Bachelorarbeit

Evaluation of cellular proteins binding to the transmembrane envelope protein gp41 of HIV-1

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Eidesstattliche Erklärung

Hiermit versichere ich eidesstattlich, dass ich die Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Braunschweig, der 05.11.2015

Tobias Kroniger

I'm a scientist and I know what constitutes proof. But the reason I call myself by my childhood name is to remind myself that a scientist must also be absolutely like a child. If he sees a thing, he must say that he sees it, whether it was what he thought he was going to see or not. See first, think later, then test. But always see first. Otherwise you will only see what you were expecting. Most scientists forget that.

Douglas Adams, *So Long, and Thanks for All the Fish*

Zusammenfassung

Das von HIV ausgelöste erwobene Immunschwäche-Syndrom (AIDS) stellt auch heute noch ein Problem dar. Im Jahr 2014 waren laut der Weltgesundheitsorganisation (WHO) 36,9 Millionen Menschen mit HIV infiziert. Heute, 30 Jahre nach der Entdeckung von HIV, gibt es zwar gute therapeutische Ansätze, eine Heilung ist aber bisher nicht in Aussicht. Während der Lebenszyklus von HIV bereits gut verstanden ist, ist noch unklar wodurch eine Immunsuppression in infizierten Menschen ausgelöst wird. Es gibt Hinweise darauf, dass eine hochkonservierte Domäne des transmembranen Hüllproteins gp41 von HIV dabei eine Rolle spielt. Jedoch konnte für gp41 bisher kein putativer Rezeptor mit immunmodulierenden Eigenschaften gefunden werden.

Im Rahmen dieser Bachelorarbeit sollte die Bindung von synthetischen Peptiden die der immunsuppressiven Domäne von gp41 entsprechen („Isu Peptid“) und rekombinant hergestelltem gp41 an humane PBMCs untersucht werden. Weiterhin sollte die Bindung des Isu Peptids an Subpopulationen von Lymphozyten und Monozyten analysiert und schließlich putative Interaktionspartner durch Immunpräzipitation isoliert und mittels Massenspektrometrie charakterisiert werden.

Es konnte ein spezifische Bindung des Isu Peptids an Monozyten und Lymphozyten festgestellt werden. Die Spezifität der Bindung wurde durch den Einsatz eines Peptids mit randomisierter Aminosäuresequenz gezeigt. Zusätzlich konnte die Spezifität der Binding an Monozyten mittels kompetitiver Hemmung demonstriert werden. Als Zielzellen des Isu Peptid wurden B Zellen, sowie klassische und intermediäre Monozyten identifizert. Eine Charakterisierung des rekombinanten gp41 ergab, dass es trimerisiert und N-glykolisiert ist und sich in der six-helix bundle Konformation befindet. Weitere Untersuchung zeigten, dass die Bindung des gp41 zu humanen PBMCs schwach, aber konzentrationsabhängig ist. Abschließend wurde eine Immunpräzipitation mit Isu Peptiden und gp41 an humanen PBMCs durchgeführt, die Proben wurden mittels Massenspektrometrie analysiert. Die Ergebnisse der Massenspektrometrie werden in wenigen Wochen erwartet.

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Abbreviations

AIDS	Acquired immune deficiency syndrome
BCA	Bicinchoninic acid
BS^3	Bissulfosuccinimidyl suberate
BSA	Bovine serum albumin
CA	Capsid
CHR	C-terminal heptad repeat
DC	Dendritic cell
ddH_2O	Double-distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
Env	Envelope
ER	Endoplasmic reticulum
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
gp	Glycoprotein
His	Histidin
HIV	Human immunodefiency virus
HSA	Human serum albumin
HTLV	Human T cell leukaemia virus
IgG	Immunoglobulin G
IN	Integrase
Isu	Immunosuppressive
k_a	Association rate
K_D	Equilibrium dissociation constant
k_d	Dissociation rate
LAV	Lymphadenopathy-associated virus
LPS	Lipopolysaccharide
LTR	Long terminal repeat

MA	Matrix
MACS	Magnetic activated cell sorting
NC	Nucleocapsid
NHR	N-terminal heptad repeat
NiNTA	Nickel-nitrilotriacetic acid
NK	Natural killer cell
PBMC	Periphal blood mononuclear cells
PBS	Phosphat buffered saline
PBS-T	PBS supplemented with 0.05 % Tween® 20
PR	Protease
RNA	Ribonucleic acid
RPMI	Rosswell Park Memorial Institute
RRE	Rev response element
RT	Reverse transcriptase
scr	Scrambled
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSC	Side scatter
ssRNA	Single-stranded RNA
TAR	Trans-activating response element
TBS-T	Tris buffered saline supplemented with 0.05 % Tween® 20
TLR	Toll-like receptor
TM	Transmembrane
TMB	3,3',5,5'-Tetramethylbenzidine
WHO	World Health Organisation

Amino acid single letter code

A	Alanine
C	Cysteine
D	Aspartic acid
E	Glutamic acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

CHAPTER 1

Introduction

The human immunodefiency virus (HIV) belongs as a member of the genus *Lentivirus* to the family of *Retroviridae*. The infection with HIV can lead to a disease called acquired immune deficiency syndrome (AIDS). With disease progression, AIDS may cause a failure of the immune system, making common infections to a serious threat. Even 30 years after the discovery of HIV, AIDS continues to be a serious health issue. The World Health Organisation (WHO) announced in 2014 that 36.9 million people were living with HIV while 70 % of these people were living in sub-Saharan Africa. In the same year, an estimated number of 1.2 million people died because of AIDS and AIDS-related diseases. Further, 2 million people were assessed to be newly infected (WHO, 2014).
In 2013 the total number of people living with HIV in Germany was determined to 80000, with 2300 newly infected patients and 550 deaths because of the consequences (Robert Koch-Institute, 2014).
Since AIDS cannot be cured, these data demonstrate the need for improved therapies or prophylactic vaccination.

1.1 The human immunodefiency virus

1.1.1 History

The pneumonia infection by *Pneumocystis carinii* is almost exclusively limited to severely immunosuppressed patients (Walzer et al., 1976). A first connection between pneumonia infection and the homosexual lifestyle was reported in 1981 (Gottlieb et al., 1981). In the following year, after several cases with identical symptoms from outside the gay community were observed, it was agreed to call the disease acquired immune deficiency syndrome (AIDS). In 1983, the french research group around Françoise Barré-Sinoussi and Luc Montagnier isolated a previously unknown lymphotrophic lentivirus from a patient with AIDS and called the virus lymphadenopathy-associated virus (LAV) (Barré-Sinoussi et al., 1983). In the same issue of Science, the research group of Robert Gallo published the isolation of a new human T cell leukaemia virus (HTLV) from an AIDS patient (Gallo et al., 1983). The group called the virus HTLV-III. Two years later, the LAV and HTLV-III were discovered to be identical (Marx, 1985), which in 1986 were renamed by the International Committee on Taxonomy of Viruses to HIV (Coffin et al., 1986).

1.1.2 Origin

HIV-1 can be divided into the three groups Outlier (O), Major (M) and New (N). It has been shown, that these groups are closely related to a strain of simian immunodefiency virus (SIV) isolated from common chimpanze (*Pan troglodytes*), indicating that *Pan troglodytes* is the source of at least three independent zoonotic transmissions, one for each HIV-1 group (Gao et al., 1999). It is assumed that SIV got transmitted to humans by direct exposure of animal blood or musocal secretions by hunting and butchering chimpanzees and other primates or by eating of so called “bushmeat” (Robinson et al., 1999; Sharp et al., 2001). The transmission from human to human of HIV-1 occurs by sexual contact, exposure of body fluids or from mother to child during birth or breast-feeding (Levy, 1993).

1.1.3 Structure and genome

The viral genome consists of two ssRNA with nine open reading frames (Figure 1.1 B), coding for 15 proteins (Table 1.1) (Frankel et al., 1998; Watts et al., 2009). With a diameter between 110–128 nm, the mature HIV particles are comparatively large (Gentile et al., 1994). In the viral capsid (CA), the two copies of positive viral ssRNA of HIV-1, which are bound to nucleocapsid (NC), are located with essential proteins such as reverse transcriptase (RT), nuclease, ribonuclease and integrase (IN). The inner side of the virus membrane is surrounded by the matrix (MA) protein (Gelderblom et al., 1987), which allows to anchor the envelope (Env) proteins (Figure 1.1 A) and is vital for virion integrity.

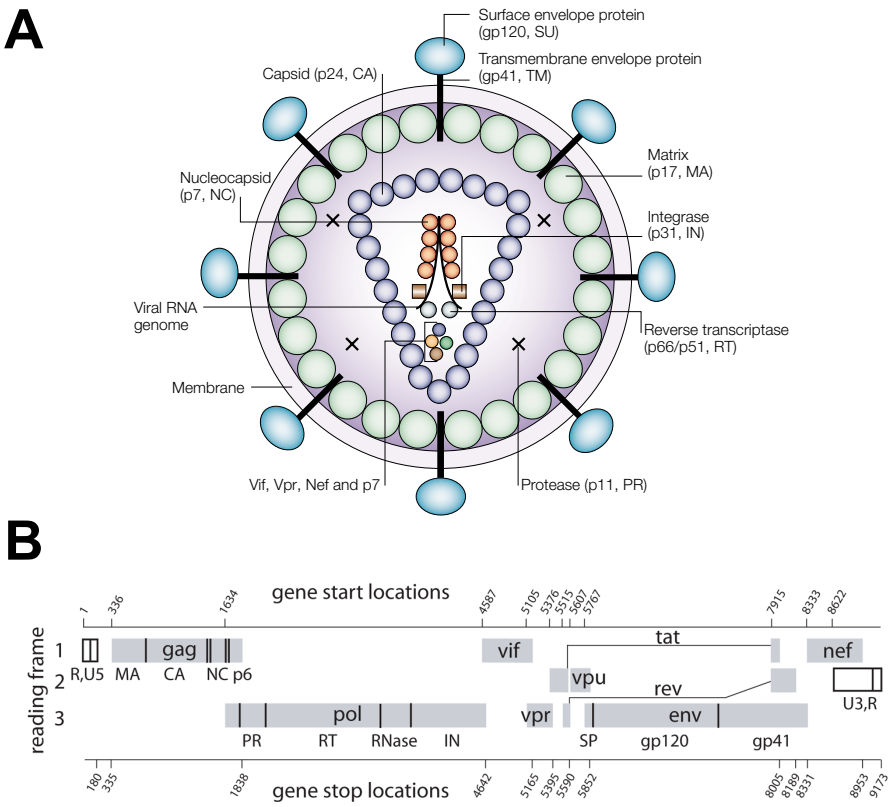

Figure 1.1: Schematic structure and genome of HIV-1. (A) Schematic structure and (B) genome of HIV-1. The genome of HIV-1 consists of 9 genes. *gag* codes for four structural proteins (MA, CA, NC and p6), *pol* codes for four enzymes (protease (PR), RT, RNase, IN), *env* codes for two envelope proteins (gp120, gp41) and *vif*, *vpr*, *vpu*, *tat*, *rev* and *nef* which code for regulatory proteins. Numbering according to NL4-3. Figure (A) taken from Robinson, 2002; Figure (B) taken from Watts et al., 2009.

Table 1.1: HIV-1 genes, proteins and functions.

Class	Gene name	Protein	Function
Structural proteins	*env*	gp41	Mediates membrane fusion between HIV and target cell (Camerini et al., 1990)
		gp120	Attaches the virus to CD4 receptor (Landau et al., 1988)
	gag	MA	Mediates viral assembly (Scarlata et al., 2003)
		CA	Forms the capsid cone, which contains the genetic material and essential proteins (Ganser-Pornillos et al., 2008)
		NC	Nucleic acid condensing and chaperoning protein, which binds viral RNA (Darlix et al., 2014)
		p6	Mediates interactions between Gag and Vpr (Paxton et al., 1993)
	pol	RT	Transcribes the viral RNA to DNA (Temin et al., 1970)
		IN	Mediates insertion of the viral DNA into the genome of the target cell (Bushman et al., 1990)
		PR	Cleaves synthesized polypeptide chains and thus initiates the maturation of infectious particles (Sadiq et al., 2012)
Regulatory proteins	*tat*	Tat	Enhances the viral transcription efficiency (Ulich et al., 1999)
	rev	Rev	Regulates the viral protein expression (Cullen, 1991)
Accessory proteins	*nef*	Nef	Downregulates CD4 (Garcia et al., 1992) and MHC Class I cell surface expression (Schwartz et al., 1996) and prevents apoptosis of infected cells (Xu et al., 2001)
	vpr	Vpr	Regulates the nuclear import of the viral DNA (Guenzel et al., 2014)
	vif	Vif	Suppresses the antiviral activity of the host (Feng et al., 2014)
	vpu	Vpu	Mediates proteasomal CD4 degradation and enhances virion release (Dubé et al., 2010)

1.1.4 HIV-1 entry and replication cycle

Since the surface envelope protein glycoprotein (gp)120 shows a high affinity ($K_D \sim 4\,nM$, Frankel et al., 1998) to the CD4 receptor, the main target of HIV-1 are $CD4^+$ cells (described in Section 1.2) (Dalgleish et al., 1984). After the viral core enters the cytoplasm of the cell through the fusion pore, the viral RT reversely transcribes the viral RNA to DNA (Coffin et al., 1997). Unlike DNA polymerases, the RT has no proofreading ability, thus mutations frequently occur. The mutation rate of the viral RT is about 3.4×10^{-5} per base pair per cycle (Mansky et al., 1995). This high mutation rate results in a high genetic diversity of the virus, where every possible single-point mutation may be found more than 10^4 times a day in an infected individual (Coffin, 1995). After the reverse transcription, the viral DNA is inserted into transcriptional active regions of the host genome (Schröder et al., 2002), where the virus uses the host transcription and translation machinery for the production of viral proteins (Walsh et al., 2011). Interestingly, it has been shown that 95 % of the $CD4^+$ T lymphocytes are bystander cells, which are not susceptible for viral integration. Consequently, these cells do not replicate the virus (Doitsh et al., 2010). When the viral DNA is integrated, the regulatory protein Tat binds to the trans-activating response element (TAR), whose structure plays an important role for the activation of the long terminal repeat (LTR) promotor (Jones et al., 1994; Kulinski et al., 2003). After the DNA is transcribed by the host, the RNA-binding protein Rev binds to a highly conserved domain, called "Rev response element (RRE)", which is present on all partially spliced or unspliced viral messenger RNA transcripts. The RRE mediates the transport of these transcripts from the nucleus to the cytoplasm (Fernandes et al., 2012). Viral RNA and associated proteins assemble at the plasma membrane (Goto et al., 1998), preferably in cholesterol-rich lipid domains, such as lipid rafts (Liao et al., 2003; Yang et al., 2015), where the viral particles bud from the host cell. In the subsequent maturation process, the viral protease cleaves five different sites within Gag and five different sites within Gag-Pro-Pol (Sundquist et al., 2012), resulting in a rearrangement of the immature virus to the mature virus, which is an essential step for the infectivity of the virus (Meek et al., 1990). A schematic overview of the HIV-1 replication cycle is illustrated in Figure 1.2.

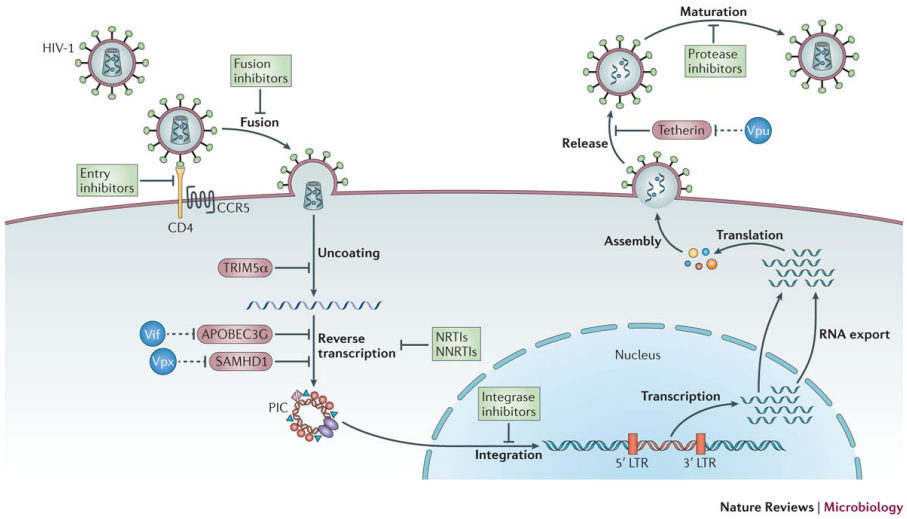

Figure 1.2: Schematic overview of the replication cycle of HIV-1. The figure shows the main steps of the HIV-1 replication cycle (bold), antiviral drug targets (green), HIV-1 restriction factors (red) and their viral antagonist (blue). CCR5 – C-C chemokine receptor type 5; CD4 – cluster of differentiation 4; HIV-1 – human immunodefiency virus 1; LTR – long terminal repeats; NRTIs – nucleoside reverse transcriptase inhibitors; NNRTIs – non-nucleoside reverse transcriptase inhibitors; PIC – pre-integration complex. Figure taken from Barre-Sinoussi et al., 2013.

1.2 The HIV-1 envelope protein

The *env* gene codes for the precurser glycoprotein gp160, which is endoproteolytical cleaved (McCune et al., 1988; Stein et al., 1990) by the cellular protease furin (Hallenberger et al., 1992). While the resulting glycoproteins gp120 and gp41 have a comparable genomic length, they highly differ in their molecular weight due to different glycosylations. While gp120 contains 24 potential sites for N-glycosylation (Leonard et al., 1990), that account for 40–50 % of its molecular weight (Leonard et al., 1990; Poignard et al., 2001), wheras gp41 has four glycosylation sites (Myers et al., 1989).

Gp120 plays a vital role in the attachment of the virus to the target cells before membrane fusion (Figure 1.3 A). The interaction with the cellular receptor CD4 results in a conformational change of gp120 (Sattentau et al., 1991; Thali et al., 1993) and an exposure of the chemokine binding domains which either interact with the co-receptor CCR5 (Deng et al., 1996; Trkola et al., 1996), CXCR4 (Bandres et al., 1997) or both (Doranz et al., 1996).

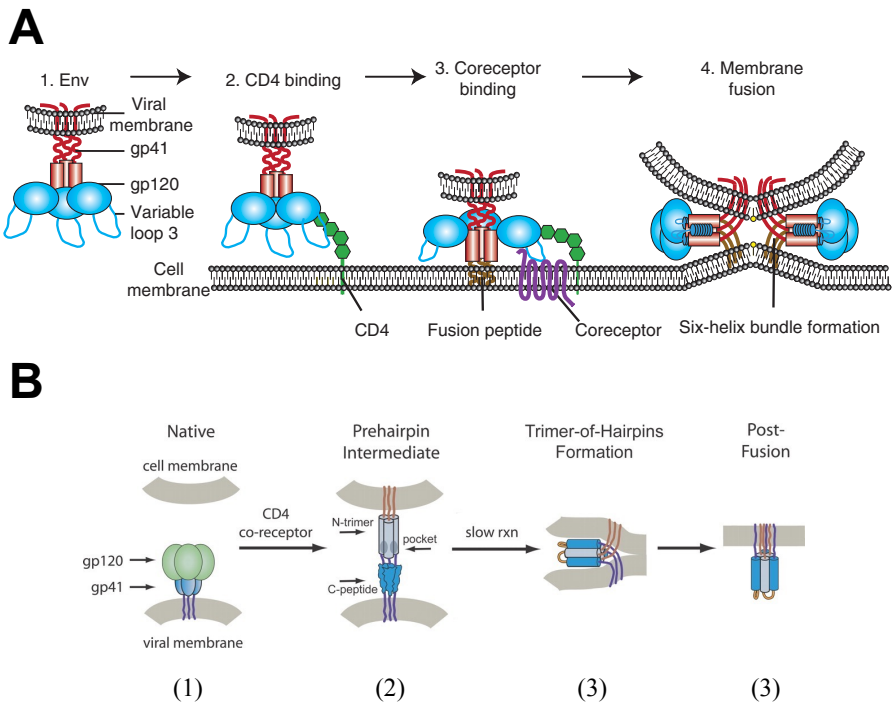

Figure 1.3: Env mediated virus and target cell membrane fusion. (A) (1) Native trimeric state of gp120 and gp41. (2) Attachement of gp120 to CD4 resulting in a conformational change of gp120 and an exposure of V3 which interacts with the coreceptor (3). Subsequently, the insertion of the fusion peptide of gp41 into the target cell membrane is triggered, followed by formation of six-helix bundle which leads to the membrane fusion of the virus and the target cell (4). Figure taken from Wilen et al., 2012. (B) Conformations of the trimeric gp41 during membrane fusion. The three different conformations of the trimeric gp41 during membrane fusion: (1) Pre-fusion; (2) prehairpin intermediate and (3) six-helix bundle. Figure taken from Welch et al., 2007

1.2.1 gp41

The transmembrane subunit gp41 of HIV-1 consists of a hydrophobic fusion peptide, a N-terminal heptad repeat (NHR) and a C-terminal heptad repeat (CHR), an immunosuppressive domain and a cysteine loop (Figure 1.4). In addition to intact viruses, for which gp41 is either exposed after binding of gp120 or after shedding of gp120 as monomeric or trimeric stumps (Pinter et al., 1989), various alternative sources for presentation of gp41 to the immune system exist. Those include cell surfaces, dendritic cells or in immune complexes (Denner, 2014).

The trimeric gp41 is essential in mediating membrane fusion between the virus and target cell membranes (Figure 1.3 B) (Camerini et al., 1990). The attachment of gp120 to CD4 and co-receptors results in a conformational changes of the pre-fusion state of gp41, which lead to the insertion of the fusion peptide of gp41 into the target cell membrane, causing an exposure of the NHR and CHR (Wilen et al., 2012). This conformation is called pre-hairpin intermediate. Subsequently, the unstable pre-hairpin intermediate changes its conformation to a six-helix bundle, bringing the viral and target cell membranes into close proximity for fusion.

1.2.2 Retroviral immunosuppression and the immunosuppressive domain

While retroviral infections are often accompanied by immunosuppression, the mechanism of induction of the immunosuppression is still unclear. Nevertheless, it has been shown that immunosuppression is observed with non-infectious virus particles and transmembrane (TM) proteins (Oostendorp et al., 1993), indicating an influence of TM proteins on the retroviral induced immunosuppression. Further, it was demonstrated that peptides corresponding to a so called "immunosuppressive (Isu) domain" have immunosuppressive properties (Cianciolo et al., 1985; Denner et al., 1994) and are highly conserved among retroviruses (Bénit et al., 2001) (Figure 1.5). The Isu domain of gp41 of HIV-1 is located between the NHR and the cysteine loop (Figure 1.4) and consists of 19 amino acids.

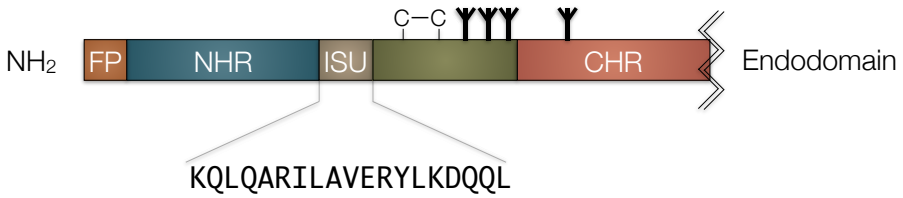

Figure 1.4: Schematic presentation of gp41 of HIV-1. NH_2 – N-terminus; FP – fusion peptide; NHR – N-terminal helical region; ISU – Isu domain; C-C – cysteine loop; CHR – C-terminal helical region.

It was shown that synthetic peptides corresponding to the Isu domain of gp41 inhibit lymphocyte proliferation, when conjugated to bovine serum albumin (BSA) (Denner et al., 1994, 1996; Ruegg et al., 1989). Furthermore, recombinant gp41 modulates an upregulation of the cytokine IL-10, while the cytokines IL-2, IFN-γ, and IL-4 are downregulated (Barcova et al., 1998; Speth et al., 2000). Nevertheless, a possible contamination by endotoxin, which also induces an IL-10 upregulation, can not be excluded, because the recombinant gp41 was produced in *E.coli*. However, glycosylated and endotoxin-free gp41, which was produced in 293T cells, induced a 700-fold higher cytokine modulation compared with synthetic Isu peptide homopolymers. Single amino acid substitutions lead to the loss of this property (Morozov et al., 2012).

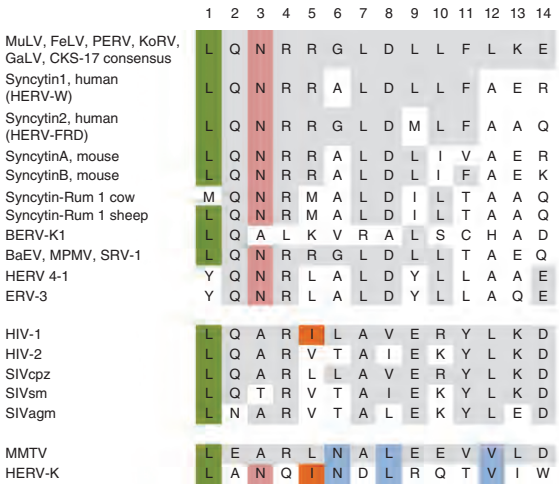

Figure 1.5: Comparison of immunosuppressive domain sequences of different retroviruses. grey – identical to first sequence of each group; green – identical in all retrovirus; pink – identical in all gammaretrovirus and HERV-K; orange – identical in HIV-1 and HERV-K; blue – identical in MMTV and HERV-K; BaEV – baboon endogenous retrovirus; BERV-K1 – bovine endogenous retrovirus-K1; CKS-17 consensus – consensus sequence of gammaretroviruses; FeLV – feline leukemia virus; GaLV – gibbon ape leukemia virus; HERV – human endogenous retrovirus; KoRV – koala retrovirus; MPMV – Mason-Pfizer monkey virus; MMTV – mouse mammary tumor virus; MuLV – murine leukemia virus; PERV – porcine endogenous retrovirus; SIVagm – simian immunodefiency virus of the african green monkey; SIVcpz – simian immunodefiency virus of the chimpanzee; SIVsm – simian immunodefiency virus of the sooty mangabey; SRV-1 – simian retrovirus-1. Figure taken from Denner, 2014.

There are several studies describing interaction partners of gp41 (a selection is presented in Table 1.2). These putative interaction partners vary in size and have been detected with different methods of detection and "bait" proteins. These "bait" proteins were either monomers of proteins corresponding to the immunosuppressive domain of gp41, Isu peptides coupled to human serum albumin (HSA) or soluble gp41 that has been produced in *E.coli*. However, a receptor that is involved in immunomodulation has not been described yet.

Table 1.2: Previously found putative receptors of gp41 or Isu peptide.

Protein	Method	Binding partner	Reference
Isu peptide	^{125}I labeled peptides	40 kDa, 60 kDa, 100 kDa	Denner et al., 1993, 1995
Isu peptide coupled to HSA	^{125}I labeled peptides	44 kDa	Qureshi et al., 1990
Isu peptide coupled to HSA	^{125}I labeled peptides	45 kDa, 80 kDa	Henderson et al., 1993
Soluble gp41 (*E.coli*)	^{125}I cell surface labeling	44 kDa, 96 kDa, 108 kDa	Ebenblicher et al., 1993
Soluble gp41 (*E.coli*)	Western blot, ^{125}I cell surface labeling	WB: 37 kDa, 55 kDa; IP: 108 kDa; Both assays: 40 kDa, 97 kDa	Chen et al., 1992

1.3 Aim of this thesis

In previous studies mainly two different immunoprecipitation approaches were used. On the one hand bacterial recombinant gp41 was used as "bait" protein, on the other hand synthetic peptides corresponding to the Isu domain were used. However, recombinant gp41, which is produced in bacterial systems, might be a problem when it comes to immunoprecipitation or cytokine arrays. Endotoxin contamination can lead to precipitation of lipopolysaccharide (LPS) receptor complex molecules (Toll-like receptor (TLR)4, CD14 and MD-2) and also induces an upregulation of IL-10 in cytokine arrays. Here, the ectodomain of gp41 has been produced in stably transfected 293 cells by the coop-

eration partner ImmunoTools (Figur 1.6). By the production in stably transfected 293 cells, it was expected that the amount of endotoxin contamination could be decreased or even prevented. In addition, the expression in eucaryotic cells may lead to the correct N-glycolysation of the gp41 antigen. Thus, the produced ectodomain of gp41 was used for evaluation of binding partners. As a second "bait" protein, similar to previous work (Table 1.2), synthetic peptides corresponding to the immunosuppressive domain of gp41 (Section 2.9) were used. These peptides were either with or without a biotin-tag, which simplifies the detection by its high affinity interaction with streptavidin. As mentioned, peptides corresponding to the Isu domain were reported to have immunosuppressive properties, which could be abrogated with single amino acid substitutions (Morozov et al., 2012). Here, the binding of Isu peptides to subpopulations of human PBMCs was examined.

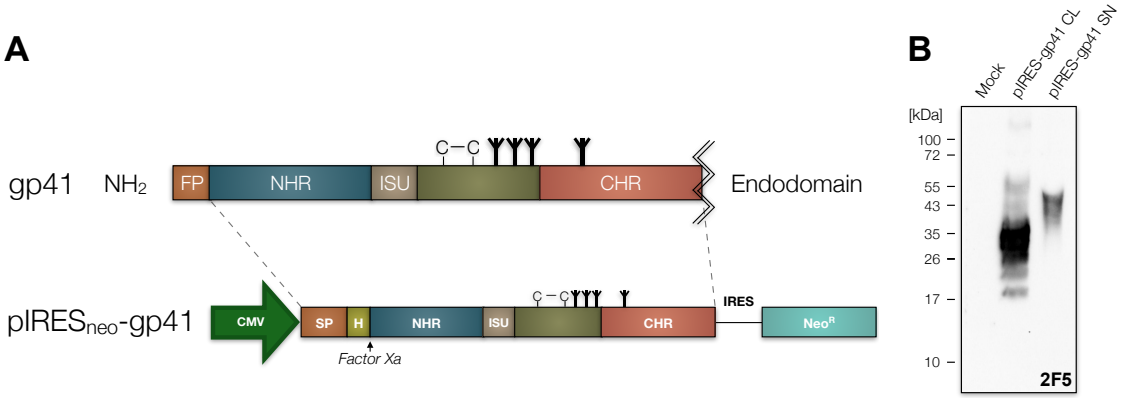

Figure 1.6: Production of gp41 in 293 cells. (A) Schematical presentation of the ectodomain of gp41 and the fragment that is cloned into pIRES vector. FP – fusion peptide; NHR – N-terminal heptad repeat; ISU – immunosuppressive domain; C-C – cysteine loop; CHR – C-terminal heptad repeat; CMV – CMV promotor; SP – signal peptide; H – 8x His-tag; Neo^R – neomicin resistance; IRES – internal ribosome entry site. (B) Western blot of the expression of gp41 in stably transfected 293 cells. The Western blot was performed after cells lysates of mock and pIRESneo-gp41 transfected cells and supernatant of pIRESneo-gp41 transfected cells were incubated with NiNTA-beads and separated on a SDS-PAGE. Gp41 expression was detected using the gp41 reactive antibody 2F5.

The aims of this bachelor thesis were:

- To study the binding of Isu homopolymers and gp41 to human periphal blood mononuclear cells (PBMC)
- To identify distinct subpopulations binding to Isu homopolymers
- Identification of binding partners of Isu homopolymers and gp41

CHAPTER 2

Materials

2.1 Chemicals and enzymes

Name	Vendor
Ammonium persulfate	Merck
β-Mercaptoethanol	Roth
BitNuclease	Biotool.com
BSA	Roth
Bromophenol blue	Roth
BS^3	Life technologies
Coomassie brilliant blue 250 G	Roth
Dimethyl sulfoxide (DMSO)	Roth
DMEM	GE Healthcare
ECL Solution	Life technologies
EndoH	NEB
Endotoxin-free water	Sigma-Aldrich
EDC	Thermo Scientific
Fetal calf serum	Biochrom AG
Glacial acetic acid	Roth
L-Glutamine	Biochrom AG
Glutathione Sepharose	GE Healthcare
HEPES	Biochrom

Name	Vendor
Pierce BCA protein assay	Thermo Scientific
PNGase	NEB
Ponceau S	Sigma-Aldrich
Rotiphorese	Roth
RPMI	GE Healthcare
SDS	Roth
Non-fat dry milk powder	Roth
Sulfo-NHS	Thermo Scientific
TEMED	Roth
Tris(hydroxymethyl)aminomethane (Tris)	Roth
Triton X-100	Roth
Trypsin	Biochrom AG
Tween 20	Roth

2.2 Instruments and equipment

Name	Vendor
Centrifuge 5415D	Eppendorf
Centrifuge 5805R	Eppendorf
ChemoCam Imager 3.2	Intas
FACSCalibur	BD Biosciences
Multiskan GO Microplate Spectrophotometer	Thermo Scientific
NanoDrop ND-1000 Spectrophotometer	Thermo Scientific
SDS-PAGE system (standard)	Höfer/Biometra
SDS-PAGE system (HT)	CBS Scientific

2.3 Buffers

	Standard Buffers
PBS	137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 7.2
PBS-T	PBS, 0.05 % Tween 20
	Buffers for SDS-PAGE and Western blotting
Anode buffer	200 mM Tris, pH 8.9
Cathode buffer	100 mM Tris, 100 mM Tricine, 0.1 % SDS, pH 8.25
Coomassie staining solution	0.25 % Brilliant blue 250 G, 10 % Glacial acetic acid, 45 % Methanol
Destaining solution	10 % Acetic Acid, 45 % Methanol
SDS Gel buffer	3 M Tris base, 0.3 % SDS, pH 8.4
4x SDS Sample buffer	300 mM Tris, 40 % Glycerine, 10 % SDS, 0.04 % Bromophenol blue, pH 8.2; added freshly: 500 mM β-mercaptoethanol
Stripping buffer	10 mM Tris, 2 % SDS, 100 mM β-mercaptoethanol, pH 6.7
Transfer buffer	50 mM Tris, 40 mM Glycine, 20 % Methanol, 0.03 % SDS
	Other buffers
Carbonate buffer (ELISA coating)	100 mM Sodium carbonate, pH 9.5: 85 mM $NaHCO_3$, 15 mM Na_2CO_3, pH 9.5
Coupling buffer	0.1 M Na_3PO_4, 10 % DMSO, 60 mM EDC, 125 mM Sulfo-NHS, pH 7.0
MACS buffer	PBS, 0.5 % FCS, 2 mM EDTA
Ponceau S solution	0.1 % Ponceau S, 5 % Acetic Acid
RIPA buffer	150 mM sodium chloride, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris, pH 8.0

2.4 Antibiotics

Antibiotic agent	Vendor
100x Penicillin-Streptomycin	PAA cell culture company

2.5 Antibodies

2.5.1 Primary antibodies

Name	Species	Concentration in $mg\,ml^{-1}$	Epitope-localisation	Special Properties
5F3	human	1.25	MPER	Oligomer specific (Yuan et al., 2009)
50-69	human	1.5	Isu + Loop	Epitope exposed in pre-hairpin intermediate and six-helix bundle (Finnegan et al., 2002)
D5	human	8.5	NHR	Binds the hydrophobic pocket of gp41 (Miller et al., 2005)
NC-1	mouse	1	NHR/CHR	Binds six-helix bundle (Jiang et al., 1998)
D50	mouse	1.73	CHR	Weak binding to pre-fusion state and fusion intermediate, strong binding to six-helix bundle (Rosny et al., 2004)
98-6	human	1.96	CHR	Barely binding to pre-fusion state, strong binding to six-helix bundle (Frey et al., 2010)
2F5	human	2.5	MPER	Strong binding to pre-fusion state and fusion intermediate, weak binding to six-helix bundle (Rosny et al., 2004)

2.5.2 Secondary antibodies

Name	Species	Concentration in $\mu g\,ml^{-1}$	Vendor
Polyclonal Anti-Mouse, Anti-Human IgG/HRP	rabbit	500	Dako
Anti-His(C-term)-HRP antibody	mouse	Dilution: 1:2000	Invitrogen

2.5.3 Fluorescence labelled antibodies

Name	Species	Vendor
α-human CD3-PerCP	mouse	BD Biosciences
α-human CD4-APC	mouse	BD Pharmingen
α-human CD8-PE	mouse	BD Biosciences
α-human CD11c-PE-Cy7	mouse	Biolegend
α-human CD14-PE	mouse	Beckmann-Coulter
α-human CD16-PerCP	mouse	Biolegend
α-human CD19-PE	mouse	Beckmann-Coulter
α-human CD56-PE	mouse	Beckmann-Coulter
Streptavidin Alexa Fluor 488 conjugate		Life technologies

2.6 Cell lines

Cell line	Additional information
C8166	Human T cells; contain defective HTLV-1 genome; form syncytia in presence of HIV
THP1-XBlue	Monocytic cell line derived from THP-1 that overexpresses TLR4 MD2 and is used as a reporter cell line for LPS stimulus

2.7 Culture media

Medium	Composition	Cells
RPMI-1640	RPMI-1640 basal medium + 2 % L-Glutamine, 1 % Penicillin-Streptomycin, 2.5 % HEPES, 10 % FCS	THP1-XBlue, C8166

2.8 Marker

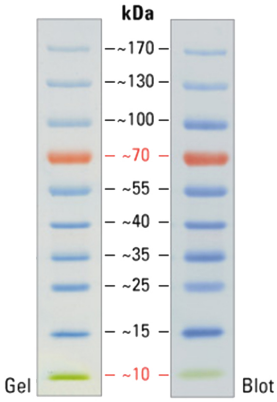

Figure 2.1: PageRuler prestained Protein ladder

2.9 Peptides

Peptide	Sequence
HIV-1 Isu	KQLQARILAVERYLKDQQL
HIV-1 Isu scrambled	LAQLRQKRVIDYQAEQLKL
HIV-1 Isu Biotin	KQLQARILAVERYLKDQQLK-Biotin
HIV-1 Isu scrambled Biotin	LAQLRQKRVIDYQAEQLKLK-Biotin

The peptide monomers were synthesized by SynPeptide Co., China. Purity 85 %.

2.10 Software

Name	Vendor
CellQuest Pro	BD Biosciences
ChemoStar gel documentation	Intas
FlowJo	Treestar, Inc
ImageJ	NIH Image
LaserGene Sequence Analysis Suite	DNAStar
Prism	GraphPad

CHAPTER 3

Methods

3.1 Cell culture

3.1.1 Defrosting of cells

The frozen cells were thawed quickly by transferring them from the nitrogen tank to a 37 °C water bath. Subsequently, the cells were transferred into 8 ml prewarmed medium and centrifuged 4 min at 800 g at room temperature. The cell pellet was resuspended in 8 ml medium and transferred to a flask with additional medium according on the cell count.

3.1.2 Cultivation and passaging of C8166 and THP1-XBlue cells

The cells were counted every two days prior the passaging. Then, the cells were centrifuged at 500 g for 5 min at room temperature and resuspended to 9×10^5 cells ml^{-1} (C8166) or 7×10^5 cells ml^{-1} (THP1-XBlue) in RPMI medium and cultivated in incubators at 37 °C and 5 % CO_2. For expansion of the cell lines, the cell count was adjusted by using additional medium.

3.1.3 Isolation of human PBMCs

15 ml of Histopaque separation medium was filled into Leucosep tubes, which contain a filter disk, and centrifuged for 1 min at 2400 g. Up to 30 ml whole blood or buffy coat was poured into the tube and centrifuged for 15 min at 2000 g in a swinging bucket rotor without break. 10 ml of the enriched fraction of PBMC were aspirated and transferred into a fresh centrifugation tube. The cells were washed with 40 ml PBS and centrifuged for 10 min at 1200 g. The cell pellet was resuspended in 10 ml 0.86 % ammonium sulfate and incubated for 20 min at 37 °C in waterbath for lysis of erythrocytes. Afterwards, the cells were washed twice with PBS and centrifuged for 10 min at 1200 g. The supernatant was discarded and the pellet was resuspended in RPMI-1640 medium. The viable cell concentration was measured by trypan blue staining. The cell concentration was adjusted by adding RPMI-1640 medium.

3.1.4 Determination of viable cell count with trypan blue staining

For determination of cell concentration and parallel viability testing, 50 µl cell suspension was mixed in a reaction tube with 50 µl trypan blue. The suspension was pipetted into a Neubauer chamber where the number of cells within four calibrated big squares was counted. The viable cell count was the result of:

$$\text{Concentration in cells ml}^{-1} = \frac{\text{Total Cells Counted} \times 2 \times 10^4}{\text{Number of squares}}$$

3.2 Protein chemical methods

3.2.1 Determination of protein concentration

The protein concentration was determined with the Pierce BCA Protein Assay. The BCA working reagent was prepared by mixing 50 parts of BCA Reagent A with one part of BCA

Reagent B. 200 µl of the working reagent per well was added to a 96-well microtiter plate and mixed with 2 µl of the examined protein. 4 µl of BSA protein standard with dilutions of 200, 400, 600, 800, 1000 and 1200 µg ml^{-1} was added to the same microtiter plate. After incubation for 30 min at 37 °C the samples were measured in duplicates using a microplate reader at 562 nm.

3.2.2 Coupling of peptides

The lyophilised peptides were resuspended at 20 mg ml^{-1} in DMSO. Then, the peptides were diluted 1:10 in coupling buffer (Section 2.3), which was prepared under steril conditions, by mixing 1 M stock solutions of Na_2HPO_4 and NaH_2PO_4 dissolved in endotoxin-free water, provided with DMSO and the required amounts of pulverized EDC and Sulfo-NHS. Polymerization was stopped after 20 min at room temperature by transfering the coupled peptides to a Slyde-A-Lyzer dialysis casette (Life technologies) with molecular weight cut-off of 10 kDa in which the coupled peptides were dialyzed extensively against PBS.

3.2.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

10 % SDS polyacrylamide separation gels with a 4 % stacking gel were used for protein separation (Schägger et al., 1987). The samples were mixed with sample buffer and boiled at 95 °C for 10 min. Along with a protein ladder (Section 2.8), the samples were loaded on a previously prepared gel for analysis. The voltage-settings were 100 V for ≈ 15 min. After the protein migration reached the separation gel, the voltage-setting was raised to 120 V for ≈ 60 min. The resulting gel was used for either Coomassie staining or Western blot analysis.

Table 3.1: SDS gel composition of stacking and separation gels according to Schägger and Jagow.

Acrylamide (% T)	4 %	10 %
Bis-Acrylamide (% C)	2.6 %	2.6 %
Gel buffer	2 ml	5 ml
aqua dest.	3.2 ml	5 ml
Acrylamide solution (30 %) 37.5 : 1	0.8 ml	5 ml
APS(10 %)	100 µl	100 µl
TEMED	10 µl	10 µl
total	6 ml	15 ml

3.2.4 Coomassie blue staining

The SDS-PAGE gel was stained by incubation in Coomassie blue staining solution for ≈ 30 min under shaking. Subsequently, the gel was washed in ddH_2O before adding the Coomassie destaining solution. When distinct proteins bands got visible, the gel was washed and documented with a contrast enhancing back-light apparatus and a ChemoCam Imager.

3.2.5 Native PAGE

Native PAGE was performed on Novex Native PAGE 4–20 % Tris-Glycine gradient gels (Life technologies) along with SERVA Native protein markers (Serva Electrophoresis GmbH). Novex Tris-Glycine Native Running Buffer (Life technologies) was used at a voltage-setting of 100 V. The resulting gel was used for Western blot analysis.

3.2.6 Glycosylation analysis

Purified gp41 was denatured for 5 min in 10 µl denaturing buffer at 95 °C. Afterwards, the denatured proteins were digested with PNGase and EndoH for 1 h at 37 °C. Subsequently, the samples were separated by SDS-PAGE and detected with Western blot.

3.2.7 Surface plasmon resonance

The surface plasmon resonance experiments in this thesis were performed with a Biacore X100 device. α-human and α-mouse IgG-Fc-specific antibodies were covalently linked to a CM5 chip. The conformation-specific antibodies 5F3, D50, 50-69, 98-6, 2F5, NC-1 and D5 were captured to the chip at 300–500 response units. Single cycle kinetics were determined by five injections of gp41 at increasing concentrations (4.5–365 nM).

3.3 Immunological methods

3.3.1 Western blotting

For semi-dry protein transfer, the SDS-PAGE gel and nitrocellulose membrane were pre-equilibrated for 10 min in transfer buffer. Two extra-thick blotting papers, with the size of the SDS-PAGE gel were soaked in transfer buffer and placed on the transfer chamber. Then, the nitrocellulose membrane, the SDS-PAGE gel and two more pieces of extra-thick blotting paper soaked in transfer buffer were placed on top. The voltage-setting for the protein transfer was 25 V for 30 min for 1 mm gels or 45 min for 1.5 mm gels.

After transfer, the nitrocellulose membrane was blocked with 3 % non-fat dry milk powder in Tris buffered saline supplemented with 0.05 % Tween® 20 (TBS-T) over night at 4 °C or 1 h at room temperature. The primary antibody 2F5 was used with 0.3 $\mu g\,ml^{-1}$ in TBS-T and incubated on the membrane for 2 h. Then, the nitrocellulose membrane was washed three consecutive times for 5 min with TBS-T on a rocking platform. Afterwards, α-human IgG-HRP conjugates were applied with a concentration of 0.1 $\mu g\,ml^{-1}$ in 3 % non-fat dry milk powder in TBS-T for 1 h followed by three consecutive 5 min washing steps with TBS-T. The membrane was stained using ECL Western Blotting Detection Kit according to the provided protocol and visualized with ChemoCam Imager.

3.3.2 Ponceau S staining

To confirm successful protein transfer, the membrane was incubated for 1–2 min in Ponceau S solution under shaking. Then, the membrane was washed three consecutive times with ddH_2O to remove unbound dye.

3.3.3 Enzyme linked immunosorbent assay (ELISA)

Monoclonal antibodies D50, 2F5 and human and mouse antibody controls were diluted to 5 µg ml^{-1} in 100 mM carbonate buffer (pH 9.6), 50 µl of these dilutions were coated per well. The wells were blocked with 5 % BSA/PBS supplemented with 0.05 % Tween® 20 (PBS-T). 50 µl per well of gp41 Ecto diluted to 2 ng µl^{-1} in 1 % BSA/PBS-T was incubated for 1 h at 37 °C. Afterwards, the plate was washed three times with PBS. Bound gp41 Ecto was detected using α-His HRP antibody (1:2000) for 1 h at 37 °C. The plates were washed five consecutive times afterwards. Finally, TMB substrate was added according to vendors instructions. The plate was measured in an ELISA reader at 450 nm.

3.3.4 Endotoxin measurement

The amount of endotoxin was measured using the EndoLISA system (Hyglos, Germany) according to the manufacturer's instructions. Briefly, an *E.coli* LPS standard was serially diluted in a range of 50-0.05 EU/ml. The LPS standards and the protein(s) of interest were mixed with binding buffer in duplicates and incubated on the LPS-specific phage coated ELISA plates for 2 h at 37 °C shaking. The plates were washed and recombinant factor C substrate was added. Fluorescence was measured after 0 min and 90 min.

3.3.5 Binding of Isu homopolymers and gp41 on PBMCs and cell lines

For analysis with FACSCalibur (i) 2.5 $\times$ 10^7 cells (binding kinetic), (ii) 1 $\times$ 10^7 cells (binding to PBMCs, competition experiment and phenotyping) or (iii) 1 $\times$ 10^6 cells (binding to cell lines) were incubated for (i) 1 h and additional 0, 20, 40 or 60 min (binding kinetic) or (ii) 2 h (binding to PBMCs, binding to cell lines, competition experiment and phenotyping) with (i) 50 µg (binding kinetic), (ii) 4 µg (Isu binding to PBMCs, binding to cell lines, competition experiment and phenotyping) or (iii) 0.1 µg, 1 µg and 10 µg (gp41 binding to PBMCs) of (i) Isu and Isu scrambled homopolymers (Isu binding kinetic, binding to PBMCs, binding to cell lines, competition experiment and phenotyping) or (ii) the recombinant produced gp41 Ecto (gp41 binding to PBMCs). Then, the samples and an untreated control were crosslinked for 30 min with 1 mM BS^3 in a final volume of 100 µl in a FACS-tube. Subsequently, the samples were analyzed with FACS.

3.3.6 Fluorescence activated cell sorting (FACS)

The crosslinked samples were washed with 5 ml PBS and centrifuged at 2000 g for 10 min. The supernatant was discarded and the cells were stained for 30 min with (i) 2 µg ml^{-1} streptavidin Alexa Fluor 488 conjugate in a final volume of 100 µl or (ii) with markers for lymphocyte and monocyte subpopulations (CD3, CD4, CD8, CD11c, CD14, CD16, CD19, CD56), which were used as recommended by the respective manufacturer. Afterwards, the cells were washed with 5 ml PBS and centrifuged at 2000 g for 10 min. The pellet was resuspended in 300 µl PBS and data was aquired with FACSCalibur and CellQuest Pro. For further analysis the software FlowJo was used.

3.3.7 Co-immunoprecipitation and mass spectrometry

6×10^7 cells were incubated for 2 h with 4 µg biotinylated Isu and Isu scrambled peptide homopolymers at 4 °C. Afterwards, the cells were washed two times with PBS and crosslinked with 1 mM BS^3 in 100 µl for 30 min at room temperature. Then, the cells were washed with 15 ml PBS and resuspended in 550 µl MACS buffer (PBS, 0.5 % FCS, 2 mM EDTA). 60 µl CD14 MACS beads were added and incubated for 15 min at 4 °C. The samples were washed with 10 ml MACS buffer, centrifuged at 1000 g for 5 min and resuspended in 1 ml MACS buffer. The column was equilibrated with 500 µl MACS buffer, before the cells were added and washed three times. The enriched monocytes were eluted by removing of the magnet and adding of 1 ml of MACS buffer. Another 500 µl were added afterwards to repeat elution. The samples were washed with 15 ml PBS for 5 min at 1000 g and resuspended in 1.5 ml, which then were splitted in three fractions of 500 µl in a 96 deep-well plate. 20 µl Neutravidine High Capacity was added per well and incubated for 30 min under shaking. The cells were lysed by addition of 500 µl RIPA buffer and 1x Pierce Protease Inhibitor Cocktail for 15 min at room temperature and centrifuged for 2 min at 1000 g. The supernatant is discarded. The deep-well plates were washed with 2 ml RIPA buffer, resuspended with 1 ml RIPA buffer and incubated at 4 °C over night. On the next day, the plate was centrifuged for 2 min at 1000 g and washed once with 2 ml RIPA buffer and twice with 2 ml 50 mM Bicarbonate buffer (pH 8.0) (LCMS grade) afterwards. Subsequently, the samples were reduced in 250 µl 50 mM ABC buffer containing 10 mM TCEP (tris(2-carboxyethyl)phosphine) and incubated for 20 min at room temperature. Next, the cysteine residues were alkylated using 25 mM iodacetamide for 20 min at room temperature in the dark. The beads were washed with ABC buffer and digested with 100 ng of chymotrypsin in 150 µl ABC buffer at 25 °C over night. The digestion was stopped by addition of 0.5 % formic acid (LCMS grade). Finally, the bead-free supernatants ($\approx$ 100 µl) were recovered and analysed with mass spectrometry.

CHAPTER 4

Results

4.1 Coupling of Isu peptides

The monomeric peptides corresponding to the Isu domain of gp41 has been synthesized by SynPeptide in China. It has been shown that Isu peptide monomers were not biologically active (Cianciolo et al., 1985; Denner et al., 2013, 1994). Therefore, the biotinylated and non-biotinylated Isu peptide monomers were coupled to Isu homopolymers. It is highly important to work precise and steril during the coupling of the peptides for two main reasons. First of all, this increases the reproducibility of the coupling reaction and secondly, depending on the experiments, endotoxin contamination may be crucial and should be avoided as good as possible. The Isu and Isu scrambled peptides have three primary amin groups that possibly react with the crosslinker mixture of EDC and Sulfo-NHS. The high diversity of crosslinked peptides can be seen by a smear occuring above the monomeric peptides (Figure 4.1 A). To prove that the peptides are endotoxin-free, a fluorescence based endotoxin assay was performed (Figure 4.1 B).

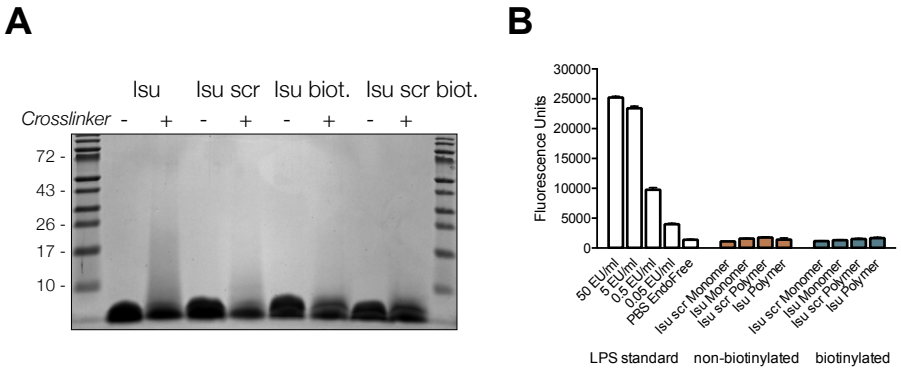

Figure 4.1: Coupling of HIV-1 gp41 Isu peptides. The peptides were coupled as described (Section 3.2.2). (A) Coomassie staining after SDS-PAGE. The amount of peptide is $\approx 30\,\mu g$ per lane. The smear in the lanes with added crosslinker indicates successful crosslinking. (B) Measurement of endotoxin amount in coupled and non-coupled samples using the EndoLISA system. In addition to the protein samples, an *E.coli* LPS standard was incubated parallel on the microtiter plate. The peptide samples are all under detection limit (0.05 EU/ml) of the EndoLISA system. Measurements were performed in duplicates.

As expected the peptide monomers with a molecular weight of 2.3–2.6 kDa exhibit distinct bands under 10 kDa, while the peptide conjugates show a continous staining to higher molecular weights, because of the crosslinking. The endotoxin assay reveals that both, monomers and polymers are under the detection limit of 0.05 EU/ml and thus are not contaminated.

4.2 Binding of Isu peptide to PBMCs and cell lines

4.2.1 FACS gating strategy

The binding of the Isu homopolymers to human PBMCs and cell lines was analyzed with FACS, which allows to examine the binding of the peptides to certain subpopulations. For the division of the subpopulations a gating strategy is needed.

Lymphocyte and monocyte populations in PBMCs were separated based on their differences in size (forward scatter, FCS) and granularity (sideward scatter, SSC) (Figure 4.2). Then, in all approaches, the gated cells were plotted against the fluorescence of Alexa Fluor 488,

which is the fluorescent dye of the streptavidin antibodies. Cells which shift into a gate, which is adjusted to the control (crosslinked and stained with 2nd antibody), indicate cells which were bound by a peptid with biotin-tag.

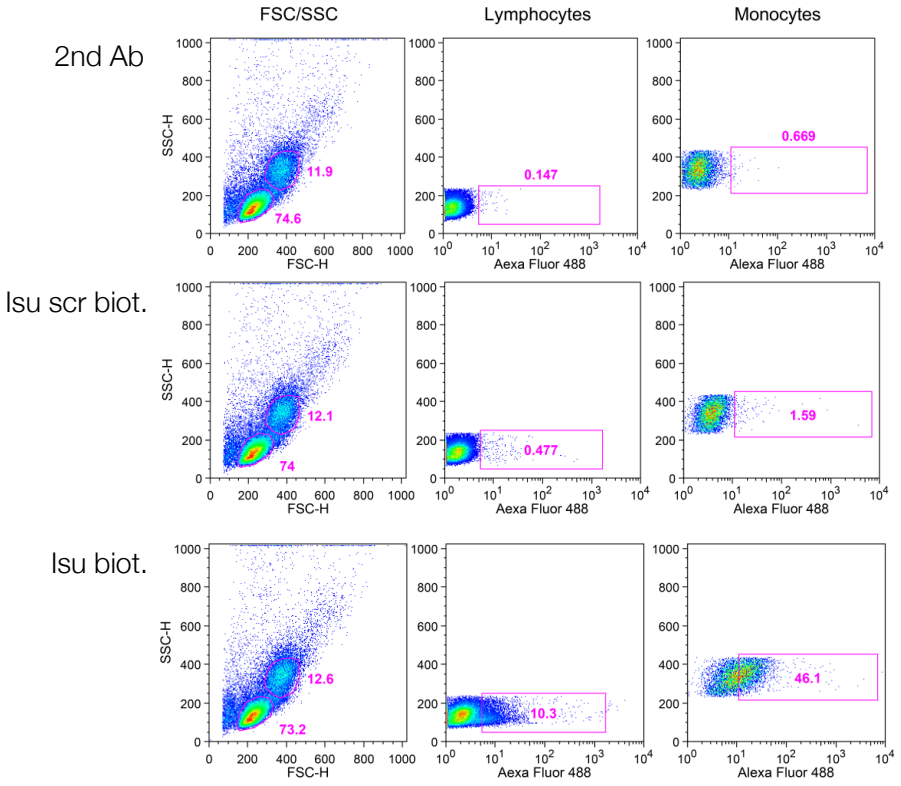

Figure 4.2: Gating strategy on human PBMCs. Separation of lymphocytes and monocytes by their differences in size and granularity. The populations of lymphocytes and monocytes were plotted against fluorescence of Alexa Fluor 488 afterwards. The number in the gate indicates the percentage of the cell population within this gate. 2nd Ab – secondary antibody control; Isu scr biot. – biotinylated Isu scrambled homopolymers; Isu biot. – biotinylated Isu homopolymer; FSC – forward scatter; SSC – sideward scatter.

4.2.2 Binding of Isu peptide homopolymers to human PBMCs

PBMCs were used for interaction studies of the Isu homopolymers. PBMCs consist of T cells, B cells, natural killer cells (NKs), dendritic cells (DCs) and monocytes and thus are essential components in the immune system. As described in the introduction, immunosuppressive properties of the Isu domain have been reported. Therefore, an effect

of a peptide corresponding to the gp41 Isu domain on human PBMCs is most likely. In addition to the Isu homopolymers, a "scrambled (scr)" peptide with the same amino acids in a randomized order is used as a specificity control. Before the binding of these peptides to PBMCs of several donors was examined, the appropriate incubation time was determined in a kinetic experiment. Consequently, the binding of biotinylated Isu and Isu scr homopolymers was examined at four time points (0, 20, 40 and 60 min) after a preincubation of 1 h at 4 °C.

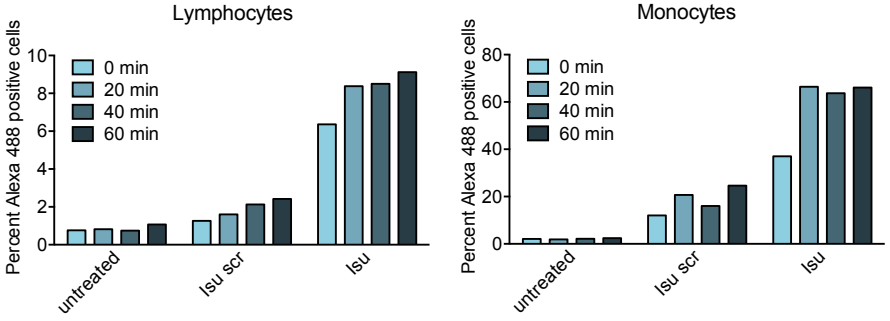

Figure 4.3: Binding kinetic of biotinylated Isu and biotinylated Isu scr homopolymers to monocytes and lymphocytes of human PBMCs. Human PBMCs were isolated from buffy coat and incubated for 1 h with biotinylated Isu and biotinylated Isu scr homopolymers. Then, the cells were crosslinked with bissulfosuccinimidyl suberate (BS^3) after 0, 20, 40 and 60 min additional incubation time.

A higher binding of Isu homopolymers to human PBMCs compared with Isu scr homopolymers can be observed (Figure 4.3). Further, the binding intensified for both homopolymers with increasing time and reaches its maximum at 1 h additional incubation time. This demonstrates that the incubation time of future experiments of the peptides should be ≈ 2 h in order to exhibit an optimal binding to the PBMCs.

Subsequently, it was analyzed whether the differences of the binding of Isu and Isu scr homopolymers to human PBMCs can be replicated with other donors. Therefore, human PBMCs of five different donors were incubated with Isu and Isu scr homopolymers for 2 h.

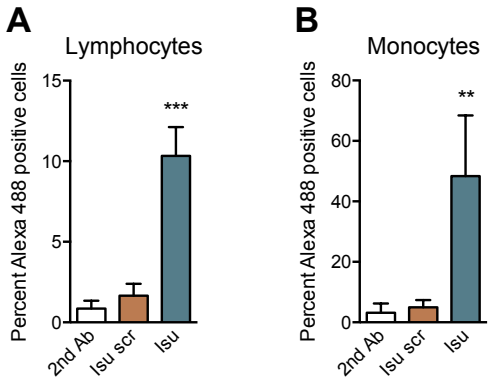

Figure 4.4: Binding of biotinylated Isu peptide on PBMCs. Specific binding of Isu homopolymers to (A) lymphocytes ($p = 0.0009$) and (B) monocytes ($p = 0.0064$) in human PBMCs. The average fluorescence of cells from five different donors incubated with Isu or Isu scrambled peptide is plotted.

The data in Figure 4.4 demonstrate the significant binding of biotinylated Isu peptide homopolymers to lymphocytes and monocytes. Similar to the result of the kinetic experiment, the average fluorescent cells incubated with biotinylated Isu homopolymers variated between 7–12 % in lymphocytes and 22–66 % in monocytes, while the fluorescence of biotinylated Isu scr homopolymers was comparable to the control, indicating a sequence-specific binding of the Isu homopolymers to human PBMCs.

The high standard-deviation, especially in the monocyte population, demonstrates high donor-dependency. Here, further analysis of the raw data revealed that the five donors can be devided in “low-binders” and “high-binders” (Figure 4.5).

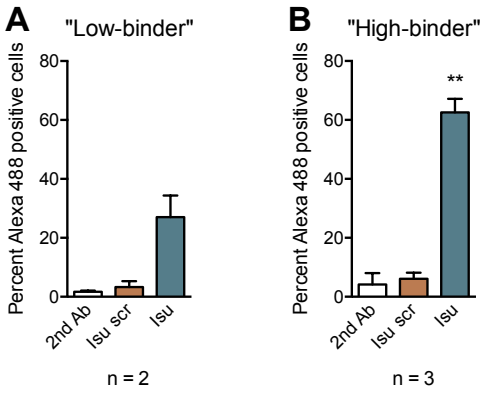

Figure 4.5: Classification of the PBMC donors in "high-binder" and "low-binder". The five donors can be separated according to the fluorescent monocytic cells. It was observed, that the monocytes of the donors either bind Isu homopolymers (A) low ($p = 0.1004$) or (B) high ($p = 0.0030$), but not in between.

In the given example, the five donors are devided into "high-binder" and "low-binder" according to the fluorecent monocytic cells. This data demonstrates that the donors either bind Isu homopolymers low (22–32 %) or high (58–67 %), but not on an intermediate level. While two of the five donors can be termed as "low-binder", the other three can be referred as "high-binder", indicating a distribution of these groups of $\approx 50\,\%$. This donor-dependency suggests a different expression of the putative receptor.

4.2.3 Binding of Isu peptide homopolymers to cell lines

Contrary to PBMCs, where a high donor-dependency was observed, it was expected that the binding of the Isu homopolymers to the monocytic cell line THP1-XBlue and the lymphocytic cell line C8166 would occur more constant, because the cell lines were cultivated under stable cell culture conditions. Further, it would be advantageous, if the results of the binding of the Isu homopolymers to PBMCs could be replicated in cell lines, because the cell line cultivation can be upscaled to large quantities, which are required for preparative isolation of the putative receptor. To examine whether the binding of Isu homopolymers to the cell lines can be compared to primary PBMCs, an experiment similar to the former one was performed.

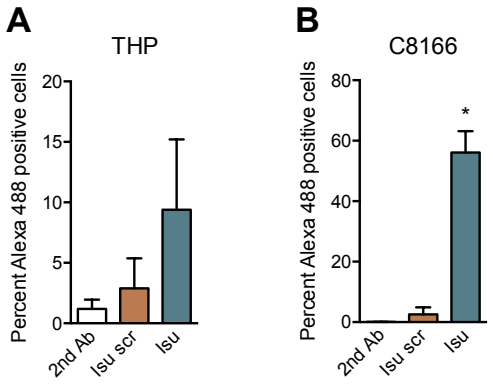

Figure 4.6: Binding of biotinylated Isu peptide to THP1-XBlue and C8166 cells. Binding of Isu homopolymers to (A) THP1-XBlue ($p = 0.2209$) and (B) C8166 ($p = 0.0399$). While the scrambled control exhibit weak binding. The average fluorescence of cells of two experiments is plottet.

In contrast to the primary PBMCs, a weak binding to the monocytic cell line THP1-XBlue (5–14 %) and a high binding to the lymphocytic cell line C8166 (51–61 %) were observed. Similar to the previous results, the binding of Isu homopolymers seems to be sequence-specific, because of the weak binding of the Isu scr homopolymers. The results indicate that C8166 might be an alternative to human PBMCs regarding preparative isolation. However, discrepancy of the binding pattern of cell lines and human PBMCs suggests a differing expression of the putative receptor, which might be caused by immortalization or cell culture conditions. Because of this discrepancy, further experiments were performed on human PBMCs.

4.2.4 Competition of biotinylated and non-biotinylated Isu peptide on human PBMCs

For further evidence of the specificity of the binding, competition experiments of biotinylated Isu homopolymers with non-biotinylated Isu homopolymers were performed on human PBMCs. In this experiment non-biotinylated homopolymers of Isu and Isu scr were preincubated in an increasing molar excess of 1x–50x with isolated human PBMCs for 20 min at 4 °C to block the putative receptors, before the biotinylated Isu homopolymers were added. If the binding of Isu homopolymers to PBMCs is specific, the binding of

the biotinylated peptides to PBMCs should decrease with increasing molar excess of non-biotinylated Isu homopolymers, but not with increasing molar excess of non-biotinylated Isu scr homopolymers.

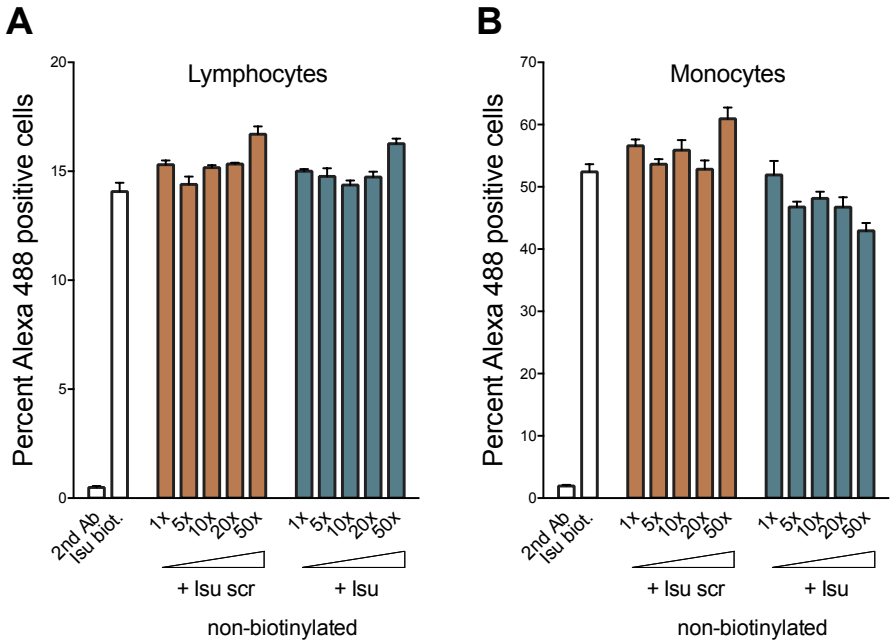

Figure 4.7: Inhibition of the binding of biotinylated Isu homopolymers to human PBMCs using non-biotinylated Isu homopolymers. Human PBMCs were isolated from buffy coat and pre-incubated with 1x, 5x, 10x, 20x and 50x molar excess of non-biotinylated Isu or Isu scr homopolymers, before the biotinylated peptides were added to the samples. The binding of biotinylated Isu homopolymers was inhibited on monocytes, but not on lymphocytes.

The results from Figure 4.7 demonstrate that non-biotinylated Isu homopolymers could inhibit the binding of biotinylated Isu homopolymers only on monocytes, but not on lymphocytes. Furthermore, the competition with non-biotinylated Isu scr homopolymers had no effect on the binding of biotinylated Isu homopolymers. This gives evidence for the specificity of the binding of Isu homopolymers to monocytes. However, it remains unclear why the binding on lymphocytes could not be inhibited in this competition experiment.

4.3 Immunophenotyping

After a specific binding of the Isu peptide homopolymers to lymphocytes and monocytes as well as a specific competitive inhibition on monocytes was observed, it had to be analyzed whether the binding of the Isu peptide homopolymers was specific to a particular subpopulation of lymphocytes or monocytes of human PBMCs. For this purpose, the binding to major lymphocyte subpopulations (B cells, $CD4^+$ cells, $CD8^+$ cells, DCs and NK cells) and to three monocyte subpopulations (classical, intermediate and non-classical, as described by Wong et al., 2011) of isolated human PBMCs was analyzed by using different subsets of surface markers. These are exclusively expressed on one of the subpopulations and might indicate the cell population which expresses the putative receptor. The cells were stained with a cocktail of surface marker antibodies conjugated with fluorochromes, which were selected by their brightness and their fluorescence channel, resulting in minimal spillover between the fluorochromes. After staining the cells, the samples were analyzed by using FACS and subsequent data processing with FlowJo.

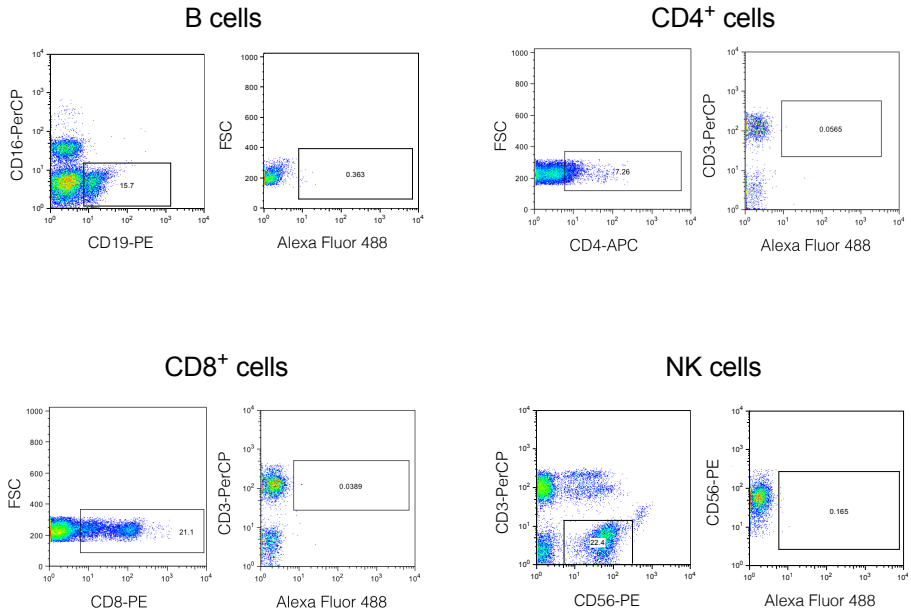

Figure 4.8: Gating of lymhpocyte subpopulations for immunophenotyping. First, the lymphocyte population were gated out of the PBMCs as described in Figure 4.2 A. Then, B cells ($CD16^-CD19^+$), $CD4^+$ cells ($CD3^+CD4^+$), $CD8^+$ ($CD3^+CD8^+$) and NK cells ($CD3^-CD56^+$) were gated as shown in this figure.

The lymphocyte subpopulations were separated by the following gating strategy: B cells ($CD16^-CD19^+$), $CD4^+$ cells ($CD3^+CD4^+$), $CD8^+$ ($CD3^+CD8^+$), DCs ($CD3^-CD11c^+$) and NKs ($CD3^-CD56^+$). Therefore, the fluorescence channels with the respective surface marker are plotted and gated according to the expression of the surface marker of the particular subpopulation (Figure 4.8). Finally, the resulting subpopulation is plotted against Alexa Fluor 488 fluorescence channel, which indicates the binding of the biotinylated peptides.

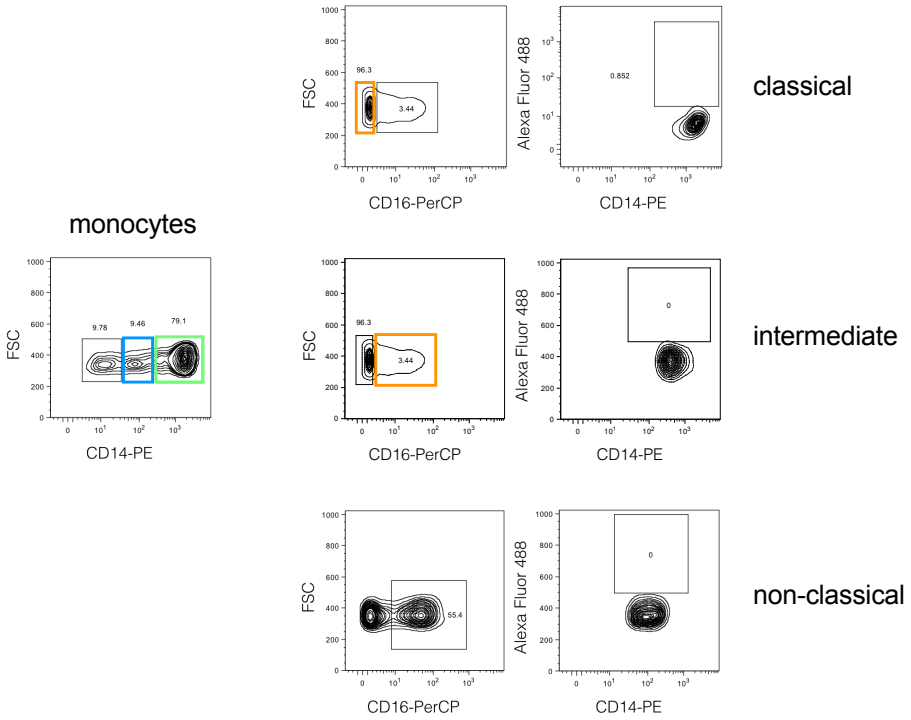

Figure 4.9: Gating of monocyte subpopulations for immunophenotyping. First, the monocyte population were gated using the FSC/SCC scatter as described in Figure 4.2 A. Then, the cells were devided based on their relative expression of CD14 and CD16. The gate of classical ($CD14^{++}CD16^{-}$) and intermediate ($CD14^{+}CD16^{+}$) monocytes, which have a high CD14 expression, is indicated in green and orange. The gate of the CD14 low expressing non-classical monocytes ($CD14^{++}CD16^{+}$) is indicated in blue.

Similar to the lymphocyte subpopulations, the monocyte subpopulations were gated according to the surface marker expression. The monocyte subpopulations were separated by the subsequent gating strategy: classical monocytes ($CD14^{++}CD16^{-}$), intermediate monocytes ($CD14^{+}CD16^{+}$) and non-classical monocytes ($CD14^{++}CD16^{+}$). The plotting procedure is the same as described before.

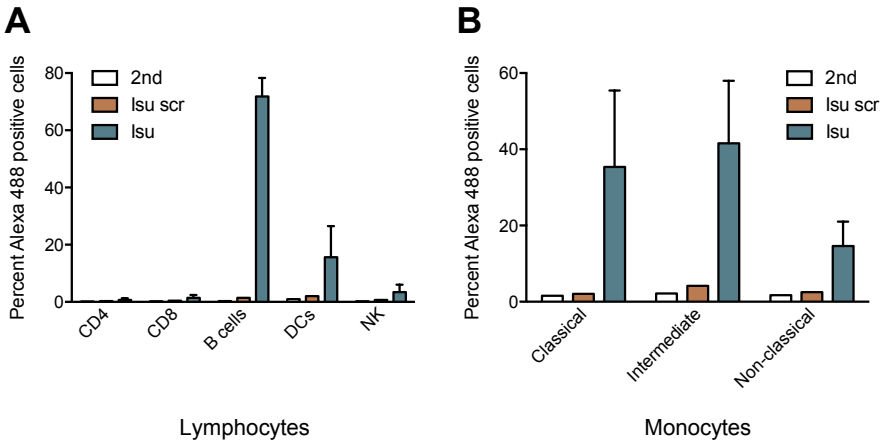

Figure 4.10: Immunophenotyping of the binding of biotinylated Isu peptide to lymphocytes and monocytes. Human PBMCs of four different donors were isolated from buffy coat and incubated with biotinylated Isu and Isu scr peptide homopolymers. Afterwards, the samples were crosslinked with BS^3. Then, the probes were stained with markers for different (A) lymphocyte and (B) monocyte subpopulations in concentrations recommended by the respective vendor.

The results show that the lymphocyte subpopulations of B cells, DCs and NKs and the monocyte subpopulations of classical, intermediate and non-classical monocytes were bound by the Isu peptide homopolymers, but not $CD4^+$ or $CD8^+$. Additionally, the specificity of the binding is demonstrated by the weak binding of the Isu scrambled homopolymers. Taken together, the Isu peptide homopolymers bound in five different donors specific to the lymphocytes and monocytes. The statistical significance of the binding of Isu homopolymers compared to Isu scrambled homopolymers indicates that the binding to human PBMCs is sequence-specific. Further evidence of the specificity of the binding has been shown in a competition experiment, where the binding only on monocytes, but not on lymphocytes could be inhibited by a non-biotinylated peptide. Finally, the immunophenotyping demonstrated that the main target of Isu peptide homopolymers are B cells and classical and intermediate monocytes.

4.4 Gp41 purification and characterization

The results above showed a significant binding of peptide homopolymers corresponding to the immunosuppressive domain of gp41 to human PBMCs. In the next step, it is of interest, whether similar results could be obtained using the recombinant produced gp41 Ecto by the cooperation partner Immunotools. Before repeating the experiments with the gp41 antigen, purification from the supernatant is neccessary. The so called "tandem-purification" consist of two steps and was performed by ImmunoTools.

In the first step of the tandem-purification, the antigen was incubated with nickel-nitrilotriacetic acid (NiNTA) beads, which bind to its 8x histidin (His) tag, followed by washing and elutions of the antigen. Then, in the second purification step, gp41 was purificated using the gp41 reactive antibody 5F3. The steps of the purification and an silver-stain of the purified gp41 are shown in Figure 4.11.

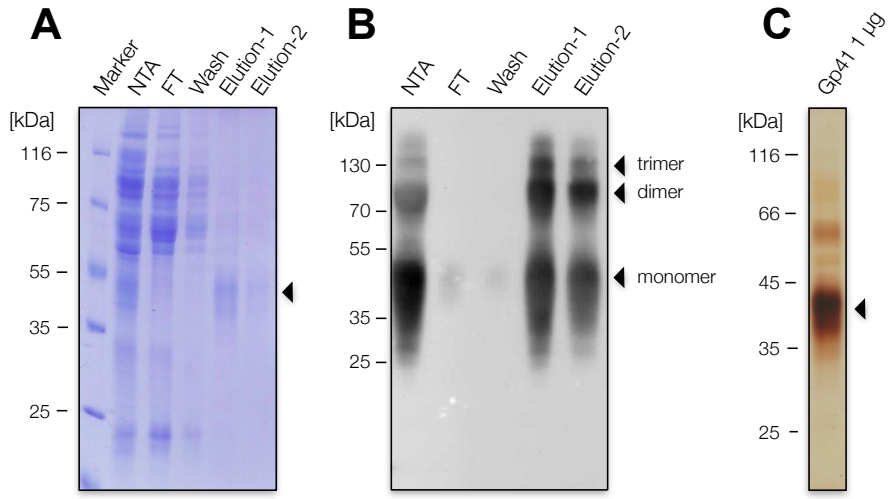

Figure 4.11: Tandem-purification of gp41. (A) SDS-PAGE and (B) Western blot of tandem-purification of gp41. The supernatants of the stably transfected cells were incubated with NiNTA-beads, washed and eluted. A second affinity purification was perfomed using the gp41 reactive antibody 5F3. Detection of gp41 in Western blot with antibody 2F5. NTA – elution of NiNTA beads; FT – flow through of 5F3 column; Wash – PBS wash; Elution-1 and Elution-2 – Elution fractions using glycine buffer. (C) Silver staining of 1 µg purified gp41.

In addition to the silver staining of the purified antigen, the SDS-PAGE and Western blot demonstrate that the purification protocol is effective. While the flow through and wash lanes of the SDS-PAGE contain a lot of protein that has been bound unspecifically by the NiNTA beads, the elution lanes seem to be very pure. Both of these observations, the successful binding and the elution of gp41 to the 5F3 column, are also proven by the Western blot. Furthermore, different assemblies of gp41 are revealed by the Western blot. Besides monomers, dimers and trimers can also be observed, leading to the question of how the oligomers are composed under non-denaturating conditions.

To answer this question, a native PAGE was performed, which suggests that the self-assembly of gp41 under non-denaturating conditions is a trimer (Figure 4.12 B). At this point it was unclear whether the gp41 is processed correctly in the endoplasmic reticulum (ER) and Golgi of the cells. Therefore, the antigen was digested with EndoH and PNGase. EndoH cleaves the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins. The cellular enzyme Golgi α-mannosidase II removes two mannose subunits from this oligosaccharide in medial Golgi region (Alberts et al., 2002), making correctly processed proteins restistant to EndoH cleavage. PNGase, on the other hand, cleaves all types of N-linked glycosylations (Figure 4.12 A). Similar to the Isu peptide homopolymers, gp41 had to be tested for endotoxin containment. Consequently, an fluorescence based endotoxin measurement of the purified gp41 was performed (Figure 4.12 C).

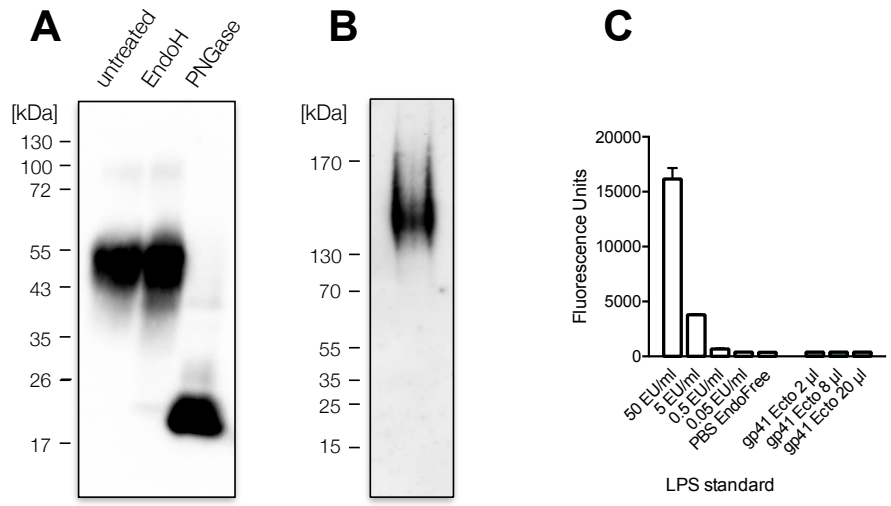

Figure 4.12: Characterization of gp41. (A) Analysis of glycolysation of denatured gp41 using digest without (untreated) or with EndoH and PNGase. Exclusive sensitivity to PNGase indicates correct processing by endoplasmatic reticulum and Golgi and modification with N-linked sugar residues. Detection of gp41 in Western blot with antibody 2F5. (B) Native PAGE of purified gp41. The migration suggests trimeric state of the gp41 antigen. Detection of gp41 in Western blot with antibody 2F5 (C) Measurement of endotoxin amount of purified gp41 Ecto. In addition to three quantities of gp41 Ecto, an *E.coli* LPS standard was incubated parallel on the microtiter plate. The samples of gp41 are all comparable to the detection limit (0.05 EU/ml) of the EndoLISA system and endotoxin-free PBS. Measurements were performed in duplicates.

The endotoxin assay revealed that the amount of endotoxin in the samples of purified gp41 is comparable to endotoxin-free PBS and the detection limit of the assay (0.05 EU/ml). Therefore, there is minimal to no endotoxin contamination during the purification process. The Western blot of the digest with EndoH and PNGase shows that gp41 is only sensitive to the digest with PNGase. This resistance to EndoH demonstrates the correct processing of gp41 through the ER and Golgi, while the band shift of the gp41 digested with PNGase indicates successful N-glycosylation.

4.5 Determination of the conformation of gp41

As mentioned in the introduction, three different conformations of gp41 are known *in vitro* (Figure 1.3 B). The conformation of the endotoxin-free, glycosylated and trimerized gp41 is

unknown. For a first hint regarding the conformation, a capture ELISA with conformation specific antibodies was performed at two different temperatures. The temperatures of choice were 37 °C in order to see the conformation at temperatures comparable to *in vivo*, while 4 °C was chosen to see the conformation at minimum molecular motion. The gp41 specific antibodies 2F5 and D50 have been reported to bind contrary to the conformations of pre-fusion state and six-helix bundle of gp41 (Rosny et al., 2004). 2F5 strongly binds to pre-fusion state of gp41, while a strong binding of D50 occurs, when gp41 is in a six-helix bundle conformation. Therefore, by the proportion of the binding of these antibodies it was expected to identify the conformation of the antigen.

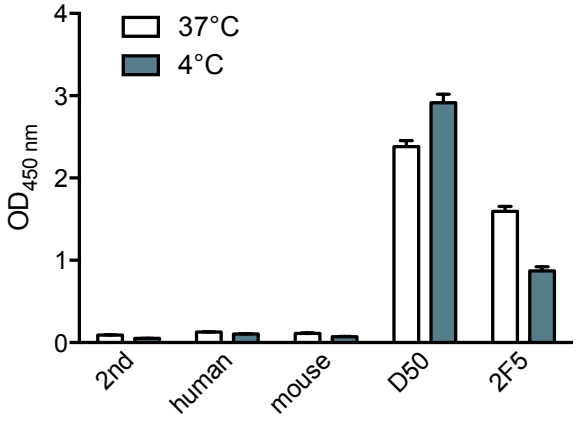

Figure 4.13: Capture ELISA with the two gp41 conformationspecific antibodies 2F5 and D50 at two different temperatures. The antibodies were coated to a microtiter plate, before gp41 Ecto was added. Bound antigen was detected with α-His HRP antibodies. The result demonstrates that the six-helix bundle specific antibody D50 exhibit at 37 °C a stronger binding compared to the pre-fusion state specific antibody 2F5. This effect is enhanced at 4 °C, indicating that the predominant conformation of gp41 Ecto is six-helix bundle

The result of the capture ELISA in Figure 4.13 demonstrates that D50 and 2F5 bind gp41 at both temperatures, while the controls show no binding. The strong binding of D50 and the weak binding of 2F5 at 37 °C indicate that the conformation of gp41 is six-helix bundle, which is stabilized when the experiment is repeated at 4 °C. To confirm this result and for further characterization of the gp41 antigen, a surface plasmon resonance measurement

(Table 4.1) was performed with five additional antibodies. All of these antibodies are conformation-specific (Section 2.5.1) and thus might give an additional clue about the conformation of the produced gp41.

Table 4.1: Surface plasmon resonance measurements of gp41 with several antibodies. The experiment was performed on a Biacore X100. The antibodies were captured on a CM5 chip at 300-500 response units. Single cycle kinetics were determined by five injections of gp41 at increasing concentration 4.5–365 nM. PF – pre-fusion state; I – intermediate state; 6HB – six-helix bundle; k_a – association rate constant; k_d – dissociation rate constant; K_D – equilibrium dissociation constant.

Antibody	Specification	k_a in $M^{-1}s^{-1}$	k_d in s^{-1}	K_D in M
5F3	Oligomer	1,1E+05	3,0E-04	2,8E-09
D50	PF weak; I/6HB strong	8,2E+04	4,1E-04	5,1E-09
50-69	I/6HB	5,8E+04	4,0E-04	7,0E-09
98-6	6HB	6,2E+04	8,1E-04	1,3E-08
2F5	6HB weak; I/PF strong	4,0E+04	7,2E-04	1,8E-08
NC-1	6HB	4,9E+04	3,7E-03	7,5E-08
D5	hydrophob. pocket	2,6E+04	2,8E-03	1,1E-07

The measurement with the Biacore X100 device revealed the association rate (k_a), dissociation rate (k_d) and equilibrium dissociation constant (K_D) of the set of antibodies (Table 4.1). The result is in line with the previous ELISA results, as a strong binding of D50 compared to 2F5 can be observed, indicating six-helix bundle conformation. Further evidence for this conformation is given by the k_a value. When the epitope of the respective antibody is accessible, the association rate should be high. Interestingly, the five antibodies with the highest k_a values are either specific for oligomers (5F3) or specific for six-helix bundle (D50, 98-6, 50-69 and NC-1), while the two antibodies with the lowest k_a values bind weakly to six-helix bundle (2F5) or not at all (D5). This suggests that, similar to the ELISA results, the predominant conformation of the recombinant produced gp41 determined by surface plasmon resonance measurement is six-helix bundle.

4.6 Binding of gp41 to human PBMCs

After basic informations about the conformation and glycosylation of the gp41 antigen were obtained, the binding of gp41 to human PBMCs was analyzed. Therefore, three different quantities of biotinylated gp41 Ecto were incubated with human PBMCs (Figure 4.14). It was expected that the percent of fluorescent cells will increase with higher amount of biotinylated gp41.

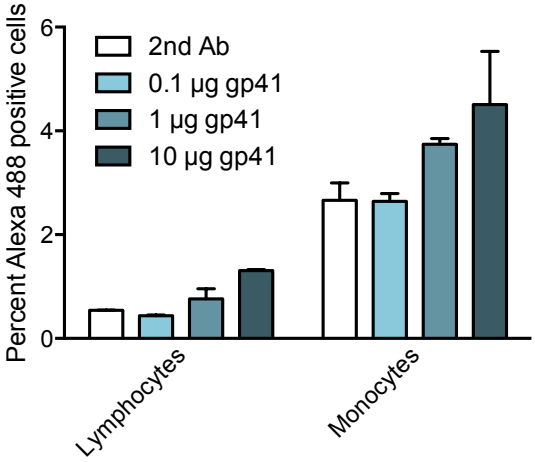

Figure 4.14: Binding of biotinylated gp41 to human PBMCs. Human PBMCs were incubated with three different quantities of biotinylated gp41. The result demonstrates a weak, but dose dependend binding of the gp41 antigen to human PBMCs. The average fluorescence of cells of two experiments is plottet.

In contrast to the Isu homopolymers, which binding variated between 7–12 % on lymphocytes and 22–66 % on monocytes, the gp41 antigen showed a weak binding to human PBMCs. However, with a higher amount of antigen, the percent of fluorescent cells increases. This indicates a dose depended binding which has to be analyzed in more detail.

4.7 Co-immunoprecipitation and mass spectrometry

As a final step, the potential binding partners had to be identified. Therefore the putative receptors of gp41 and Isu homopolymers were co-immunoprecipitated as described (Section 3.3.7). Unfortunately the results of the mass spectrometry have not arrived in time to be included in this bachelor thesis.

CHAPTER 5

Discussion

5.1 Binding of Isu peptide to PBMCs and cell lines

In this thesis a significant binding of Isu homopolymers to human PBMCs, compared to the scrambled control, has been shown, giving evidence for a sequence-specific binding. Further, the successful inhibition of biotinylated Isu homopolymers with non-biotinylated homopolymers on monocytes was demonstrated. Similar results has been obtained using the CS3 peptide (=Isu peptide) conjugated to HSA or fluorescein isothiocyanate (FITC) labelled HSA on RH9 cells (Qureshi et al., 1990). The lack of inhibition on lymphocytes and differences in binding between donors, as it was seen in the presented results, might be explained by different receptors or different affinities to the non-biotinylated and biotinylated homopolymers. Interestingly, a donor dependency regarding the ability to induce of IL-10 release was previously shown by Denner et al., 2013.

It was furthermore demonstrated, that in four different donors Isu homopolymers highly bound to classical and intermediate monocytes and B cells. Previously, a binding of soluble gp41 to $CD19^+$ B cells (47 %) and $CD14^+$ monocytes (44.2 %) in human PBMCs has been reported (Chen et al., 1993). This supports the presented data of this thesis and indicates that the binding of soluble gp41 to $CD19^+$ B cells and $CD14^+$ monocytes is caused by the Isu domain of gp41. Additionally an upregulation of IL-10 accompanied

with a downregulation of IL-2, IFN-γ and IL-4 in monocytes, but not B, T, or NK cells after incubation with bacterial produced soluble gp41 was reported (Barcova et al., 1998). IL-10 and α-IL-10 antibodies have contrary effects on the IL-2 production. While IL-10 inhibits IL-2 production, α-IL-10 antibodies reverse this effect. In this case, the α-IL-10 antibodies almost completely reversed the effect of IL-10 on the production of IL-2, suggesting a regulatory role of IL-10. Thus, the strong binding observed on monocytes could indicate a suppression of other immune cells, such as proliferation of T lymphocyte (Denner et al., 1994, 1996; Ruegg et al., 1989) or B lymphocytes (Denner et al., 1996) by release of IL-10. The high binding of Isu homopolymers to B cells furthermore indicate that the inhibition of B lymphocyte proliferation may be due to a direct effect.
By the abscence of endotoxins and by using a scrambled biotin control the uptake through LPS receptor complex (TLR, CD14 and MD-2) and biotin receptor can be excluded.

5.2 Gp41 characterization and binding to human PBMCs

The purification of the gp41 antigen was described. It was shown that the antigen was correctly processed through ER and Golgi and is N-glycosylized, trimerized and endotoxin-free. Further, it was examined whether the antigen changes its conformation with increasing temperature, as it has been reported (Henderson et al., 2006; Melikyan et al., 2000). In contrast to the conformational changes to six-helix bundle with increasing temperatures that has been reported, the predominant conformation of the examined antigen was at all measured temperatures the six-helix bundle. Anyhow, the conformation is stablized with decreasing temperature, indicating that the conformational changes of the examined antigen are due to molecular motion. Taken together with the surface plasmon resonance measurement, the predominant conformation of the gp41 produced in stably transfected 293 cells is six-helix bundle.
Interestingly, the binding of biotinylated gp41 to human PBMCs was much weaker, compared with biotinylated Isu homopolymers. A possible explanation is that the six-helix

bundle conformation does not or weakly bind to the putative receptor. Further, the method of detection may lead to a wrong interpretation of the results. While the biotinylated Isu homopolymers contain many possible interaction partners for the fluorescence labelled streptavidine per molecule, gp41 contains one, resulting in a weaker fluorescent signal compared to biotinylated homopolymers. Therefore, a much higher amount of gp41 is needed to create a comparable signal of the Isu homopolymers. To exclude this possibility, further analysis is needed.

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APPENDIX A

Surface plasmon resonance sensorgrams

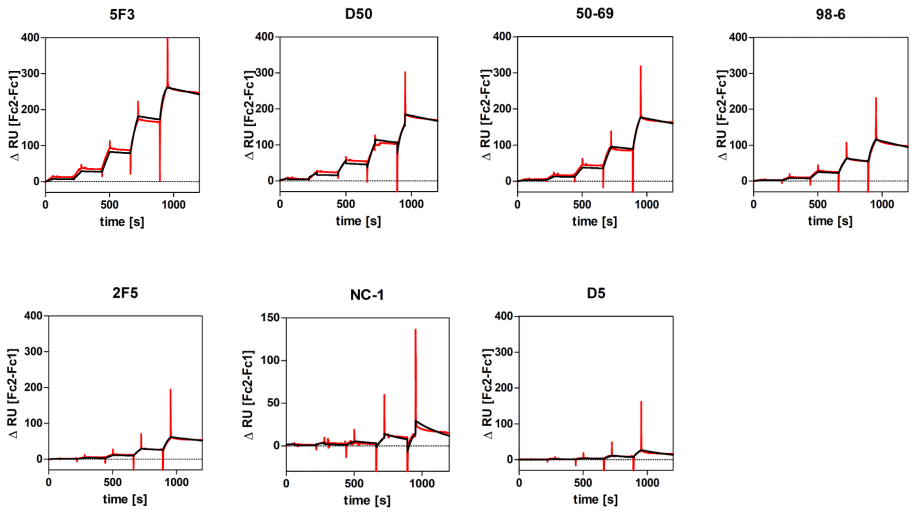

Figure A.1: Sensorgrams of surface plasmon resonance measurement of several conformation-specific antibodies of gp41. Human and mouse conformation-specific IgGs were covalently linked to a CM5 chip. Single cycle kinetics were determined by five injections of gp41 at increasing concentrations (365–4.5 nM). The antigen was carried in a continuous flow of running buffer. The resulting sensorgram was plotted against Δ-RU which is the substraction of flow cell 2 (with antigen) and flow cell 1 (negative control)

APPENDIX B

Publications and posters

Publications

Parts of this work have been published in the following manuscripts

1. Mühle, M, T Kroniger, K Hoffmann and J Denner (2015): 'Binding of the immunosuppressive domain of HIV-1 to human monocytes'. *In preparation.*

Poster

Parts of this work have been presented on a poster at the following meetings

1. Mühle, M, T Kroniger, K Hoffmann and J Denner (2015): 'Binding of the immunosuppressive domain of HIV-1 to human monocytes'. *Retropath 2015*, 27th workshop on retroviral pathogenesis, Wolfsburg, Mühlheim an der Ruhr, Germany, August 24-27, 2015

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