III. MATERIAL AND METHODS:

3.1 Material

3.1.1 Blood samples

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

3.1.2 Biologic substances

Mitogenic activators:

Phytohemagglutinin (PHA)	(Sigma, Deisenhofen)
Calcium-ionophore (Ca-ionophore)	(Sigma, Deisenhofen)
Phorbol-12-myristate-13-acetate (PMA)	(Sigma, Deisenhofen)
Concavalin A (ConA)	(Sigma, Deisenhofen)

Antigens:

Equine herpesvirus-1, A IV (inactivated)	(Dr. F. Steinbach)
Tetanus toxoid #191A/B	(List Biological Laboratories, USA)

Antibiotics:

Ampicillin	(AppliChem, Darmstadt)
Blasticidin	(Invitrogen, Carlsbad, USA)
Gentamycin	(Biochrom, Berlin)

Competent bacteria:

Top10 competent bacterial cells	(Promega, Madison, USA)
JM109 high efficiency competent cells	(Promega, Madison, USA)

Insect cell line:

High Five stable insect cell line for expression	(Invitrogen, Carlsbad, USA)

Recombinant biological substances:

Reference recombinant equine Interleukin 4 (rec.eqIL-4)	(Endogen, Rockford, USA)

Molecular biology enzymes and reagents:

DNase I, RNase-free	(Roche Diagnostics, Mannheim)
RNasin ribonuclease inhibitor	(Promega, Madison, USA)
Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase RNase H minus, point mutant	(Promega, Madison, USA)
Avian myeloblastosis virus (AMV) reverse transcriptase	(Promega, Madison, USA)
Taq DNA polymerase	(Promega, Madison, USA)
pGEM-T easy vector system I	(Promega, Madison, USA)
T4 DNA ligase	(Promega, Madison, USA)
EcoR1 restriction enzyme	(Promega, Madison, USA)

10 mM dNTPs mix	(Promega, Madison, USA)
Nuclease free water	(Fermentas, St. Leon-Rot)
O'GeneRuler™ 100bp DNA Ladder	(Fermentas, St. Leon-Rot)
PageRuler prestained protein MW Ladder, RTU	(Fermentas, St. Leon-Rot)
Ethidium bromide	(Sigma, Deisenhofen)
Agarose molecular biology grade	(Roth, Karlsruhe)
HEPES (4, (2-hydroxyethyl)-1-piperazineethanesulfonic acid)	(Roth, Karlsruhe)

3.1.3 Kits

RNA extraction kits:

Nucleospin® RNA II kit	(Macherey-Nagel, Düren)
SV total RNA isolation system	(Promega, Madison, USA)

Gel elution and PCR clean-up kits:

Wizard SV Gel and PCR Clean-Up System	(Promega, Madison, USA)
Nucleospin® Extract kit	(Macherey-Nagel, Düren)
Wizard Plus Minipreps DNA Purification System	(Promega, Madison, USA)

Cloning vector:

pGEM®-T Easy Vector System	(Promega, Madison, USA)

Expression systems:

PIB/V5-His TOPO ®TA expression kit	(Invitrogen, Carlsbad, USA)

Sequencing kit:

ABI BigDye® Terminator v3.1 cycle sequencing ready reaction kit	(ABI, Darmstadt)

Silver staining kit:

SilverSNAP® Stain Kit II	(PIERCE, Rockford, USA)

3.1.4 Media, buffers and solutions

Luria Bertani (LB) medium with ampicillin: (Invitrogen)

Bacto®-Tryptone	10 g/L
Bacto®-Yeast Extract	5 g/L
NaCl	5 g/L

pH was adjusted to 7.5 with NaOH and autoclaved to be sterilized. The medium was allowed to cool to 55°C and ampicillin was added (final concentration 100µg/ml). For LB plates, 15 g agar was included prior to autoclaving.

IPTG stock solution, 0.1M:

IPTG		1.2 g	(Promega, Madison, USA)
H_2O	to	50 ml	

Filter sterilized through a 0.2 µm filter unit and store at 4°C.

X-Gal:

X-Gal	50 mg/ml in dimethylformamide (DMF)	(Promega, Madison, USA)

Glucose, 2M: (Athena Enzyme System, Baltimore, USA)

Glucose		180.16 g
H_2O	to	500 ml

Filter- sterilized through a 0.2 µm filter unit and aliquots stored stable for 1 year at –20°C.

Sterile glycerol: (Merck-Schuchardt, Hohenbrunn)

Glycerol 99% autoclave sterilized and stored sterile at room temperature to be used for preservation of cultures containing positive bacterial clones.

Mg^{++}stock solution, 2M:

$MgCl_2$-$6H_2O$		101.5 g
$MgSO_4$-$7H_2O$		123.3 g
d. H_2O	to	500 ml

Filter sterilized through a 0.2 μm filter unit.
Note: Filter-sterilizing units should be prerinsed with distilled water before use to remove any toxic material

SOC medium:

Bacto®-tryptone		2.0 g	
Bacto®-yeast extract		0.5 g	
NaCl (1M)		1 ml	
KCl (1M)		0.25 ml	
Mg^{++} Stock		1 ml	Mg^{++} stock: (1M $MgCl_2$; 1 M $MgSO_4$)
Glucose (2M)		1 ml	
Filter-sterilized H_2O	to	100 ml	

Bacto-tryptone, Bacto-yeast extract, NaCl and KCl were added to the distilled water then stirred to dissolve, autoclaved and cooled to room temperature. Mg^{++}stock (2M) and glucose (2M) stock were added, each to a final concentration 20 mM. The complete medium was filtered through a 0.2 μm filter unit and pH was adjusted to 7.0.

3.1.5 Laboratory equipments and instruments

Flow cytomery:

FACSCalibur with multiwell autosampler (Becton Dickinson, Heidelberg)
CELLQuest Pro® software (Becton Dickinson, Heidelberg)

Centrifuges:

Eppendorf cooling centrifuge 5417R with (FA-45-24-11) rotor (Eppendorf, Hamburg)
Kendro Multifuge 3S-R with BIOshield® Rotor 7500 6435
and Highplate® Rotor 7500 6444 (Heraues, Berlin)

Incubators:

Shaking incubator (Johanna Otto GmbH, Hechingen)
Automatic CO_2 incubator (Forma Scientific LABEQUIP, Ontario, Canada)

Thermal cyclers:

TGradient®cycler (Biometra, Göttingen)
Mx3005P Real Time PCR System (Stratagene, La Jolla, CA, USA)

Other equipments:

UV-Transiluminator (Biometra, Göttingen)

NIKON TS100 inverted Microscope (Nikon, Dusseldorf)

TB1 Thermal block (Biometra, Göttingen)

Spectrophotometer (Thermospectronic) (BioMate, Rochester, NY, USA)

Mini-Protean3 system (Bio-Rad, München)

Bio-View UV transilluminator	(Biostep, Jahnsdorf)
Heidolph Reax Top vortex mixer	(Heidolph, Kelheim)
Eppendorf micropipettes, 10, 100 and 1000µl	(Eppendorf, Barkhausenweg, Hamburg)
Eppendorf tubes and pipetting tips	(Eppendorf, Barkhausenweg, Hamburg)
Julabo F12 Cooling water bath	(Labortechnik GmbH, Seelbach)
Consort E835 electrophoresis power supply	(PEQLab, Erlangen)
PowerPac 200 electrophoresis power supply	(Bio-Rad, München)

3.1.6 Standard chemicals

Where not listed extra standard chemicals and media were obtained through Merk (Darmstadt), Sigma (Diesenhofen), Roth (Karlsruhe), Fluka (Neu-Ulm), Biochrom (Berlin) and Pharmacia (Freiburg). Sterile tissue culture disposable wares were obtained from Becton Dickinson (Heidelberg) and Nunc (Wiesbaden).

3.2 Methods

3.2.1 Isolation of peripheral blood mononuclear cells and monocytes

Solutions and media used for peripheral blood mononuclear cells (PBMC) isolation:

Biocoll (Ficoll®) (density; 1.090 g/ml and 1.077 g/ml) (Biochrom, Berlin)

HEPES (4, (2-hydroxyethyl)-1-piperazineethanesulfonic acid) 15 mM

Complete RPMI (RPMI, Biochrom, supplemented with glutamine 2 mM, 10% bovine calf serum (FCS) and gentamycin [50 µg/ml]).

Phosphate Buffered Saline (PBS), pH 7.4

NaCl		137.0 mM (8 g)
KCl		2.7 mM (0.20 g)
Na_2HPO_4 X2 H_2O		8.0 mM (1.42 g)
KH_2PO_4		1.8 mM (0.24 g)
d.H_2O	to	1000 ml

Blood was collected from healthy animals (African buffalo, European bison, Nubian giraffe, Asian elephant, domestic horse, Indian rhinoceros, Hartmann's mountain zebra and Grevy's zebra) at the Tierpark-Berlin and the Horse Clinic of the Faculty of Veterinary Medicine, Free University Berlin. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient separation over Biocoll (1.090) and thereafter, over Biocoll (1.077). Isolated PBMC were washed 3-times with sterile PBS and re-suspended in complete RPMI.

Monocyte isolation:

In order to obtain monocytes, PBMC were suspended at 10^7/ml in complete RPMI (3.2.1). Thereafter, they were incubated in plastic tissue culture plates at 37°C for an hour in

humidified atmosphere containing 5%CO_2 tension. Monocytes were left to adhere to the plate whereafter lymphocytes could be washed off using fresh complete RPMI.

3.2.2 Flow cytometry analysis

Horse monocytes were incubated with *in vitro* expressed eqIFNγ and eqIL-4 (3.2.4) for various times and in different concentrations. Thereafter the effects on equine monocytes were analysed using flow cytometry. Anti-equine MHC II (VMRD, clone eqT2, isotype mIgG2a) and anti-human CD14 antibodies (Biometec, clone big 12, isotype mIgG1) were used. Monocytes were spun down in a microcentrifuge (1200 rpm for 5 min), supernatant was removed and the cells were resuspended in 50 µl cold PBS. Unconjugated first monoclonal antibody mAb was added to the monocytes. Samples were incubated on ice in the dark for 30 min, washed once with 1ml PBS and spun down. Monocytes were resuspended in 50 µl PBS and PE-labelled anti-mouse IgG as secondary conjugated antibody (Jackson Immuno-research) was added for another 30 min. After this, samples were washed again with PBS. Labelled cells were fixed through resuspension in 200 µl paraformaldehyde (2.5% in PBS), stored at 4°C until being analysed by flow cytometry on a FACSCalibur with CELLQuest software (Becton Dickinson) parallel to nontreated monocytes and isotype negative controls.

3.2.3 Cloning of IFNγ and IL-4 of various species

Schematic diagram for the cloning of cytokines:

PBMC isolation & activation
↓
RNA extraction & Reverse transcription
↓
(Nested) PCR (to span ORF)
↓
PCR product gel electrophoresis
↓
DNA purification
↓
Ligation/Transformation
↓
Plasmid extraction
↓
Sequencing
↓
Re-cloning
(In case of expression only)
↓
Detection PCR for confirmation of selected clones
↓
Transfection/Gene expression

In vitro stimulation of peripheral blood mononuclear cells:

Mitogens were used for non-specific polyclonal stimulation of PBMC. Either ConA (5 μg/ml) or PHA (5 μg/ml) was added to freshly isolated PBMC. Mitogen stimulated PBMC cultures were incubated at 37°C in humidified atmosphere of 5% CO_2 tension for 24 hours. PBMC were harvested and washed twice with sterile PBS before pellets were stored at –80°C until RNA extraction.

3.2.3.1 RNA extraction from isolated PBMC

Total cellular RNA was extracted using the RNA isolation Nucleospin RNA II kit (Macherey-Nagel). This combines the disruptive and protective properties of guanidine thiocyanate (GTC) and β-mercaptoethanol to inactivate the ribonucleases, and in association with SDS, acts to disrupt nucleoprotein complexes, allowing the RNA to be released into solution and isolated free of proteins. The solution contains large amounts of chaotropic ions and so appropriate binding conditions were created to favour the adsorption of the RNA to silica membranes. Contaminating DNA was destroyed by a DNase I solution directly applied during the silica membrane binding. Washing steps with buffers removed salts, metabolites and macromolecular cellular components. RNA was finally eluted under low ionic strength conditions with RNase free water and kept in –80°C until reverse transcription.

3.2.3.2 Reverse transcription

Random hexamer primers were used for mRNA reverse transcription (Promega). RNA was transcribed into cDNA using random hexamer primers and a reaction mix set-up below:

RNA primer mix	Volume	Enzyme mix	Volume
Random hexamer primers RNA Nuclease free water	1 μl =20 pmol 3 μl 6 μl	5X Buffer dNTPs 10 mM RNasin M-MLV Nuclease free water	4.00 μl 1.25 μl 1.00 μl 400U (1 μl) 2.75 μl

In case of use of AMV, 20U (2 μl), instead of M-MLV, was added and the water volume was reduced to 1.75 μl.

RNA primer Mix (10 μl) was heated in a thermal block for 5 minutes at 95°C for denaturation and thereafter, kept on ice for 5 minutes followed by quick spin and addition of the enzyme mix. This mix was incubated in thermal blocks for an hour at 37°C (42°C in case of AMV) followed by 5 minutes at 95°C for termination of the reaction. cDNA was stored at –20°C until further use.

3.2.3.3 Polymerase chain reaction (PCR)

Design of primers for IFNγ and IL-4:

cDNAs containing the open reading frames (ORF) of IFNγ and IL-4 from previously reported sequences of different mammalian species (equine, bovine, mice and human) were aligned using MacVector software (Accelrys, Cambridge, UK) to design two pairs of primers for each target. The outer primer pair was chosen to flank an area outside the ORF and the inner primer pair designed to cover the complete ORF. Wobble positions were used to allow their use over a wide range of species; K= G or T, W= A or T, Y= C or T

Species	IFNγ GenBank accession no.	IL-4 GenBank accession no.
Equine	D28520	AF305617
Bovine	M29867	M77120
Mouse	K00083	M25892
Human	M37265	M13982

IFNγ outer primers: (Metabion, Martinsried)

V3: 5' AAGATCAGCTACCTCCTTKGGACCTG 3' Tm 65.6°C
R3: 5' GATTTAAAAATTCAAATATTGCAGGCAG 3' Tm 57.8°C
Product size 619 bp with optimal annealing temperature 55.0°C

IFNγ inner primers: (Metabion)

V4: 5' ATGAAATATACAAGTTATWTCTTRGCTTT 3' Tm 56.1°C
R4: 5' TTATTTYGAYGCTCTCCGGCCTCGAA 3' Tm 64.8°C
Product size 501 bp with optimal annealing temperature 54.2°C

IL-4 outer primers: (Metabion)

Primer 1: 5' AATAGAGATACTATTAATGGGTCTCACC-3' Tm 57.2°C
Primer 2: 5' CTCATAAATTAAAATATTCAGCTTCAAC-3' Tm 56.3°C
Product size 447 bp with optimal annealing temperature 53.1°C

IL-4 inner primers: (Metabion)

Primer 1: 5' ATGGGTCTCACCTACCAGCTGAT 3' Tm 61.5°C
Primer 2: 5' TCAACACTTGGAGTATTTCTCCTTCA 3' Tm 59.3°C
Product size 408 bp with optimal annealing temperature 54.6°C

IFNγ detection primers: (specific for horse sequence) (Metabion)

Primer 1 (eqIFNγ intv): 5' CAGAGCCAAATCGTCTCCTTCTAC 3' Tm 65.2°C
Primer 2 (eqIFNγ intr): 5' TCTTCCGCTTCCTCAGGTTAGC 3' Tm 64.0°C
Product size 256 bp with optimal annealing temperature 54.3°C

IL-4 detection primers: (specific for horse sequence) (Metabion)

Primer 1 (eqIL-4intV): 5' CCAACTGATTCCAGCTCTGGTC 3' Tm 64.0 °C
Primer 2 (eqIL-4intR): 5' ACAGTACAGCAGGTCCCGTTTG 3' Tm 64.0 °C
Product size 318 bp with optimal annealing temperature 56.5°C

PCR reactions were carried out using the Taq-polymerase system (Promega) according to the manufacturer's instructions and basic protocols and with the following reaction mix and cycling parameters:

Reagent	Volume in 50µl PCR
10X PCR Buffer	5 µl
dNTPs 10 mM	0.5 µl
Taq-polymerase	2.5 U (0.5 µl)
Forward primer (20 pmol)	1 µl
Reverse primer (20 pmol)	1 µl
Nuclease free water	38 µl
cDNA	4 µl

An initial step of denaturation at 95°C for 5 min, followed by 35 cycles of (30 sec at 95°C, 30 sec at optimal annealing temperature and one min at 72°C for extension), thereafter one cycle of 10 min at 72 °C for final extension.

Sequencing primers: (Promega)

T7: 5' TAATACGACTCACTATAGGG 3'
SP6: 5' GATTTAGGTGACACTATAG 3'

A SP6/T7 PCR reaction was carried out according to the basic protocols above. Here 17 µl of boiled bacterial lysate were applied and amount of nuclease free water was reduced accordingly. The annealing temperature was set at 51°C.

3.2.3.4 Gel electrophoresis

Tris-acetate EDTA (TAE) 1X:
0.040 M Tris-acetate
0.001 M EDTA
Ethidium bromide 10 mg/ml Stock

PCR products were run through a 1% agarose gel (in TAE buffer) containing Ethidium bromide (5 µl/100ml agarose solution). In parallel, a 100 bp DNA ladder (Fermentas) was applied to determine the size of the DNA. DNA fragments were visualized through UV light.

3.2.3.5 Purification of the DNA

In case of presence of only one band of the expected size, the remainder of the PCR product (40 µl) underwent clean-up using the Wizard SV Gel and PCR Clean-up System (Promega). DNA binds to the membrane-based minicolumn and impurities were removed using the membrane wash solution. Elution of the DNA was carried out using nuclease-free water.

The concentration of DNA was determined diluting 4 µl by the addition of 76 µl distilled water (i.e. 1: 20) and using spectrophotometer measurement of absorption at 260 nm (A_{260}). The A_{260}/A_{280} should be between 1.7 and 1.9 as smaller ratios indicate the presence of

contaminating proteins. An OD of 1 corresponds to 50 μg/ml for dsDNA and 40 μg/ml for ssDNA and RNA, and 20 μg/ml for oligonucleotides (Sambrook et al., 1989).

To obtain concentration of nucleic acid in μg/ml, the following equation was used:

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

In case of the presence of more than one band in the PCR, the band of interest was excised, and the DNA separated from agarose and Ethidum bromide through the use of Wizard SV Gel and PCR clean-up system.

3.2.3.6 Preparation of competent bacteria

TfBI

100 mM $RbCl_2$		2.41 g
45 mM $MnCl_2$		1.13 g
35 mM potassium acetate		0.69 g
10 mM $CaCl_2$		2 ml of 1M stock solution
0,5 mM HCl		200 μl of 0.5M solution
15% Glycerine		30 ml
pH adjusted to 5.8	with	0.2M glacial acetic acid
dd.H_2O	to	200 ml

Sterile filtration of TfBI and stored at –20ºC

TfBII

10mM MOPS		0.105 g
75mM $CaCl_2$		0.45 g
10mM $RbCl_2$		0.06 g
15% Glycerine		7.5 ml
pH adjusted to 7.0 with		0.1 M NaOH
d.H_2O	to	50 ml

Sterile filtration of TfBII and stored at –20ºC

Stretches of XL-1 or top10 frozen stocks were placed on LB agar plates and incubated overnight at 37 ºC. One colony was picked into 5ml LB broth and incubated overnight at 37ºC with shaking. One ml of the overnight broth culture was transferred to 200ml LB medium and incubated at 37 ºC with shaking until the culture had a density of: OD_{600} = 0.45 - 0.5. XL1 cells were then aliquoted in 50 ml tubes and incubated on ice for 10 minutes before being centrifuged at 3000 rpm in a minifuge for 10 minutes at 4ºC. Each cell pellet was resuspended in 7ml ice cold TfBI and incubated for 10 minutes on ice before a spin down at 2000 rpm for 5 minutes at 4ºC. Carefully, each pellet was resuspended in 2ml ice cold TfBII. 50μl aliquotes were then aliquoted in cold Eppendorf tubes and immediately put in liquid nitrogen before storage at –80°C.

3.2.3.7 Ligation and transformation

The pGEM-T easy and PIB/V5-His TOPO vectors contain a 3´ terminal thymidine overhang to both ends. Thereby, providing a compatible overhang for PCR products generated by Taq

polymerases, as these add a single deoxyadenosine in a template-independent fashion to the 3´-ends of the amplified fragments. The pGEM-T easy vectors also contain T7 and SP6 RNA polymerase promoter sequences flanking the cloning region. Insertional inactivation of the alpha-peptide allows recombinant clones to be directly identified by colour screening on indicator plates as white colonies contain a DNA insert while blue colonies do not.

Purified PCR products were ligated inside the vector in a total volume of 10 µl ligation reaction as below. This reaction mix was incubated at room temperature for 1 hour or at 4°C overnight.

Reagent	Volume
2X ligation buffer	5 µl
Vector	1 µl
Ligase	1 µl
DNA elute	1.5 µl
Nuclease free water	1.5 µl

For transformation, 2 µl of the ligation reaction were added to 50 µl thawed competent bacteria and incubated on ice for 20 minutes. Then, bacteria were heat shocked for 45-50 seconds in 42°C and as soon as possible were put on ice for 2 minutes. Thereafter, 950 µl SOC media was added and incubated at 37°C for 1 hour with 150 rpm shaking. Transformed bacteria were grown overnight on LB plates with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and IPTG (isopropylthio-β-D-galactoside) containing ampicillin. X-Gal turns blue when catalyzed by β-galactosidase, the gene encoded by the pGEM-T Easy plasmid. If the ligation was carried out successfully, this gene will be interrupted. After overnight incubation at 37°C, white colonies were selected using a sterile toothpick. A colony was boiled in water bath for 10 minutes, centrifuged for 5 minutes at 2000 rpm before supernatant was taken into the a PCR reaction. The toothpick with the remaining bacteria was put into 3ml LB ampicllin broth for over-night incubation 37°C with shaking 150 rpm/min. The next day, plasmid extraction could be performed and the correct plasmids were stored at –20°C.

3.2.3.8 Clone analysis

Extraction of the plasmids of transformed bacterial cells was carried out using Wizard SV Minipreps DNA Purification System (Promega), a silica membrane-based system, based on principles related to those described earlier.

Restriction Enzyme Analysis (REA):

The restriction enzyme EcoR I was used to determine the composition of extracted plasmids. The analytical restriction enzyme reaction was performed in a total volume of 20 µl on 0.2-1.5 µg of substrate DNA.

The restriction enzyme digestion reaction mix contained:

Sterile nuclease free water	14 µl
Restriction enzyme 10X buffer	2 µl
BSA, Acylated (1mg/ml)	2 µl
DNA sample 0.2-1.5 µg, in water	1 µl
Restriction enzyme (2-10) U	1 µl

3.2.3.9 Sequence analysis

The universal T7/SP6 primer pair was also used in the chain terminating sequencing PCR, stopping the extension with big-dye kit from Applied Biosystems (ABI). DNA concentration of purified gene ORF was determined using a spectrophotometer (3.2.3.5). Thereafter, the template concentration required for sequencing PCR was calculated as followed:

nanogram = template length/15. It is, however, better to use 2-3 times more in order to produce sufficient signals at the end of sequences. For the same plasmid, two sequencing reactions, one containing forward primer T7 and the other containing the reverse primer SP6 were carried out in a 0.2 ml PCR tube as follows:

Reagent	**Volume in 10 µl PCR**
5X sequencing buffer	2 µl
ABI Big dye kit	1 µl
Either T7 primer 10µM	1 µl =10 pmol
Or SP6 primer 10µM	1 µl =10 pmol
DNA of extracted plasmid	To be estimated
Nuclease free water	Added to 10 µl

The PCR reaction was carried out as 35 cycles of (96ºC for 10 seconds, 50ºC for 5 seconds and 60ºC for 4 minutes).

In case of direct sequencing from the PCR products, the inner primer pair was used instead of T7/SP6 and the PCR product instead of the plasmid.

After the sequencing PCR, 10 µl PCR reaction, 8 µl H_2O and 2 µl SDS 2.2 % were mixed well and heated immediately in a thermal block at 98°C for 5 min. After short centrifugation, Sodium acetate 0.5 M (30 µl) was added and mixed well. Absolute ethanol (125 µl) was then added and the mix centrifuged at top speed (16000 rpm) for 20 minutes. Supernatant was discarded and the pellet was washed by addition of 180 µl ethanol 70% and thereafter, centrifuged at 16000 rpm for 8 minutes and the supernatant discarded again. Pellets were air-dried at room temperature with their lids opened. Pellets were resuspended in 20 µl nuclease free water and placed in the ABI Prism 3130 genetic analyzer. Sequencing was carried out in collaboration with the workgroup evolution genetics, IZW. Data were analysed using MacVector software package and the obtained 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 analysis using MEGA3 software.

3.2.3.10 Preservation of transformed bacterial clones

Bacterial clones shown to contain the target plasmids were preserved for further use. Sterile glycerol was added in a ratio of 1:3 (i.e. 3 ml LB ampicillin broth containing bacterial culture grown to the log phase to be actively increasing were added to 1.5 ml glycerol). Thereafter, vortexed to disperse glycerol and aliqouted in 1.5 ml cryotubes then stored at –80°C.

3.2.4 Expression of recombinant equine cytokines (IFNγ and IL-4)

Both cytokines were expressed using the expression vector pIB/V5-His TOPO with the insect cell line High Five (Invitrogen). Cloning was performed such as described above (3.2.3.7). The cloning success was determined performing a PCR with a vector specific sequencing forward primer and the cytokine antisense specific reverse primer (3.2.3.3). Such expression system uses the signal sequences of the equine cytokine-specific mRNAs. In order to have bioactive cytokines, the V5-His tag was avoided using the cytokine-specific stop codon. Lipofectamine^{TM} 2000 (Invitrogen) is a proprietary formulation for the transfection of nucleic acids into eukaryotic cells. The cells were selected for gene expression by adding blasticidin.

Prior to transfection, 5 x 10^4 High Five cells were plated in a 24-well tissue culture plates with 500 µl of growth medium without antibiotics, so that cells were 90-95% confluent at the time of transfection. Lipofecatmine was gently mixed and 2 µl were diluted in 50 µl Opti-MEM I medium and incubated for 5 minutes at room temperature. Diluted DNA was combined with diluted Lipofectamine, gently mixed, and incubated for 20 minutes at room temperature. The complexes were added to each well containing cells and media and mixed gently by rocking the plate. The following day selective antibiotic, Blasticidin (10 µg/ml) was added.

To check the transcription of the equine cytokine specific mRNA, the RNA was prepared (3.2.3.1) and reverse transcribed with cytokine specific internal primers (3.2.3.2).

3.2.5 SDS-poly acrylamide gel electrophoresis and protein staining

To analyse the equine IL-4 protein expression, sodium dedocyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) and silver staining were performed.

Concentration of equine IL-4 expressed proteins:

To overcome the low-concentration of protein in insect cell supernatants, trichloroacetic acid/deoxycholate (TCA/DOC) was used to concentrate expressed eqIL-4 for SDS-PAGE.

100% TCA solution	2% Na-deoxycholate (DOC) in distilled water
Acetone	Laemmli sample treatment buffer

1/100 volume of 2% DOC was added to one volume eqIL-4 insect cell supernatant and kept on ice for 15 min. Then, 1/10 volume 100% TCA was added and kept on ice for 1 hour. Thereafter, the sample was centrifuged at 10.000 xg for 10 min at 4ºC, followed by washing the pellet in acetone in order to get rid of residual TCA. Thereafter, the pellet was re-solubilised adding Laemmli sample treatment buffer to the protein. If the sample changed to yellow, TCA still remained (resulting in reduced pH) and proteins would probably not migrate towards the anode. This problem could be overcome by adding a few microlitres of 1M Tris, pH 8.8 until the colour changed back to blue (basic pH).

SDS-PAGE solutions

1.5M Tris-HCl pH 8.8/SDS:

SDS	0.20 g
1.5M Tris-HCl pH 8.8	50.00 ml

3% Acrylamide pre-mix:

Acrylamide 40%, 29:1	3.80 ml
0.5M Tris-Hcl pH 6.8/SDS	10.00 ml
50% sucrose	6.00 ml
Bromophenol Blue 1%	500.0 µl
H_2O_{bidest} to	50.00 ml

8% Resolving gel:

Acrylamide 40%, 29:1	2.00 ml
1.5M Tris-HCl pH 8.8/SDS	1.70 ml
50% Sucrose	1.30 ml
$H_2O_{bidest.}$	4.948 ml
10% Ammonium persulfate	43.0 µl
TEMED	9.0 µl

Stacking gel:

Acrylamide 3% pre-mix	4.00 ml
10% Ammonium persulfate	28.00 µl
TEMED (stir quickly)	6.00 µl

2x SDS gel-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 just before the buffer was used)

10x MOPS running buffer:

0.2 M MOPS (pH 7.0)
20 mm sodium acetate
10 mM EDTA (pH 8.0)
Filtered and stored in the dark.

Protein ladder Marker

PageRuler^{TM} prestained protein ladder (Fermentas)

The denatured polypeptides bind SDS and become negatively charged. SDS-polypeptide complexes migrate through polyacrylamide gels in accordance with the size of the

polypeptide. Polyacrylamide gels are composed of chains of polymerized acrylamide that are cross-linked by bisacrylamide. Effective range of separation of SDS-Polyacrylamide gels depends on the concentration of polyacrylmide used to cast the gel and on the amount cross-linking. The mini-protean3 system (Bio-Rad) was used. Two glass plates with integrated spacers were put in the casting frame. 8% resolving gel mix with freshly added ammonium persulphate and TEMED was poured between the glass plates and then layered with 0.3 M Tris-HCl pH 8.8/SDS and left for 30 minutes at room temperature for polymerization. Tris-HCl over-lay was removed and stacking gel premix with freshly added ammonium persulphate and TEMED was poured, followed by hanging a plastic comb for 30 minutes at room temperature during polymerization. TCA/DOC concentrated proteins with Laemmli buffer were heated at 100ºC for 10 minutes before loading to SDS-PAGE. A PageRuler prestained protein ladder was also loaded. Thereafter, electrophoresis was conducted at 60V until the blue band of the loading buffer indicated that the sample reached the resolving gel. Then, electrophoresis voltage was increased to 100V. Electrophoresis was stopped when the samples front reached the end of the resolving gel.

Silver staining for SDS-PAGE gel:

SDS-PAGE gels were silver stained using the SilverSNAP stain kit II (PIERCE), containing sensitizer, enhancer, stain and developer. The additional materials required were ethanol, acetic acid and ultra pure water.

Solution preparations:

Fixation solution: 30% ethanol: 10% acetic acid solution (i.e. 6:3:1 water: ethanol: acetic acid).
Sensitizer working solution: 1 part sensitizer with 500 parts ultra pure water.
Stain working solution: 1 part enhancer with 50 parts stain.
Developer working solution: 1 part enhancer with 50 parts of developer.
Stop solution: 5% acetic acid was used as a stop solution.

SDS-PAGE gels were washed twice in ultra pure water for 5 minutes. Thereafter, gels were soaked in fixation solution for 15 minutes, before the solution was discarded, replaced and the fixation step repeated for another 15 minutes. Gels were rinsed twice in 10% ethanol for 5 minutes followed by two washes in ultra pure water for 5 minutes each. Thereafter, incubated in sensitizer working solution for exactly one minute and washed twice with ultra pure water for 1 minute each. Gels were soaked for 30 minutes in stain working solution. Gels were rinsed quickly with two changes of ultra pure water for 20 seconds each and incubated in developer working solution until protein bands appeared (2-3 minutes). When the desired band intensity was reached, the developing solution was discarded and replaced by the stop

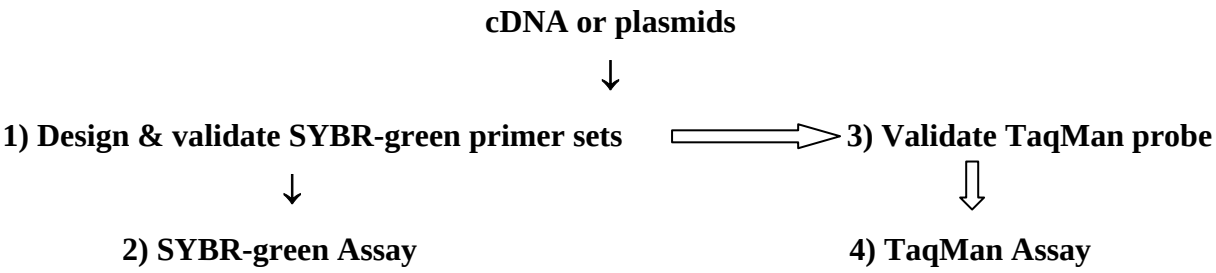
solution. Thereafter, gels were washed briefly before transfer to an un-used stop solution for 10 minutes.

3.2.6 Real-time PCR

The TaqMan chemistry uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR. TaqMan probes are linear oligonucleotides, designed to allow close proximity of the fluorophore and quencher in the intact, unstructured probe. The fluorophore is usually at the 5´ end of the probe, and the quencher is either internal or at the 3´end. As long as the probe is intact, no fluorescence is observed from the fluorophore. During the extension step of the PCR, the polymerase displaces the TaqMan probe by 3 or 4 nucleotides and the 5´nuclease activity of the DNA polymerase cuts the fluorophore from the quencher. When fluorescence signal from a PCR reaction is monitored in real-time, the results can be displayed as an amplification plot. C_t is defined as the cycle at which fluorescence is determined to be statistically significant above background. The more template that is initially present, the fewer the number of cycles it takes to reach the point where the fluorescence signal is detectable above background.

A real time PCR may also be quantified using intercalating dye (SYBR-green) analogous to Ethidium bromide, or by alternative probe technologies such as molecular beacons.

Real-time PCR development scheme:



PCR primers and the TaqMan probe for equine IFNγ and IL-4 were designed using Primer Express Software (ABI), or by Metabion and TIB MOLBIOL through their programmes. 6-carboxy-fluorescein (FAM) was used as a reporter dye with non-fluorescent quencher (NFQ). The ABI TaqMan probe was conjugated to a minor groove binder (MGB) to form hyper-stabilized duplex with complementary DNA giving higher melting temperature (T_m). The conjugation of a MGB allowed the design of shorter probes with increased specificity at elevated hybridization temperatures (Kutyavin et al., 2000). Primer sets were designed to overlay a putative intron-exon junction to eliminate signal from genomic DNA contamination, with amplicon lengths of 80 to 200 bp. The T_m was pre-defined at 60°C.

Alternatively, standard TaqMan probes with FAM as a reporter and 4-(4'-dimethylaminophenylazo)-benzoic acid (DABCYL) as a dark quencher were synthesized by TIB MOLBIOL, or by Metabion with 6-carboxy-tetramethyl-rhodamine (TAMRA) as a quencher dye.

Real-time PCR eqIFNγ primers and TaqMan probe: (specific for horse sequence)

ABI designed primers and probe set:

Forward primer (EQIFNG-EX3F) 5`-CCAGGTCATTCAAAAGAGCATGGA-3`
Reverse primer (EQIFNG-EX3R) 5`-CTGGTGCTGCTGTTAAAGAACTT-3`
Probe (EQIFNG-EX3M1) 5`-FAM-TCAAGGAGGACCTGTTCGTT-NFQ-MGB-3`

TIB MOL BIOL designed primers and probe set:

Forward primer (IFNγ-Se): 5`-TGAAGAACTGGAAAGAGGATAGTGAC-3`
Reverse primer (IFNγ-Rev): 5`–GCTGCTGTTAAAGAACTTAACGAA-3`
Probe (IFNγ-TM): 5`-FAM-TCCTCCTTGATGGTGTCCATGCTCTT-DABCYL-3`

Real-time PCR eqIL-4 primers and TaqMan probe: (specific for horse sequence)

ABI designed primers and probe set:

Forward primer (EQIL4-EX2F) 5`-GACAGGTCCTTGATCAAAGAATGC-3`
Reverse primer (EQIL4-EX2R) 5`-TTGCCATGCCCTTGAGGTT-3`
Probe (EQIL4-EX2M) 5`-FAM-CCTGTCCAGTCCGCTCAG-NFQ-MGB-3`

Metabion designed primers and probe set:

Forward primer (eqIL-4Qv): 5`-AGAATGCCTGAGCGGACTGG-3`
Reverse primer (eqIL-4Qr): 5'-TGCTCTTCTTGGCTTCATTCACAG-3`
Probe (eqIL-4-S): 5`FAM-AGCAGGTCCCGTTTGCCATGC–TAMRA-3`

TIB MOLBIOL designed primers and probe set:

Forward primer (eqIL-4vor): 5`-TTCGTGCATGGAGCTGACTG-3`
Reverse primer (eqIL-4rück): 5`-CCGCTCAGGCATTCTTTGATC-3`
Probe (eqIL-4-TM): 5`-FAM-TGCCTTTGCTGGCCCGAAGAACAC- DABCYL-3`

Real-time quantitative PCR can provide both absolute and relative quantification during the exponential phase of amplification. In order to achieve comparative values, the use of a housekeeping gene was required.

18S rRNA primers (for normalization in QPCR): (ABI)

Forward primer: 5`-CGGCTACCACATCCAAGGAA-3`
Reverse primer: 5`-GCTGGAATTACCGCGGCT-3`
Probe: 5`-VIC-TGCTGGCACCAGACTTGCCCTC-MGB-3`

SYBR-green is an intercalating dye, which binds only to double stranded DNA giving an increase in fluorescence as the amount of PCR product increases. Since SYBR-green chemistry detects all double-stranded DNA, including non-specific reaction products, a well-optimized reaction is essential for SYBR-green based assays.

Real-time PCR reagents:

Brilliant QPCR Master Mix 2X	(Stratagene)
Brilliant SYBR-green PCR Master Mix 2X	(Stratagene)
Passive reference dye (ROX)	(Stratagene)

Carboxy-*x*-rhodamine (ROX) is a reference dye that can be used to compensate changes in fluorescence between wells caused by slight volume differences (pipetting errors) in reaction tubes. This allows for the dRn (Baseline-corrected normalized fluorescence) analysis, in contrast to dR (Baseline-corrected raw fluorescence) to be measured.

Each reaction using a TaqMan probe was prepared by adding components in the order listed:

Reagent	Volume
Brilliant QPCR master mix	12.5 µl
ROX (30 nM)	0.375 µl
Forward primer (20 pM)	1 µl
Reverse primer (20 pM)	1 µl
TaqMan probe (2.5-5.0 pM)	1 µl
Sample	10 µl

Real time PCR cycling parameters

Two-step amplification:

Initial denaturation at 95°C for 10 min to activate Taq polymerase was followed by 40 cycles each of, denaturation at 95°C for 30 seconds and annealing plus extension at 60°C for 1 min.

Three-step amplification:

An initial step at 95°C for 10 min, followed by 40 cycles of 30 seconds at 95°C, 1 min at 58°C for annealing and 30 seconds at 72°C for extension.

Alternatively, a reaction using SYBR-green was prepared by adding the following components:

Reagent	Volume
SYBR-green mix	12.5µl
Forward primer (20 pM)	1 µl
Reverse primer (20 pM)	1 µl
ROX (30 nM)	0.375 µl
cDNA	10 µl

Quantitative SYBR-green PCR thermal cycling conditions comprised of a three-segment protocol as follows: segment I (1 cycle) as an initial step at 95°C for 10 min, segment II (40 cycles each of) 95°C for 30 seconds, 55°C for 1 min and 72°C for 30 seconds and segment III (1 cycle) 95°C for 1 min, 55°C for 30 seconds and slowly up to 95°C for 30 seconds.

3.2.6.1 Real-time PCR optimization

Gene specific primers and probes for horse IFNγ and horse IL-4 were designed independently. Primers and probe concentrations were first optimized on a positive control, here plasmids containing the cDNA. Thereafter, standard curves were analysed to determine efficiency, precision and sensitivity. A SYBR-green dissociation curve was performed on cDNA from horse lymphocytes to analyse specificity of the primers. PCR reactions for IFNγ and IL-4 were additionally examined by agarose gel electrophoresis to confirm that only one PCR product was synthesized.

A standard curve is a linear relation by plotting the threshold cycle against the logarithm of the known amount of the initial template. The equation of the line that best fitted the data is determined by regression analysis. A standard curve was generated from datasets obtained from a serial (log10) dilution of positive control (plasmids).

The slope of the curve is directly related to the average amplification efficiency throughout the cycling reaction. The equation that relates the slope to amplification efficiency used by the software was: PCR efficiency = $10^{(-1/\text{slope})}$–1, and a value between –3.1 and –3.6 (corresponding to 90-110% reaction efficiency) was considered satisfactory. To analyse the precision of the standard curve, R Squared (RSq), correlation coefficient value was calculated by the software from individual data points obtained. If all the data were perfectly on a line, the Rsq value is 1.00. An Rsq value ≥ 0.985 is generally considered acceptable.

A normalizer is a gene with little to no variance in expression due to treatment of cells. The 18S rRNA TaqMan set of primers and VIC-MGB probe are known to cross-react with numerous species even beyond mammals (ABI, pers. comm.). It was therefore chosen in relative gene expression QPCR experiments. The 18S rRNA was included in comparative gene expression assays to reduce the variations due to sample-to-sample differences in total amount of the input of cDNA, here for example based on differences in the RNA extraction or reverse transcription. The ratio between the 18S content in calibrator samples (e.g. prior stimulation) and test samples (e.g. after stimulation) was defined as the normalization factor.

To determine the relative quantitation of gene expression, the comparative C_t method was applied (Whetstine and Matherly, 2001). For each sample, a threshold cycle (C_t) was determined for the genes of interest (i.e. IFNγ and IL-4) as well as for the housekeeping gene (i.e. 18S rRNA).

The software calculated relative gene expression as $\Delta\Delta C_t$ where the changes in relative gene expression of target were determined by evaluating the expression $2^{(-\Delta\Delta Ct)}$ whereby,

$\Delta\Delta C_t = \Delta C_t$ (sample i.e. cells after treatment) – ΔC_t (calibrator i.e. cells without treatment).

$\Delta C_t = C_t$ (gene of interest e.g. IFNγ) – C_t (normalizer i.e.18S rRNA).

3.2.6.2 Estimation of plasmids copy number

In order to determine the minimal number of plasmid molecules detectable by QPCR, the spectrophotometric measurement of nucleic acid concentration was applied for DNA quantification (3.2.3.5), where 1 unit at 260 nm corresponds to 50 μg DNA per ml. Further calculation was based on the following formula for dsDNA (applied using the Biomath Calculator, Promega):

$$\mu g\ DNA \times \frac{pmol}{660pg} \times \frac{10^6 pg}{1\mu g} \times \frac{1}{N} = pmol\ DNA$$

Where, N = number of nucleotides (here size of plasmid) and 660 pg/pmol is used as the average molecular weight of a nucleotide pair.

IFNγ stock plasmid concentration was determined as:

3.5 kb (plasmid) at 35 ng/μl = 15 pmol/ml = 15.082 x 10^{-12} mol/ml = 9.0 x 10^9 molecules/μl

IL-4 stock plasmid concentration was determined as:

3.4 kb (plasmid) at 25 ng/μl = 11 pmol/ml = 11.043 x 10^{-12} mol/ml = 6.6 x 10^9 molecules/μl

3.2.6.3 Lymphocyte stimulation and co-stimulation

For lymphocyte stimulation, Ca-ionophore and PMA were added to freshly isolated PBMC at different concentrations according to the experimental design.

In order to determine the optimal duration of Phorbol myristate acetate (PMA) activation, horse PBMC were stimulated with 0.1 μg/ml cell suspension for different times (1-18 hours), before cellular RNA was extracted and reverse-transcribed using the random hexamer primers. Levels of IFNγ were determined using absolute quantitative real-time PCR.

Another important aim was the determination of the optimum and the suboptimum PMA concentrations. Therefore, different PMA concentrations (1.00-0.0001 μg/ml cell suspension) were applied for 8 hours. Stimulated PBMC cultures were incubated at 37°C in humidified atmosphere of 5% CO_2 tension. PBMC were harvested and washed twice with sterile PBS before pellets were stored at –80°C until RNA extraction. Whole RNA was extracted, reverse-transcribed and thereafter mRNA expression levels of IFNγ were determined in absolute

quantitative real-time PCR TaqMan protocol. In both cases, the comparability of data was checked using 18S rRNA as a control.

Horse PBMC were also stimulated with one Ca-ionophore concentration 1.00 μg/ml cell suspension along different times 2-24 hours. Stimulated PBMC cultures were incubated at 37°C in humidified atmosphere of 5% CO_2 tension. PBMC were harvested and washed twice with sterile PBS before pellets were stored at –80°C until RNA extraction. The cellular RNA was extracted, reverse transcribed and levels of IFNγ were determined in absolute QPCR using TaqMan protocol.

In order to optimize the Ca-ionophore concentration in QPCR, horse PBMC were stimulated with variable Ca-ionophore concentrations (10.00-0.001 μg/ml cell suspension) for a fixed time (2 hours). Whole RNA was extracted, reverse-transcribed and levels of IFNγ were determined (as a readout for activation) in absolute quantitative real time PCR TaqMan protocol.

Objecting to optimize PBMC stimulation, horse PBMC were primed with combined mitogenic stimuli of PMA and calcium-ionophore (Ca-ionophore). PMA (0.001 μg/ml) was applied for 6 hours stimulation prior to addition of Ca-ionophore (1 μg/ml) for further 2 hours.

The polyclonal anti-human CD28 (R&D systems) was tested for its co-stimulatory effect with horse PBMC. To determine the optimum anti-human CD28 concentration, PMA and Ca-ionophore, were used again at suboptimal concentrations; PMA (0.00025 μg/ml) and Ca-ionophore (0.1 μg/ml). PMA (0.00025 μg/ml) was firstly added into culture with varying concentrations of CD28 (0.5, 1.00 and 2.00) μg/ml and after 6 hours Ca-ionophore (0.1 μg/ml) was added for 2 hours. The non-activated horse PBMC used as the experiment calibrator and PBMC with only anti-CD28 or anti-CD69 (R&D systems) as negative controls. Thereafter, the activated cells were harvested and levels of IFNγ and IL-4 were determined (normalized to 18S rRNA) in relative quantification real-time PCR.

In an alternative activation procedure, a tissue culture plate was coated with goat anti-mouse IgG (20 μg/ml) in sterile PBS and incubated for 18 hours at 4°C. Thereafter, the plate was washed twice with sterile PBS and blocked with bovine serum albumin (BSA) 3% for an hour at 37°C, followed by 2 washes with sterile PBS then anti-equine CD3 mAb was added at different concentrations for 18 hours at 4°C. Thereafter, plates were washed twice with sterile PBS followed by one time wash with RPMI (Müller, 1998). Thereafter, PBMC suspension

was added accompanied with the polyclonal anti-human CD28 1μg/ml for variable time serial sampling. The non-activated horse PBMC were used as the calibrator and PBMC with only anti-CD28 as the negative control. Cultured horse PBMC were extensively washed with sterile PBS and harvested. Total cellular RNA was extracted, reverse transcribed, and mRNA expression levels of both IFNγ and IL-4 were determined and normalized to 18S rRNA in relative quantification real-time PCR.

Equine herpesvirus-1 (EHV-1) was used as a model antigen to test memory Th1 response and tetanus toxoid a model antigen of a Th2 response (as it was supposed that the horses were immunized against *EHV-1* and *Clostridium tetani* toxins).

Inactivated *EHV-1* was used in different concentrations (10, 20, 50, 100, 200 and 400 μl) corresponding to 10^4 – 4 x 10^5 PFU/ml, along beside the co-stimulation by anti-CD28 (1μg/ml). The non-activated horse PBMC were used as calibrator and PBMC with only anti-CD28 as negative controls. Total cellular RNA was extracted and reverse-transcribed using the random hexamer primers. mRNA expression levels of IFNγ and IL-4 were determined and normalized to 18S rRNA levels in relative quantification real-time PCR.

Tetanus toxoid (List Biological Laboratories) was used at different concentrations (2.00, 1.00, 0.5, 0.1 and 0.01) μg/ml with anti-CD28 (1 μg/ml) for different durations of 6-96 hours. The nonactivated horse PBMC were used as calibrator and co-stimulant only (i.e. anti-CD28) with nonactivated PBMC as the test negative control. mRNA expression levels of IFNγ and IL-4 were determined by relative quantification real-time PCR.