TIRC7.

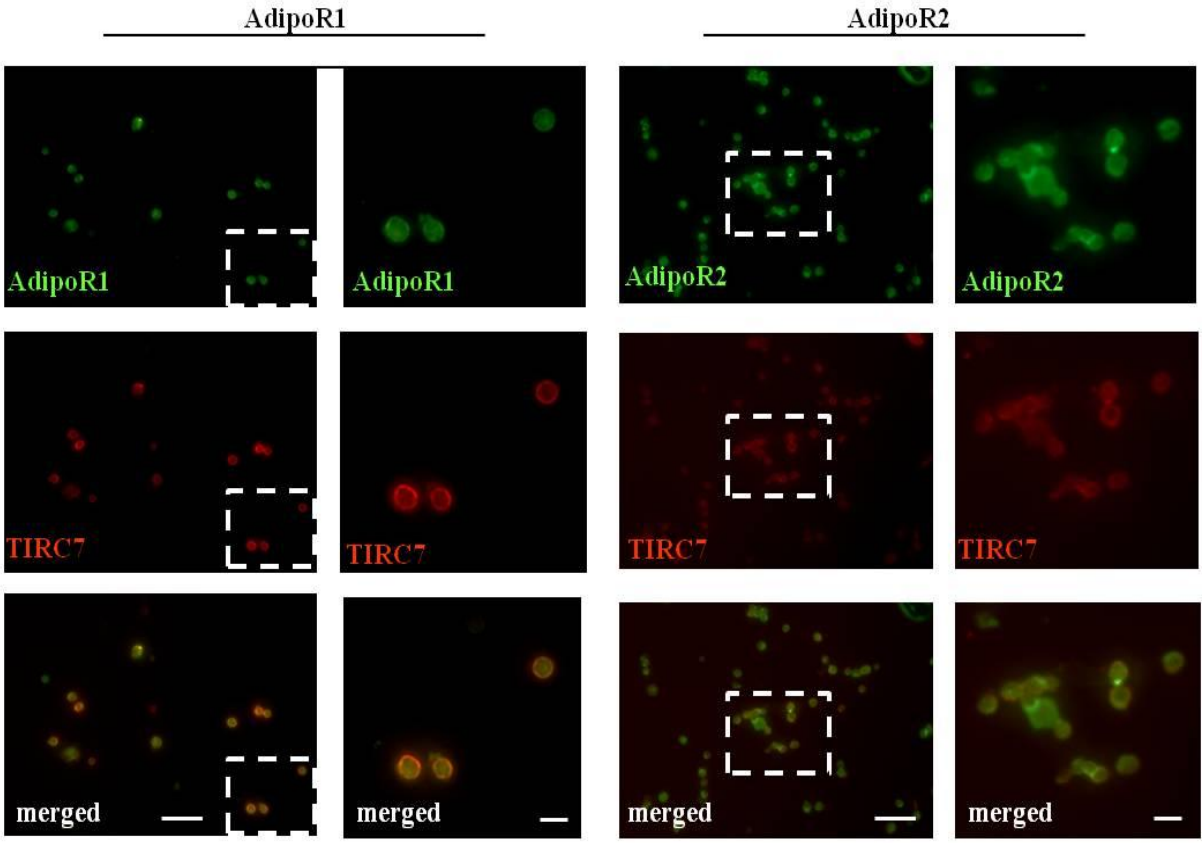

Figure 11: Intracellular co-localizations of AdipoRs with TIRC7 in human T cells. Staining of AdipoR1 and AdipoR2 (green) and TIRC7 (red) labeled cells was detected by confocal fluorescence microscopy. Dotted areas indicate co-stained, yellow structures (scale bar 20µm) taken and shown right in detail (scale bars 10µm; magnification, 63x). One representative co-staining is shown.

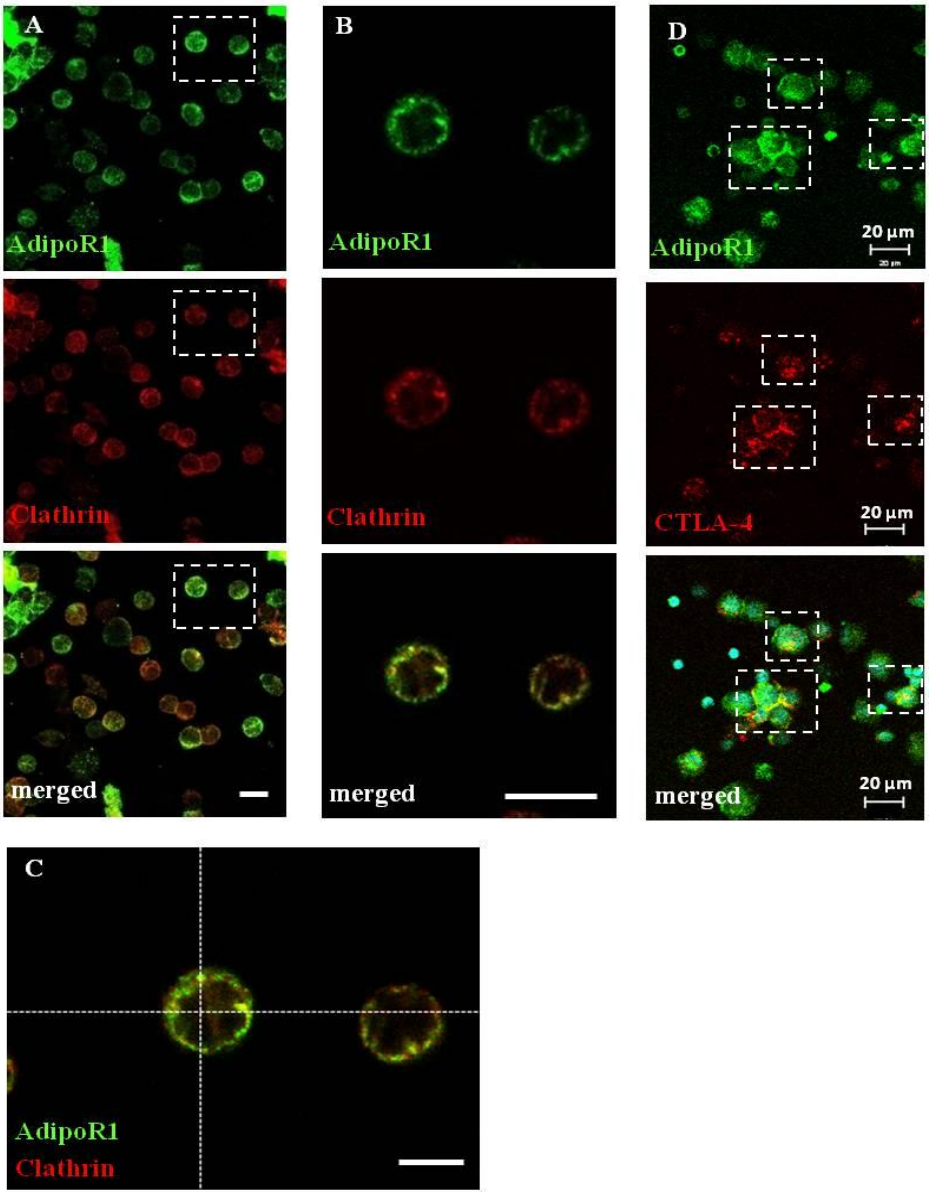

Figure 12: Intracellular co-localization of AdipoR1 with clathrin-coated vesicles and CTLA-4. Immunofluorescence staining was detected by confocal microscopy of $CD3^+$ T cells. (A) General view in a merged z-image stack and (B) single optical planes (z-section, 0.4µm thick) taken at the mid-section of the cells are shown (scale bars 10µm). (C) Orthogonal sections across the z-image stack from the cells shown in (A) dotted area (scale bars 5µm). (D) General view of the cells. Dotted area indicates co-stained structures (magnification, 63x). One representative co-staining is shown. Published in [112].

4.1.2 AdipoRs Expression on murine T cells

Next we analyzed expression patterns of AdipoRs in murine T cells. Therefore mice splenocytes of male C57BL//6 WT mice aged 8-10 weeks were removed, stained for CD3e characterizing T cells, and analyzed for AdipoRs distribution by flow cytometry. Similar to human T cells analysis revealed that both receptors are predominantly expressed intracellular (Fig. 13). Only a minor fraction of freshly isolated murine splenic T cells displayed AdipoRs on the cell surface. Whereas the intracellular staining showed that more than 90% of T cells store the receptors in the cell plasma solely <15% expose AdipoR1 and AdipoR2 on the cell surface (Fig. 13 and 14).

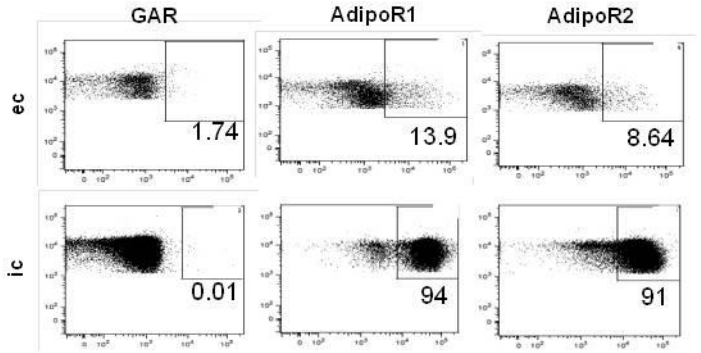

Figure 13: Distribution of AdipoRs in murine CD3e$^+$ T cells. Representative dot plots of extra- (e.c.) and intracellular (i.c.) staining of AdipoRs in murine T cells are shown. GAR indicates negative control and numbers the percentages of positive cells.

For functional analysis of APN effects we also used APN-KO mice. Intra- and extracellular staining of both receptors on T cells from APN-KO mice showed no differences in the expression pattern of AdipoRs compared to WT mice (Fig. 14). Surface AdipoR1 and AdipoR2 expression was detected on a minor fraction of T cells, respectively, whereas receptor expressions were identified in the cytoplasm of >90% of T cells of APN-KO mice. Thus, the absence of APN in the APN-KO mice had no influence on the expression pattern of AdipoRs on T cells.

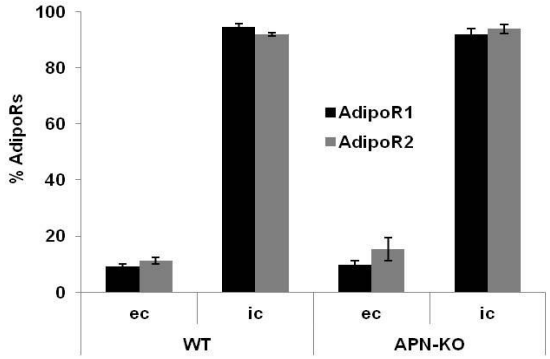

Figure 14: Expression of AdipoRs on murine CD3e$^+$ T cells of WT and APN-KO mice. Represented are frequencies of AdipoR1 (black bars) and AdipoR2 (grey bars) on *ex vivo* T cells analyzed by flow cytometry. AdipoRs were stained extra- (e.c.) and intracellular (i.c.) (mean±SEM; n=5).

4.1.3 AdipoRs regulation on T cells

Analysis of the potential function of APN on T cells requires the knowledge about the regulation of AdipoRs. To elucidate the regulation of AdipoRs T cells were first stimulated with Staphylococcal enterotoxin B (SEB) to generate antigen reactive T cells.

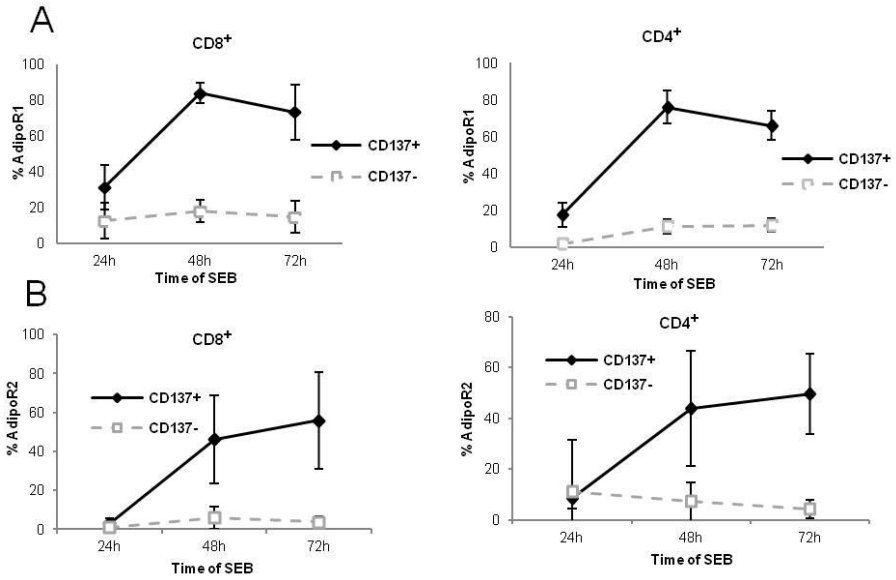

Figure 15: Regulation of AdipoRs on SEB stimulated human CD4$^+$ and CD8$^+$ T cells. Surface expression of (A) AdipoR1 and (B) AdipoR2 was analyzed on activated T cells detected by CD137 expression at various time points via flow cytometry. (n=4). Published in [112].

SEB is known as a superantigen. It activates a large number of T cells because it is not processed and presented via MHC class II binding groove but cross-links MHC class II to the TCR therefore causing polyclonal T cell activation which can be detected by CD137 expression. AdipoRs surface expression was determined via flow cytometry in activated $CD4^+$ and $CD8^+$ T cells characterized by CD137 upregulation at various time points of SEB stimulation (Fig. 15). After 48h of stimulation both receptors were upregulated on $CD137^+$ $CD4^+$ and $CD8^+$ T cells (Fig. 15A and B) however, AdipoR1 revealed higher expression levels compared to AdipoR2 (Fig. 15A). In contrast non activated $CD137^-$ $CD4^+$ and $CD8^+$ T cells showed no upregulation following stimulation with SEB. In addition, activated $CD4^+$ and $CD8^+$ T cells upregulated the inhibitory molecule CTLA-4 within 48h of SEB incubation whereas expression on $CD137^-$ T cells was not detectable (Fig.16).

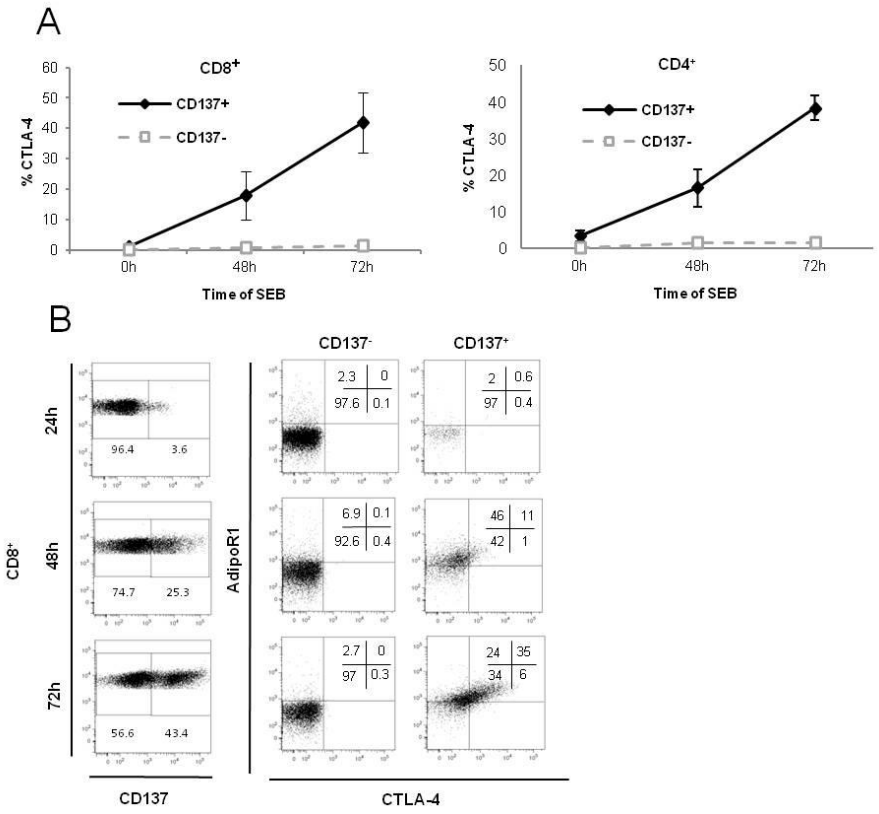

Figure 16: Co-expression of AdipoR1 and CTLA-4 on human $CD137^+$ T cells. FACS data illustrating (A) the surface expression of CTLA-4 on activated $CD137^+$ $CD4^+$ and $CD8^+$ T cells (n=6) and (B) the co-expression of AdipoR1 and CTLA-4 on $CD137^+$ CD8+ T cells (n=4) at various time points of SEB stimulation. Numbers indicate percentages of positive cells. Published in [112].

In accordance to the fluorescence microscopy data CTLA-4 expression correlated with the upregulation of AdipoRs (AdipoR2 not shown) on the surface of antigen specific T cells (Fig. 16B). Figure 16B demonstrates the co-expression of AdipoR1 and CTLA-4 on the surface of activated $CD137^+$ $CD8^+$ T cells following SEB incubation. Similar results were obtained for AdipoR1 on $CD137^+$ $CD4^+$ T cells and AdipoR2 on CD8+ and $CD4^+$ T cells (data not shown).

To confirm these results we performed additional experiments with short term T cell lines generated against a HLA-A2-binding influenza peptide. At day 9, two days after restimulation with influenza matrix protein, antigen specific T cells were detected by influenza tetramer displaying frequencies of 12.1% as shown in Fig 17. HIV tetramer staining was used as a negative control (0.1%). Co-staining revealed that the majority of influenza tetramer positive $CD8^+$ T cells co-expressed AdipoR1 (83%) whereas only a minor population of the influenza negative cells expressed AdipoR1 on their surface (8.41%). 13% of T cells expressed CD137 after 9 days of culture. Analysis of $CD8^+$ $CD137^+$ T cells revealed that almost 70% of them are influenza specific T cells and 86.4% of the influenza specific T cells expressed surface AdipoR1. Interestingly 34.8% of $CD8^+$ $CD137^+$, but influenza negative T cells showed also an AdipoR1 expression. Influenza expanded cells stained with HIV tetramer showed also 13% of activated $CD137^+$ T cells not specific for influenza. 56% of $CD137^+$ T cells expressed AdipoR1 surface receptor.

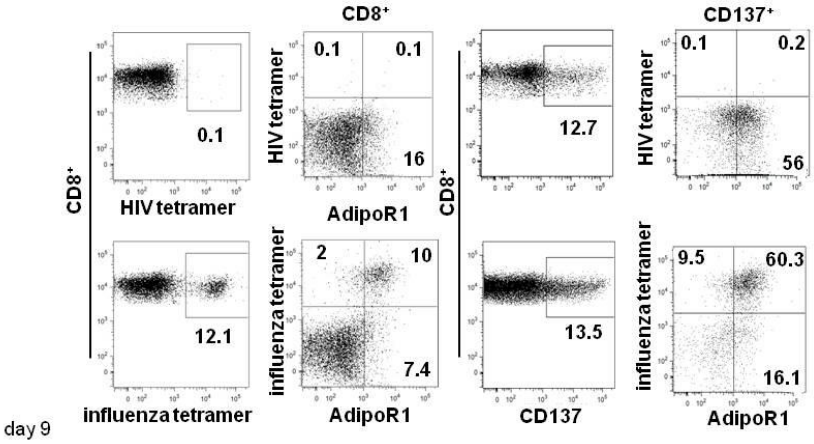

Figure 17: AdipoR1 surface expression on influenza activated human T cells detected by tetramer staining. Representative dot plot of influenza specific T cells visualized by tetramer staining at day nine is shown. Majority of influenza specific T cells express AdiopoR1. Co-expression of influenza tetramer and AdipoR1 was detected on $CD8^+$ $CD137^+$ T cells. HIV tetramer staining served as a negative control (upper panel). Numbers indicate percentages of positive cells. Data were obtained by flow cytometry. Partially published in [112].

Raised short term influenza specific T cell lines were additionally characterized by IFNγ production on day 6 after 6 hours of restimulation with influenza peptide. Controls were restimulated with HIV peptide (Fig. 18).

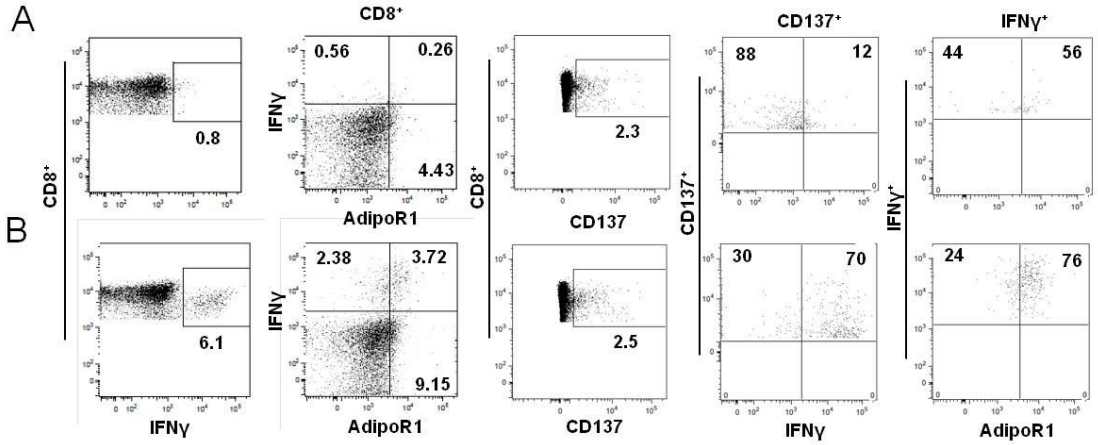

Figure 18: AdipoR1 surface expression on influenza activated human T cells detected by IFNγ staining. Shown are representative dot plots of influenza specific T cells visualized by IFNγ staining at day six. (A) Influenza specific T cells restimulated with HIV. Cultures served as negative controls for cytokine staining (B) Influenza specific T cells restimulated with influenza peptide. Numbers indicate percentages of positive cells.

As shown in Figure 18B 6.1% of $CD8^+$ T cells in the generated cultures expressed IFNγ. Co-staining revealed that 61% of IFNγ producing T cells expressed surface AdipoR1 whereas only 9.7% of $IFN\gamma^-$ $CD8^+$ T cells exposed AdipoR1 on the cell surface. Controls restimulated with HIV peptide showed no IFNγ production but displayed a minor fraction of 4.5% $IFN\gamma^-$ $CD8^+$ T cells expressing AdipoR1 (4.5%). 70% of $CD137^+$ $CD8^+$ T cells expressed IFNγ and were characterized as influenza specific T cells which exhibited 76% of surface Adipor1 expression. Summarized, AdipoRs are upregulated upon antigen activation on antigen specific and antigen activated $CD137^+$ T cells

To evaluate the dynamics of AdipoR1 surface upregulation on antigen specific T cells, extracellular expression of AdipoR1 was determined on influenza specific $CD8^+$ T cells assayed by CD137 expression 24h up to nine days after peptide stimulation (Fig. 19). AdipoR1 was detected on up to 50% of $CD8^+CD137^+$ T cells within 72h of stimulation.

Surface expression was increased after 48h of stimulation and could by detected until day nine on influenza-specific $CD8^+CD137^+$ T cells whereas extracellular expression of AdipoR1 on $CD137^-$ T cells was not induced.

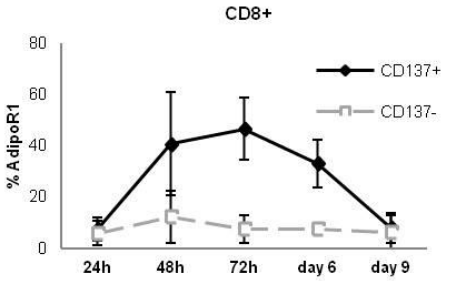

Figure 19: Time course of human AdipoR1 surface expression. AdipoR1 surface expression on $CD137^-$ and $CD137^+$ $CD8^+$ influenza activated T cells. AdipoR1 expression was detected by flow cytometry following influenza peptide stimulation (n=4). Published in [112].

Similar to human T cells resting murine T cells of WT and APN-KO mice revealed predominantly intracellular AdipoRs expression (Fig. 14), Coxsackie virus B3 (CVB3) infection induced an upregulation of both receptors in WT and APN-KO animals on the cell surface (Fig. 20). AdipoR1 was detected on mean 30%, AdipoR2 on mean 40% of murine T cells. There was no difference in the fold induction of receptor expression between WT and APN-KO mice.

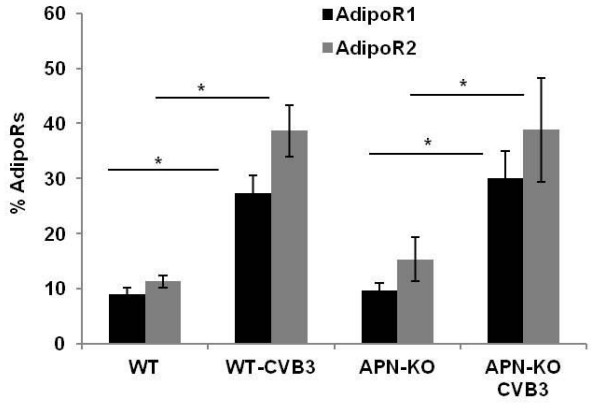

Figure 20: Regulation of AdipoRs on murine T cells. Surface expression of AdipoR1 (black bars) and AdipoR2 (grey bars) was analyzed by flow cytometry on T cells of uninfected and infected WT and APN-KO mice (n=5; *$p<0.05$). Data shown are mean±SEM.

4.1.4 Functional Effects of APN on T cells

Next we studied the effects of APN on generation, proliferation and cytokine production on antigen specific T cells. Influenza specific T cell lines were raised from PBMCs in the presence or absence of APN added together with influenza peptide (Fig. 21). The cell numbers of influenza specific T cells were identified by CD8 and CD137 staining at various time points analyzed by flow cytometry. Whereas total cell counts of the cultures were not impaired by the presence of physiological concentrations of APN (+APN: mean 1.6 x 106; -APN: mean 1.2 x106) the cell numbers of $CD137^+$ $CD8^+$ T cells were diminished in the presence of APN at day 6 and day 9 (Fig. 21).

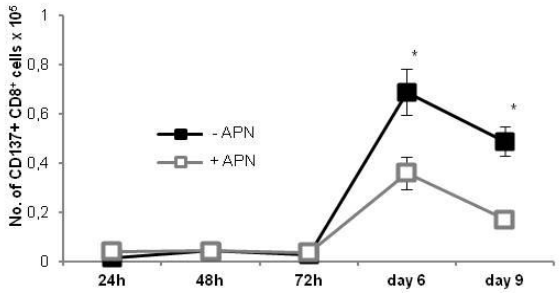

Figure 21: Influence of APN on the generation of human antigen specific T cells. Counts of influenza specific T cells assayed by CD8 and CD137 staining and analyzed by flow cytometry. Cell numbers were determined in the absence (black squares) and presence (white squares) of APN at the indicated time point (n=6; *$p<0.05$ for days 6 and 9). Published in [112].

AdipoRs expression was, however, not influenced by APN (Fig. 22). Measurement of surface AdipoR1 expression after 24h, 48h and 72h of SEB stimulation revealed no impact of APN neither on total nor on $CD137^+$ $CD4^+$ and $CD8^+$ T cells. In accordance, lower numbers of antigen specific, cytokine producing T cells were detected at day 9 in cell lines raised in the presence of APN (Fig. 23 left). Following six hours of restimulation with influenza peptide at day 9 the numbers of IFNγ-, TNFα- and IL-2-producing cells were significantly decreased in cultures incubated with APN.

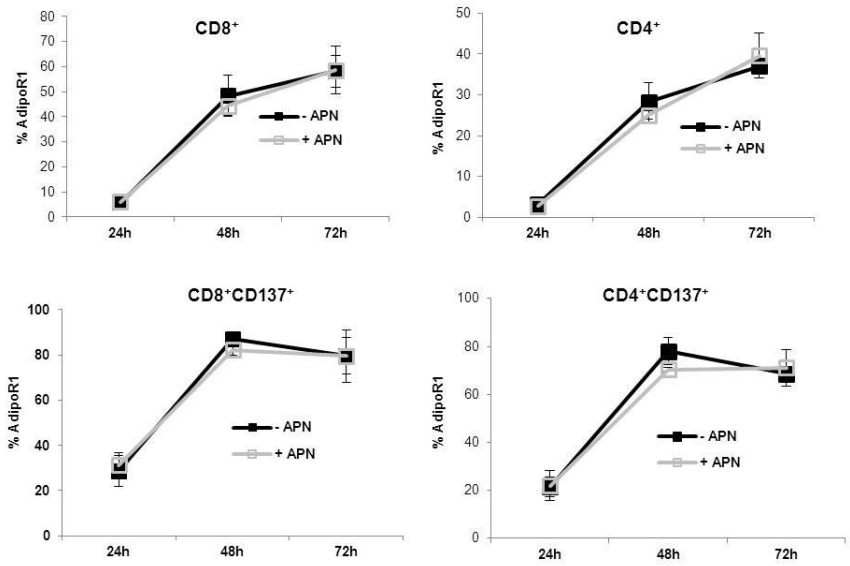

Figure 22: Influence of APN on AdipoR1 expression of SEB activated T cells. Frequencies of AdipoR1 expression following SEB stimulation on total (upper panel) and CD137$^+$ (lower panel) CD8$^+$ and CD4$^+$ T cells. PBMCs were stimulated in the presence (grey lines) and absence (black lines) of APN (n=3). Data shown are mean±SEM.

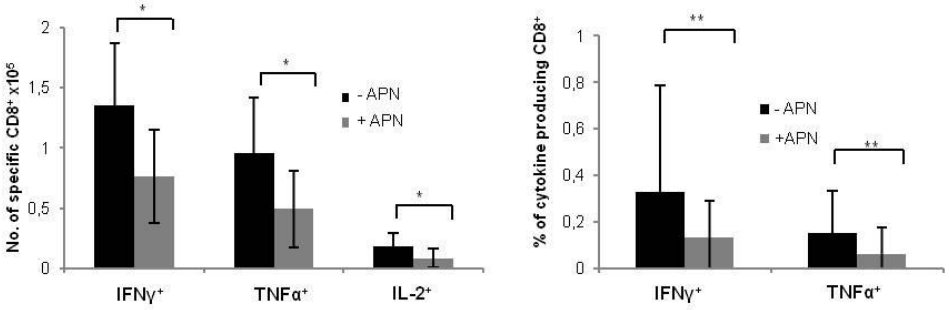

Figure 23: APN impact on cytokine producing human CD8$^+$ T cells and on *ex vivo* cytokine production. (Left) Numbers of influenza specific T cells determined by IFNγ, TNFα and IL-2 production in the absence (black bars) and presence (grey bars) of APN were detected by intracellular cytokine staining (n=6; *p<0.05). (Right) Frequencies of influenza specific T cells producing IFNγ and TNFα in response to 24h peptide stimulation in freshly isolated PBMCs in the absence (black bars) and presence (grey bars) of APN (n=7; **p<0.01). Data presented are mean±SD. Published in [112].

Further, the direct effect of APN on specific T cell cytokine production was studied *ex vivo*. Therefore freshly isolated PBMCs were preincubated with APN and stimulated with influenza peptide for 24h. Influenza specific T cell responses were assessed by cytokine production of CD8$^+$ T cells detected by flow cytometry (Fig. 23 right). Frequencies of influenza specific IFNγ as well as TNFα producing CD8$^+$ T cells were diminished in the presence of APN.

These data show that APN exerts a direct inhibitory effect on antigen-induced cytokine production.

To study the inhibitory molecular mechanism of APN on the generation of antigen activated T cells we analyzed the influence of APN on apoptosis and proliferation. The impact on both processes was assayed in CD3/CD28 polyclonal activated human T cells. BrdU incorporation and Annexin expression in anti CD3/CD28 activated T cell, defined as $CD3^+$ $CD137^+$, were determined by flow cytometry. As shown in Figure 24, APN had no effect on the proliferation and apoptosis of $CD137^-$ T cells whereas apoptotic cell death was increased in $CD137^+$ T cells (Fig. 24A).

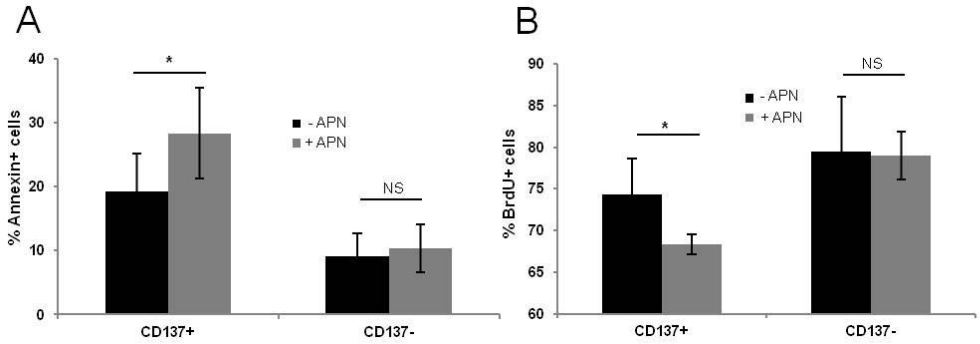

Figure 24: APN influence on apoptosis and proliferation of human CD3/CD28 activated T cells. (A) Apoptosis of $CD137^+$ and $CD137^-$ T cells was assayed by annexin staining and measured by flow cytometry. Percentages of apoptotic cells in the absence (black bars) and presence (grey bars) of APN are shown. (B) Proliferation of $CD137^+$ and $CD137^-$ T cells was determined by BrdU incorporation and flow cytometry. Impact of APN on proliferation is shown by grey bars. (n=6; *$p<0.05$; NS-not significant). Data shown are mean±SD. Published in [112].

In line with the pro-apoptotic effect, APN inhibited the proliferation of $CD137^+$ T cells (Fig. 24B) but not of $CD137^-$ T cells. Similar but less pronounced results were obtained using influenza specific $CD137^+$ T cells (Fig. 25). In the presence of APN significantly higher frequencies of apoptotic $CD8^+$ $CD137^+$ T cells were detected on day 4 as compared to non-treated cultures. However, the proliferation of influenza specific T cells was not decreased by APN.

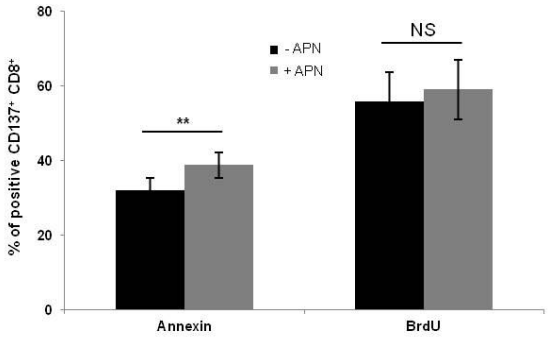

Figure 25: APN influence on apoptosis and proliferation of human influenza activated T cells. Frequencies of apoptotic and proliferating influenza specific $CD137^+$ $CD8^+$ T cells analyzed by flow cytometry. Black bars indicate cultures in the absence of APN and grey bars show results of cultures incubated with APN. Data shown are mean±SEM (Annexin n=7; **p<0.01; BrdU n=3; NS-not significant).

To determine whether the presence of APN attenuates the antigen activated T cell response *in vivo*, we comparatively analyzed the T cell response in CVB3 infected APN-KO and WT mice. T cell responses, defined as frequencies of $CD137^+$ cells, were analyzed at day 3 and 7 after infection (Fig. 26).

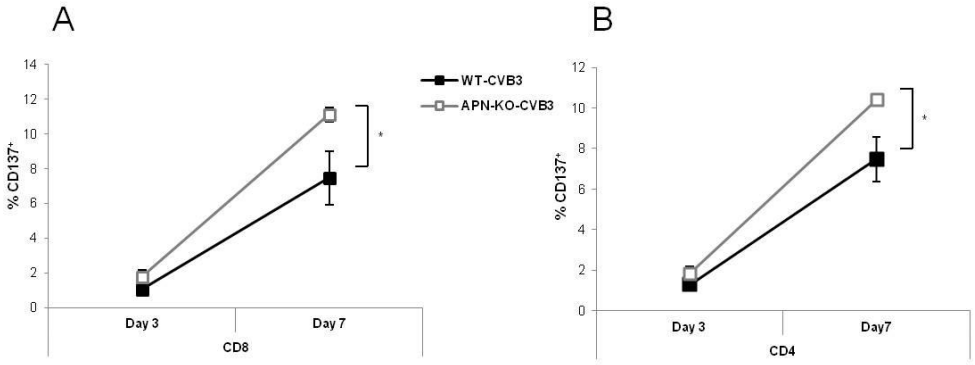

Figure 26: Influence of APN deficiency on murine antigen activated T cells. Frequencies of (A) $CD8^+$ $CD137^+$ and (B) $CD4^+$ $CD137^+$ T cells of infected WT and APN-KO mice were determined by flow cytometry. Splenocytes were isolated on day 3 and 7 after infection Data shown are mean±SEM (n=5; *p<0.05). Published in [112].

In line with the human *in vitro* data, both, the numbers of $CD8^+$ $CD137^+$ (Fig. 26A) and $CD4^+$ $CD137^+$ (Fig. 26B) T cells were significantly higher in APN-KO mice as compared to WT mice on day 7of infection

In summary, APN negatively influenced the generation of antigen specific T cells *in vitro* and *in vivo* via the inhibition of proliferation and the enhancement of apoptosis.

4.2 Immunomodulatory effects of APN on NK cells

4.2.1 AdipoRs expression on human NK cells

First the distribution of AdipoRs was determined using flow cytometry on human $CD56^{+}$ peripheral blood NK cells. Remarkably, the majority of $CD56^{dim}$ NK cells expressed both AdipoRs while only a minor fraction of $CD56^{bright}$ NK cells showed a surface expression (Fig. 27A-C).

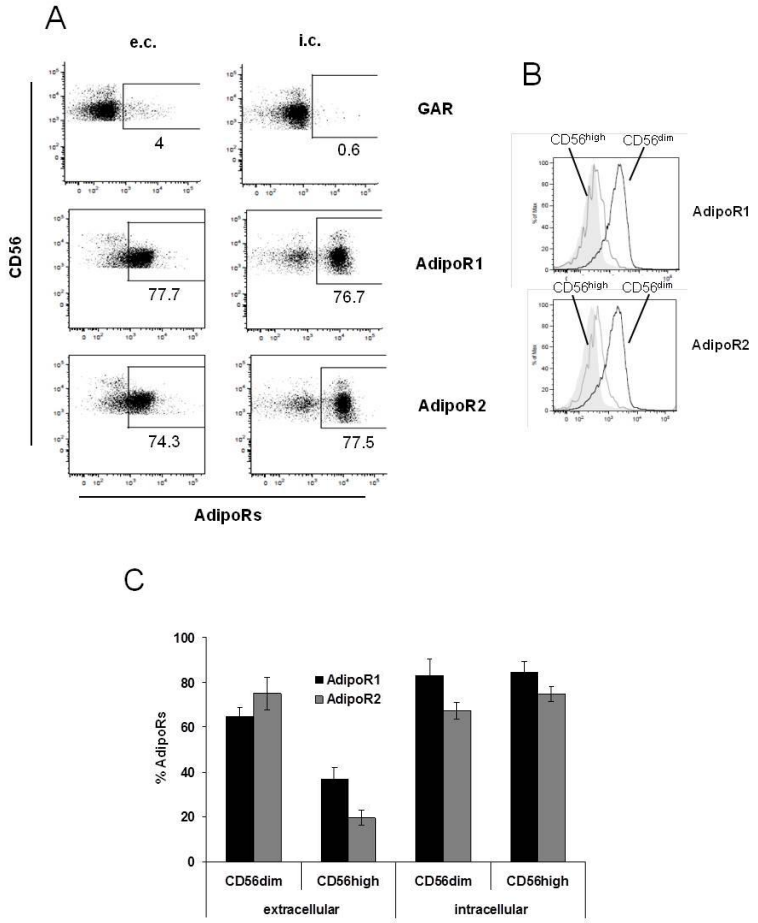

Figure 27: AdipoRs expression on human $CD56^{+}$ NK cells. (A) Extra- (e.c.) and intracellular (i.c.) staining of AdipoR1 and AdipoR2 on $CD56^{+}$ NK cells visualized by flow cytometry. One representative dot plot is shown. GAR indicates negative control and numbers represent the frequencies of positive cells. (B) Representative histograms of extracellular staining of AdipoR1 (upper panel) and AdipoR2 (lower panel) on $CD56^{dim}$ and $CD56^{high}$ NK cells. Filled grey area represents negative control. (C) Mean frequencies of e.c. and i.c. AdipoRs expression on $CD56^{dim}$ and $CD56^{bright}$ NK cells. Data shown are mean±SEM. (AdipoR1 n=8; AdipoR2 n=4). Published in [113].

Contrary, intracellular staining revealed the expression of both receptors in the majority of $CD56^{dim}$ and $CD56^{bright}$ NK cells. (Fig. 27C) but a small fraction of both NK cell subsets remained negative for the extracellular as well as for the intracellular expression of AdipoRs (Fig. 27A).

4.2.2 AdipoRs expression on murine NK cells

Flow cytometry analysis of AdipoRs expression on murine splenic $CD3e^{-}$ $Nkp46^{+}$ NK cells exhibited a similar expression pattern as it was illustrated for human (see Fig.8) and murine (see Fig.13) T cells. AdipoR1 and AdipoR2 expression was mainly limited to the cytoplasm of the analyzed cells. As shown in Figure 28A AdipoRs were expressed in the cytoplasm of nearly all $Nkp46^{+}$ NK cells of WT mice while AdipoR1 surface expression was detected on 14.5%±1 (mean±SEM) of murine NK cells of WT mice and mean 10.2%±1.3 (mean±SEM) of NK cells of APN-KO mice. Mean 15.5%±1.6 (mean±SEM) of NK cells of WT mice and mean 12.9%±3 (mean±SEM) of NK cells of APN-KO mice expressed the AdipoR2 extracellularly (Fig. 28A and B).

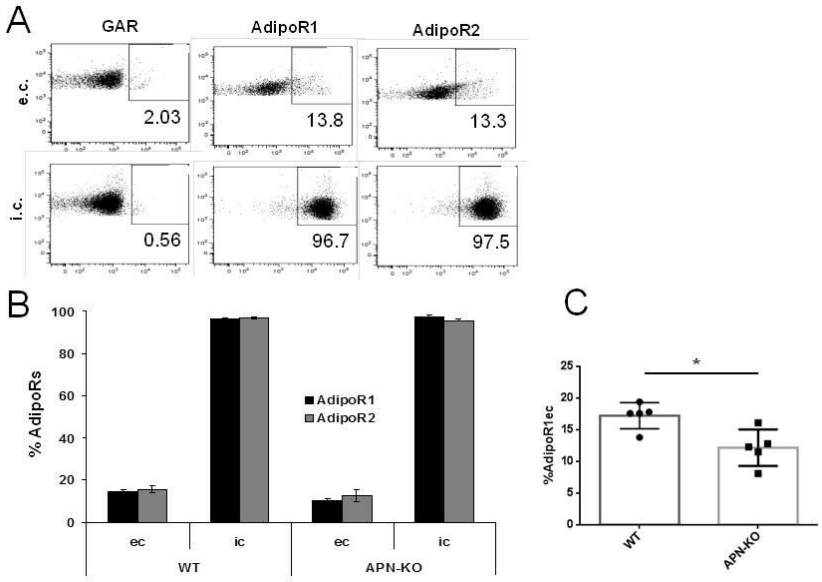

Figure 28: AdipoRs expression on murine splenic $Nkp46^{+}$ NK cells. (A) Representative dot plots of AdipoRs expression on WT mice. Numbers indicate percentage of positive cells. GAR represents negative control. (B) Average of extracellular (e.c.) and intracellular (i.c.) AdipoRs expression on $Nkp46^{+}$ NK cells of WT and APN-KO mice. (C) Surface AdipoR1 expression on NK cells of WT and APNKO mice. Data are mean±SEM (n=5; *$p<0.05$). Partially published in [113].

Whereas the absence of APN had no influence on the intracellular expression levels of AdipoRs and the extracellular expression of AdipoR2 (Fig. 28B), AdipoR1 expression was significantly diminished in APN-KO mice as compared to WT mice (Fig. 28C).

Murine NK cells are subdivided by the expression of CD11b and CD27 into four subsets characterizing their maturation status as described in section 1.4. Given that extracellular receptor expression could depend on developmental status of Nkp46^{+} NK cells, we used flow cytometry to analyze AdipoR1 distribution on the four subsets of murine NK cells in WT and APN-KO mice. Figure 29 shows that the immature NK cell subset CD11b^{low} CD27^{low} of WT as well as of APN-KO mice revealed significant higher AdipoR1 surface expression as compared to the more mature subpopulations.

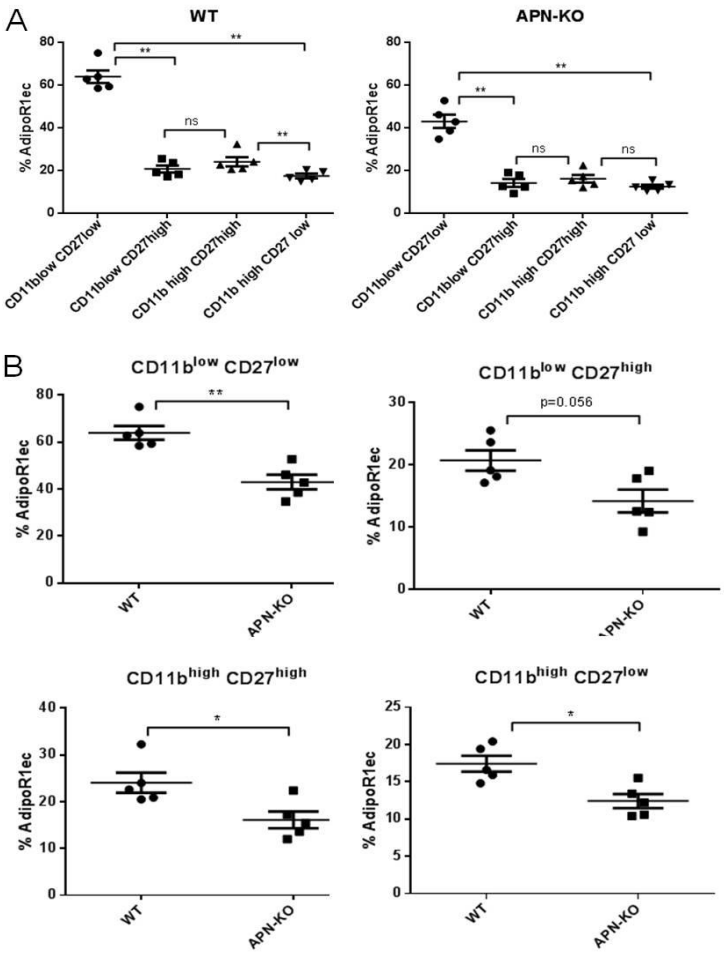

Figure 29: Surface AdipoR1 distribution on murine NK cell subsets of WT and APN-KO mice. (A) Surface AdipoR1 expression during developmental stages of murine Nkp46^{+}NK cells of WT and APN-KO mice. (B) Comparison of extracellular AdipoR1 expression during particular maturation stages of murine NK cells of WT and APN-KO mice. Data are mean±SEM (n=5; *p<0.05).

During progression of murine NK cell maturation surface AdipoR1 expression significantly dropped down in both mice strains (Fig. 29A). Comparing AdipoR1 surface expression on NK cell subsets studies have showed that APN-KO mice exhibited a lower receptor expression during all maturation stages as compared to WT mice (Fig. 29B). Looking at the immature NK cell subset $CD11b^{low}$ $CD27^{low}$ 63%±3 (mean±SEM) of NK cells of WT mice showed AdipoR1 surface expression compared to 42%±3 (mean±SEM) of NK cells of APN-KO mice.

4.2.3 AdipoRs regulation on NK cells

Whereas AdipoRs surface expression on T cells could be induced by activation, neither incubation with IL-2 nor with TLR-L was able to upregulate AdipoRs extracellularly on human $CD56^{bright}$ NK cells *in vitro* (data not shown).

In contrast, CVB3 infection of WT and APN-KO mice resulted in an upregulated surface expression of both AdipoRs on murine $NKp46^{+}$ cells of WT and APN-KO mice (Fig.30). Similar results were also obtained for murine $CD3e^{+}$ T cells (see Fig. 20).

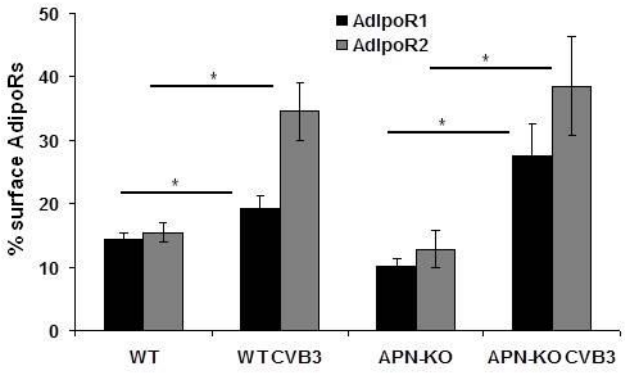

Figure 30: Regulation of surface AdipoRs expression on murine $Nkp46^{+}$ NK cells. Surface AdipoRs expression in uninfected and CVB3 infected murine NK cells was analyzed by flow cytometry (n=5 uninfected; n=4 infected; *p<0.05) Data are mean±SEM. Published in [113].

4.2.4 Functional effects of APN on NK cells

4.2.4.1 Human NK cells

To examine the functional effect of APN on human $CD3^{-}CD56^{+}$ NK cells we analyzed TLR-L induced IFNγ secretion using intracellular cytokine staining. Freshly isolated PBMCs were

primed with IL-12, preincubated with APN and stimulated with LPS and R848 for 24h to provoke cytokine production. $CD3^{-}CD56^{+}$ NK cells within PBMC cultures were determined for frequencies of IFNγ secretion.

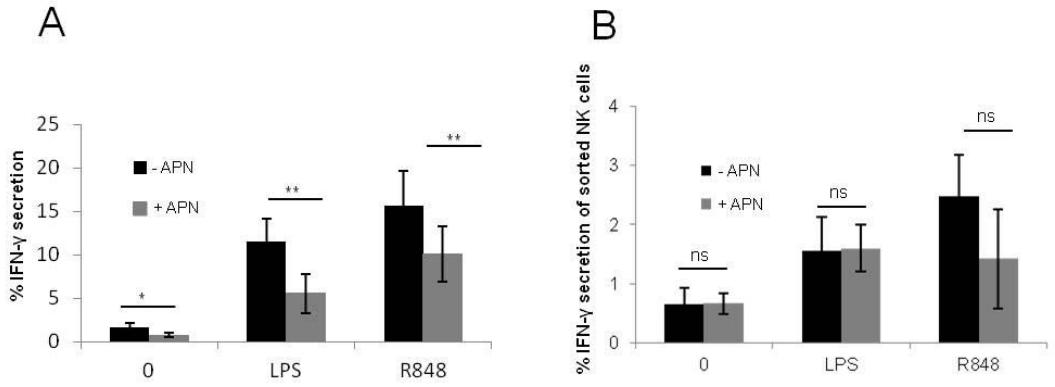

Figure 31: Functional effects of APN on TLR-L induced IFNγ secretion of human NK cells. (A) Frequencies of LPS and R848 induced IFNγ secretion of human NK cells within PBMCs (n=6; *p<0.05; ** p<0.01). (B) Frequencies of TLR-L induced IFNγ secretion of sorted human NK cells. Black bars indicated cultures raised in the absence of APN, grey bars represents cultures raised in the presence of APN (n=5; ns- not significant) Data were obtained by flow cytometry and are mean±SEM. Published in [113]

Independent of APN, LPS and R848 significantly increased the IFNγ expression of NK cell (Fig. 31A). However, APN incubation resulted not only in a decreased TLR-L induced IFNγ response but also affected the spontaneous IFNγ production in human $CD56^{+}$ NK cells.

To find out if the inhibitory effect of APN on IFNγ secretion is directly, this assay was performed also with sorted human NK cells (Fig. 31B). Generally, IFNγ production of sorted NK cells was lower (Fig.31B) as compared to IFNγ secretion of NK cells cultured with PBMCs (Fig. 31A). APN preincubation did not diminish spontaneous or LPS induced IFNγ secretion of sorted $CD56^{+}$ NK cells. R848 stimulated IFNγ production was lower, but this difference was not significant (Fig. 31B).

To further determine the influence of APN on human NK cells the effects of APN on NK cell degranulation and cytotoxicity were studied and analyzed by flow cytometry. Degranulation of human $CD56^{+}$ NK cells was analyzed by degranulation assay using mAb for the degranulation marker CD107a (Fig. 32A). IL-2 known as inducer of degranulation and cytotoxicity was used as a positive control. Human PBMCs were stimulated with APN, IL-2

or simultaneously with APN and IL-2 for 20h and incubated with K562 target cells in an effector to target ratio of 1:1 and 1:2.

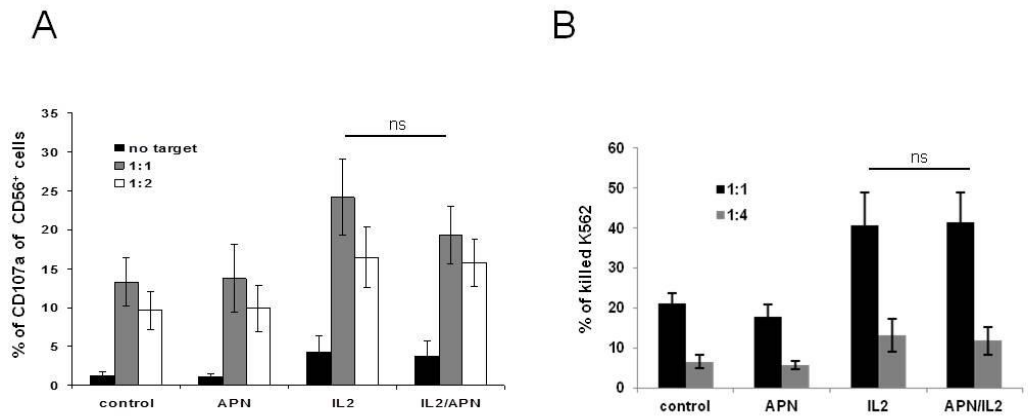

Figure 32: **APN influence on human NK cell degranulation and cytotoxicity.** (A) Frequencies of $CD107a^+$ human NK cells treated with APN, IL-2 or simultaneously with APN and IL-2 and incubated without (black bars) and with K562 target cells at indicated ratios (n=3; ns-not significant). (B) Frequencies of K562 target cells killed by sorted human NK cells. NK cells were cultured with APN, IL-2 or simultaneously with APN and IL-2 and incubated at effector to target ratios of 1:1 (black bars) and 1:4 (grey bars) (n=5; ns-not significant). Control indicates non treated cultures. Data are mean±SEM. Published in [113].

As shown in Figure 32A IL-2 induced $CD56^+$ NK cell degranulation whereas APN had no impact on frequencies of $CD107a^+$ NK cells as compared to non-treated controls. The co-incubation with APN had no influence on IL-2 induced degranulation. Degranulation was dependent on effector to target ratio in all cases. Cytotoxicity was determined by the cytometric analysis of fluorescence labeled K562 target cells killed by sorted NK cells (Fig. 32B). Again, APN had no effect on frequencies of killed target cells while IL-2 enhanced cytotoxicity as compared to non-treated and APN treated NK cell cultures. Induction was dependent on effector to target ratio. APN had no influence on neither basal nor IL-2 induced cytotoxicity of sorted human NK cells.

Taken together, APN had no effect on spontaneous or IL-2 induced degranulation and cytotoxicity of human $CD56^+$ NK cells.

4.2.4.2 Murine NK cells

To study the functional effects of APN *in vivo* we compared NK cells of uninfected and CVB3 infected APN-KO and WT mice. We studied NK cell subsets and receptor composition, cytotoxicity and cytokine release. The determination of murine NK cells within splenocytes by flow cytometry revealed elevated percentages of NK cells defined as $CD3e^-$ $Nkp46^+$ $DX5^+$ cells in spleens of APN-KO mice (Fig. 33A and B).

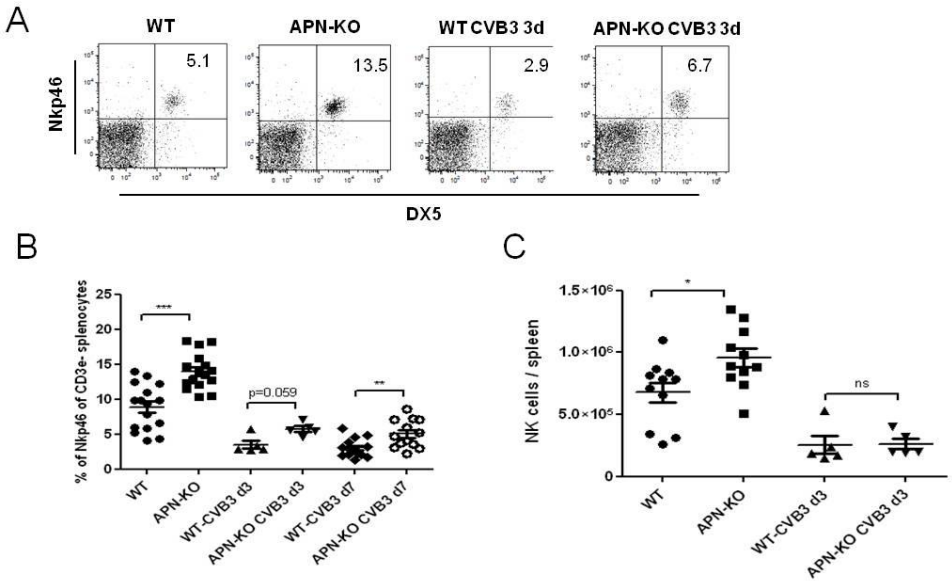

Figure 33: Increased NK cell frequencies and numbers in APN-KO mice. (A) Representative dot plots of $CD3e^-$ $Nkp46^+$ $DX5^+$ splenic NK cells of uninfected and three day infected WT and APN-KO mice. Numbers indicate frequencies of positive cells. (B) Percentages of splenic $CD3e^-$ $Nkp46^+$ NK cells in uninfected and infected WT and APN-KO mice (n=16 uninfected; n=5 three days after infection; n=12 seven days after infection; **p<0.01; *** p<0.001). (C) Absolute NK cell numbers per spleen calculated by flow cytometry of uninfected and infected WT and APN-KO mice (n=11 uninfected; n=5 infected; *p<0.05; ns-not significant). Published in [113].

Increased percentages of NK cells were observed in APN-KO mice independent of infection. CVB3 infection resulted in diminished frequencies of splenic NK cells. Still three and seven days after infection NK cells of APN-KO mice remained higher as compared to frequencies of WT mice. Analysis of absolute splenic NK cell numbers also revealed higher cell counts in uninfected APN-KO mice as compared to WT mice (Fig. 33C). Upon CVB3 infection we could not observe higher NK cell numbers in spleens of APN-KO mice. To further analyze the effects of APN deficiency on murine NK cell composition we comparatively determined

NK cells subsets characterizing maturation status by CD11b and CD27 expression in spleens of uninfected and CVB3 infected WT and APN-KO animals (Fig. 34). In uninfected as well as in seven days infected animals the terminally differentiated $CD11b^{high}$ $CD27^{low}$ subset represented the major cell fraction within splenic $Nkp46^{+}$ NK cells as measured by flow cytometry. Surprisingly the $CD11b^{high}$ $CD27^{high}$ subset representing the most potent effector NK cells was decreased in uninfected APN-KO mice (Fig. 34A) whereas APN-KO animals showed elevated frequencies of these effector NK cells upon CVB3 infection as compared to WT mice (Fig. 34B). Frequencies of the immature and naïve $CD11b^{low}$ $CD27^{low}$ murine NK cell subset did not differ between uninfected and infected WT and APN-KO animals but were increased upon CVB3 infection in spleens of both mouse strains.

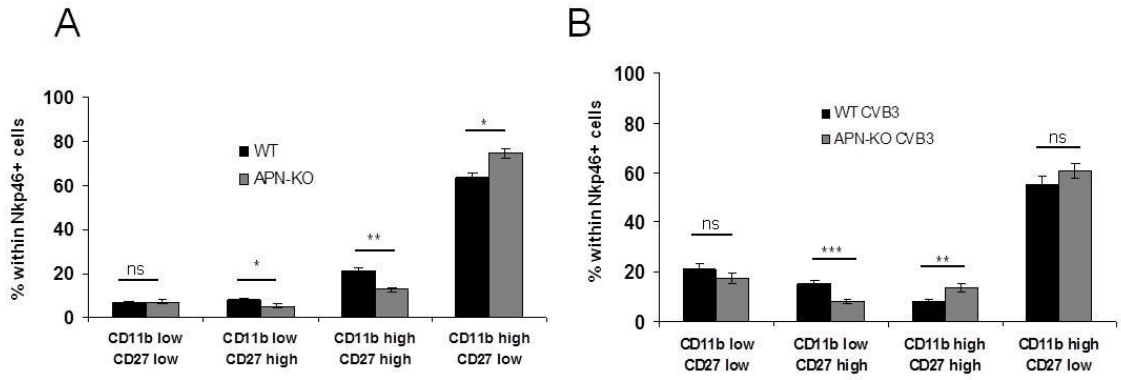

Figure 34: Murine NK cell subsets of uninfected and infected WT and APN-KO mice based on CD11b and CD27 expression. (A) Frequencies of splenic $NKp46^{+}$ NK cell subset in uninfected WT and APN-KO mice (n=5). (B) Frequencies of splenic $NKp46^{+}$ NK cell subset in seven day CVB3 infected WT and APN-KO animals (n=14). Data are mean±SEM (ns-not significant; *$p<0.05$; **$p<0.01$; ***$p<0.001$). Published in [113].

Next the degranulation of uninfected and infected WT and APN-KO animals was studied by CD107a expression and measured by flow cytometry. Splenocytes were removed, counted and used for CD107a degranulation assay. CD107a expression was dependent on effector to target ratio (Fig. 35). As shown in Figure 35 NK cell degranulation was strongly increased upon CVB3 infection as compared to uninfected animals. Seven days after infection CD107a expression declined to similar levels as shown for NK cells of uninfected mice. In line with the decreased frequencies of effector NK cells in APN-KO mice (Fig. 34A) degranulation was diminished in uninfected APN-KO mice as compared to WT mice (Fig. 35 left).

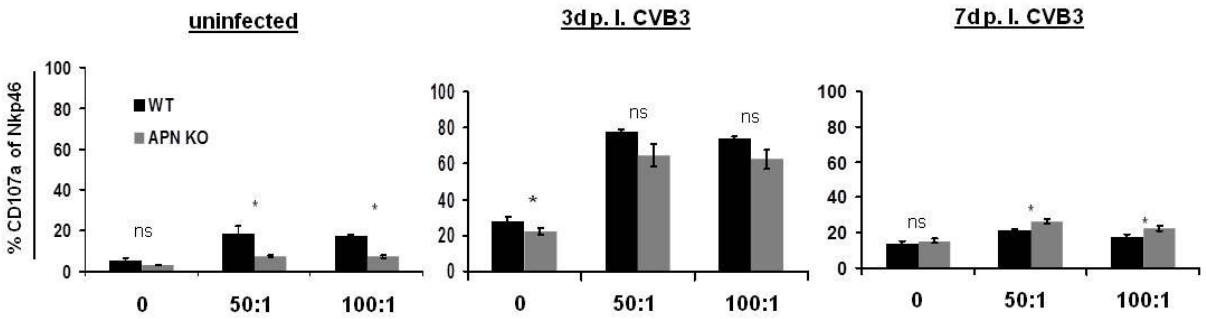

Figure 35: Degranulation of murine NK cells of uninfected and infected WT and APN-KO animals. Frequencies of degranulating $Np46^{+}$ NK cells within splenocytes of uninfected (left), three days infected (middle; 3d p.I.) and seven days infected (right; 7d p.I.) WT and APN-KO mice. CD107a secretion was analyzed in the absence (0) and in the presence of YAC-1 target cells in an effector to target ratio (splenocytes to YAC-1 cells) 50:1 and 100:1. Data are mean±SEM (n=5; ns-not significant; *$p<0.05$). Published in [113].

Interestingly, degranulation of APN-KO mice was significant higher compared to WT mice seven days after CVB3 infection (Fig. 35 right) consistent with the increased proportion of $CD11b^{high}$ $CD27^{high}$ effector NK cells at day seven (Fig. 34B).

To further determine the mechanism underlying the impaired ability to degranulate of NK cells of APN-KO animals we studied the expression of NK cell activating receptors NKG2D and CD94 characterizing a subset with a higher proliferative and cytotoxic ability. Consistent with the degranulation data of uninfected APN-KO mice NKG2D expression was diminished in splenic and peripheral NK cells of uninfected APN deficient animals compared to WT mice (Fig. 36A and B). Upon CVB3 infection NKG2D was strongly downregulated on splenic and peripheral NK cells of WT and APN-KO mice at day three and seven (Fig. 36A-C), but was not lower in APN-KO mice.

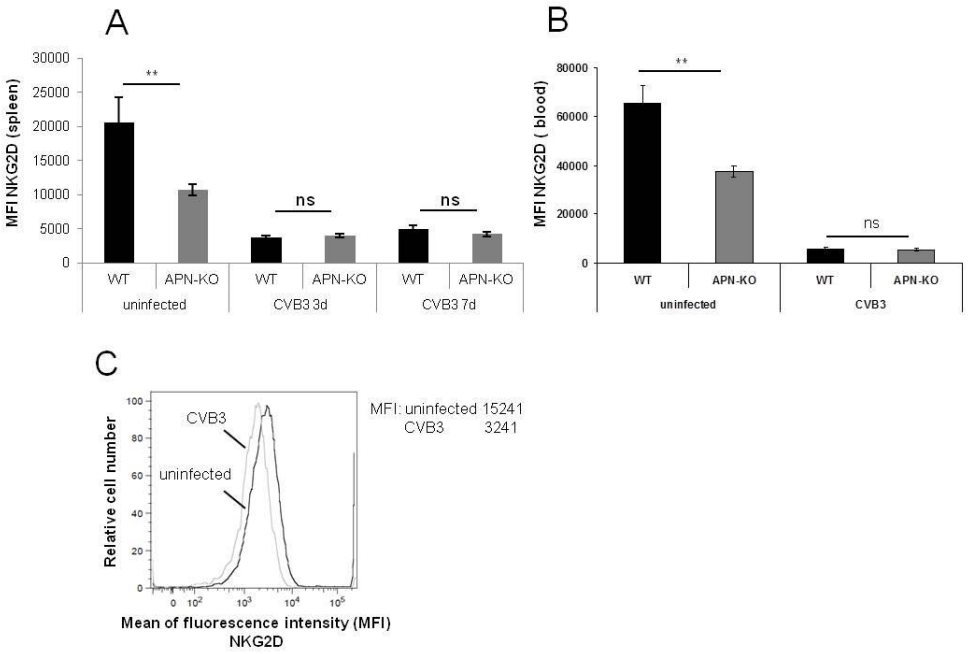

Figure 36: NKG2D expression on NK cells of uninfected and infected WT and APN-KO animals. Mean fluorescence intensity (MFI) of NKG2D in uninfected and CVB3 infected WT and APN-KO splenic (A) (uninfected n=5; infected n=7) and peripheral (B) NK cells (uninfected n=10; infected n=9; ns-not significant; **p<0.01). Data are mean±SEM. (C) Representative histogram of MFI NKG2D on splenic NK cells. Black line represents NK cells of uninfected and gray line of CVB3 infected animals on day seven. Published in [113].

$CD94^{high}$ cells are defined as a high proliferative and cytotoxic subset within murine $CD3^{-}$ $Nkp6^{+}$ NK cells. Similar to NKG2D expression in WT and APN-KO mice (Fig. 36) analysis of CD94 by flow cytometry revealed lower frequencies of $CD94^{high}$ NK cells in uninfected as well as in CVB3 infected APN-KO mice. In contrast, CD94 was not modulated upon CVB3 infection (Fig. 37).

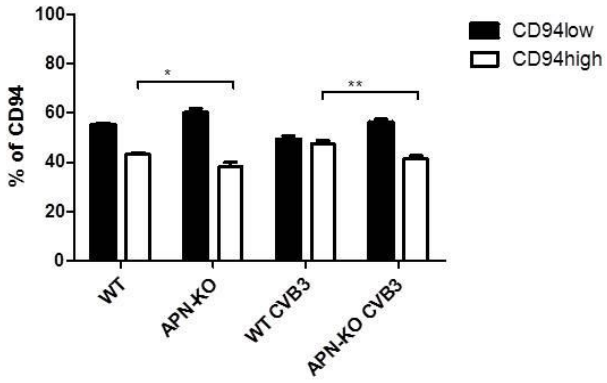

Figure 37: CD94^{high} and CD94^{low} subsets of murine NK cells in uninfected and infected WT and APN-KO mice. Percentages of CD94^{high} and ^{low} subsets of splenic CD3^{-} Nkp46^{+} NK cells analyzed by flow cytometry. Infected animals were sacrificed at day seven (n=5, uninfected and n=14 infected; *p<0.05; **p<0.01). Data are mean±SEM. Published in [113].

NK cell functionality is also defined by the ability of cytokine release. IFNγ expression of splenocytes of infected WT and APN-KO mice were determined by flow cytometry (Fig. 38). As demonstrated in Figure 38 CVB3 infection resulted in increased levels of NK cell secreted IFNγ. In line with degranulation and phenotype data infected APN-KO mice showed elevated frequencies of IFNγ producing NK cells.

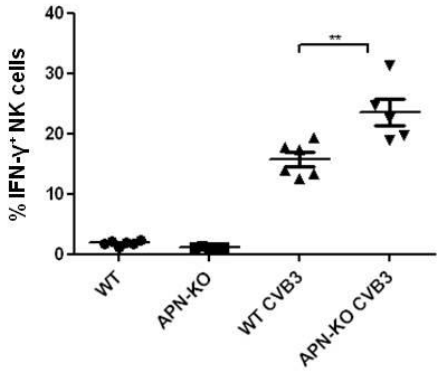

Figure 38: IFNγ production by NK cells of uninfected and infected WT and APN-KO mice. Frequencies of IFNγ producing murine CD3^{-} Nkp46^{+} NK cells assayed in uninfected and seven day infected WT and APN-KO mice (n=6 WT; n=5 APN-KO; **p<0.01). Published in [113].

5 DISCUSSION

Several previous *in vitro* and animal studies have already highlighted the importance of APN as an immunomodulator inhibiting the function of monocytes, macrophages and epithelial cells. Little has been known concerning its influence on T and NK cell function so far. This work investigated the effects of Adiponectin on T and NK cells *in vitro* and in adiponectin deficient mice exposed to CVB3 infection.

5.1 APN and T cells

T cells belonging to the adaptive immune system are the key players of anti-viral immunity. They are triggered when pathogens invade cells, are involved in the regulation of the adaptive immune response and are responsible for the specific elimination of pathogens.

In a first report Pang and Narendran showed the expression of both APN receptors on a small population of T cells [25]. To extend these findings we analyzed AdipoRs expression on T cells using qRT-PCR, flow cytometry and immunofluorescence. In accordance, both receptors were expressed only on a small fraction of T cells. Investigations of receptor expression in $CD3^+$ T cells and CD3 depleted PBMCs by qRT-PCR demonstrating high amounts of AdipoRs mRNA in $CD3^+$ T cells prompted us to examine a potential intracellular depot of AdipoRs. Intracellular flow cytometry analysis revealed that the majority of peripheral and bone marrow $CD4^+$ and $CD8^+$ T cells stored AdipoRs within the cytoplasm. Further, immunofluorescence staining of AdipoR1 and AdipoR2 demonstrated an internal storage in clathrin coated vesicles. Moreover, AdipoRs were co-expressed with TIRC7 and CTLA-4 in $CD3^+$ T cells. Cytoplasmatic storage and clathrin dependent intracellular trafficking has been illustrated not only for TIRC7 and CTLA-4 [94, 114] but also for co-stimulatory proteins, including EGF-R and IFNγ [115, 116]. Regulation, induction and control of surface exposition of intracellular stored molecules require activating signals for membrane transport. Like described for TIRC7 and CTLA-4 [117] AdipoRs were exposed upon antigen activation on the surface of activated $CD137^+$ T cells. Surface exposure occurred simultaneously with CTLA-4 surface expression within 72 hours and was detected up to nine days after antigen stimulation, an effect not observed on $CD137^-$ T cells. Interestingly, analysis of influenza specific T cells characterized by tetramer and IFNγ staining and SEB activated T cells revealed an activation dependent upregulation of AdipoRs. AdipoRs surface expression was

not only induced upon TCR specific stimulation but also by polyclonal T cell activation.

To test the functional consequence of AdipoRs upregulation on activated T cells we investigated the influence of APN on antigen activated $CD137^+$ T cell. *In vitro* APN incubation resulted in a diminished generation of antigen activated T cells following antigen stimulation and assessment by CD137 and cytokine staining. While the expansion of antigen activated $CD137^+$ and cytokine producing $CD8^+$ T cells was reduced in antigen activated cultures the presence of APN had no effect on $CD137^-$ T cells. APN was found to affect $CD137^+$, not $CD137^-$, T cells by exerting an anti-proliferating and apoptosis-promoting effect thereby leading to a diminished generation of antigen activated T cells. Engagement of upregulated CD137 on T cells is known to mediate co-stimulatory and anti-apoptotic functions, promoting T cell proliferation and T cell survival [91] by NFκB activation [118]. The upregulation of AdipoRs on $CD137^+$ T cells enabling APN action on these cells provides receptors to negatively influence T cell expansion and to retract immune responses. Anti-proliferating effects of APN were also described in endothelial cells [44], cancer cells [53, 54] myeloid [55] and myelomonocytic progenitor cells [79] where APN was found to downregulate anti-apoptotic gene transcription including the *bcl-2* gene after 48h. Otherwise pro-apoptotic genes like *p53*, *bak* and *bax* were not affected [55]. The pro-apoptotic action on $CD137^+$ T cells is in line with previous data demonstrating increased cell death caused by APN in hepatocellular carcinoma cells [48], gastric cancer cells [49] and endometrial carcinoma cells [50]. Further, APN exerted a direct inhibitory effect on antigen induced T cell cytokine production diminishing IFNγ and TNFα secretion in PBMCs cultures *in vitro*. A direct inhibitory APN effect has also been described for LPS induced TNFα [55], IL-6 [67] and IFNγ [68] secretion in macrophages constitutively expressing AdipoRs. APN diminished cytokine secretion was caused by a downregulation of mRNA transcription in consequence of suppressed NFκB translocation [67]. Given that the maximum of AdipoRs upregulation could be detected 72h after antigen stimulation on T cells the decreased cytokine production is suggest to be provoked or at least amplified by an APN dependent reduction of cytokine secretion of cells in the PBMC culture, mostly monocytes. Many effector functions of T cells are modulated by cells of the innate immune system [90] displaying abundant surface AdipoRs expression. To explore the influence of other PBMCs population on the reduced T cell cytokine secretion it would be necessary to perform these experiments in isolated $CD3^+$ T cells. Further, the measurement of AdipoRs upregulation in isolated and antigen stimulated

$CD3^+$ T cells and the blockade of AdipoRs by mAb would provide evidence of direct inhibitory function of APN on T cell cytokine secretion.

Consistent with the human data analysis of murine AdipoRs expression revealed a minor fraction of $CD3e^+$ T cells expressing surface AdipoR1 and AdipoR2. Expression was predominantly restricted to the cytoplasm of the analyzed cells. Moreover, like activated human T cells murine splenic T cells upregulated both receptors upon CVB3 infection. In contrast to studies by Bauche *et al*. where APN deficiency was shown to result in higher AdipoR2 expression levels compared to WT mice in adipose tissue [119] there was no difference in the AdipoRs expression pattern of resting and virus activated T cells of WT and APN-KO mice suggesting no influence of APN on the AdipoRs expression and regulation in murine T cells. Diet induced obesity was shown to upregulate murine AdipoR1 and AdipoR2 in the liver and AdipoR1 in muscle [120] whereas acute exercise increased murine AdipoR1 mRNA levels in liver and muscle [121]. To date there exist no further data about the expression and regulation of AdipoRs in murine T cells. The obtained murine data provide evidence of a similar AdipoRs regulation mechanism in T cells like we have shown for human T cells. In both cases antigen activation was followed by an upregulation of surface AdipoRs on T cells whereas resting T cells store both receptors intracellular.

Similarly to the *in vitro* data describing APN as a negative T cell regulator, APN-KO mice revealed significantly higher frequencies of antigen activated $CD137^+$ T cells upon CVB3 infection. In the murine system the engagement of CD137 as a second signal for T cells is required for the T cell IL-2 production in response to antigen activation, enhances IFNγ production and promotes T cell expansion and survival [92]. Higher frequencies of $CD137^+$ T cells in infected APN-KO mice result in an increased activation status of the immune system. In line with these findings several studies in APN-KO mice demonstrated beneficial effects of APN under inflammatory conditions. APN-KO mice are shown to be more susceptible to colitis [83] liver fibrosis [81] and sepsis provoked by increased cytokine levels [84]. Further, APN deficiency implicated acute cardiac allograft transplant rejection followed by higher infiltration of $CD4^+$ and $CD8^+$ T cells as well as increased TNFα and IFNγ levels [88]. APN administration in these disease models attenuated disease severity [80, 81]. The mechanism underlying the immunosuppressive effect of APN on T cells *in vivo* is most likely both directly and indirectly through innate immune cells. The generation of conditional knock-out of AdipoRs in PBMCs subsets except T cells would shed light on the direct effects of APN on T cells *in vivo*. As this is difficult to perform, a conditional knockout of AdipoRs on T cells

would provide an evidence of the indirect effects of APN.

Our findings may also provide an explanation for the observation that obesity is associated with diminished APN production but elevated numbers of T cells, increased T cell activation and IFNγ expression in adipose tissue [122] leading to a chronic inflammatory response. The state of low-grade inflammation includes an increased expression of pro-inflammatory cytokines among others, TNFα and IL-6 which in turn suppress APN transcription. [18, 22]. Low levels of systemic APN concentrations perpetuate without attenuating mediators of inflammation and the activated immune status but rather promote inflammation, generating a self-sustained inflammatory loop.

Although APN was shown to exert immunosuppressive function in animal models elevated APN serum levels found in chronic autoimmune diseases such as rheumatoid arthritis and inflammatory bowel disease [121] lead to the postulation of a dual effect of APN. APN has been shown to activate inflammatory processes including IL-6 and matrixmetalloproteinase-1 (MMP-1) upregulation via the p38 MAP kinase pathway [123]. On the contrary APN has exhibited potential anti-inflammatory effects in a mouse model of rheumatoid arthritis. The delivery of adiponectin resulted in significantly reduced disease severity and decreased mRNA levels of pro-inflammatory cytokines [124, 125]. Increased adiponectin levels were also found in individuals suffering from inflammatory bowel disease exerting pro-inflammatory effects by inducing cytokine production [126]. However, in experimental models of colonic inflammation, adiponectin-deficient mice have been reported to be either protected or more susceptible [83, 127]. Based on our data an alternate hypothesis is that APN is upregulated in autoimmune diseases through yet unknown factors in response to inflammation and acts as negative regulator to directly or indirectly modulate pathologically activated T cell response. Further, circulating and local adiponectin is composed of four isoforms hence it might be the ratio between them, rather than their respective concentrations, which determines the final action of this adipokine [23]. Whereas the HMW isoform is considered to exert pro-inflammatory actions, the LMW isoform is responsible for the anti-inflammatory effects of adiponectin [22]. Thus, another explanation for increased APN levels acting in a pro-inflammatory way is the upregulation of a distinct adiponectin isoform in chronic autoimmune diseases shifting the ratio of the present oligomers and provoking pro-inflammatory effects. In contrast to disorders typically associated with excess adiposity, like type 2 diabetes and cardiovascular diseases, APN levels are elevated in disorders unrelated to

increased adipose tissue suggesting the presence of a different adiponectin regulation mechanism and/or its isoforms with opposite functions.

In summary this study describes a novel function of APN as a cytokine negatively regulating T cell expansion and function attenuating an overwhelming T cell response. In contrast to other immunoregulatory receptor-ligand interactions being restricted to the interplay between immune cells, APN is abundant in human plasma and may exhibit an important role in systemic T cell regulation as well as provides an additional link between the immune system and metabolism.

5.2 APN and NK cells

Natural killer cells are innate effector lymphocytes involved in the first defense against infected or malignant cells. They act directly through mediating cellular cytotoxicity and secretion of cytokines essential for the clearance of infectious pathogens [98] and indirectly through regulation of the immune system [128].

Besides the expression of AdipoRs on T cells Pang and Narendran also demonstrated the presence of both receptors on a subset of human NK cells suggesting an immunomodulatory role of APN for NK cells [25]. In agreement, we found surface AdipoRs expression on the majority of $CD56^{dim}$ subset of human blood NK cells, whereas the majority of $CD56^{bright}$ NK cells were negative on the surface expressing both receptors intracellularly. However, a small fraction of both NK cell subsets lacked both surface and intracellular expression. Like shown for T cells displaying an intracellular storage of AdipoRs and surface upregulation upon TCR stimulation we supposed and confirmed a similar cytoplasmatic depot in $CD56^{bright}$ NK cells. We could not further upregulate AdipoRs surface expression neither by IL-2 nor TLR-L stimulation on $CD56^{dim}$ NK cells. As $CD56^{bright}$ NK cells are proposed as precursors of the $CD56^{dim}$ subset [129, 130] they exhibit a different surface receptor phenotype resulting in distinct functions. $CD56^{bright}$ NK cells have been shown to have low to absent expression of killer cell immunoglobulin-like receptors (KIRs), but high levels of CD94-NKG2A inhibitory receptors, whereas the opposite is true for $CD56^{dim}$ NK cells [105]. Further, both subsets show a different repertoire of chemokine receptors and adhesion molecules [131]. Maturation dependent NK cell receptor expression was also shown in murine NK cells [107]. AdipoRs expression may therefore be developmentally regulated during maturation being absent on the surface of $CD56^{bright}$ and transducing their signals only to $CD56^{dim}$ NK cells.

In line with the anti-inflammatory effects of APN observed on DCs, epithelial cells, monocytes and macrophages [36, 55, 68, 70] APN was observed to inhibit TLR-L induced IFNγ production in human NK cells in PBMCs cultures. A suppression of TLR response and reduction of IFNγ was also described in human macrophages [68, 69]. However, the inhibitory effect of APN on IFNγ production of NK cells was indirect, as it was not seen in TLR-L stimulated sorted human NK cells thus mediated indirectly by other cells and/or factors. NK cells express constitutively receptors for monocyte- and macrophage-derived cytokines (monokines) hence their responsiveness can be modulated by monocytes and macrophages [105]. APN diminished monokine secretion by accessory cells could result in decreased NK cell IFNγ production. Since APN was shown to downregulate C-C chemokine receptors (CCR) type 2 and CCR5 in monocytes [132] it also could impair the levels of NK cell monokine receptors reducing binding options and stimulatory effect of monokines on IFNγ secretion. Consistently, APN was shown to inhibit TLR family induced NFκB signaling in macrophages [69] thus resulting in the suppression of NFκB target genes including chemokines and chemokine receptors. Additionally, the absence of AdipoRs on the potent cytokine producing $CD56^{bright}$ NK cells [105, 106] corroborated the indirect action of APN on human NK cells. Interestingly, degranulation and cytotoxicity assessed by CD107a secretion and *in vitro* killing assay were not influenced by APN. Differential mechanism were postulated for the regulation of NK cell IFNγ production and cytotoxicity [133, 134] Our results are in accordance with the study of *O'Shea et al.* in which APN did not inhibit human NK cell cytotoxicity but partially restored the unfavorable effects of cigarette smoke [109]. On the contrary APN previously was shown to act as a negative regulator of NK cell cytotoxicity and IFNγ production in murine NK cells [71]. Differences between both studies may be attributed to unphysiological high concentration of APN used *in vitro* by Kim *et al*. The inhibitory effect was observed at 20µg/ml APN while lower amounts had no effects on neither NK cell cytotoxicity nor on IFNγ secretion in their study.

Interestingly, we identified a major difference between human and murine NK cells surface AdipoRs expression, which we observed only on a minor subset of resting murine NK cells.

Most murine NK cells stored AdipoRs intracellular and upregulated surface AdipoRs expression upon infection indicating an immunomodulatory role of APN mostly in activated murine NK cells, and resembling the upregulation of AdipoRs on human and murine T cells upon antigen activation. Surface AdipoR1 distribution was decreased in APN-KO mice

compared to WT mice during all maturation stages. Moreover, AdipoR1 was regulated developmentally revealing the highest AdipoR1 expression on the immature $CD11b^{low}$ $CD27^{low}$ NK cell subset in both mice strains. As shown for human NK cells also murine NK cells display maturation dependent receptor expression [107]. Interestingly, on the contrary to human NK cells, in the murine system the immature $CD11b^{low}$ $CD27^{low}$ NK cell subset showed the highest receptor expression suggesting the most prominent influence of APN not only on activated NK cells but on the first developmental stages of murine NK cells.

To further study the potential immunoinhibitory role of APN on NK cell function we compared CVB3 infected WT and APN-KO mice. Unexpectedly, we detected however, increased NK cells with a lower frequency of effector NK cells and diminished cytotoxic function in uninfected APN deficient mice suggesting a role of APN in NK cell maturation. In this regard a modulatory effect of APN on monocyte precursors and B lymphopoesis has been described in humans [55, 79]. In line with the lower frequency of $CD11b^{high}CD27^{high}$ NK cell subset representing the most potent effector NK cell subset [107, 108] we observed diminished NK cell degranulation in uninfected APN-KO mice. In addition, expression of NKG2D, an activating receptor required for NKG2D-dependent cytolytic activation [135-137] was decreased in APN-KO mice. Further the $CD94^{high}$ NK cell subset possessing a higher proliferative and cytotoxic potential [138] was diminished in APN-KO mice. Upon viral infection the frequency of the $CD11b^{high}CD27^{high}$ NK cell subset as well as cytotoxic function and IFNγ production were, however, restored, suggesting that APN plays a role in NK cell maturation and distribution but is not crucial for NK cell function. The general downregulation of NKG2D expression on NK cells we observed upon CVB3 infection is in accordance with the study by Muntasell *et a*l. [139] who similarly observed downregulation of NKG2D in response to CMV infection while the percent of degranulating NK cells triggered by NKp46 increased upon infection.

The effect of APN on activated NK cells cannot be adequately addressed in the APN-KO model since APN-KO mice had less functional NK cells per se. Based on the enhanced NK cell function observed upon infection in APN-KO mice one may, however, speculate that this is due to the lack of an inhibitory signal provided by APN.

In summary, our work describes APN as an indirect negative NK cell regulator in humans. This finding is in line with a number of previous studies having underscored the importance of APN as an immunoregulator for macrophages and as shown here for T cells *in vitro* as well as in various animal models. Moreover we show that compared to humans murine NK cells

differentially express AdipoRs and in human and murine NK cells were regulated developmentally. Further, APN-KO mice have lower frequencies of effector NK cells whose functionality is somehow restored upon infection. Thus in addition to its anti-inflammatory effects APN influences also number and differentiation status of NK cells.

Our data implicate APN as anti-inflammatory molecule with immunosuppressive properties and therapeutic potential. Of special interest in this regard is the observation that APN inhibits anti-viral memory and effector T cell and NK responses *in vitro* and in our animal model of autoimmune myocarditis (EAM), a model of human inflammatory cardiomyopathy (DCMi) [140]. In line with this APN exerted anti-inflammatory effects in patients with DCMi. Elevated APN concentrations were associated with better hemodynamic outcome in patients with DCMi. Also the effect of APN on regulatory T cell function which similar to conventional T cells store AdipoRs mostly intracellular (own unpublished results) may play an important role in immunoregulation.

Taken together these data suggest that APN up-regulation may be a promising therapeutic approach to ameliorate pathological inflammation.

6 REFERENCES

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7 Attachments

7.1 Publications

Bobbert P., Scheibenbogen C., Jenke A., Kania G., **Wilk S.,** Krohn S., Stehr J., Kuehl U., Rauch U., Erikson U., Schultheiss H.P., Poller W. and Skurk C. (2011)
Adiponectin expression in patients with inflammatory cardiomyopathy indicates favourable outcome and inflammation control. *European Heart Journal* 9, 1134-1147

Wilk S., Scheibenbogen C., Bauer S., Jenke A., Rother M., Guerreiro M., Kudernatsch R., Goerner N., Poller W., Elligsen-Merkel D., Utku N., Magrane J., Volk H.D. and Skurk C. (2011)
Adiponectin is a negative regulator of antigen activated T cells. *European Journal of Immunology.* 41, 2323-2332

Wilk S., Jenke A., Stehr J., Yang C.A., Bauer S., Goeldner K., Kotsch K., Volk H.D., Poller W., Schultheiss H.P., Skurk C. and Scheibenbogen C. (2013)
Adiponectin modulates Natural killer cell function. Accepted for publication in *European Journal of Immunology.* DOI: 10.1002/eji.201242382.

Jenke A., **Wilk S**., Rother M., Stehr J., Poller W., Eriksson U., Schultheiss H.P., Scheibenbogen C. and Skurk C. (2012)
Adiponectin inhibits Toll-like receptor 4 mediated cardiac inflammation and injury. In review in *Cardiovascuklar Research*

7.2 Posters

"Adiponectin receptor is a negative T cell regulator co-expressed with CTLA-4".
International Symposium 2010: New molecular Pathomechanisms and novel therapeutic Approaches in Heart failure. January 2010 Berlin

“Adiponectin receptors are co-expressed with CTLA-4 and their ligand Adiponectin is a negative regulator of antigen activated T cells”.
40th Annual Conference of the German society of Immunology. September 2010 Leipzig

“Immunmodulatory effects of Adiponectin on Natural Killer cells”.
International Symposium 2011: Novel cardiovascular therapies based on the modulation of cell migration and cell differentiation. February 2011 Berlin

“Adiponectin is a negative regulator of antigen activated T cells”.
International Symposium 2011: Novel cardiovascular therapies based on the modulation of cell migration and cell differentiation, February 2011, Berlin and. European Society of Cardiology Congress. September 2011, Paris. Winner in category moderated posters, presented by Carsten Skurk

Involved in:
“Adiponectin inhibits Toll-like receptor signaling-indication for inflammation control”.
International Symposium 2011: Novel cardiovascular therapies based on the modulation of cell migration and cell differentiation. February 2011, Berlin

“Adiponectin inhibits Toll-like receptor 4 mediated Cardiac Inflammation and Injury”
American College of Cardiology. March 2012 Chicago

“Cardioprotection and immunmodulation by Foxo3s as master regulator of Adiponectin”
Expertise of the Deutsche Forschungsgemeinschaft of the Sonderforschungsbereich Transregio 19 named „Inflammatory Cardiomyopathy-Molecular Pathogenesis and Therapy, Project B7. September 2012, Tübingen

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