III. MATERIAL AND METHODS

3.1 MATERIAL

3.1.1 Monoclonal antibodies

A panel of 534 mAbs against different human leukocyte CD molecules was evaluated in this study for its cross reactivity with equine leukocytes. 379 mAbs (Appendix, table 16) were submitted through the HLDA8 animal homologues section (including 4 isotype controls). Further (non-HLDA8) 155 mAbs (Appendix, table 17) were submitted directly from companies to be included in this study.

3.1.2 Polyclonal antibodies:

Table 1: Anti-human polyclonal antibodies

Specificity	Source	Isotype	Catalog No.
huIL-2 Rα (CD25)	R&D systems, Wiesbaden	Goat IgG	AF-223-NA
huCD28	R&D systems, Wiesbaden	Goat IgG	AF-342-PB

3.1.3 Conjugated secondary antibodies:

Table 2: List of conjugated secondary antibodies

Specificity	Conjugate	Source	Stock conc.	Code No.
Donkey Anti-goat IgG (H+L)	Alkaline Phosphatase	Dianova, Hamburg	0.6mg/ml	705-055-147
Donkey Anti-goat IgG (H+L)	Fluorescein (FITC)	Dianova, Hamburg	1.5mg/ml	705-095-147
Donkey Anti-mouse IgG (H+L)	Alkaline Phosphatase	Dianova, Hamburg	0.6mg/ml	715-055-150
$F(ab')_2$ fragment donkey Anti-mouse IgG (H+L)	R-Phycoerythrin (PE)	Dianova, Hamburg	0.5mg/ml	715-116-150
$F(ab')_2$ fragment goat anti-rat IgG (H+L)	R-Phycoerythrin (PE)	Dianova, Hamburg	0.6mg/ml	112-116-143
Donkey Anti-mouse IgM	Fluorescein (FITC)	Dianova, Hamburg	1,5mg/ml	715-095-140

Legend to table 2:
All secondary antibodies were supplied as freeze-dried powder and reconstituted according to manufacturer instructions in deionized water and Glycerol 1:1 volume and stored at –20ºC. For flow cytometry, 50 µl of 1:200 PBS (pH 7.4) diluted conjugated 2^{ry} Ab was used for each staining. For western blot, 10 ml of 1:10.000 TBST diluted 2^{ry} Ab were used for each staining.

3.1.4 Blood samples:

Fresh blood samples (~500 ml/sample) were collected from healthy horses (*Equus caballus)* of different breed and sex and the following zoo animal species: Somali wild ass (*Equus africanus somalicus*), Grevy's zebra (*Equus grevyi*), Hartmann's mountain zebra (*Equus zebra hartmannae*), Greater one-horned rhinoceros (*Rhinoceros unicornis*), Asian elephant (*Elephas maximus*), European bison *(Bison bonasus*), African buffalo (*Syncerus caffer*), Nubian giraffe (*Giraffa camelopardalis*) into sterile blood bags (Baxter, Munich) with citrate solution.

3.1.5 Cytokines:

Different equine recombinant cytokines, GM-CSF, IL-4, and IFNα were used for *in vitro* differentiation of monocytes toward DCs or macrophages (all provided by Dr. Steinbach & Dr. Mauel, equine immunology group).

3.1.6 Enzymes and reaction kits:

DnaseI, Rnase-free	Roche
Recombinant RNasin® Ribonuclease inhibitor	Promega
M-MLV reverse transcriptase Rnase H minus Point mutant	Promega
Taq DNA polymerase	Promega
*EcoR*I restriction enzyme	Promega
NucleoSpin® RNA extraction kit	Macherey-Nagel
Wizard®SV Gel and PCR Clean-Up System	Promega
pGEM®-T Easy vector system I	Promega
WIZARD® plus minipreps DNA purification system	Promega
BigDye Terminator v3.1 Cycle Sequencing Kit®	ABI
pIB/V5-His TOPO® TA expression kit	Invitrogen

3.1.7 Cell lines and bacterial stocks:

Mammalian cell lines:	
eCAS	Dr. A.Werners, Utrecht (Werners et al., 2004)
EqT8888	Dr. D. Horohov, Kentucky (Hormanski et al., 1992)
Insect cells :	
High five insect cells	Invitrogen
Bacterial stocks:	
E.coli XL1-Blue	Stratagene
E.coli Top10	Stratagene

3.1.8 Chemicals, reagents, and consumables:

VLE-RPMI 1640	Biochrom
Express Five® serum free medium (SFM)	Invitrogen
Fetal calf serum (FCS)	Biochrom
L-glutamine, 200 mM	Fluka
Hepes buffer, 1M	Biochrom
Gentamycin	Biochrom
Amphotericin B, 250µg/ml	Biochrom
Blasticidine	Invitrogen
Biocoll, ρ 1.090 and ρ 1.077	Biochrom
Phorbol 12-Myritate 13-Acetate (PMA)	Sigma
Ca-Ionophor A23187	Sigma
Phythaemagglutinin (PHA)	Sigma
Lipopolysaccharide (LPS)	Sigma
LB agar	Invitrogen
LB broth base	Invitrogen
Isopropyl-beta-D-thiogalactopyranoside (IPTG)	Fermentas
5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-Gal)	Fermentas
Ampicillin (Sodium salt)	AppliChem
Glycerol	MERK
PCR Nucleotide Mix (10mM each dNTP)	Promega
Nuclease-Free Water	Promega
Agarose	Promega
Ethidium bromide	Promega
6x gel loading buffer	Promega

Lipofectamine™ 2000	Invitrogen
Protein G 4 fast flow®	Amersham
EZ-link® Sulfo-NHS-Biotin reagent	Pierece
Immunopure® strpetavidine-AP	Pierce
CDP-Star® chemilumencense substrate	Roche
10x Luminescence buffer	Prionics AG
Hybond™-ECL™ nitrocellulose membrane	Amersham
Kodak® Biomax light film (5 in. x 7 in.)	Kodak
Kodak® Lx2 x-ray developer	Kodak
MOPS	ROTH
Sodium dodecyl sulphate	MERK
5-Bromo-4-Chloro-3-Indolylβ- D-Galactopyrinoside	Sigma
Ethanol, pure (99.9%)	ROTH
EDTA	MERK

3.1.9 Electrophoresis markers:

Table 3: List of DNA and protein size and molecular weight ladders

Ladder	Source
GeneRuler™ 100bp DNA ladder	Fermentas
1kb DNA ladder	Promega
PageRuler™ Prestained protein Ladder	Fermentas
ECL Western Blotting Molecular Weight Marker	Amersham Biosciences
Magic Mark™ XP western standard	Invitrogen

3.1.10 Primers:

A set of primers (Table 5, diagram 1) were delineated from human and other animals sequences to amplify CD28 ORF in horse and other wild animals. cDNAs and ORF of CD28 of known reported GenBank published sequences (Table 4) of different mammalian species (human, bovine, feline, and mouse) were aligned using MacVector® software. The most conserved regions flanking the CD28 ORF were choosen to design the outer primer pair, V4R4, with specific wobble positions to allow its flexible usage over a wide range of mammalian species. The V1R1 primer pair was designed to cover the complete ORF. The inner primers, V2R2 and V5R5 were designed to detect the most conserved region inside the CD28 ORF. Expression primers V3R3 were designed by addition of a Kozak sequence to the 5' end of V1 primer and deletion of the native stop codon from R1 primer. All specific primers were supplied by Metabion, Germany.

Table 4: GenBank accession numbers (AN) of mRNA sequences used for designing CD28 primers

Species	CD28 mRNA GenBank AN
Human (*Homo sapiens*)	NM-006139
Bovine (*Bos taurus*)	X93304
Cat (*Felis catus*)	AB025316
Dog (*Canis familiaris*)	AF259962
Mouse (*Mus musculus*)	NM-007642

Table 5: Sequence and location of different primers used for RT, PCR, and sequencing PCR of CD28 ORF.

Primer	Sequence	Amplicon length	Annealing temperature	Gene position
RT Primer:				
Random	5' d(N6) 3' [N = A,C,G,T]	Random	25ºC	Multiple
Outer primer for amplification (nested):				
V4 R4	5'-ACCCTAGCCCATCGTYAGSACA- 3' 5'-GCTCGCWTCTGGATAGGCGTCC- 3'	713 bp	51ºC	Flanking CD28 ORF
V1 R1	5'-ATGCTCAGGCTGCTCTTGGCTC-3' 5'-TCAGGAGCGATAGGCTGCGAAG-3'	663 bp	55ºC	Amplify CD28 ORF
Inner primer nested PCR (detection):				
V2 R2	5'-AACAAGATTTTGGTGAAGCAGTCGC- 3' 5'-CGGGGAGTCATGTTCATGTAGTCAC- 3'	536 bp	55ºC	Inside CD28 ORF
V5 R5	5'-CAATAATGCGGTCAACCTCAGC-3' 5'-GCGTAGAAGACTCAGTAAACGGATG-3'	359 bp	55ºC	Inside CD28 ORF
Expression vector CD28 cloning primer:				
V3 R3	5'-ACCATGCTCAGGCTGCTCTTGGCTC-3' 5'-GGAGCGATAGGCTGCGAAGTC-3'	666 bp	55ºC	Amplify CD28 ORF
Sequencing primers:				
OpIE2 F OpIE2 R	5'-CGCAACGATCTGGTAAACAC-3' 5'-GACAATACAAACTAAGATTTAGTCAG-3'	908 bp	50ºC	
T7 SP6	5'-TAATACGACTCACTATAGGG-3' 5'-TATTTAGGTGACACTATAG-3'	840 bp	51ºC	

Legend to table 5:
-V4R4 wobble bases: Y = Pyrimidine (C or T), S = G or C, W = A or T
-A Kozak translation initiation sequence was designed into the forward PCR primer, V3, providing an Adenine base at position -3 (in front of the ATG start codon) for proper initiation of translation of equine CD28 ORF.
-The native stop codon (5' TCA) was removed from R3 primer and replaced by a GGA sequence to maintain the frame through the DNA encoding the C-terminal peptide of the expression vector for detection of the expressed protein with anti-V5 mAb.

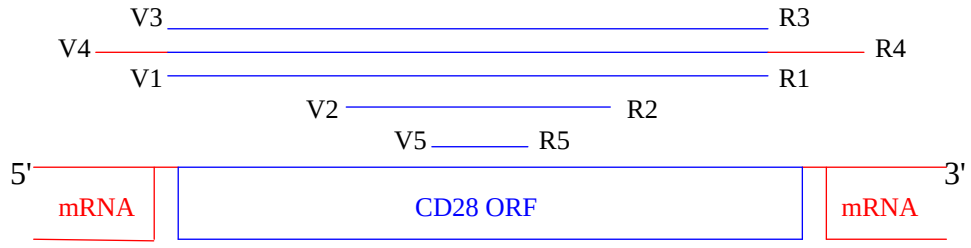

Diagram 1: Location of forward (V) and backward (R) primers around and within CD28 ORF.

3.1.11 Vector systems:

pGEM®-T Easy vector system: (Promega)

A 3,015 kb plasmid vector with 3' terminal thymidine overhangs at both ends of the plasmid insertion site to improve the efficiency of ligation of PCR products generated by classical thermostable polymerases which often add a single deoxyadenosine, in a template-independent fashion, to the 3' ends of the amplified fragments. T7 and SP6 RNA polymerase promotors flank the multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide through ligation of the PCR product allows recombinant clones to be directly identified by colour screening on indicator plates (blue/white selection) as white colonies contained the desired insert while blue colonies did not.

pIB/V5-His TOPO® TA expression vector: (Invitrogen)

A 3.5 kb plasmid vector for protein expression in insect cells, having the following features:

- 3' terminal thymidine overhangs at both ends of the insertion site.
- Topoisomerase I bound to the vector for efficient and rapid ligation of the insert.
- EM7 promotor for expression of ampicillin resistance in *E.coli*.
- *OpIE2* promotor for high-level, constitutive expression of the inserted ORF.
- Blasticidine resistance gene for selection of stable insect cell lines.
- Optional C-terminal peptide containing the V5 epitope and 6xHis tag for detection and purification of fusion protein.

3.1.12 Laboratory equipments and instruments:

FACS Calibur with multiwell autosampler, CELLQuest Pro® and MPM3® softwares	Becton Dickinson
Kendro Multifuge 3S-R with BIOshield® 7500 6435 and Highplate® 7500 6444 rotors	Heraeus
Eppendorf® cooling centrifuge 5417R with (FA-45-24-11) rotor	Eppendorf
TGradient®cycler	Biometra
Horizontal DNA electrophoresis chamber with casting tray and 8, 10, and 14 teeth combs	peQlab
PowerPac 200 electrophoresis power supply	BioRad
Consort E835 electrophoresis power supply	PeQlab
TB1 Thermal block	Biometra
ABI Prism® 3130 xI Genetic Analyser	ABI
Shaking incubator	Johana Otto GmbH
Automatic CO_2 incubator	Forma Scientific
Thermospectronic spectrophotometer	Biomat
Mini-Protean3 system	Bio-Rad
Autoradiography cassette	Sigma
Bio-View UV transilluminator	Biostep
Heidolph Reax Top vortex mixer	Heidolph
Julabo F12 Cooling water bath	Julabo
NIKON TS100 inverted Microscope	NIKON
ST3-Plate shaker	ELMI ltd.
Heidolph Duomax 1036 shaker	Heidolph
Sartorious BL1505 digital balance	Sartorious
Eppendorf® micropipettes, 10, 100, and 1000µl	Eppendorf
Eppendorf® tubes and pipetting tips	Eppendorf

3.2 METHODS:

3.2.1 Isolation of leukocytes and subsequent culture of primary cells:

Buffers and solutions:

PBS, pH 7.4:
137 mM NaCl
2.7 mM KCl
8 m M Na_2HPO_4
1.8 mM KH_2PO_4

Biocoll, ρ 1.090 and ρ 1.077

Fresh blood samples (~500 ml/sample) were collected into sterile blood bags with citrate solution (Baxter, Munich, Germany). Blood was slightly diluted with room temperature sterile PBS, pH 7.4 (40 ml blood + 10 ml PBS), aliquoted into sterile 50-ml conical centrifuge tubes and centrifugated for 15 min at 1800 rpm (660 xg), 20ºC (Kendro Multifuge 3S-R, BIOshield® Rotor 7500 6435), to obtain buffy coats. After removing the upper plasma layer, the buffy coat area was collected into another centrifuge tube and an equal volume of room-temperature PBS was added. Using 50-ml tubes again, the diluted buffy coat was layered on top of 16.5 ml Biocoll (Biochrom, Berlin, Germany, ρ 1.090) for separating leukocytes from erythrocytes. The gradient was centrifuged 20 min at 1600 rpm (520 xg), 20ºC. Thereafter, the upper layer (plasma) was removed and the leukocyte fraction (including much of Biocoll solution) was collected carefully in order not to lose cells. Half of the leukocytes were diluted again with PBS and subjected to a second gradient centrifugation with Biocoll (ρ 1.077) for 20 min at 1600 rpm (520 xg), 20ºC to isolate PBMC. These were collected and mixed with the leukocyte fraction resulting in a PBMC enriched pan-leukocyte preparation that was finally washed with PBS, pH 7.4 and centrifuged for 5 min at 1200 rpm (360 xg), 20ºC. The cell pellet (which still included a lot of platelets) was resuspended in sterile cold PBS to a final concentration of 1-2 x 10^6 cells/ml.

Activation of PBMC:

Solutions and media:

PHA-M,1mg/ml:	PHA-M was dissolved in 1 ml serum-free RPMI and aliquots were stored at -80ºC
PMA, 0.1 mg/ml:	PMA was dissolved in 1 ml DMSO and aliquots were stored at -80ºC
Ca-Ionophor A23187, 1mg/ml:	A23187 was dissolved in 1 ml DMSO and aliquots were stored at -80ºC
LPS, 1mg/ml:	LPS was dissolved in 1 ml serum-free RPMI and aliquots were stored at -80ºC

VLE-RPMI 1640 medium

For analysis of CD markers known to be expressed on activated human cells only, equine leukocytes were isolated as mentioned above and resuspended in RPMI medium

supplemented with 10% FCS, 0.1mg/ml Gentamycin and 10mM Hepes buffer and transferred to sterile tissue culture dish. Activation of leukocytes, T cells, and B cells was performed using Phorbol 12-Myritate 13-Acetate (PMA) at 20ng/ml plus the Ca-Ionophor A23187 at 500ng/ml for 12 hours. Alternatively, phytohaemagglutinin (PHA) at 5µg/ml (for T-lymphocytes) or LPS (for B-cells) at 1µg/ml for 2 days was used (all reagents from Sigma, Germany).

Isolation of monocytes from PBMC using adherence method:

Additionally, monocytes were also isolated via adherence, such as described previously (Steinbach et al., 2005). Briefly, isolated PBMC were washed three times with sterile PBS to remove platelets as far as possible. PBMC were then pelleted by centrifugation for 5 min at 1200 rpm (390 xg) 4°C, resuspended in 8 ml RPMI medium supplemented with 10% FCS, 0.1mg/ml Gentamycin and 10mM Hepes buffer and transferred to sterile tissue culture dish, incubated for one hour at 37ºC, 5% CO_2 in a humidified incubator. After incubation, non-adherent PBMC were washed out with room temperature RPMI and transferred to a new tissue culture plate and incubated for one hour at 37ºC, 5% CO_2 humidified incubator and new medium added to adherent monocytes (1^{st} collection). Three to five collections of adherent monocytes could be achieved by this way.

Differentiation of monocytes into dendritic cells (DC) and macrophages (Mϕ):

Monocytes isolated by adherence as described above were differentiated into dendritic cells (DC) and macrophages (MΦ) using the protocol described by Mauel et al. (2006). Briefly, monocytes were cultured in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS), 0.1mg/ml Gentamycin and 10mM Hepes buffer. For differentiation, equine granulocyte macrophage colony stimulating factor (GM-CSF), 200U/2ml and interleukin-4 (IL-4), 50U/2ml were added to the monocytes culture to obtain MoDC after 5 days at 37ºC, in a 5% CO_2 humidified incubator. To obtain mature DC, iDCs were treated with EqIFNα (10µl/ml) and LPS (1µg/ml) and incubated for one day. For differentiation towards MΦ, adherent monocytes were isolated after overnight incubation with lymphocytes and supplemented with EqGM-CSF (250U/ml) and LPS (1µg/ml) for two days.

3.2.2 Cell culture and cryo-preservation of cell lines:

Mammalian cell lines:

The equine cell lines eCAS and EqT8888 were cultured in RPMI-1640 supplemented with

10% FCS, 0.1mg/ml Gentamycin and 10mM Hepes buffer. Both cell lines were passaged the day before assay to ensure analysis in log-scale growth phase. eCAS monolayer was passaged at confluency and was diluted 1:2 in order to maintain log phase growth. eCAS monolayer was incubated with 2 ml of Trypsin-EDTA at 37ºC for 4-5 minutes. Detached cells then were resuspended in 10 ml of supplemented RPMI and centrifuged at 200 xg for 5 min. Supernatant was discarded and cell pellet resuspended in 8 ml of supplemented RPMI plus 2 ml conditioned RPMI (old culture supernatant) and divided into two flasks prior to adding further medium. EqT8888 suspension cultures were propagated in 25 cm^2 tissue culture flask. Propagation of these non-adherent cells did not require trypsin.

For cryopreservation, 2-4 x 10^6 viable eCAS or EqT8888 cells were centrifuged at 400 xg for 5 minutes before resuspension in 1 ml of RPMI-1640 supplemented with 20% FCS and 5% DMSO (freezing medium) in pre-cooled cryotubes. Gradient freezing was performed by incubating cells for 1 hour at -20°C then transferred to -80°C for 24-48 hours before storage in liquid nitrogen. For recovery of frozen cells, cryovials were removed from liquid nitrogen and thawed rapidly in 37°C water bath. Cells that were just thawed were transferred rapidly to 10 ml of room temperatured supplemented RPMI-1640. Cell suspension was centrifuged at 400 xg for 5 minutes and supernatant containing traces of DMSO was discarded. Cell pellet was resuspended in 4 ml of RPMI and incubated at 37°C, 5% CO_2.

Insect cells:

Express Five® serum free medium (SFM)

High five cells were maintained in confluency by subculturing in Express Five® serum free medium (SFM) supplemented with 18mM L-glutamine, 10 µg/ml Gentamycin and Amphotericin B (Fungizone), 0.25 µg/ml, and incubation at 27°C. High five adherent cell culture was passaged at confluency or slightly after when a few cells started to detach from the bottom of the flask. Subculturing was performed by sloughing the cells. Dislodged cells were then diluted at 1:5 in order to maintain log phase growth.

For cryopreservation, 3 x 10^6 viable High Five™ cells were centrifugated at 400-600 xg for 10 minutes at room temperature. Supernatant (conditioned medium) was removed and used for preparing the freezing medium (42.5% conditioned Express Five® SFM, 42.5% fresh Express Five® SFM (with L-glutamine), 10% DMSO, and 5% FCS). Cell pellet was resuspended in 1ml freezing medium before aliquoted in sterile pre-cooled cryovials. Gradient freezing was performed by incubating cells for 1 hour at -20°C, and then transferred to -80°C for 24-48 hours before storage in liquid nitrogen. For recovery of frozen cells, cryovials were removed

from liquid nitrogen and thawed rapidly in 37°C water bath. Cells that were just thawed were transferred rapidly to 4 ml of fresh Express Five® SFM (with L-glutamine) and incubated at 27°C.

3.2.3 Immunofluorescence staining of equine leukocytes:

One colour single label surface staining of leukocytes:

Buffer, solutions and reagents:

PBS, pH 7.4	S.3.2.1
1^{ry} mAbs	Appendix, tables 16 and 17
Conjugated 2^{ry} Abs	Table 2

2% Paraformaldehyde, pH 7.4:
10 gram paraformaldehyde were dissolved in 250 ml, 60°C prewarmed, 2x phosphate buffer saline stock. 250 ml deionized water were added and the solution was filtered and pH adjusted to 7.0-7.4 before storage in dark at 4°C.

A panel of 534 mAbs (Tables 16 and 17) was screened for cross reactivity with equine leukocytes surface antigens by single colour surface staining and flow cytometry. All steps were done on ice. 100 µl of a 10^6 leukocyte/ml in PBS solution were aliquoted into each well of a 96-well plate (Becton Dickinson, Heidelberg, Germany). To each well, 1µg of a tested mAb was added and mixed with cell suspension by swirling the plate at 600 rpm for 30 seconds in plate shaker before incubation on ice for 30 min. Thereafter, cells were pelleted by centrifugation at 1200 rpm (250 xg) for 5 minutes at 4°C (Kendro Multifuge 3S-R, Highplate® Rotor 7500 6444). Supernatant was discarded and the cells were washed once in 100µl cold PBS (pH 7.4), and stained thereafter for 30 min. at 4°C in the dark with secondary phycoerythrin (R-PE)-labelled donkey anti-mouse IgG (H+L) $F(ab')_2$ fragments, absorbed against human serum proteins and various other species but not rodents. In addition, secondary R-PE-labelled goat anti-rat IgG (H+L) $F(ab')_2$ fragments absorbed against human, bovine, equine and rabbit serum proteins were used for analysis of the rat mAbs and a secondary FITC-labelled donkey anti-mouse IgM (µ-chain) anti-serum absorbed against human, bovine, equine and rat serum proteins for analysis of the mouse IgM mAbs. Finally, the cells were washed again and resuspended in 200µl of cold 2% paraformaldehyde. The cells were kept at 4°C in the dark until analysis within days.

Anti-equine CD4 (CVS4) and CD8 (CVS8) were used as positive control antibodies and to control compensation via FITC and PE labelling. Negative isotype controls for matching mouse and rat isotypes were also included as part of the study. Most mAbs of the HLDA8 panel were unconjugated. For the directly conjugated mAbs, FITC-labelled isotype muIgG1 and PE-labelled isotype muIgG2a were used as control. All antibodies were tested at least

three times independently and mAbs failed to provide clear negative or positive results were tested further.

Two colour double label cell surface staining of equine leukocytes:

Two-colour flow cytometry was applied to analyze the subpopulations of lymphocytes detected using positive mAbs defined with single colour labelling. Only mAbs that stained a subpopulation of lymphocytes in the screening phase were included here. In addition, a few questionable mAbs were tested. When one Ab was not conjugated and the second one was directly conjugated, the single colour protocol was applied with indirect staining of the mAb to be tested considering 15 min incubation time and three washings with 100µl cold PBS (pH 7.4) between all steps. Free binding sites of the secondary antibodies were then blocked by incubation with 1-5 µg of isotype control mAb matching the respective mAb under investigation in the dark at 4ºC for 15 min. Three times washing with 100 µl cold PBS (pH 7.4) to get ride of excess (unreacted) mAbs was performed. Second colour labelling was then performed by incubation with 1-5µg of directly conjugated mAb for 15 min in the dark at 4ºC, followed by a final washing step and fixation. When two mAbs were of different origin (rat vs. mouse, or IgG vs. IgM), two indirect labellings could be performed consecutively, using isotype-specific negative control mAbs for blocking after first labelling. Alternatively, if two directly conjugated mAbs were used, both mAbs were incubated with leukocytes at the same time before washing and fixation.

One colour intracellular antigen staining of equine leukocytes:

Buffer, solutions and reagents:

PBS, pH 7.4	S.3.2.1
1^{ry} mAbs	Appendix, tables 16 and 17
2^{ry} conjugated Abs	Table 2
2% Paraformaldahyde	S.3.2.3 (one colour single label surface staining of leukocytes)
96% EtOH	

Intracellular staining had to be performed for some antigens, such as CD68. The cells were pelleted for 5 min at 1200 rpm in 1.5 ml Eppendorf® tube, washed once with PBS (pH 7.4) and fixed thereafter with ice-cold (-18°C) 96% EtOH for 5min. Thereafter the cells were washed again in PBS and used for staining such as described in one colour single staining protocol.

3.2.4 Flow cytometeric analysis of equine leukocytes:

Cells were analyzed using a FACS-Calibur flow cytometer (Becton-Dickinson, Heidelberg, Germany) equipped with a Multi-Well Autosampler (MAS), using Cell Quest Pro® and MPM-3® softwares. Data acquisition and analysis were performed on the basis of a protocol

described before (Steinbach & Thiele, 1994). Both FSC amplifier gain and SSC voltage were adjusted to display the scatter properties of equine leukocyte populations. Granulocytes, lymphocytes and monocytes were identified according to their scattergramme profiles, using linear FSC vs. SSC. For data acquisition, wide gates were used, whereas for analysis narrow gates were drawn to exclude unwanted debris, doublets etc. In order not to miss rare granulocyte populations (basophils and eosinophils), two additional gates were used, based to reports in the human literature. Likewise, for the analysis of activated lymphocytes, special emphasis was drawn to a region along the linear axis of PBL, where bigger cells about to divide should appear. For each defined cell population a FL1 vs. FL2 acquisition to analysis dot blot with quadrant marker and quadrant statistics window was created. Non-specific binding of mAbs together with cellular autofluorescence were considered background fluorescence and set to a background signal of 10^0-10^1 for lymphocytes. For the acquisition and analysis of platelets, log-scale scattergrammes were used, where platelets were the predominant population. Acquisition was set to record 50.000 events for each mAb.

3.2.5 Extraction of total cellular RNA from activated PBMC:

To analyze CD28 ORF of horse and wild life equines, RNA was extracted from activated PBMC using the NucleoSpin® RNA kit (Macherey-Nagel, Germany) based on silica membrane technology. Briefly, $5x10^6$ or 30 mg activated PBMC cell pellet was lysed by incubation in 350 µl buffer RA1 and 3.5 µl mercaptoethanol. Cell lysate was then cleared and its viscosity reduced by filtration through filter units. Cleared cell lysate was mixed with 350 µl of 70% ethanol and loaded to silica membrane columns. Finally, membrane desalting buffer was added and the column centrifuged to dry the membrane. To digest cellular DNA, 95 µl DNase reaction mixture were applied directly onto column and incubated at room temperature for 15 min. Residues of DNase I were removed by washing the column with 200µl buffer RA2. After further washes RNA was eluted in 40 µl nuclease-free H_2O.

3.2.6 Spectrophotometric determination of nucleic acid amount:

Absorbance of nuclease-free water diluted nucleic acid samples was measured at 260 nm after re-zeroing the spectrophotometer with the diluent. An OD of 1 corresponds to 50µg/ml dsDNA, 40µg/ml ssDNA or RNA, and 20 µg/ml in case of oligonucleotides (Sambrook et al., 1989). To obtain the concentration in µg/ml, following equations were followed:

dsDNA, µg/ml = OD_{260nm} x 50 x dilution factor

ssDNA/RNA, µg/ml = OD_{260nm} x 40 x dilution factor

Olignucleotides, µg/ml = OD_{260nm} x 20 x dilution factor

To calculate molar concentration of olignucleotides, the following equation was used:

$$\frac{OD_{260nm} \text{ x } 100 \text{ x dilution factor}}{\text{Number of nucleotides}} = X\ [\mu M]$$

3.2.7 Reverse transcription (RT):

For first strand cDNA synthesis, reverse transcription reaction was performed on total cellular RNA extracted from activated horse and wild life animals PBMC using random hexamer primer (Promega, Germany). Briefly, 2µg of total RNA and 0.5µg of random hexamer primer were added in a sterile 1.5 ml RNase-free microcentrifuge tube and the total volume completed to 10µl by RNase-free deionized H_2O. RNA/primer mix was then heated in a thermal block to 96°C for 5 minutes to melt secondary structures within the RNA template. After immediate cooling of the tube on ice to prevent reforming of secondary structures, a brief spin to collect tube contents at the tube bottom was performed before adding the following enzyme mixture (10µl) to the RNA/primer mixture:

Component	Volume	Final conc.
5X M-MLV Reaction Buffer	4µl	1x
dNTPs, 10mM	1µl	0.5 mM
Recombinant RNasin® Ribonuclease Inhibitor	1µl	25 units
M-MLV RT	1µl	400 units
Nuclease-Free Water	3µl	

The RT mixture (20 µl) was incubated at 25°C for 10 minutes at a thermal block to anneal the hexamer primer to RNA templates before the temperature was raised to 37°C for 60 minutes for reverse transcription. To degenerate the remaining reverse transcriptase, RT mixture was heated to 95ºC for 5 minutes thereafter.

3.2.8 Polymerase chain reaction (PCR):

For amplification of target sequences, 50 µl polymerase chain reactions were performed. In 0.5 ml nuclease-free PCR tube the following components were added in the order shown:

Component	Volume	Final conc.
10X Reaction Buffer (with 15mM $MgCl_2$)	5µl	1X
PCR Nucleotide Mix (10mM each dNTP)	0.5µl	100µM
up-stream primer, 10µM	2µl	0.4µM
down-stream primer, 10µM	2µl	0.4µM
Taq DNA Polymerase, 5U/µl	0.5µl	2.5U
cDNA	5 µl	
Nuclease-Free Water	35 µl	

The PCR reaction mix was substracted to the following thermal protocol in a TGradient® cycler (Biometra, Germany):

	Temp.	Duration	No. of cycles
Initial denaturation:	96°C	3 min.	one
Amplification:	96°C (denaturation)	30 sec.	25
	*NN°C (annealing)	30 sec.	
	72°C (extension)	30 sec.	
Final extension:	72°C	10 min.	one

* NN°C = specific primer annealing temperature (Table 5)

3.2.9 Horizontal agarose gel electrophoresis:

Buffer:

1x Tris-acetate-EDTA (TAE) buffer, pH 8.0:
40 mM Tris-acetate
1 mM EDTA

Size of the PCR product was analyzed by electrophoresis through agarose gel. Briefly, agarose powder (Promega) was mixed with TAE electrophoresis buffer to 1% (W/V) concentration and heated in a microwave oven until melted. Ethidium bromide was added to the agarose solution (final concentration 0.5 µg/ml). After cooling to about 60ºC, the solution was poured into a casting tray containing a sample comb and allowed to solidify at room temperature. The comb was then removed and the gel covered with TAE buffer. DNA samples were mixed with 6x gel loading buffer and pipetted into sample wells. In a separate well, 100 bp PageRuler DNA ladder® (Fermentas) was loaded and electrophoresis performed at 60 V. To visualize DNA, the gel was placed on ultraviolet transilluminator and photographed.

3.2.10 Purification of PCR products:

The Wizard®SV Gel and PCR Clean-Up System (Promega) is based on the ability of DNA to bind to silica membranes in the presence of chaotropic salts. Briefly, after electrophoresis, the DNA fragment of interest was excised in a minimal volume of agarose using a clean scalpel and transferred to a weighed microcentrifuge tube. Membrane binding solution was added in a ratio of 10µl/10mg of agarose gel slice then incubated at 60ºC for 10 minutes or until complete dissolving of the gel slice. The dissolved gel mixture was transferred to SV minicolumns and incubated for 1 minute at room temperature before being centrifuged in a microcentrifuge at 16.000 xg (14.000 rpm) for 1 minute. Then, the SV column was washed by adding 700 µl of membrane wash solution. The washing step was repeated using 500µl

membrane wash solution. Then, the column was transferred to a clean 1.5 ml microcentrifuge tube and 25 µl of nuclease-free water was used to elute the DNA which stored at –20ºC.

3.2.11 Cloning of CD28 into pGEM–T® Easy cloning vector:

pGEM®-T Easy vector system was used for cloning of CD28. To ligate purified PCR product (3.2.10) to pGEM®-T Easy vector, 2X Rapid Ligation Buffer was vortexed vigorously and the ligation reaction was set to 10 µl total reaction volume and mixed by pipetting before incubation for 2 hours at room temperature.

Component	Volume
2X Rapid Ligation Buffer, T4 DNA Ligase	5µl
pGEM –T® Easy cloning vector (3015bp)	1µl (50ng)
purified CD28 ORF	xµl*
T4 DNA Ligase (3 Weiss units/µl)	1µl
deionized water to a final volume of	10µl

*volume of purified PCR product (insert) was determined to achieve an insert:vector ratio of 3:1 using the following equation:

$$\frac{\text{ng of vector X kb size of insert}}{\text{kb size of vector}} \text{ X insert:vector molar ratio = ng of insert}$$

Preparation of competent XL-1 bacteria:

Solutions and media:

TfBI:

100 mM $RbCl_2$	2.41 g
45 mM $MnCl_2$	1.13 g
35 mM $KC_2H_3O_2$	0.69 g
10 mM $CaCl_2$	2 ml (of 1M stock solution)
0.5 mM HCl	200µl (of 0.5M solution)
15% Glycerine	30 ml

pH adjusted to 5.8 with 0.2 M glacial acetic acid
deionized H_2O to 200 ml
then filter-sterilised and stored at –20ºC

TfBII:

10mM MOPS	0.105 g
75mM $CaCl_2$	0.45 g
10mM $RbCl_2$	0.06 g
15% Glycerin	7.5 ml

pH adjusted to 7.0 with 0.1 M NaOH
deionized H_2O to 50 ml
then filter-sterilised and stored at –20ºC

LB agar plate:
32 g of LB agar (Invitrogen) were dissolved in 1 litre deionized H_2O then autoclaved for 15 minutes. The medium allowed to cool down to 50ºC before poring 8-10ml of medium into 21mm Petri dishes and the agar left to harden.

LB broth:
20 g of LB broth base (Invitrogen) were dissolved in 1 litre deionized H_2O and autoclaved for 15 minutes.

XL-1 (frozen stocks) were streaked on LB agar plates using a sterile platinum loop and incubated at 37ºC overnight. One colony was picked into 5 ml LB broth and incubated overnight at 37ºC with shaking (~150 rpm/min). One ml of the overnight broth culture was transferred to 200 ml LB medium and incubated at 37ºC with shaking till OD_{600} was 0.45-0.5. XL-1 culture was then aliquoted in 50 ml tubes and incubated on ice for 10 minutes before centrifuged at 300 rpm in Kendro Multifuge 3S-R with a pre-cooled BIOshield® Rotor 7500

6435 for 10 minutes at 4ºC. Then, each cell pellet was resuspended in 7 ml ice cold TfBI and incubated for 10 minutes on ice, before again centrifuged at 2000 rpm for 5 minutes at 4ºC. Carefully, each cell pellet was resuspended in 2 ml ice cold TfBII. 50 µl aliquots of cell suspension were then aliquoted in pre-cooled Eppis and immediately put in liquid nitrogen before storing at –80ºC.

Transformation of competent XL-1:

Medium:

SOC medium:

2% Tryptone
0.5% Yeast extract
10 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
20 mM glucose

20 g tryptone, 5 g yeast extract and 0.5 g NaCl were dissolved in 950 ml deionized water. To dissolved components, 10 ml of 250 mM KCl solution (1.86 g of KCl dissolved in 100 ml deionized H_2O) were then added and pH adjusted to 7.0 with 5 N NaOH (~ 0.2 ml). The volume was adjusted to one litre with deionized H_2O then sterilized by autoclaving. Sterile solution of 2 M $MgCl_2$ (19 g of $MgCl_2$ dissolved in 90 ml of deionized H_2O then sterilised by autoclaving) was added to a final concentration of 10 mM. Filter sterilised 1 M glucose was added to a final concentration of 20 mM.

LB/ampicillin/IPTG/X-Gal plates:
32 g of LB agar (Invitrogen) were dissolved in 1 litre deionized H_2O and autoclaved. The medium was allowed to cool down to about 50ºC before adding ampicillin to a final concentration of 100 µg/ml then 8-10 ml of medium were pored into 21mm Petri dishes and left to harden at room temperature. Thereafter, 100µl of 100mM IPTG and 20µl of 50mg/ml X-Gal were spread over the surface to absorb for 30 minutes at room temperature.

Frozen XL-1 competent cells were removed from storage and placed in an ice bath until just thawed then mixed by gentle flicking of the tube. 50 µl of thawed XL-1 competent cells were transferred to the ligation reaction (3.2.11) and mixed by gentle flicking of the tube before incubation in ice for 20 minutes. Thereafter, a heat-shock was applied for 45-50 seconds in a water bath (or thermal block) at 42ºC without shaking, followed by immediate incubation on ice bath for two minutes. 950 µl SOC medium were then added to the tube and incubated at 37ºC for 1.5 hour with shaking (~150 rpm/min). To select transformed bacterial colonies, 200µl of transformed culture were plated onto LB/ampicillin/IPTG/X-Gal plates and incubated at 37ºC overnight before storage for 24 hours at 4ºC to facilitate colour reading. White transformant colonies indicated positive transformation with disrupted β-galactosidase.

3.2.12 Extraction of cloned plasmids from positive *XL-1* transformants:

Cloned plasmids were purified using WIZARD® plus minipreps DNA purification system (Promega). A single white colony of CD28 transformed *XL-1* was picked from LB selective plates into 10 ml LB broth with 100µg ampicillin/ml and incubated at 37ºC overnight with shaking. 5 ml of bacterial culture were harvested by centrifugation for 5 minutes at 10.000 xg.

Supernatant was discarded and the cell pellet resuspended in 250 µl of cell resuspension solution. 250 µl of cell lyses solution were added next and mixed gently, prior to incubation at room temperature until the cell suspension cleared (max. 5 min.). 10 µl of alkaline protease to inactivate proteases were added and mixed by inverting the tube. Then, incubation for 5 minutes at room temperature was performed before 350 µl of neutralizing solution were added and mixed. Bacterial lysates were cleared by centrifugation at 14.000 xg for 10 minutes and cleared supernatant (lysate) was transferred to WIZARD® mini-column and centrifuged at maximum speed for 1 minute at room temperature. Then, the column was washed by 750 µl of washing solution and centrifuged at maximum speed for 1 min. The washing step was repeated. WIZARD® mini-column silica membrane was then dried by centrifugation for 2 minutes and plasmid was eluted in 50 µl nuclease free water. Part of eluted plasmid was forwarded to restriction enzyme digestion to confirm the presence of CD28 ORF insert while another part was forwarded for sequencing after confirmation of positive insertion by RE digestion.

Restriction enzyme (RE) digestion of CD28 constructs:

The multiple cloning site of pGEM-T® Easy vector is flanked by recognition sites for the restriction enzymes *Eco*RI, *Bst*ZI, and *Not*I, thus providing single-enzyme digestions to isolate the insert and confirm its size by agarose gel electrophoresis. To digest CD28 constructs with *EcoRI*, a restriction enzyme mix of 20 µl total volume was pipetted:

Component	Volume
Sterile deionized water	14.3µl
10X buffer	2µl
Acetylated BSA, 10µg/µl	0.2µl
DNA, 1 µg/µl	2.5µl
EcoRI (10U/µl)	1µl

The restriction digestion reaction was incubated at 37ºC for 1 hour and the size of the digest was analyzed by agarose gel electrophoresis (3.2.9).

3.2.13 Demonstration of cloned fragments by SP6-T7 PCR:

Alternative to restriction enzyme digestion, DNA inserts could be demonstrated by PCR from transformed colonies using primers to SP6 and T7 promoters (Table 5). To expose plasmid DNA, thermal lysis of one white colony picked from LB selective plate was performed in 10µl of deionized water by boiling for 10 minutes. Bacterial cell lysates were cooled down for 5 minutes on ice before centrifugation at 16.000 rpm for 10 minutes to collect cell debris. Supernatant, containing plasmid DNA, was used as template for SP6-T7 PCR using

conditions described in chapter 3.2.8. Insert size was confirmed by agarose gel electrophoresis (3.2.9).

3.2.14 Chain terminating sequencing PCR:

Reagents and solutions:

BigDye® Terminator v3.1 cycle sequencing kit.
2.2% SDS:
1.1 g SDS dissolved in 50 ml deionized H_2O.
70% ethanol:
35 ml of absolute ethanol were diluted with 15 ml nuclease free H_2O.

CD28 ORF was first amplified by PCR (as in step 3.2.7) using primers annealing to T7 and SP6 promoters as described above. Amplified fragment was purified (3.2.10) before two chain terminator single strand sequencing PCRs using The BigDye® Terminator v3.1 Cycle Sequencing Kit (ABI) were then applied, using in different tubes the T7 primer or the Sp6 primer to analyze the complete sequence of CD28 ORF. DNA concentration of purified CD28 ORF was determined using a spectrophotometer (3.2.6), before calculating the optimal template concentration required for sequencing PCR as nanogram = template length/15.
PCR mix was performed at room temperature in a 0.2 ml PCR tube as follows:

Component		Volume
5x sequencing buffer		2µl
T7 **or** Sp6 primer (10 µM)		1µl
Big dye		1µl
DNA template		xµl
deionized water	to	10µl

The following thermal cycles were applied:

96ºC	10 sec.	35 cycles
50ºC	5 sec.	
60ºC	4 min.	

Directly before sequencing, amplified DNA was purified. Therefore the 10µl PCR reaction, 8µl H_2O and 2µl SDS 2.2 % were mixed well and heated in a thermal block to 98ºC for 5 min. Spun shortly before 30µl of 0.5M Sodium acetate were added and mixed well by pipetting. Then, 125µl of absolute ethanol were then added and the mix centrifuged at top speed (16.000 rpm) for 20 minutes. Supernatant was discarded and the pellet was washed by the addition of 180 µl of 70% ethanol. Pellets were air-dried at room temperature with their covers opened. Pellets were resuspended in 20µl nuclease free water and placed in an ABI Prism 3130 xI Genetic Analyser. Data were analyzed using MacVector® software package. Obtained CD28 sequences were analyzed for local similarities with GenBank published sequences using the

Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast) before further analyses using MEGA3 software.

3.2.15 Preservation of transformed bacterial clones:

Medium:

LB/ampicillin broth:
20 g of LB broth base (Invitrogen) were dissolved in 1 litre deionized H_2O and autoclaved for 15 minutes. The medium was allowed to cool down to 50ºC before adding ampicillin to 100 µg/ml final concentration.

To prepare stock cultures of transformed bacteria, positive XL-1 clones (steps 3.2.12, 3.2.13, and 3.2.14) were grown in LB/ampicillin broth over night at 37ºC with shaking (~150 rpm/min). To 0.85 ml of bacterial culture, 0.15 ml of sterile glycerol were added and the culture was vortexed to evenly disperse glycerol. Cultures containing glycerol were aliquoted in 1ml cryotubes and stored at –80ºC.

3.2.16 Expression of equine CD28 using pIB/V5-His TOPO® TA expression kit:

To express equine CD28, the cloned ORF cDNA was amplified from pGEM-T® Easy plasmid (step 3.2.11) using V3R3 expression primers (Table 5) which have the same sequence of V1R1 primers except that the forward primer (V3) contains an added Kozak translation initiation sequence which included an **A** at position –3 (**A**CCATG) for proper start of translation. To include the C-terminal peptide for detection of expressed protein with V5 antibodies and purification using the 6x His-tag, the native stop codon was removed by the backward primer (R3). PCR was performed as mentioned in step 3.2.8.

TOPO® cloning reaction and transformation:

Medium:

LB/ampicillin agar plates:	S. 3.2.11 (transformation of competent XL-1)
LB/ ampicillin broth:	S. 3.2.15
Express Five® SFM	

pIB/V5-His-TOPO® expression vector, was supplied linearized with a single 3' thymidine (T) overhangs for TA cloning and with a bound topoisomerase for efficient ligation. V3R3 amplicon was cloned into pIB/V5-His-TOPO® expression vector as follows:

Component		Volume
Purified PCR product (V3R3 amplicon)		0.5 to 4 µl
Salt solution (1.2M NaCl; 0.06M $MgCl_2$)		1µl
Nuclease free H_2O	to final volume of	5µl
TOPO® vector		1µl

The reaction was gently mixed and incubated for 5 minutes at room temperature.

XL-1 competent cells were transformed (3.2.11) with the cloned vector pIB/V5-His-TOPO® vector and selected on ampicillin LB agar plates. Recombinant plasmids from XL1 transformants were analyzed by PCR for the presence and orientation of CD28 ORF inserts. PCR was performed (3.2.8) using the vector specific forward sequencing primer and backward R3 primer. Plasmids were extracted from PCR positive clones (3.2.11) and sequenced to confirm that equine CD28 ORF was cloned in frame, using vector specific sequencing primers (Table 5) as described above (3.2.14).

Lipid mediated transfection using Lipofectamine™ 2000 (Invitrogen) was applied to transfect High five insect cells with equine CD28 constructs. High five™ insect cells with high viability were passaged (3.2.2) one day before transfection to maintain log phase growth and 80-90% cell density. To 50µl of Express Five® serum free medium (SFM), 0.8µg of TOPO® CD28 constructs were added and mixed gently. In a separate tube, 2µl of Lipofectamine® 2000 were diluted in 50 µl Express Five® SFM. After five minute incubation, diluted DNA was added, mixed gently and incubated at room temperature for 20 minutes before the DNA-lipid complex was added to the high five cell suspension in 24 well tissue culture plates. A positive control expression vector plus four negative controls representing Lipofectamine™ 2000, High Five™ insect cells, pIB/V5-His-TOPO® expression vector and CD28 ORF amplicon were included in other wells of the 24 well tissue culture plates. Transfected cells were incubated at 27ºC overnight and medium was replaced with Express Five® SFM containing 18mM L-glutamine, 10mg/ml Gentamycin and 0.25 µg/ml Amphotericin B. To select positive transfectants, Blasticidine was added to a final concentration 40 µg/ml. To test CD28 mRNA transcription, total cellular RNA was extracted (3.2.5) and RT-PCR (methods 3.2.7 and 3.2.8) performed using V5R5 detection primers (Table 5, diagram 1). Transfected cells were incubated for two weeks to allow selection of stable transfectants. To test for expression of CD28 protein, pellets of CD28 transfectants and untransfected high five cells (negative control) were lysed with SDS sample loading buffer and analyzed by SDS-PAGE and western blot (3.2.18) using anti-V5 mAb which detects the N-terminal 14 amino acids epitope of the expression construct. Alternatively, polyclonal anti-CD28 antibody (Table 1) was applied.

3.2.17 Immunoprecipitation of leukocyte surface antigens:

Buffers and reagents:

PBS: S.3.2.1

Triton-X lysis buffer:
150mM NaCl
1% TritonX-100
50mM Tris-HCl pH 8.0
Complete Mini EDTA protease inhibitor (Roche) 1 tablet/10 ml lyses buffer

Protein G 4 fast flow®
EZ-link® Sulfo-NHS-Biotin reagent
Immunopure® strpetavidine-AP
CDP-Star® chemilumencense substrate

Immunoprecipitation is a technique for analytical separation of target antigens from crude cell lysates. By combining immunoprecipitation with other techniques, such as SDS-PAGE and immunoblotting, it can be used to detect antigens and determine relative molecular weights. Immunoprecipitation is a technique by which an antigen is isolated by binding to a specific antibody attached to a sedimentable matrix. Protein G Sepharose is an affinity medium for the efficient and rapid isolation of antibodies from various solutions due to their specificity for the Fc region of IgG from a wide range of mammalian species.

To prepare a 50% Protein G 4 fast flow® (Amersham Biosciences) slurry, 75 µl of the slurry was washed three times with ice cold 500µl lysis buffer by centrifugation at 12000 xg for 30 seconds. Then, an equal volume (75µl) of lyses buffer was added to obtain 50% slurry. Equine cell suspensions were centrifuged at 300 rpm/min for 5 minutes and washed twice with PBS pH 8.0 (alkaline pH) two times to get rid of the medium before re-susupension in 1ml PBS pH 8.0. To biotinylate cell surface proteins, 1mg of EZ-link® Sulfo-NHS-Biotin reagent (Pierce) was added to 1 ml of cell suspension (~25 x 10^6 cells/ml in PBS pH 8.0) and incubated for 30 min at 4ºC to prevent active internalization of the biotin reagent. Biotinylated cells were then washed twice with PBS (pH 7.2-7.4) with 100mM glycine to bind and remove excess biotin reagent. A further washing step was performed without glycine. After biotinylation, cells could be preserved at -80ºC or used directly for IP.

For lyses of the biotinylated cells, 500 µl of ice-cold Triton X-100 cell lysis buffer were added. Then, the cell suspension was incubated at 4ºC for 30 minutes with gentle mixing. Cell lysate was centrifuged at 12000 xg for 10 minutes at 4ºC to remove particles and supernatant was transferred to a new tube. Cell lysate was pre-cleared by adding 100µl of 50% Protein G 4 fast flow® sepharose slurry with gentle mixing, using MacsMix® cell mixer (Miltenyi Biotech) for one hour at 4ºC. After centrifugation at 12000 rpm at 4ºC, supernatant was transferred to a new tube. To couple antigen and antibody, 1 µg of candidate cross-reactive mAb was added to 500 µl of pre-cleared cell lysate, and incubated for one hour at 4ºC with gentle mixing. To precipitate the immune complexes, 50µl of 50% Protein G 4 fast flow®

sepharose were added and incubated at 4ºC for 1 hour with gentle mixing prior to centrifugation at 12000 xg for 30 seconds at 4ºC. The pellet was then washed three times with 500µl ice cold lysis buffer and supernatant was carefully discarded to avoid loosing beads. To analyze the immun-complexes, beads were resuspended in 30 µl of SDS sample loading buffer, heated at 95ºC for 3 minutes in a thermal block to denature immune complex/Protein G and centrifuged at 14000 xg for 1 minute at 4ºC. Supernatant containing the separated antigen and antibodies was analyzed by SDS-PAGE (3.2.18) and membrane blotting followed by application of Immunopure® streptavidin-AP (Pierce) and detection with CDP-Star® chemilumencense substrate (Roche) as described in step 3.2.19.

3.2.18 SDS-Poly acrylamide gel electrophoresis (SDS-PAGE):

Buffers and solutions:

1.5M Tris-HCl pH8.8/SDS:

SDS	0.2 g
1.5M Tris-HCl pH 8.8	50 ml

2x SDS sample-loading buffer:

100 mM Tris-HCl (pH 6.8)
4% (w/v) SDS
0.2% (w/v) bromophenol blue
20% glycerol
200 mM β-Mercaptoethanol (added before the buffer was used)

3% Acrylamide pre-mix:

Acrylamide 40%, 29:1	3.8 ml
0.5M Tris-Hcl pH 6.8/SDS	10 ml
50% sucrose	6 ml
Bromophenol Blue 1%	500µl
$H_2O_{bidest.}$ to	50 ml

8% Resolving gel:

Acrylamide 40%, 29:1	2 ml
1.5M Tris-HCl pH 8.8/SDS	1.7 ml
50% Sucrose	1.3 ml
$H_2O_{bidest.}$	4.948 ml
10% Ammonium persulfate	43 µl
TEMED	9µl

Stacking gel:

Acrylamide 3% pre-mix	4ml
10% Ammonium persulfate	28µl
TEMED	6µl

10x MOPS running buffer:

0.2 M MOPS (pH 7.0)
20 mm sodium acetate
10 mM EDTA (pH 8.0)
sterilised by filtration and kept in dark.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was used for the analysis of immunprecipitated CD molecules (3.2.17) and the expressed equine CD28 (3.2.16). The denatured polypeptides bind SDS and become negatively charged. Because the amount of SDS should be proportional to the molecular weight of the polypeptide, SDS-polypeptide complexes migrate through polyacrylamide gels in accordance with their size. To cast SDS-Polyacrylamide gels, components of Mini-Protean 3 system (Bio-Rad) were assembled. 8% resolving gel mix with freshly added ammonium persulphate and TEMED was poured between the glass plates and layered with 0.3 M Tris-HCl pH 8.8/SDS for 30 minutes at room temperature for polymerization. Tris-HCl over-lay was decanted and stacking gel premix with freshly added ammonium persulphate and TEMED poured. 30 µl of immunoprecipitation

elutes (3.2.17) or 7 µl of equine CD28 insect cell lysates (3.2.16) were heat-denatured at 100ºC for 10 minutes prior to loading SDS-Polyacrylamide gels. 4 µl of page ruler™ prestained protein ladder (Fermentas) were loaded in one lane and electrophoresis was conducted at 60 V until the blue band of the loading buffer reached the resolving gel where upon electrophoresis voltage was increased to 100. Electrophoresis was stopped when the sample front reached the end of the resolving gel.

3.2.19 Western Blot and immunostaining (Immunoblot):

Buffers and reagents:

Transfer buffer:
- 48 mM Tris base
- 39 mM glycine
- 20% methanol

Blocking buffer:
- 10 mM Tris-Cl (pH8.0)
- 150 mM NaCl
- 0.05% (v/v) Tween-20
- 1% (w/v) gelatine [blocking agent]

TBST, pH 7.4:

Tris base		3g
NaCl		8g
KCl		0.2g
$H_2O_{bidest.}$	to	1000 ml

pH adjusted to 7.4 with HCl
Tween 20 added to 0.05%

Alkaline phosphatase conjugated 2nd Abs — S. table 2
Immunopure® strpetavidine-AP (Pierce)
CDP-Star® chemilumencense substrate (Roche)

Electrophoretically separated proteins (3.2.15) were transferred from polyacrylamide gels to protein binding nitrocellulose membranes (Hybond™-ECL™, Amersham). Biotinylated equine leukocyte CD molecules were probed with alkaline phosphatase conjugated streptavidin. When weak signals were obtained, the precipitating cross reactive human mAb was re-used for probing the transferred protein in combination with a secondary alkaline phosphatase conjugated antibody. In all cases, the enhanced chemiluminescence (ECL) detection was used and signals emitted from the enzymatically dephosphorylated product were captured on Kodak® Biomax light films (5 in.x 7 in.).

To transfer proteins, Hybond™-ECL™ nitrocellulose membranes (6cm x 8cm) were equilibrated for 10 minutes in transfer buffer. Criterion Blotter® (BioRad) transfer unit was filled with pre-chilled (+4ºC) transfer buffer. A transfer sandwich was assembled by placing the membrane on Whatman paper which had been moistened with transfer buffer. Thereafter, the polyacrylamide gel was removed from the two glass plates and placed on the nitrocellulose membrane before the gel was overlaid with further moistened Whatman paper. The transfer cassette was then placed into the transfer unit with the gel towards the negative pole and the membrane towards the positive pole. Protein transfer was performed at 490mA for one hour at 4ºC with continuous cooling. Before immunostaining of transferred proteins,

blocking of the unoccupied sites on the nitrocellulose membrane was performed by incubation with 25 ml of blocking buffer for 30 min at room temperature with gentle agitation (Duomax 1036 shaker, Heidolph). Blotted immunoprecipitated proteins were stained by incubating the blocked membrane with 9.3µg (2µl) of Immunopure® strpetavidine-AP in 10 ml TBST for one hour at room temperature with gentle agitation. Membrane was washed 4 times, 15 minutes each, in 50 ml PBS (pH 8.0) with 0.1% Tween-20 to remove unbound streptavidin-AP.

To detect CD28 expression, membrane blots of electrophoresed transfected insect cell lysates were stained by incubation with 2µg anti-V5 mAb or anti-human CD28 polyclonal Ab in 10ml TBST for one hour at room temperature with gentle agitation. Then, membrane was washed 3x for 5 minutes in 50 ml TBST to remove unbound Ab. Blotted membranes were incubated with 10 ml of 1:10.000 dilution of 2^{nd} antibodies, anti-mouseAP or anti-goatAP. Incubation with 2^{nd} Abs was performed for 30 minutes at room temperature with gentle agitation, followed by 5x washing for 5 minutes with 50 ml TBST at room temperature with gentle agitation to get rid of unbound 2^{nd} Abs.

For detection, the membranes were equilibrated for 10 minutes in 10 ml luminescence buffer (Prionics). Thereafter, membranes were placed on a glass plate and 3 ml of 1:100 freshly diluted CDP-Star® chemilumencense substrate (Roche) in luminescence buffer were distributed evenly on the membrane and incubated for 5 minutes in the dark at room temperature. Excess substrate was removed with soft tissue and the membrane was placed in foil in an autoradiography cassette before exposure to Kodak® Biomax light film (5 in.x 7 in.). Light films were developed manually using 1:5 diluted Kodak® Lx2 x-ray developer applying standard photographic procedures until optimal signals were visualised.