

AUS DEM  
LEIBNIZ-INSTITUT FÜR ZOO- UND WILDTIERFORSCHUNG (IZW)  
IM FORSCHUNGSVERBUND BERLIN E.V.

# **Analysis of monoclonal antibody cross-reactivity with leukocytes from equids and cloning of CD28**

Inaugural-Dissertation  
zur Erlangung des Grades eines  
**DOKTORS DER VETERINÄRMEDIZIN**  
an der Freien Universität Berlin

vorgelegt von  
**SHERIF MAHMOUD MOHAMED MOHAMED IBRAHIM**  
Tierarzt (B.V.Sc, M.V.Sc.) aus Kairo/Ägypten

Berlin 2007  
Journal-Nr.: 3144

**Gedruckt mit Genehmigung  
des Fachbereiches Veterinärmedizin  
der Freien Universität Berlin**

**Dekan:** **Prof. Dr. Leo Brunnberg**  
**Erster Gutachter:** **PD Dr. Falko Steinbach**  
**Zweiter Gutachter:** **Prof. Dr. Armin Saalmüller**  
**Dritter Gutachter:** **PD Dr. Jakob Walter**

***Deskriptoren (nach CAB-Thesaurus):***

**horses, leukocytes, immunoglobulins, monoclonal antibodies, antigens, CD28 (MeSH),  
immunoprecipitation tests, flow cytometry, DNA cloning, gene expression**

**Tag der Promotion: 01.10.2007**

**To my mother, brothers, wife and daughters**

## Table of Contents

<b>ABBREVIATIONS</b>	3
<b>I. REVIEW OF LITERATURE</b>	6
<b>II. AIM OF THE STUDY</b>	19
<b>III. MATERIAL AND METHODS</b>	20
<b>3.1 MATERIAL</b>	20
3.1.1 Monoclonal antibodies	20
3.1.2 Polyclonal antibodies	20
3.1.3 Conjugated secondary antibodies	20
3.1.4 Blood samples	20
3.1.5 Cytokines	21
3.1.6 Enzymes and reaction kits	21
3.1.7 Cell lines and bacterial stocks	21
3.1.8 Chemicals, reagents, and consumables	21
3.1.9 Electrophoresis markers	22
3.1.10 Primers	22
3.1.11 Vector systems	24
3.1.12 Laboratory equipments and instruments	24
<b>3.2 METHODS</b>	25
3.2.1 Isolation of leukocytes and subsequent culture of primary cells	25
3.2.2 Cell culture and cryo-preservation of cell lines	26
3.2.3 Immunofluorescence staining of equine leukocytes	28
3.2.4 Flow cytometric analysis of equine leukocytes	29
3.2.5 Extraction of total cellular RNA from activated PBMC	30
3.2.6 Spectrophotometric determination of nucleic acid amount	30
3.2.7 Reverse transcription (RT)	31
3.2.8 Polymerase chain reaction (PCR)	31
3.2.9 Horizontal agarose gel electrophoresis	32
3.2.10 Purification of PCR products	32
3.2.11 Cloning of CD28 into pGEM-T <sup>®</sup> Easy cloning vector	33
3.2.12 Extraction of cloned plasmids from positive <i>XL-1</i> transformants	34
3.2.13 Demonstration of cloned fragments by SP6-T7 PCR	35
3.2.14 Chain terminating sequencing PCR	36
3.2.15 Preservation of transformed bacterial clones	37
3.2.16 Expression of equine CD28 using pIB/V5-His TOPO <sup>®</sup> TA expression kit	37
3.2.17 Immunoprecipitation of leukocyte surface antigens	39
3.2.18 SDS-Poly acrylamide gel electrophoresis (SDS-PAGE)	40
3.2.19 Western Blot and immunostaining (Immunoblot)	41

<b>IV. RESULTS</b>	43
<b>4.1 Cross-reactivity analysis of a large panel of mAbs with horse leukocytes</b>	44
4.1.1 Single colour flow cytometry	45
4.1.1.1 Dot plots analysis of HLDA8 mAbs	47
4.1.1.2 Dot plots analysis of non-HLDA8 mAbs	56
4.1.2 Two-colour flow cytometry of mAbs that cross reacted with horse Lymphocytes	64
4.1.3 Flow cytometric analysis of human mAbs using eCAS and EqT8888 equine cell lines	69
4.1.4 Estimation of the molecular weight of candidate CD molecules using immunoprecipitation	73
<b>4.2 Cross-reactivity analysis of mAbs (defined positive with horse leukocytes) with wild equids using single colour flow cytometry</b>	81
<b>4.3 Cloning of CD28 from horse and zoo animal species</b>	92
4.3.1 RT-PCR of CD28	92
4.3.2 Sequence analysis of CD28	93
<b>4.4 Anti-human polyclonal antibodies as another tool to analyze horse (<i>Equus caballus</i>) leukocytes</b>	100
4.4.1 Analysis of anti-human CD28 polyclonal Ab	100
4.4.2 Analysis of anti-human CD25 polyclonal Ab	102
<b>V. DISCUSSION</b>	104
5.1 Cross-reactivity analysis of a large panel of mAbs with horse leukocytes	104
5.2 Flow cytometric analysis of human mAbs using eCAS and EqT8888 equine cell lines	118
5.3 Preliminary analysis of cross-reactivity of mAbs (defined positive with horse leukocytes) with wild equids leukocytes using single colour flow cytometry	120
5.4 Cloning and sequence analysis of CD28 from horses and zoo animal species	121
5.5 Anti-human polyclonal antibodies used as another tool to analyze cross-reactivity	123
<b>VI. SUMMARY/ZUSAMMENFASSUNG</b>	126/128
<b>VII. REFERENCES</b>	130
<b>VIII. APPENDIX</b>	148
<b>PUBLICATIONS</b>	156
<b>ACKNOWLEDGMENTS</b>	157
<b>SELBSTÄNDIGKEITSERKLÄRUNG</b>	158

**ABBREVIATIONS**

aa	amino acid (s)
Ab	antibody
Act. PBMC	activated PBMC
Ag	Antigen
H+L	Heavy + Light chain
AP	Alkaline phosphatase
APC	Antigen Presenting Cell
Approx.	Approximate
bp	base pairs
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
CD	cluster of differentiation
CDP	Disodium 2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)-tricyclo [3.3.1.1 <sup>3,7</sup> ]decan}-4-yl)-1-phenyl phosphate
CR2	Complement receptor type 2
CR3	Complement receptor type 3
GM-CSF	Granulocyte Monocyte colony-stimulating factors
DC	Dendritic cells
dH <sub>2</sub> O	Distilled water
DL	Double labelling
DNA	Deoxyribonucleic acid
cDNA	complementary Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
dNTPs	deoxyribonucleoside triphosphate
ds	double stranded
EDTA	Ethylene Diamine Tetra Acetic acid
ELAWI	Equine leukocyte antigen workshop number one
ELAWII	Equine leukocyte antigen workshop number two
ELISA	Enzyme linked immunosorbent assay
Eq	Equine
IL	interleukin
WC	workshop cluster
EtOH	Ethanol
FACS	fluorescence-activated cell sorter
Fc	Fragment crystallizable part of antibody
FCS	Fetal calf serum
Fig.	Figure
FITC	Fluorescein isothiocyanate
FL-1	Fluorescence-1
FL-2	Fluorescence-2
FSC	Forward scatter
g	centrifugal force/gram
h	Hour
H <sub>2</sub> O	Water
H <sub>2</sub> O <sub>bidest.</sub>	Bidistilled water
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxidase
HEPES	4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid
HLDA8	8 <sup>th</sup> human leukocyte differentiation antigen workshop
HSC	Haematopoietic stem cells
hu	human
ICAM	Intracellular adhesion molecule
iDC	Immature dendritic cells
IFN	Interferon
Ig	Immunoglobulin
IgA	Immunoglobulin-A
IgD	Immunoglobulin-D
IgE	Immunoglobulin-E
IgG	Immunoglobulin-G

---

IgM	Immunoglobulin-M
IL-2R $\alpha$	Interleukin-2 receptor alpha chain
IP	Immunoprecipitation
IPTG	Isopropyl- $\beta$ -D-Thiogalactopyranoside
IUCN	The World Conservation Union, Gland, Switzerland
Kb	kilo-base
kDa	kilo Daltons
LB	Luria Bertani
LDL	Low density lipoproteins
LPS	Lipopolysaccharide
$\mu$	micro
m	milli
M	Molar (mole per litre)
MΦ	Macrophage
mA	milli ampere
mAb	Monoclonal antibody
mAk	Monoklonale Antikörper
max.	Maximum
MHC	Major Histocompatibility Complex
min.	minute
ml	millilitre
M-MLV	Moloney Murine Leukemia Virus
MMR	Macrophage mannose receptor
Mo	monocytes
MoDC	Monocyte-derived dendritic cells
MoMΦ	Monocyte-derived macrophages
MOPS	3-(N-morpholino) propanesulfonic acid
MPS	Mononuclear-phagocyte system
mRNA	messenger RNA
mu	mouse
MW	Molecular weight
ng	Nano gram
NK cells	Natural killer cells
nm	nanometer
OD	Optical density
ORF	Open Reading Frame
PBL	Peripheral blood lymphocytes
PBMo	Peripheral blood monocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PE	phycoerythrin
PHA	Phytohaemagglutinin
PMA	Phorbol-12-Myristate-13-Acetate
poly (I:C) acid	polyinosinic-polycytidylic acid
R	Receptor
RE	restriction enzyme
RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	Round per minute
RT	Reverse transcription
SDS	Sodium Dodecyl Sulphate
PAGE	Poly Acrylimde Gel Electrophoresis
Sp.	Species
SSC	Side scatter
TAE	Tris acetate EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline with tween
Tc	cytotoxic T cells
TCA	Trichloroacetic acid

TCR	T cell receptor
TEMED	Tetramethylethylenediamin
TfB	Transformation buffer
Th	T helper
TLR	Toll like receptor
Tm	Melting temperature
TNF	Tumour Necrosis Factors
U	Unit
UV	Ultraviolet light
V	Volt
vs	Versus
V/V	volume/volume
W	watt
WB	Western blot
W/V	Weight/volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside