Analysis of Foal LAK Cell Function

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Declaration in lieu of oath

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Abstract

Neonatal foals are at increased risk for a range of infectious disease including pneumonia caused by facultative intracellular gram-positive soil bacterial pathogen *Rhodococcus equi*. *R. equi* is a common cause of bronchial pneumonia in foals less than six months of age. Foals less than four weeks of age are particularly susceptible to infection with the bacteria. This is due in part to the naïvety of the neonate's immune system, as well as functional deficiencies in their cell-mediated immune responses. *R. equi*-infected cells are recognized by antigen-specific and non-specific cytotoxic cells that are a source of the interferon-gamma (IFNγ) that is essential for resistance to this bacterium as it increases bactericidal activity of macrophages. Although there are some reports that natural killer cell activity is reduced in neonates, others report that lymphokine-activated killer (LAK) cell activity is similar to that of adult horses. Nevertheless, foals have a diminished ability to produce IFNγ. The discrepancy between LAK activity and reduced IFNγ production has not been addressed. The aim of this research project is to characterize LAK cells generated from interleukin-2 (IL-2)-stimulated peripheral blood samples from adult horses and foals aged one day to twelve months. LAK activity is determined using fluorescence-activated cell scanning (FACS) analysis and gene expression quantitated by real-time polymerase chain reaction (qPCR). The goal of this research project is to gain further insight in the relationship between LAK activity in foals and the ability of these cells to produce IFNγ. The data generated during the course of this study demonstrates that foals' peripheral blood mononuclear cells (PBMCs) exhibited greater cytotoxicity in the LAK assay as well as an increased expression of IFNγ messenger ribonucleic acid (mRNA) and related genes when compared to the PBMCs of adult horses. These results indicate that foals are capable of responding to a high dose of IL-2 by generating LAK activity and producing IFNγ. As such, the susceptibility of foals to *R. equi* does not appear to be the result of a deficiency in this innate immune response.

Kurzfassung

Neonatale Fohlen haben ein erhöhtes Risiko für eine Reihe von Infektionskrankheiten einschließlich durch das fakultativ intrazelluläre gram-positives bakterielles Pathogen *Rhodococcus equi* verursachte Pneumonie. *R. equi* ist eine häufige Ursache für Bronchialpneumonie bei Fohlen, die jünger als sechs Monate sind. Fohlen, die jünger als vier Wochen sind, sind besonders anfällig für Infektionen mit diesem Bakterium. Dies ist zum Teil auf die Naivität des Immunsystems des Neugeborenen sowie auf funktionelle Defizite in ihrer zellvermittelten Immunreaktionen zurückzuführen. *R. equi*-infizierte Zellen werden durch antigenspezifische und unspezifische zytotoxische Zellen erkannt, welche eine Quelle für Interferon-Gamma (IFNγ) sind. IFNγ ist für die Resistenz gegenüber diesem Bakterium von großer Bedeutung, da es die bakterizide Aktivität von Makrophagen erhöht. Obwohl es einige Berichte gibt, dass die Aktivität von natürlichen Killerzellen bei Fohlen reduziert ist, berichten andere, dass die Lymphokin-aktivierte Killer (lymphokine-activated killer = LAK) Zell-Aktivität ähnlich wie bei erwachsenen Pferden ist. Trotzdem haben Fohlen eine verminderte Fähigkeit, IFNγ zu produzieren. Diese Diskrepanz zwischen LAK-Aktivität und reduzierter IFNγ-Produktion wurde noch nicht untersucht. Ziel dieses Forschungsprojektes ist, LAK-Zellen aus Interleukin-2 (IL-2)-stimulierten peripheren Blutproben von erwachsenen Pferden und Fohlen im Alter von einem Tag bis zwölf Monaten zu charakterisieren. Die LAK-Aktivität wird unter Verwendung von der Fluoreszenz-aktivierter Zellscanning (fluorescence-activated cell scanning = FACS)-Analyse bestimmt. Die Genexpression wird durch eine Echtzeit-Polymerase-Kettenreaktion (real-time polymerase chain reaction = qPCR) quantifiziert. Das Ziel dieses Forschungsprojektes ist, einen Einblick in die Beziehung zwischen der LAK-Aktivität in Fohlen und der Fähigkeit dieser Zellen IFNγ herzustellen zu gewinnen. Die im Laufe dieser Studie gewonnenen Daten zeigen, dass die mononukleäre Zellen des peripheren Blutes (peripheral blood mononuclear cells = PBMCs) von Fohlen im Vergleich zu PBMCs von erwachsenen Pferden eine größere Zytotoxizität im LAK-Assay sowie eine erhöhte Expression von IFNγ Messenger Ribonukleinsäure (messenger ribonucleic acid = mRNA) und verwandten Genen aufweisen. Diese Ergebnisse zeigen, dass Fohlen in der Lage sind, auf eine hohe Dosis von IL-2 mit der Erzeugung von LAK-Aktivität zu reagieren und IFNγ zu produzieren. Somit scheint die Anfälligkeit von Fohlen zu *R. equi* nicht das Ergebnis einer mangelhaften Immunantwort des angeborenen Immunsystems zu sein.

Table of Content

1 Introduction and scope

1.1 Objective

The aim of this research project is to characterize LAK cells generated from interleukin-2 (IL-2)-stimulated peripheral blood samples from adult horses and foals aged one day to twelve months. LAK activity is determined using fluorescence-activated cell scanning (FACS) analysis and gene expression quantitated by real-time polymerase chain reaction (qPCR). The goal of this research project is to gain further insight in the relationship between LAK activity in foals and the ability of these cells to produce IFNγ.

1.1.1 Foal Immunity to *Rhodococcus equi*

The immune system of the foal, like that of neonates of other species, is deficient compared to adults [1]. The lack of immunologic memory (i.e. their naïve status) and the diminished immunologic capacities in the neonatal foal lead to an increased susceptibility to the facultative intracellular gram-positive bacterial pathogen *Rhodococcus equi*. *R. equi* lives in soil and has simple growth requirements [2]. The lung is the organ which is affected in most cases, as the most common infection route is via inhalation of contaminated dust particles. Alveolar macrophages phagocytose the inhaled *R. equi* [3]. There, *R. equi* prevents the fusion of the phagosome with the lysosome and acidification of the phagosome. This leads to an inhibition of the respiratory burst. *R. equi* can then multiply within the phagosome, protected from the immune system by the cell that is supposed to kill it [4]. 48 hours later, the macrophage is killed by necroptosis [2]. Foals between one and five months of age are susceptible to this infection with *R. equi*, leading to a life-threatening pyogranulomatous pneumonia [5]. Extrapulmonary lesions are also present in some cases. As *R. equi* is a ubiquitous bacterium in equine environments, foals are exposed and likely infected with this pathogen from their day of birth. Through this exposure, which does not necessarily lead to pyogranulomatous pneumonia, foals develop immunity to this bacterium within the first year of life. The lifelong immunity against *R. equi* of adult horses is related to a type 1 memory response that involves the activation of macrophages through the production of IFNγ by *R. equi*-specific cluster of differentiation 4 positive ($CD4^+$) Type 1 helper T (T_h1) cells. *R. equi*-infected cells are recognized and lysed in a major histocompatibility complex (MHC) class I unrestricted manner by $CD8^+$ cytotoxic T lymphocytes (CTL). These and other antigen-non-specific cytotoxic cells may be a source of the IFNγ that is essential for resistance to this bacterium as it increases bactericidal activity of macrophages.

1.1.2 Blood of vertebrates

Blood is a body fluid with a pH that ranges from 7.35 to 7.45 and circulates around the vessels in the body by the pumping action of the heart [6]. Blood transports necessary substances, including nutrients and oxygen, to the cells of the body, while simultaneously transporting metabolic waste products away from those cells.

The blood itself is comprised of blood cells suspended in plasma. [7].

Approximately 55 % of the blood fluid is plasma, which is made of 92 % water. Plasma itself can be distinguished into serum (itself comprised of approximately 90 % water) and fibrin/fibrinogen. Other components of the serum include proteins, electrolytes, and low molecular weight electrolytes. Fibrin/Fibrinogen plays an important role in blood coagulation.

The blood cells are red blood cells (erythrocytes), which are the most numerous and responsible for oxygen transport; white blood cells (leukocytes), which are largely responsible for the activity of the adaptive immune system; and platelets (thrombocytes), which play an important role in wound healing.

Of particular interest to this research project are the peripheral blood mononuclear cells, which show LAK activity following stimulation with IL-2.

1.1.3 Peripheral blood mononuclear cell (PBMC)

The term “peripheral blood mononuclear cell (PBMC)” describes any peripheral blood cell with one round nucleus [8]. PBMCs are composed of cells involved in the innate immune response as well as in the adaptive immune response [9], including lymphocytes (T cells, B cells, natural killer cells) and monocytes [8]. By comparison, erythrocytes and platelets have no nuclei, and granulocytes (neutrophils, basophils, and eosinophils) have multi-lobed nuclei, and are therefore not PBMCs.

1.1.4 Lymphokine activated killer (LAK) cells

LAK cells are defined as IL-2-activated cytotoxic cells capable of lysing natural killer cell (NK)-resistant cell lines and fresh tumor targets in a non-MHC-restricted manner [10]. LAK cells kill all types of NK-resistant tumors, but do not require any tumor present for activation [11]. Nevertheless, LAK cells show little cytotoxicity against normal cells. LAK cells are a unique population of cytolytic cells, which are distinct from CTLs and NK cells [12]. LAK cells can become activated during any immune response in which the lymphokine cascade leads to ample IL-2 production [11].

LAK cells can be obtained from any sample of lymphoid tissue, including PBMCs from patients with or without cancer, through incubation with IL-2 [11]. It is unknown precisely which PBMCs become LAK cells, as T cells, NK cells, and macrophages all express an IL-2 receptor on their cell surface [13]. Both the LAK cell and its precursor are non-adherent, which strongly argues against a monocytic origin, and thus against macrophages as progenitors of LAK cells. Furthermore, LAK cells show different surface receptors than their possible progenitors [10].

In the horse it has been shown that in short-term stimulation, LAK activity is mediated by NK cells, whereas in longer incubation periods, LAK activity is mediated by $CD8^+$ cells [12]. This delay in activation is due to different affinity receptors for IL-2 on these two cell populations. So, the effector population is phenotypically heterogeneous [10].

1.1.5 Interleukin-2 (IL-2)

IL-2 is a cytokine produced by T_h cells that is required for T cell proliferation during immune response [14]. Recombinant equine IL-2 has a molecular weight of about 14.9 kDa. It is produced in yeast (and thus does not contain endotoxin) and is naturally folded and post-translationally modified.

T cell activation is triggered through the activation of the T cell receptor, which leads to a rapid hydrolysis of inositol phospholipids to diacylglycerol and inositol phosphates by phospholipase C [15]. Diacylgycerol activates protein kinase C and inositol phosphates. Inositol phosphates trigger a release of calcium, which leads to the activation of T_h cells. A cellular response of the T cells is the production and secretion of IL-2.

IL-2 signals through a heterotrimeric receptor complex consisting of IL-2-specific IL-2 receptor alpha (IL-2Rα (CD25)), IL-2 receptor beta (IL-2Rβ, CD122) and a common gamma chain (IL-2Rγ, γ_c), which span the cell membrane and transmit into the cell to distribute the biochemical signals to the cell interior (see Figure 1) [16].

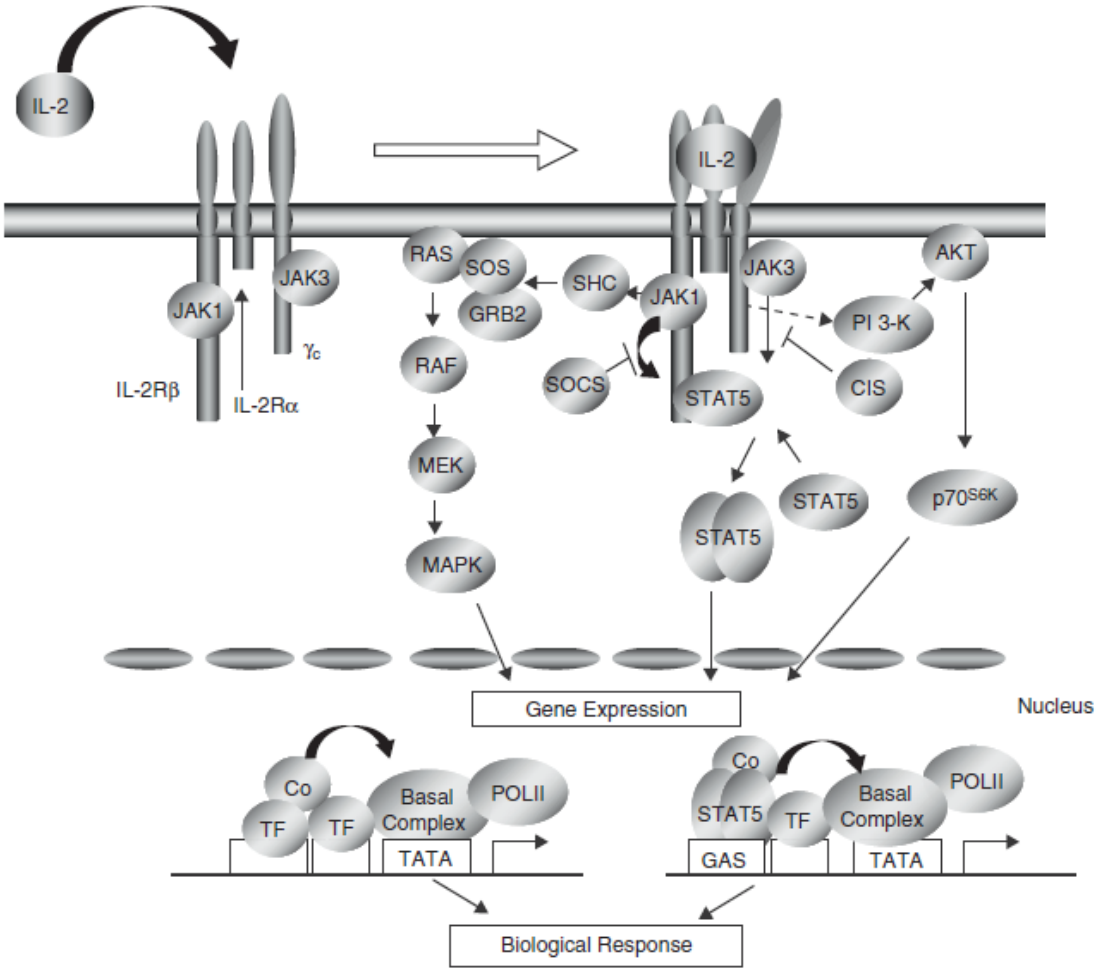


Figure 1: Signaling pathways of IL-2 [16]: The IL-2 binding to the IL-2 receptor activates three intracellular signaling pathways: JAK-STAT pathway, RAS-MAP kinase pathway, PI-3K/AKT pathway

The low affinity IL-2Rα receptor is only expressed on activated cells, whereas the IL-2Rβ and IL-2Rγ, which form an intermediate affinity receptor, are expressed constitutively on the surface [17]. When the T cell becomes activated, the IL-2Rα is expressed and forms a high affinity receptor together with the intermediate affinity receptor. The binding of IL-2 to the α-chain recruits the β-chain and finally the γ_c-chain, but only the β- and γ_c-chain participate in signaling. The β-chain is complexed with the enzyme Janus kinase 1 (JAK1) and the γ_c-chain with Janus kinase 3 (JAK3).

The binding of IL-2 to the IL-R leads to the internalization of the high affinity receptor, whereby IL-2, IL-2Rβ, and γ_c are quickly degraded, but IL-2Rα is brought back to the cell surface. Furthermore, a heterodimerization of the IL-2β and the γ_c cytoplasmic domains occur. This activates three intracellular signaling pathways [16]: the JAK-STAT (Janus kinase-Signal transducer and activator of transcription) pathway, the RAS-MAP kinase (rat sarcoma-mitogen activated protein kinase) pathway, and the PI-3K/AKT (phosphoinositol 3-kinase/protein kinase B) pathway. These pathways mediate cell survival, proliferation, differentiation and cell death.

1.1.5.1 JAK-STAT pathway

When IL-2 binds to the IL-2 receptor, the Janus kinases 1 and 3 (JAK1 and JAK3) become activated, which leads to an autophosphorylation of tyrosine residues [18]. Now, the signal transducer and activator of transcription 5 (STAT5) molecules in the cytoplasm attach to the phosphorylated residues. Afterwards, JAK phosphorylates the attached STAT5 proteins. This leads to a dimerization of the STAT5 proteins. This STAT5-dimer enters the nucleus, binds onto the promoter region of the DNA (deoxyribonucleic acid) and promotes transcription of certain mRNAs (messenger ribonucleic acid).

1.1.5.2 RAS-MAP kinase pathway

The autophosphorylation of tyrosine residues on the IL-2Rβ through JAK1 promotes the recruitment of the SHC (sarcoma homology 2 domain containing transforming protein 1) adaptor protein leading to activation of the RAS-MAP kinase pathway [19].

GRB2 (growth factor receptor binding protein 2) activates SOS (son of sevenless). SOS catalyzes RAS-GDP (rat sarcoma-guanosine diphosphate) to RAS-GTP (rat sarcoma-guanosine-5'-triphosphate), which is an activated form of the monomeric G-protein RAS (rat sarcoma) that is attached to the cytoplasm membrane.

RAS starts a kinase cascade, where the initial signal is amplified through dimerization of the activated molecule:

1. RAS activates RAF (rat fibrosarcoma; MAPKKK = mitogen-activated protein kinase kinase kinase).
2. RAF activates MEK (MAPKK = mitogen-activated protein kinase kinase).
3. MEK activates ERK (extracellular signal-regulated kinases, MAPK = mitogen-activated protein kinase).

ERK enters the nucleus and activates transcription factors leading to gene expression.

1.1.5.3 PI-3K/AKT pathway

The autophosphorylation of tyrosine residues on the γ_c-chain through JAK3 activates a PI-3K (phosphatidylinositol-4,5-bisphosphate 3-kinase) [16]. PI-3K catalyzes PIP_2 (phosphatidylinositol 4,5-bisphosphate) to PIP_3 (phosphatidylinositol (3,4,5)-trisphosphate). PIP_3 activates AKT. AKT activates RHEB (rat homolog enriched in brain), which activates $p70^{S6K}$ (p70S6 kinase). $p70^{S6K}$ interacts and activates the translation factor S6K (ribosomal S6 kinase). S6K binds at the large subunit of ribosomes and activates the translation of mRNA into protein.

1.1.5.4 Termination of IL-2 signaling

The termination of IL-2 signaling is mediated by two gene families [16]: the SOCS (suppressor of cytokine signaling proteins) inhibit JAK1 on IL-2Rβ, whereas CIS (cytokine-inducible SH2 (sarcoma homology 2-containing protein)) blocks JAK3 on the γ_c-chain. As a result, the JAK-STAT pathway as well as the RAS-MAP kinase pathway and the PI 3-K/AKT pathway are inhibited.

1.1.5.5 Cause for decreased IL-2 levels in foals and its consequence

Neonatal T cells show reduced T cell receptor mediated activity, due to inefficient activation of phospholipase C [20]. This is revealed through the direct stimulation of neonatal T cells with Phorbol 12-Myristate 13-Acetate (PMA), which circumvents the receptor-mediated activation of IL-2 signaling pathway [21]. This phenomenon occurs as neonatal foals have fewer T cells expressing MHC class II antigen [22]. There is an age-dependent maturation of the T cell population, as the number of T cells expressing MHC class II molecules increases up through four months of age.

Furthermore, neonatal T cells exhibit T_h2 responses more often as a higher amount of IL-4 and less often as multifunctional T_h1 cytokines (like IFNγ, tumor necrosis factor alpha (TNFα) and IL-2). The level of IFNγ mRNA expression increases with age and is comparable to that of adults at the age of three months [23]. This finding is supported as neonatal T cells express a lower amount of high affinity IL-2 receptors on their surface [21].

1.1.5.6 Relevant gene expression for this study

Beta-Glucuronidase (βGus) is a homotetrameric hydrolase in the lysosome that degrades glycosaminoglycans, including heparan sulfate, dermatan sulfate, and chondroitin-4,6-sulfate [24]. It will be used as a housekeeping gene in this study for the normalization of PCR data.

Perforin is one of the main cytolytic proteins of cytolytic granules and leads to transmembrane tubules in the target cell [25]. It is a key effector molecule for T cell- and NK cell-mediated cytolysis. Because of this, it can non-specifically lyse a variety of target cells.

Granzyme B is a serine protease which is secreted by NK cells and CTLs [26]. Once it is active, it induces apoptosis in the target cell. Other functions of this protein are to process cytokines and to degrade extracellular matrix proteins. It is implied that granzyme B plays a role in chronic inflammation and wound healing.

IFNγ is a soluble homodimeric cytokine that is secreted by cells of the innate and of the adaptive immune systems and binds to the interferon gamma receptor to fight viral and microbial infections [27]. A mutation in this gene leads to an increased susceptibility to viral, bacterial and parasitic infections and to several autoimmune diseases.

TNFα is a multifunctional pro-inflammatory cytokine within the TNF superfamily [28]. TNFα plays a huge role in various biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation.

1.1.6 Target cell killing

1.1.6.1 EqT8888 target cells

EqT8888 is an equine-transformed lymphoid cell line derived from an anaplastic undifferentiated lymphosarcoma from a 2-year-old Arabian filly [12]. EqT8888 cells are non-adherent, polymorphic-shape and -size leukemic cells. The detailed lineage of this cell line is unclear [29].

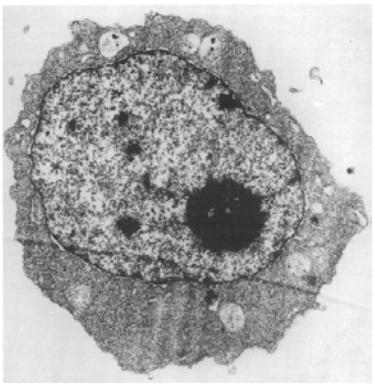


Figure 2: EqT8888 transmission electron picture (Magnification 160000x) [12]

EqT8888 is a NK cell-resistant cell line, as it expresses MHC class I molecules on its surface [30]. However, LAK cells are able to induce apoptosis in the EqT8888 cell line [12].

1.1.6.2 Apoptosis

Apoptosis is a normal physiologic process of programmed cell death that occurs in multicellular organisms during embryonic development as well as in maintenance of tissue homeostasis [31]. It is a coordinated process of loss of plasma membrane asymmetry and attachment, blebbing of the cell membrane, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation and global mRNA decay. This is a highly regulated process, as apoptosis cannot be stopped once it has begun. Killing of tumor cells by CTLs, NK cells or LAK cells depends on apoptosis induction, which is mediated by granule exocytosis and engagement of death receptors [32].

Granule exocytosis pathway

The granules contain mainly perforin and granzymes [32]. After an infected cell is recognized, CTLs secrete perforin and granzyme B via exocytosis. Perforin forms pores that penetrate the membrane of the infected cell. Through these pores, granzyme B can enter into the cytosol of the infected target cell. There are three pathways that are involved in the killing of target cells by granzyme B: the direct activation of caspase-3, the activation of the intrinsic mitochondrial pathway, and the caspase-independent pathway.

Direct activation of caspase-3

Once granzyme B enters the target cell, it mimics initiator caspases of the extrinsic pathway [33]. This leads to the activation of caspase-3, which is able to cleave ICAD (inhibitor of caspase-activated DNase), the inhibitor of the pro-apoptotic protein CAD (caspase-activated DNase). As ICAD is inactivated, CAD is activated and transfers into the nucleus to degrade the DNA.

Activation of the intrinsic mitochondrial pathway

Granzyme B also activates the intrinsic mitochondrial pathway [33]. This occurs in one of two ways: by acting directly upon the mitochondrial membrane to increase its permeability so that cytochrome c can be released in the cytosol; the cytochrome c release can also be triggered through activation of the pro-apoptotic protein BID (BH3 (Bcl-2 (B cell lymphoma 2) homology domain 3) interacting-domain death agonist). BID can interact with BAX (Bcl-2 (B cell lymphoma 2)-associated X protein). BAX enters the mitochondrial membrane and initiates the release of cytochrome c into the cytosol.

In both intrinsic mitochondrial pathways, outflowing cytochrome c leads to an asymmetric change of the membrane. Furthermore, it interacts with APAF-1 (apoptotic protease activating factor-1). Cytochrome c and APAF-1 together form the apoptosome. The apoptosome activates the procaspase-9 to its active form caspase-9 (initiator caspase). Caspase-9 activates the procaspase-3 to caspase-3 (executor caspase). Caspase-3 activates, leading to apoptosis.

Caspase-independent pathway

Granzyme B can enter directly into the nucleus and activate the caspase substrates independent of caspases [32].

Death receptors

Apoptosis via death receptors (FAS, TNF-R and TRAIL-R) is possible as CTL, NK cells and LAK cells can express TNF-ligands, lymphotoxin ligands, Fas-L, and TRAIL [32].

FAS pathway

FAS-L (first apoptosis signal-ligand) binds to the FAS-R (first apoptosis signal-receptor) and activates its attached FADD (FAS associated death domain), so that it can associate with another FADD, which is located on the FADD adaptor protein [34]. The FADD adaptor protein forms a complex with the FAS-R. The FADD adaptor protein has a DED (death effector domain), which can associate with a DED on procaspase-8. This whole complex is known as DISC (death inducing signaling complex), which contains FAS-L, FAS-R, FADD, FAD adaptor protein and procaspase-8. Next, caspase-8 becomes activated. Caspase-8 is an initiated caspase that activates caspase-3. Caspase-3 activates BID. The further order of events is equal to the steps after the activation of BID in pathway 2 of the granule exocytosis pathway.

TNF-R pathway

TNFα or lymphotoxin binds to TNF-R (tumor necrosis factor receptor), which leads to caspase activation via the intermediate membrane proteins TRADD (TNF receptor-associated death domain) and FADD [35]. TRADD leads to an indirect activation of transcription factors, which triggers apoptosis in a caspase-independent manner. FADD acts as described before in the FAS pathway, and activates caspase-8, which in turn activates caspase-3. Caspase-3 leads to apoptosis in the target cell.

TRAIL-R pathway

TRAIL (TNF-related apoptosis-inducing ligand) binds to DR-4 (death receptor-4) and DR-5 (death receptor-5), which recruits the FAS adaptor protein [36]. Thus, DISC is formed and leads to apoptosis through the activation of capsase-8 and capsase-3, as described before in the FAS pathway.

1.2 Research problem

Neonatal foals are at increased risk for a range of infectious disease including pneumonia caused by *Rhodococcus equi* [1]. This is due in part to the naïvety of the neonate's immune system, as well as functional deficiencies in their cell-mediated immune responses. Overall, the neonatal immune system is deficient against viral, bacterial and fungal infections compared to that of adults. This deficiency is caused by a lack of preexisting memory T and B cells, as well as a reduced T cell response [37]. Therefore, neonatal T cells can be distinguished from those of adults as they produce low amounts of IL-2 and IFNγ, but high amounts of IL-4 in response to primary stimulation *in vitro* [38]. The reason for this bias, as well as their ability to produce LAK effector cells, is still unclear [39].

The potential of NK cells and LAK cells to lyse infected cells is an important effector mechanism in the initial response to intracellular pathogens. As those cells are an important source of IFNγ, they can also contribute to the resistance via the activation of infected macrophages. However, increased expression of IL-10 by dendritic cells has been described for foal antigen-presenting cells, which could negatively impact IFNγ production [40]. Nevertheless, foals have a diminished ability to produce IFNγ [39]. Although there are some reports that NK cell activity is reduced in neonates, others specify that LAK activity is similar to adult horses.

The discrepancy between LAK activity and reduced IFNγ production has not been addressed. Here it is proposed to address this question using an equine model. The goal of this research project is to gain further insight in the relationship between LAK activity in foals and the ability of these cells to produce IFNγ.

1.3 Hypothesis

The hypothesis of this project is that there will be robust LAK activity in the neonatal PBMC cultures on a scale similar to adult PBMCs. Furthermore, increased expression of LAK activity-associated genes, like perforin and granzyme B, should occur. However, in comparison to adult LAK cells, no production of IFNγ by those cells is expected.

1.4 Expected results

The outcome of this project should support the original observations that the susceptibility of young foals to *R. equi* is the result of a dysregulation of IFNγ mRNA expression. The detailed mechanism for the decreased production of IFNγ in neonates is unknown. The determination of whether neonate LAK cells can produce IFNγ would give insight into the possible mechanism of this defect.

1.5 Relevance

As mentioned previously, infectious diseases are a main reason of morbidity and mortality among young foals. The veterinary expenses and the loss of a valuable animal is a financial burden to the industry.

2 Materials

2.1 Experimental setup

As a first step of this project, the optimal concentration of recombinant equine IL-2 as well as the optimal stimulation period (one to five days) to induce the gene expression of IFNγ, granzyme B, perforin, IL-2Rα and TNFα is determined using adult horse blood samples. Two Monoject™ Blood Collection Tubes with green stoppers, which collect 3 mL of whole blood and contains sodium heparin to prevent clotting of the blood sample, are drawn for each horse to be used for *in vivo* stimulation. One of the tubes is stimulated with recombinant equine IL-2, while the other serves as an unstimulated control. After the incubation period, the blood samples are transferred into Tempus™ Blood RNA Tubes. The Tempus™ Blood RNA tube contains a proprietary RNA stabilization reagent, which allows for long-term storage of whole blood samples for RNA isolation. The RNA (ribonucleic acid) is purified from the whole blood sample using the iPrep™ purification system. Following the determination of the RNA yield by spectrophotometry, complementary DNA (cDNA) is reverse transcribed following the laboratory standard reverse transcription protocol, followed by qPCR to determine gene expression. Appendices 1, 2, 4, 5, and 6 contain detailed descriptions of this procedure.

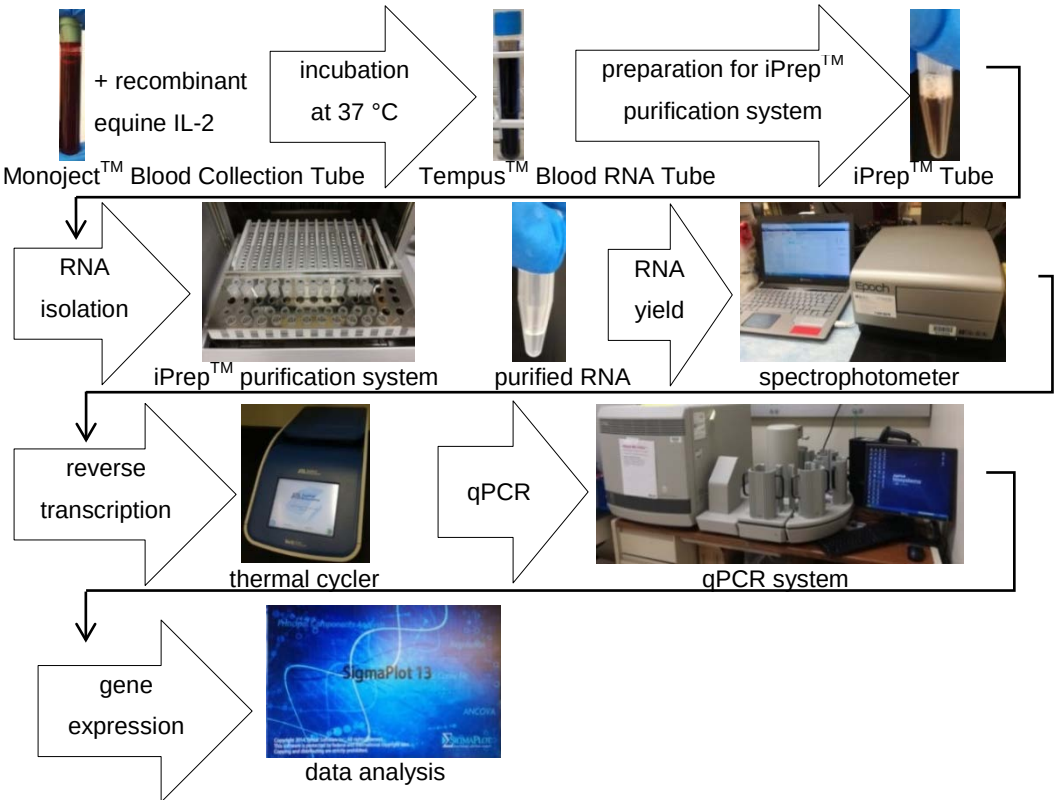


Figure 3: Assay set-up to determine the optimal concentration of recombinant equine IL-2 and of incubation time: The RNA is isolated from drawn blood by the iPrep™ purification system, followed by a reverse transcription reaction and qPCR.

The incubation period and IL-2 concentration are optimized similarly for the LAK assay. PBMC samples are isolated from mares and foals using a density gradient centrifugation protocol. The isolated PBMCs are used for the LAK assay, as well as to determine the gene expression level in recombinant equine IL-2 stimulated and unstimulated samples. Appendix 8 contains a detailed description of this procedure.

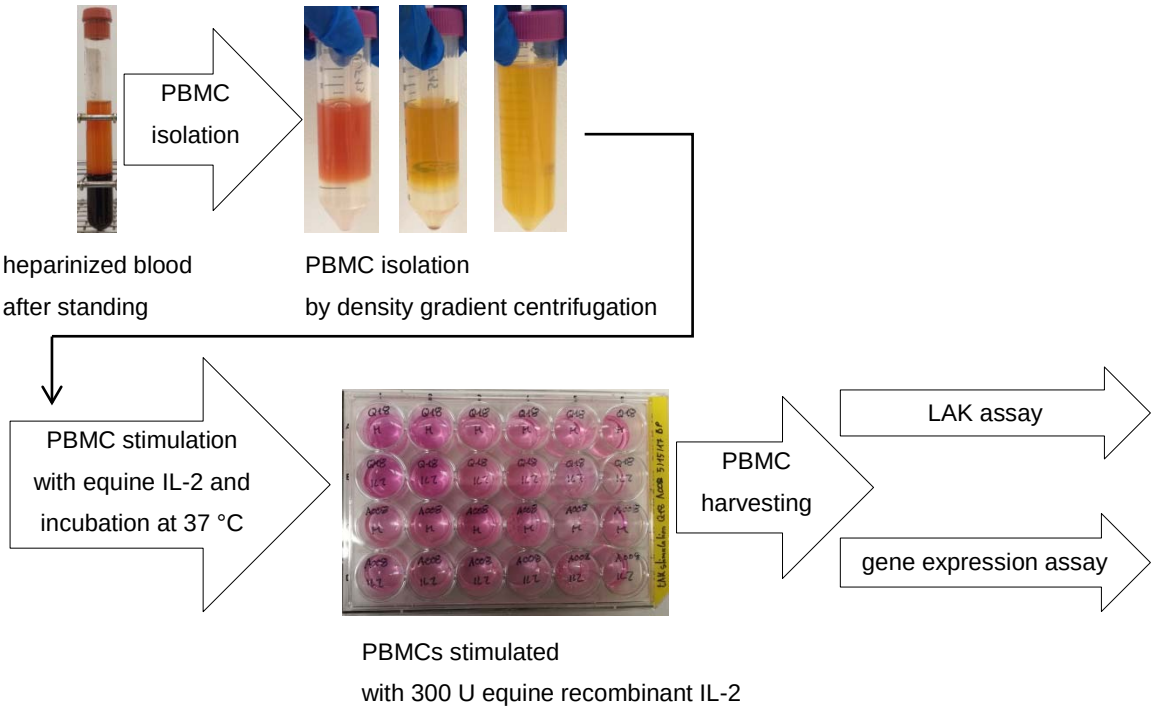


Figure 4: PBMC isolation and stimulation with recombinant equine IL-2: PBMCs are isolated by density gradient centrifugation followed by stimulation with equine recombinant IL-2 to generate LAK cells

For the LAK assay, the PBMCs are plated on a flat-bottomed 24 well plate, whereby half of the wells are stimulated with 300 units (equivalent to 900 ng) of recombinant equine IL-2. After 3.5 days of incubation, the LAK assay is performed. The stimulated PBMCs are incubated with 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) -stained EqT8888 target cells in a v-bottomed 96 well plate for two hours at 37 °C and 5 % carbon dioxide (CO_2). Finally, the apoptotic cells are stained with R-phycoerythrin (PE)-Annexin V. Hence, the target cells are CFSE and PE-Annexin V positive as they undergo apoptosis, which is detected by flow cytometry. Appendices 7 and 9 contain detailed descriptions of this procedure.

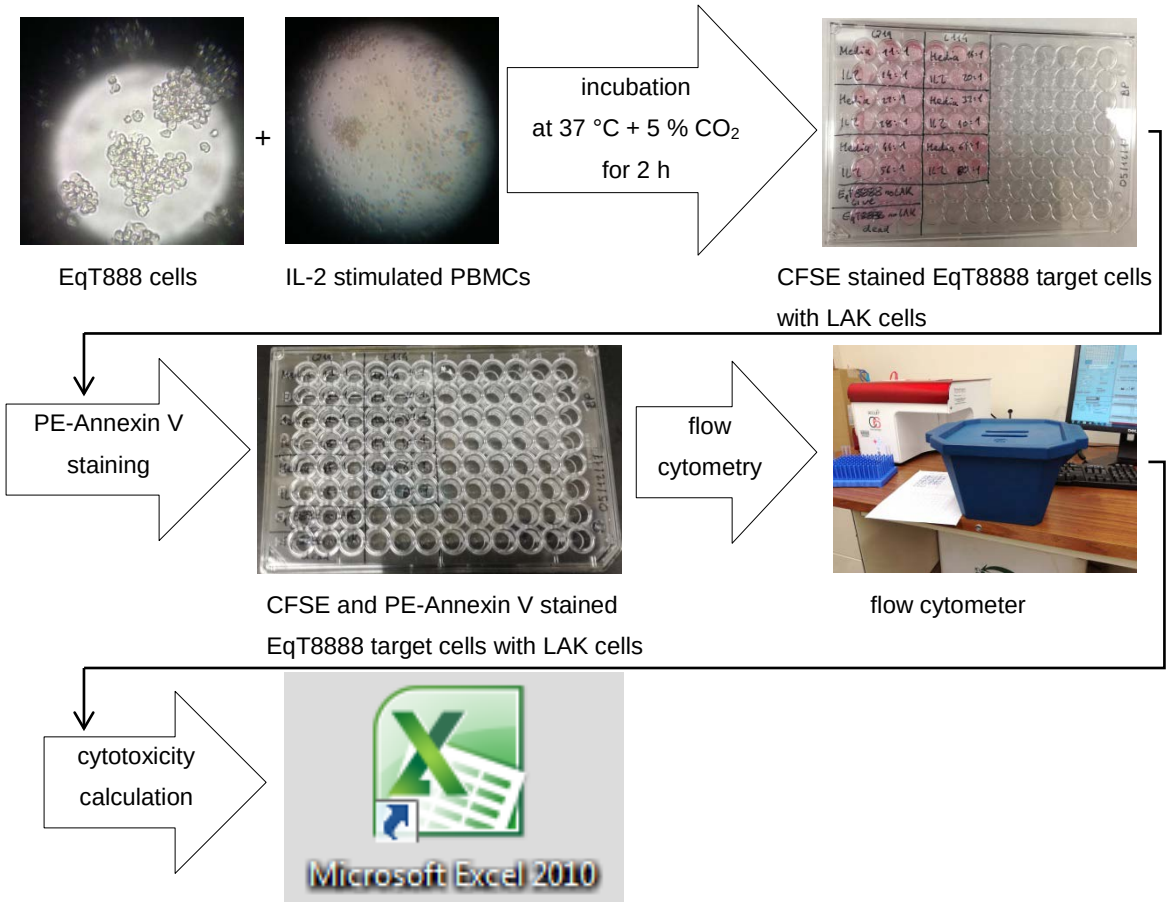


Figure 5: LAK assay: Incubation of CFSE stained EqT8888 target cells with IL-2 stimulated PBMCs to induce apoptosis of target cells, which is determined by flow cytometry

To measure the gene expression from PBMC cultures, RNA is isolated using the single-step-method using TRIzol® reagent. The PBMCs are harvested and resuspended in TRIzol® reagent solution to lyse the cells and liberate the RNA. The RNA is extracted using a phenol-chloroform isolation technique. Again, the RNA yield is determined by spectrophotometry, and following reverse transcription, the qPCR is performed to determine gene expression. Appendices 3, 4, 5, and 6 contain detailed descriptions of this procedure.

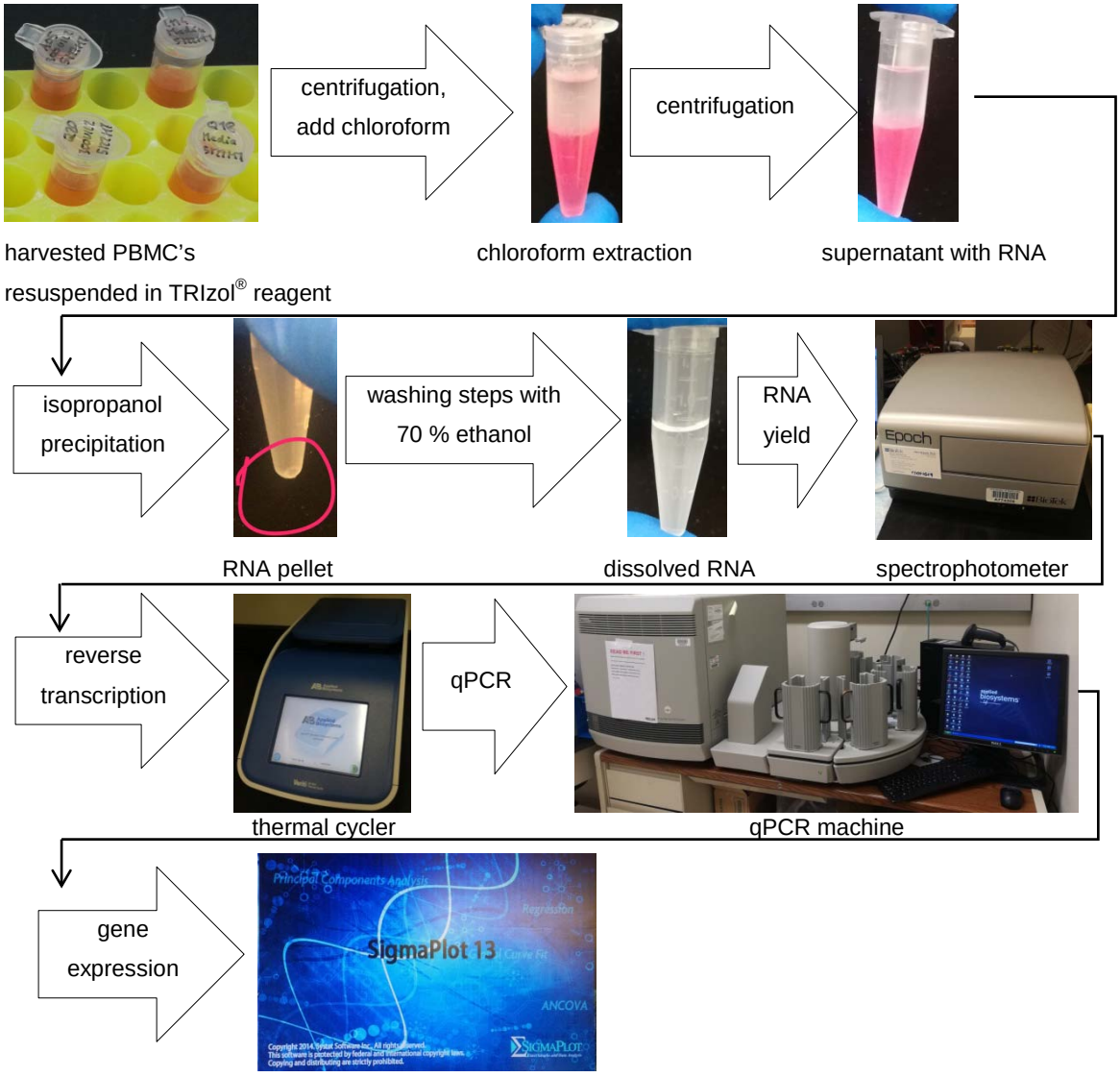


Figure 6: Gene expression assay: Extraction of RNA by the single-step-method with TRIzol followed by a reverse transcription reaction and qPCR

2.2 List of materials

Most equipment and tools necessary for this experiment are part of the basic equipment of an immunological laboratory. Therefore, only the substances and specific equipment required for this experiment are listed below (see Table 1 to Table 8).

Table 1: Instruments

Instruments	
Monoject™ Blood Collection Tube with green stopper	Coviden; cat. no.: 8881320256
Monoject™ Blood Collection Tubes with red stoppers	Coviden; cat. no.: 8881301819
Tempus™ Blood RNA Tube	Greiner bio-one; cat. no.: 4342792
iPrep™ Purification Instrument	Invitrogen; cat. no.: IS-10000
Gen5 Software	Applied Biosciences; version: 2.03
Microplate Spectrophotometer	BioTek Epoch™
Veriti® 96-Well Thermal Cycler	Applied Biosciences; cat. no.: 4375786
ep*Motion*® 5070	Eppendorf; cat. no.: 5070007001
7900HT Fast Real-Time PCR System with 384-Well Block Module and Automation Accessory	Applied Biosciences; cat. no.: 4329001
LinRegPCR Software	Academic Medical Center Amsterdam; Version: 2014.3
SigmaPlot13 Software	Systat Software; version: 13.0
Microsoft Office *Excel 2010*	Microsoft; version: 2010
NAPCO series 8000 WJ CO_2 Incubator	ThermoFisher Scientific; model no.: 3595
T75 flask	Greiner Bio-one; cat. no.:658170
TPP® tissue culture flat-bottom 24 well plates	Sigma-Aldrich; cat. no.: TPP 92424
Nunc® MicroWell™ v-bottom 96 well plates	ThermoFisher Scientific; cat. no.: 249570
ViCell XR cell viability Analyzer	Beckman Coulter; model. no.: 0AG44185
Inverted Routine Microscope	Nikon; model: Eclipse TS100
Flow Cytometer	BD; model: Accuri C6

Table 2: Reagents for RNA isolation with iPrep™ purification system

Reagents for RNA isolation with iPrep™ purification system	
RNase *AWAY*® Reagent	Molecular BioProducts; cat. no.: 7005-11
HyClone™ Dulbecco's Phosphate Buffered Saline solution	GE Healthcare; cat. no.: SH30028.03
Viral Lysis Buffer	Invitrogen; cat. no.: 46-6370
iPrep™ PureLink® Total RNA Kit	Invitrogen; cat. no.: IS-10006
Elution Buffer “1 M Tris; pH = 8.0”	Ambion; cat. no.: AM9855G

Table 3: Reagents for RNA isolation with the single-step-method with TRIzol®

Reagents for RNA isolation with the single-step-method with TRIzol®	
RNase *AWAY*® Reagent	Molecular BioProducts; cat. no.: 7005-11
TRIzol® Reagent	Ambion; cat.no.: 1559601
Chloroform	Sigma-Aldrich; cat. no.: 496189
Isopropanol (2-propanol)	ThermoFisher Scientific; cat. no.: A516
Ethanol (Ethyl Alcohols pure)	Sigma-Aldrich; cat. no.: E7023

Table 4: Reagents for reverse transcription reaction and qPCR

Reagents for reverse transcription reaction and qPCR	
600 u Avian Myeloblastosis Virus (AMV) Reverse Transcriptase and 5 X Avian Myeloblastosis Virus (AMV) buffer	Promega; cat. no.: M9004
25 mM Magnesium Chloride Solution	Promega, cat. no.: A3511
10 mM dNTP Mix	Promega; cat. no.: U1515
10,000 u RNasin Plus RNase Inhibitor	Promega; cat. no.: N2615
500 µg/mL Oligo(dT) 15 Primer	Promega; cat. no.: C1101
2 X SensiFAST™ Probe Hi-ROX Mix	Bioline Reagents; cat. no.: BIO-86020
RNase-free water	MP Biomedicals; cat. no.: 821739

Table 5: Reagents for EqT8888

Reagents for EqT8888	
RPMI 1640 medium	Life technologies; cat. no.: 72400-047
AIM V® Medium	Life technologies; cat. no.: 12055-091
MEM Non-Essential Amino Acids Solution	Life technologies; cat. no.: 11140-050
Sodium Pyruvate	Life technologies; cat. no.: 1136070
Gentamycin	Life technologies; cat. no.: 15750-060
2-mercaptoethanol	Thermo Fisher Scientific: cat. no.: 35602
Fetal bovine serum (FBS)	Sigma Aldrich; cat. no.: 16A313
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich; cat. no.: D2650
CFSE	Sigma-Aldrich; cat. no. 21888
PE-Annexin V	BD; cat. no.: 556421
10 X Anexin V binding buffer	BD; cat. no.: 51-66121E

Table 6: Reagents for PBMCs

Reagents for PBMCs	
Ficoll-Paque Plus	GE Healthcare; cat. no.: 17-1440-03
Roswell Park Memorial Institute (RPMI) 1640 medium	GE Healthcare; cat. no.: SH30096.01
Fetal equine serum (FES)	Biowest; cat. no.: S0960
2-mercaptoethanol	Thermo Fisher Scientific: cat. no.: 35602
Recombinant equine IL-2	Kingfischer Biotech; cat. no.: RP0006E

Table 7: Primer/probe sets used for gene expression measurements

Primer/probe sets	
Beta-glucuronidase (β-GUS)*	ThermoFisher Scientific; cat. no.: 4331182; Assay ID: Ec03470630_m1
IFNγ*	ThermoFisher Scientific; cat. no.: 4331182; Assay ID: Ec03468606_m1
Granzyme B	ThermoFisher Scientific; cat. no.: 4331348; Assay ID: AICSWBJ
Perforin	ThermoFisher Scientific cat. no.: 4331348; Assay ID: AIVI53H
TNFα*	ThermoFisher Scientific; cat. no.: 4331182; Assay ID: Ec03467871_m1
IL-2Rα (CD25)*	ThermoFisher Scientific; cat. no.: 4331182; Assay ID: Ec03469221_m1

*The sequences are proprietary, so ThermoFisher Scientific will not be able to provide that information. Granzyme B and perforin are custom sequences.

Table 8: Custom primer/probe information

Custom primer/probe information			
Name	**Forward primer**	**Reverse primer**	**Probe**
Granzyme B	GGACCCGAAGGAA AAGAAGTCTT	CCTGGATCAC GTTCTTACACACAAG	CCGGAG TCCCCCTTAAA
Perforin	GCTTCAG CAGCGACTCAGT	CGTGCAC CAGGCGAAA	ACTGTAGAA GCGACACTCC

3 Methods

3.1 Horses

16 horses (eight foals and eight adult horses, all owned by the University of Kentucky) are used for the initial study to determine optimal IL-2 dosage and culture incubation period. An additional ten horses (five foals and five mares, also owned by the University of Kentucky) are used for the development of the LAK cytotoxicity assay. 24 horses (18 foals and six mares, owned either by the University of Kentucky or by a nearby thoroughbred farm) are used to determine variations in the gene expression in the different aged horses. The 18 foals are divided into three age groups (birth to six weeks, six weeks to six months and six months to one year). A detailed description of the horses used in this study can be found in Appendix 1.

3.2 Sample collection

Peripheral venous blood (3 mL) for the validation of the IL-2 stimulation assay is collected into Monoject™ Blood Collection Tubes, which is a 10.25 mm x 64 mm sterile blood collection tube with a glycerin coated green stopper that contains sodium heparin to prevent blood coagulation [41], by local veterinarians. Peripheral venous blood (15 mL) for isolation of PBMCs is collected into Monoject™ Blood Collection Tubes with red stoppers (with added sodium heparin to prevent coagulation).

3.3 Validation of the IL-2 stimulation assay

3.3.1 Optimal amount of recombinant equine IL-2

The goal of this validation is to determine the optimal amount of recombinant equine IL-2 for stimulating the whole blood cultures. Therefore, Monoject™ Blood Collection Tubes are stimulated with different amounts of recombinant equine IL-2 and incubated at 37 °C for 24 hours (see Appendix 1).

3.3.2 Optimal incubation time

Once the optimal amount of recombinant equine IL-2 is determined, whole blood cultures are incubated at 37 °C and sampled over five days to examine differences in gene expression during the incubation period (see Appendix 1).

3.4 iPrep™

iPrep™ PureLink® Total RNA Kit is suitable for the extraction and purification of total RNA using the sensitive Dynabeads® MyOne™ Silane and the rapid and automated iPrep™ Purification Instrument within 30 to 45 minutes [42]. The purified total RNA can be used for sensitive downstream applications including gene expression studies such as reverse transcription and quantitative real-time PCR.

The Dynabeads® MyOne™ Silane are 1 µm monodisperse magnetic beads with a highly specific silica-like surface chemistry [42]. The total RNA isolation is achieved by the use of a magnetic bead-based purification procedure. The lysed samples are mixed with Dynabeads® MyOne™ Silane whereby the RNA binds to the beads. The RNA-bound magnetic beads and the lysate are separated by magnetic separation. To remove contaminants, the beads are thoroughly washed with wash buffers. At the end, the total RNA is eluted in elution buffer.

The iPrep™ Purification Instrument is a benchtop, automated nucleic acid purification instrument with integrated magnetic and syringe unit as well as a platform, capable of purifying nucleic acid from up to 13 samples using a magnetic bead-based technology [42]. The purification parameters (e.g. buffer volume, mixing steps and incubation time) are controlled though a pre-programmed iPrep™ Protocol Card.

3.4.1 RNA isolation from whole blood samples

The RNA isolation from the Tempus™ Blood RNA Tubes is done using the iPrep™ PureLink® Total RNA Kit according to the laboratory standard protocol (see Appendix 3). The blood sample is washed with 3 mL 1 x PBS and the RNA pelleted. The pelleted RNA is resuspended in 600 µL viral lysis buffer and purified by the iPrep™ purification system.

3.4.2 RNA isolation from PBMCs

The RNA isolation from PBMCs is performed with the single-step-method with TRIzol® reagent. TRIzol® reagent contains guanidinium thiocyanate, which lyses the cells and simultaneously inactivates RNases, and phenol, in which RNA dissolves [43]. The phase separation occurs after chloroform is added, followed by centrifugation [44]. The upper aqueous phase contains RNA and the lower chloroform phase contains DNA and proteins. The RNA is precipitated with isopropanol. Following two washing steps with 70 % ethanol, the RNA is purified and ready for further use.

The laboratory's standard protocol for RNA isolation is detailed in Appendix 4.

3.4.3 RNA Yield

The quantification of the RNA concentration is done through the spectrophotometric measurement at 260 nm, which is the absorption maximum of nucleic acids [45].

The laboratory's standard protocol is used (Appendix 5).

3.5 Synthesis of first strand cDNA

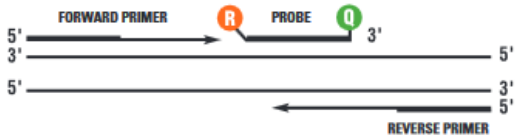
Single stranded cDNA is generated through the reverse transcription synthesis of DNA from an RNA template [46]. During this process, short primers, complementary to the 3' end of the RNA, attach to the RNA and a reverse transcriptase synthesizes a single-stranded cDNA. This single-stranded cDNA can be used directly as a template for the (quantitative real-time) PCR.

The procedure used is the laboratory's standard protocol (Appendix 6).

3.6 Quantitative real-time PCR (qPCR)

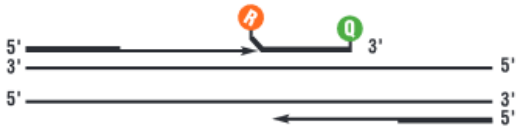
The TaqMan® probe-based assay allows generating a measurable fluorescent signal, which is detected with a 7900HT Fast Real-Time PCR System [47].

1. Polymerization



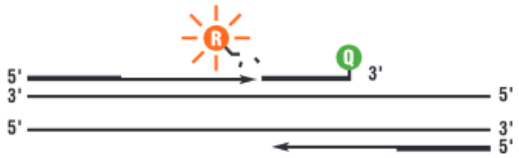
A fluorescent reporter (R) dye and a quencher (Q), are attached at the 5' and 3' end, respectively, of a TaqMan® probe.

2. Strand displacement



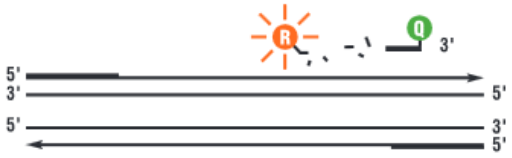
The emission of the reporter dye is absorbed by the quencher if the probe is still intact.

3. Cleavage



The reporter dye is cleaved from the probe during the extension cycle by the DNA polymerase.

4. Polymerization completed



Once it is separated from the quencher, the reporter dye reports its characteristic fluorescence.

Figure 7: The four steps of the TaqMan® probe-based assay: polymerization, strand displacement, cleavage and polymerization completed [47]

The fluorescence in each well is measured by a laser and spectrally resolved by a spectrograph and charge-coupled device (CCD) camera, which collects the fluorescence emission from each sample.

The data analysis is done by relative quantification, which is suitable for gene expression studies. Samples are measured in terms of a threshold cycle (CT), the number of PCR thermal cycles required to reach a threshold level of fluorescence specific to the detection limits of the PCR platform. To normalize differences including sample volume and temperature variability, an endogenous control (in this assay the housekeeping gene β-GUS) is used. Expression levels are calculated relative to a calibration sample, generally either a non-treated control sample or pre-treatment time point.

The protocol is based on [48] Liu et. al. (2011) and is slightly modified (Appendix 7).

3.7 Statistical analysis

All data are analyzed for statistical significance using the software *SigmaPlot13*, whereby statistical significance is set at $P < 0.050$. ANOVA (analysis of variance) is used to determine statistically significant differences among different factors. Correlations among the results of the different mRNA expressions are determined using the Pearson product moment correlation.

3.8 EqT8888 target cells

The EqT8888 cells, which are used as target cells in the LAK assay, are maintained in suspension in RPMI-1640 media supplemented with 10 % FBS at 37 °C and 5 % CO_2 in air. The cells are monitored daily under the microscope and split as needed. Furthermore, the cells are split the day before the LAK assay. This is necessary to ensure that the cells are in the log-phase of growth and multiplication prior to CFSE staining. The cultivation media with 10 % DMSO is used as freezing media for the EqT8888 cells for storage in liquid nitrogen.

Cell viability is measured using trypan blue dye exclusion method. Dead cells take up trypan blue dye, due to cell membrane permeability [49]. Viable cells do not take the dye up and remain unstained. The contrast between dyed cells and unstained cells is measured to determine viability.

The method for culturing the EqT8888 cells is described in Appendix 8.

3.9 PBMC isolation

PBMCs are isolated through density gradient centrifugation using Ficoll-Paque Plus, a hydrophilic polysaccharide that separates layers of blood [50]. In this process, the blood is separated into a top layer of plasma, followed by a layer of PBMCs and a bottom fraction of polymorphonuclear cells (like neutrophils and eosinophils) and erythrocytes. In the denser and the PBMC fraction, basophils are detected.

PBMC isolation is performed using procedures taken from the laboratory standard protocol (see Appendix 9).

3.10 LAK activity

3.10.1 LAK cell generation

LAK cells are generated following the laboratory's standard protocol (see Appendix 10). PBMCs are incubated with 300 U (equivalent to 900 ng) recombinant equine IL-2 for four days. LAK activity is determined using the EqT8888 target cells.

3.10.2 LAK cell flow cytometric assay

The physical and chemical characteristics of a single cell in a suspension can be measured by flow cytometry [51]. When a single cell, transported in the laminar sheath flow, passes a laser light beam, an optical signal is emitted, detected by a laser, and converted to an electrical signal. Advantages of this technology include the ability to rapidly analyze large numbers of cells, in great detail with regard to cell size and complexity. Forward scatter measures the diffraction of the light at a flat angle and is influenced by the volume of the cell. The side scatter is a measure of the refraction of light at right angles and depends on the cell granularity, size, and structure, as well as on the amount of vesicles in a cell.

Fluorescence-activated cell scanning (FACS)

Therefore, the cells are coupled to a fluorescence dye, which releases fluorescence when it passes the laser. The choice of the emission wavelength is done by the use of filters. In this way, it is possible to measure the fluorescence from multiple dyes in a single sample.

5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE)

5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) is an amine-reactive fluorescent probe that forms green fluorescent conjugates on deacetylation [52]. It is used as a stain in flow cytometry. The excitation maximum is 492 nm and the emission maximum is 517 nm. Therefore, a blue laser with an excitation wavelength of 488 nm is used.

R-phycoerythrin-Annexin V (PE-Annexin V)

Annexin V is a 35-36 kDa calcium dependent phospholipid-binding protein that binds with a high affinity to cells, which show the membrane phospholipid phosphatidyl-serines on their surface to the external cellular environment [53]. This process occurs in apoptosis due to the loss of the plasma membrane.

R-phycoerythrin (PE) is found in red algae, where it is an accessory photosynthetic pigment. PE is *in vitro* a 240-kDa protein with 23 phycoerythrobilin chromophores per molecule. This property makes it to one of the brightest fluorochromes for flow cytometry. Therefore, a blue laser with an excitation wavelength of 488 nm is used.

The protocol is based on [48] Liu et. al. (2011) and is slightly modified (Appendix 11).

4 Results

4.1 Validation of the IL-2 stimulation assay

Table 9 shows the doses of IL-2 and their referring stock volume (see Equation 2 and Equation 3).

Table 9: Tested units of IL-2 and their referring mass and stock volume

U_{wanted}	m_{IL-2}	V_{stock}
30 U IL-2	90 ng	9 µL
100 U IL-2	300 ng	30 µL
300 U IL-2	900 ng	90 µL
1000 U IL-2	3000 ng	300 µL

4.1.1 Optimal amount of recombinant equine IL-2

4.1.1.1 Blood samples from adult horses

The RNA yield from blood samples of adult horses stayed stable within each horse among the different treatments. The RNA yields ranged between 5.560 ng/µL and 18.493 ng/µL. The RNA yields of the different adult horses can be found in Appendix 12.

The gene expression of granzyme B, IFNγ, IL-2Rα, perforin and TNFα of the adult horses J06, N123 and O121 is measured by qPCR to determine the optimal amount of recombinant equine IL-2.

As shown in Table 10, the results of the OneWayANOVA test (group: treatment) show a statistically significant ($P < 0.050$) difference in the different treatments (media, 30 U, 100 U, 300 U, 1000 U recombinant equine IL-2) in the IFNγ mRNA expression. For IFNγ mRNA expression there is a statistically significant difference ($P = 0.041$) between the treatment with 300 U recombinant equine IL-2 and the unstimulated media sample (see also Figure 8). This is determined by pairwise multiple comparison procedures Holm-Sidak method.

Table 10: Results of OneWayANOVA of adult horses about different treatments (media, 30 U, 100 U, 300 U, 1000 U recombinant equine IL-2): statistically significant difference only for IFNγ noted by asterisk (*)

	P value	P < 0.050
Granzyme B	0.228	no
IFNγ	0.041*	yes
IL-2Rα	0.082	no
Perforin	0.410	no
TNFα	0.732	no

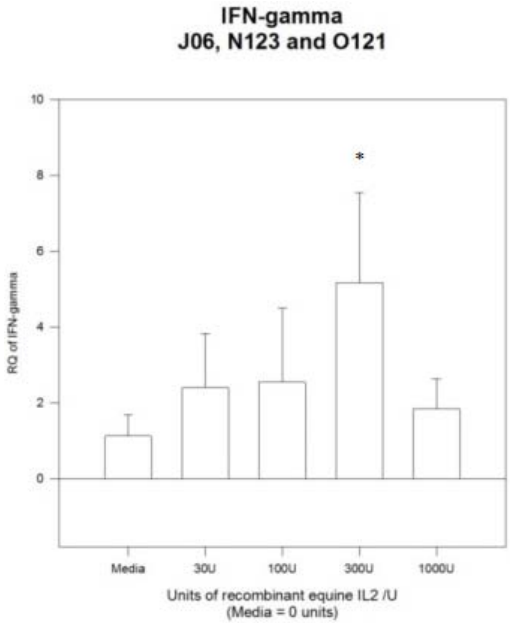


Figure 8: Relative quantification of IFNγ of adult horses about different treatments (media, 30 U, 100 U, 300 U, 1000 U recombinant equine IL-2): statistically significant difference for treatment with 300 U recombinant equine IL-2 noted with an asterisk (*)

4.1.1.2 Blood samples from foals

The RNA yield from blood samples of foals stayed stable within each horse among the different treatments. The RNA yields ranged between 4.493 ng/µL and 50.683 ng/µL. The RNA yields of the different foals can be found in Appendix 12.

The gene expression of granzyme B, IFNγ, IL-2Rα, perforin and TNFα of the foals Q15, Q17, Q18, Q19 and Q20 is measured by qPCR to determine the optimal amount of recombinant equine IL-2.

As shown in Table 11, the OneWayANOVA test (group: treatment) shows a statistically significant ($P < 0.050$) effect from the different treatment concentrations (media, 30 U, 100 U, 300 U, 1000 U recombinant equine IL-2) on the mRNA expression for granzyme B and IFNγ.

Table 11: Results of OneWayANOVA about different treatments of foals (media, 30 U, 100 U, 300 U, 1000 U recombinant equine IL-2): statistically significant difference for granzyme B and IFNγ noted by asterisks (*)

	P value	P < 0.050
Granzyme B	0.017*	yes
IFNγ	0.033*	yes
IL-2Rα	0.127	no
Perforin	0.799	no
TNFα	0.239	no

A pairwise multiple comparison procedures Holm-Sidak method is performed with the genes, where a statistically significant treatment effect occurs (see Table 12).

Table 12: Results of pairwise multiple comparison procedures Holm-Sidak method of foals about granzyme B and IFNγ (media, 30 U, 100 U, 300 U, 1000 U recombinant equine IL-2): statistically significant difference for granzyme B at 1000 U IL-2 (equal to 300 U IL-2) and IFNγ at 300 U IL-2 noted by asterisks (*)

	Comparison	P	P < 0.050
Granzyme B	1000 U IL-2 vs media	0.006*	yes
	1000 U IL-2 vs 300 U IL-2	0.605	no
	100 U IL-2 vs media	0.175	no
IFNγ	300 U IL-2 vs media	0.034*	yes
	300 U IL-2 vs 100 U IL-2	0.875	no
	1000 U IL-2 vs media	0.056	no

For granzyme B mRNA expression there is a statistically significant difference between the treatment with 1000 U recombinant equine IL-2 and the unstimulated media sample. Furthermore, it is shown that there is no difference between the treatment with 1000 U recombinant equine IL-2 and 300 U recombinant equine IL-2 (see Figure 9).

For IFNγ mRNA expression there is a statistically significant difference between the treatment with 300 U and the unstimulated media sample, whereas no statistically significant difference between the treatment with 300 U recombinant equine IL-2 and 100 U recombinant equine IL-2 occurs. However, it can be said that there is no statistically significant difference between the treatments with 1000 U compared to the unstimulated media sample (see Figure 9).

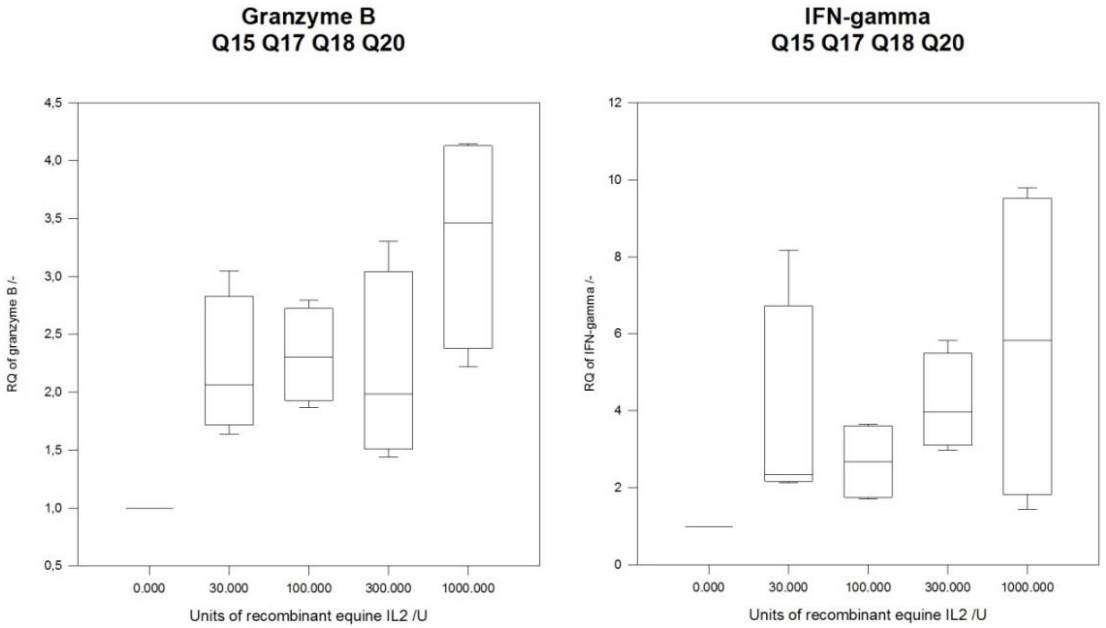


Figure 9: Box plot of granzyme B and IFNγ in foals across different treatments (media, 30 U, 100 U, 300 U, 1000 U recombinant equine IL-2): statistically significant difference for granzyme B at 1000 U IL-2 (equal to 300 U IL-2) and IFNγ at 300 U IL-2

4.1.2 Optimal incubation time

4.1.2.1 Blood samples from adult horses

The RNA yield from the Tempus™ Blood RNA Tubes appeared to decay over the course of the incubation period. After one day of incubation the maximum RNA yield is 49.162 ng/µL whereas after five days of incubation the maximum RNA yield dropped to 4.367 ng/µL. The RNA yields of the different adult horses can be found in Appendix 12.

A OneWayANOVA test (group: time point) is performed only with the data of the untreated media samples over time (1-5 days). No statistically significant difference is detected (see Table 13), which establishes that there is no significant change in gene expression among untreated samples over the course of the incubation period.

Table 13: Results of OneWayANOVA of adult horses of untreated media samples at different time points (1-5 days): no statistically significant difference

	P value	P < 0.050
Granzyme B	0.995	no
IFNγ	0.988	no
IL-2Rα	0.891	no
Perforin	0.858	no
TNFα	0.991	no

Then, a TwoWayANOVA test (factor A: day; factor B: treatment) is performed to determine if there is a difference in the day of the treatment and the treatment itself and on what day the difference occurs. Table 14 shows that there is, in all cases, except for perforin, a statistically significant difference for the factor "treatment" as the P-value is lower than 0.050, which means 300 U recombinant equine IL-2 induces a significant change in gene expression.

Table 14: Results of TwoWayANOVA about different days (1-5 days) and treatments (media, 300 U recombinant equine IL-2) of adult horses: statistically significant difference for factor "day" only for granzmye B; statistically significant difference for factor "treatment" in all cases, except for perforin; statistically significant difference for factor "day x treatment" for granzyme B and IL-2Rα. Statistically significant results are noted with asterisks (*)

Source of variation	Granzyme B	IFNγ	IL-2Rα	Perforin	TNFα
Day	0.006*	0.064	0.083	0.197	0.090
Treatment	0.004*	0.034*	0.012*	0.708	0.037*
Day x Treatment	0.006*	0.052	0.038*	0.179	0.089

There is a statistically significant difference in the day of the treatment for some, but not all of the cytokines of interest (see Table 14 and Table 15), but as Figure 10 shows there is a trend between those two parameters, which suggest that the treatment is most effective on the fourth day.

Table 15: Pairwise multiple comparison procedures Holm-Sidak method of adult horses of granzyme B and IL-2Rα about factor "day within 300 U IL-2" (statistically significant difference between IL-2 treated and untreated sample) and "treatment within day 4" (statistical significance)

	Comparison	P
Granzyme B	300 U IL-2 vs. media	< 0.001
	D4 vs. D2	
	D4 vs. D1	
	D4 vs. D3	
	D4 vs. D5	0.004
IL-2Rα	300 U IL-2 vs. media	< 0.001
	D4 vs. D5	0.001

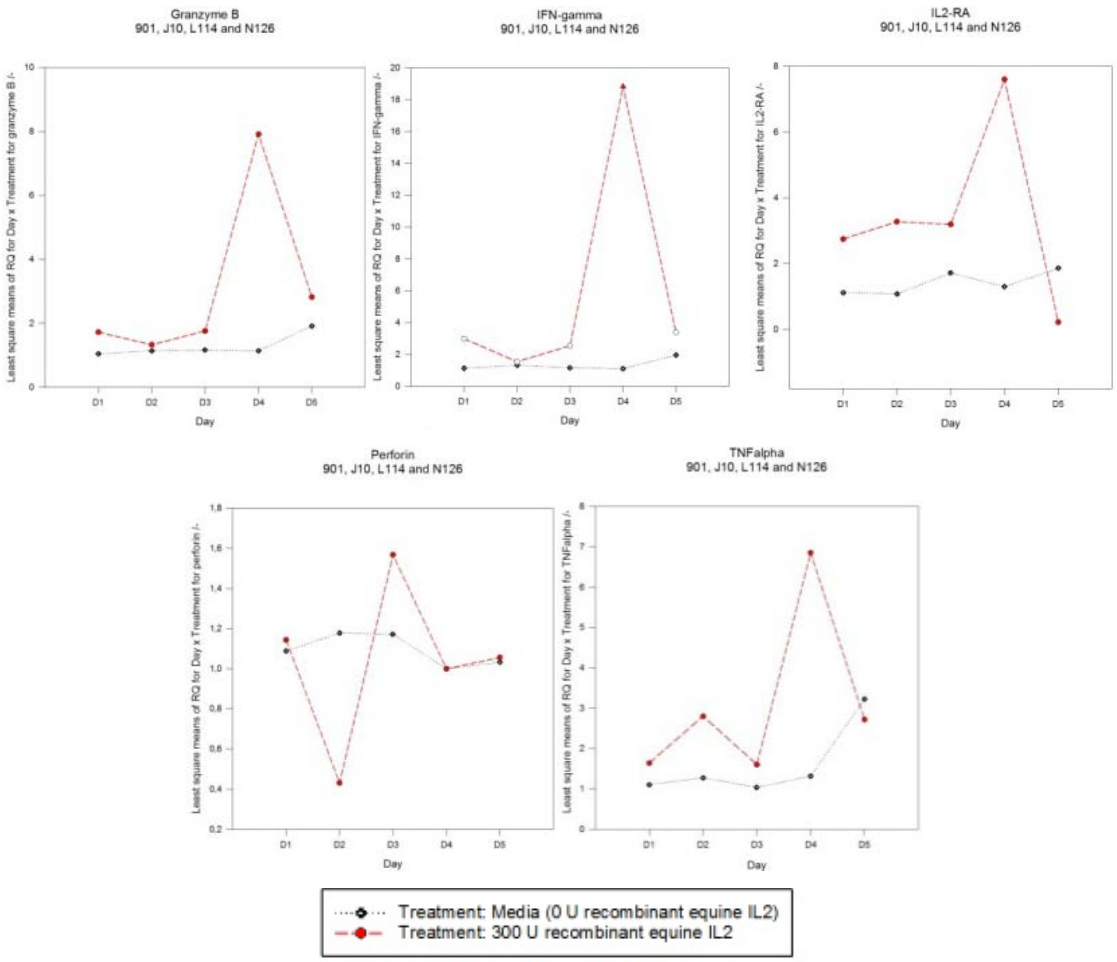


Figure 10: Least Square Means for Day x Treatment of adult horses (as a representation of variance among sample groups): no statistically significant difference, but a trend that suggests that the four days of stimulation with IL-2 are most effective

4.1.2.2 Blood samples from foals

As before, there appears to be degradation in the RNA yield from the Tempus™ Blood RNA Tubes over the course of the incubation period. After one day of incubation the maximum RNA yield is 83.379 ng/µL, whereas after five days of incubation the RNA yield dropped to 3.002 ng/µL. The RNA yields of the different foals can be found in Appendix 12.

A OneWayANOVA test (group: time point) is performed only with the data of the untreated media samples over time (1-5 days). No statistically significant difference is detected (see Table 16), which again confirms that there is no statistically significant change in gene expression among the unstimulated samples over the course of the incubation period.

Table 16: Results of OneWayANOVA of foals of untreated media samples at different time points (1-5 days): no statistically significant difference

	P value	P < 0.050
Granzyme B	0.986	no
IFNγ	0.992	no
IL-2Rα	0.988	no
Perforin	no statistics possible	
TNFα	0.937	no

A ThreeWayANOVA test (factor A: day; factor B: treatment) is performed to determine if there is a difference between the foals themselves, between the days of the incubation period, and between the treatment groups. As seen in Figure 11, each foal reacted differently to the stimulation with IL-2. Therefore, only qualitative data could be gained from this sample set. It seems that a peak in gene expression is achieved either on the second or on the fourth day of incubation. This qualitative result, combined with the statistically significant data points seen in the adult horse data, informed the decision to incubate for four days to generate LAK cells in the later experiments.

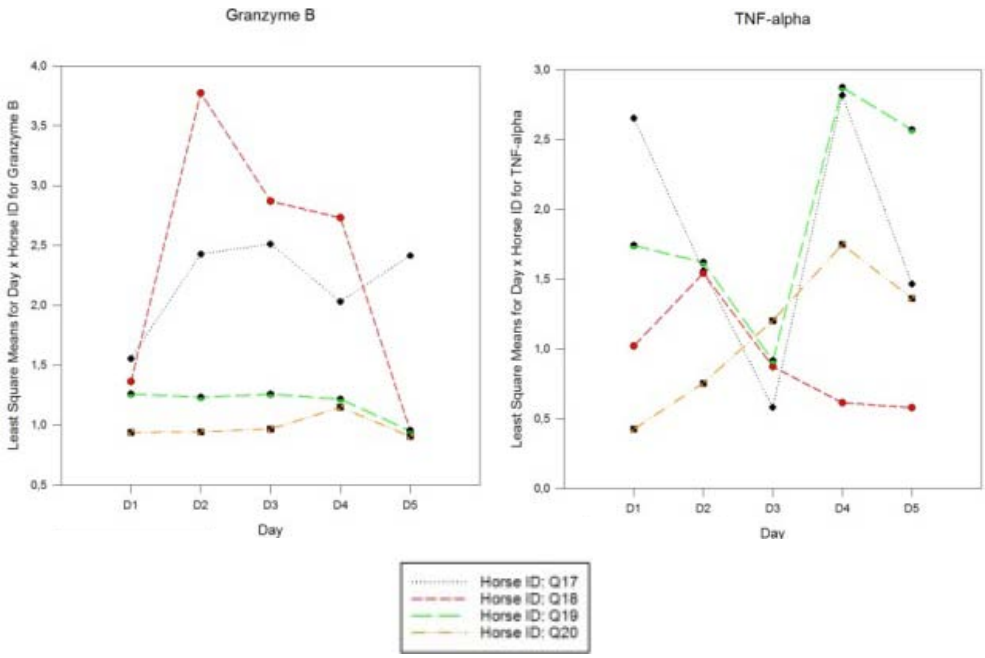


Figure 11: Least Square Means for Day x Treatment of foals (as a representation of variance among sample groups): gain of qualitative data only, as each foal reacts different to IL-2 stimulation

4.2 LAK assay

The purpose of the LAK assay is to measure the LAK activity in foal and adult horse PBMCs culture. The PBMC cultures are stimulated with 300 U recombinant equine IL-2 and incubated at 37° C and 5 % CO_2 for 3.5 days. An untreated sample serves as a reference sample to determine the naturally-occurring cytotoxicity over the incubation period. The thawed PBMCs had a viability of at least 96.0 %. However, the overall loss of viable PBMC's is approximately 83.5 % following the incubation period. The EqT8888 target cells are passaged the day before the LAK assay is performed. The viability of the EqT8888 target cells that are used in the LAK assay is at least 92.5 %.

4.2.1 Gating

Figure 12 shows how cell populations are gated to focus on events of interest.

The first gate (R1) is drawn to determine the target cell population by using dead EqT8888 target cells as a positive control and live EqT8888 target cells as a negative control. Within this cell population (R1) the brightest CFSE-stained cells are gated (M1). This gate (M1 in R1) is plotted with the CFSE stain on the x-axis against the PE-Annexin V stain on the y-axis. Finally in this graph (M1 in R1), the gate is moved as close as possible around the positive control (killed EqT8888 cells), which are both CFSE and PE-Annexin V positive. Hence, the upper right quadrant of this graph is the area in which the killed EqT8888 cells are located.

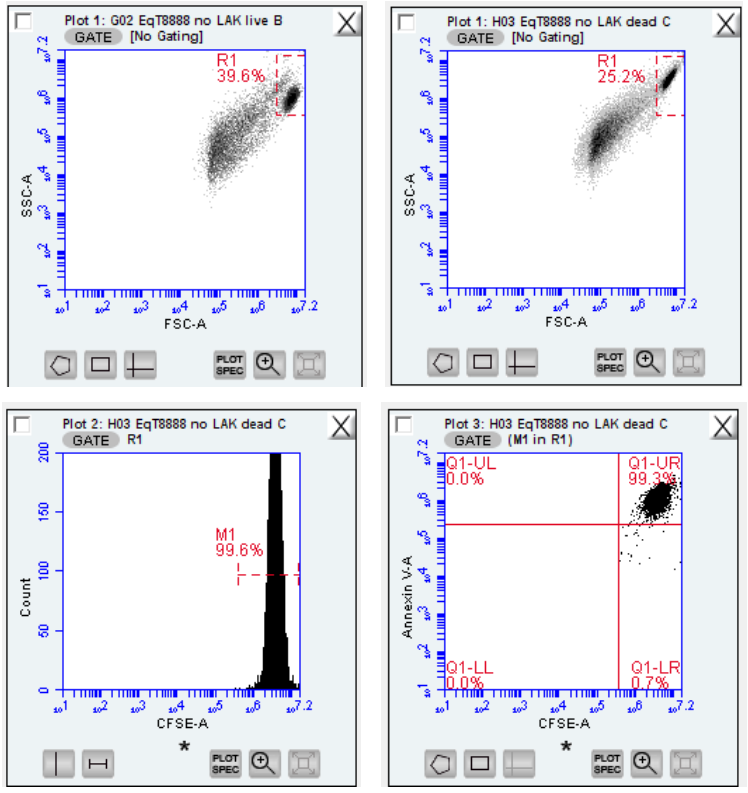


Figure 12: Process of gating: gating within the target cell population (first row) on the brightest CFSE stained cells and adjustment of the quadrants based on positive control (second row)

Based on the negative control (live EqT8888 cells) the color compensation is set (see Figure 13), by increasing the compensation in the FL1 and FL2 channels until the target cell population showed a circular shape. Color compensation refers to the virtual modification of CFSE and PE-Annexin V fluorescence readings (by subtracting a percentage of the CFSE reading from the Annexin V reading, and vice versa) in order to establish distinct cell populations. After establishing the color compensation parameters, the graph showed two distinct cell populations.

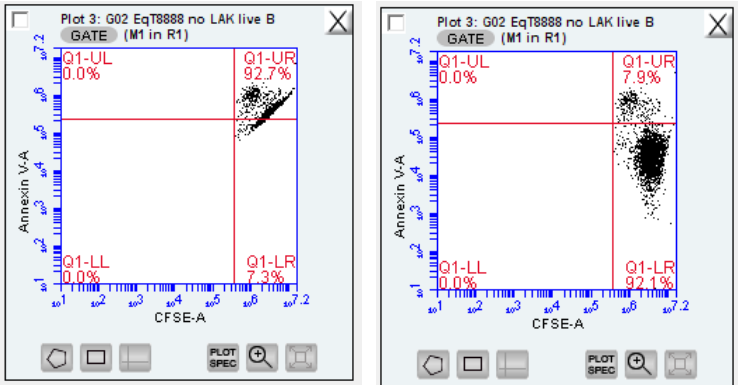


Figure 13: Set-up of color compensation based on negative control: increase of FL1 and FL channels until cell population had a circular shape

4.2.2 E:T ratios and cytotoxicity

The apoptotic activity of effector LAK cells can be measured as a function of the ratio of effector to target tumor cells (E:T ratio). For each horse, three different E:T ratios (the highest possible E:T ratio and two levels less than that: one half and one quarter of the highest E:T ratio) are measured for each horse. When the measurement process is started with the lowest E:T ratio, the LAK assay seems to work as intended, as the cytotoxic activity of the LAK cells increased with increasing E:T ratios. However, after changing the order of measurements, the cytotoxic profile of the E:T ratios changed. Now, the lowest E:T ratio showed the highest cytotoxic activity. This suggests that the duration of the incubation, rather than the E:T ratio, is the true predictor of cytotoxic activity. In order to confirm this irregularity, a set of samples of two horses are measured in opposite direction – the foal sample set is measured from the highest E:T ratio to the lowest E:T ratio, while the adult horses samples are measured from the lowest E:T ratio up to the highest E:T ratio. As shown Figure 14, the resulting curves show an opposite increase in the cytotoxicity. Thus, the assay is not reliable for comparing different E:T ratios.

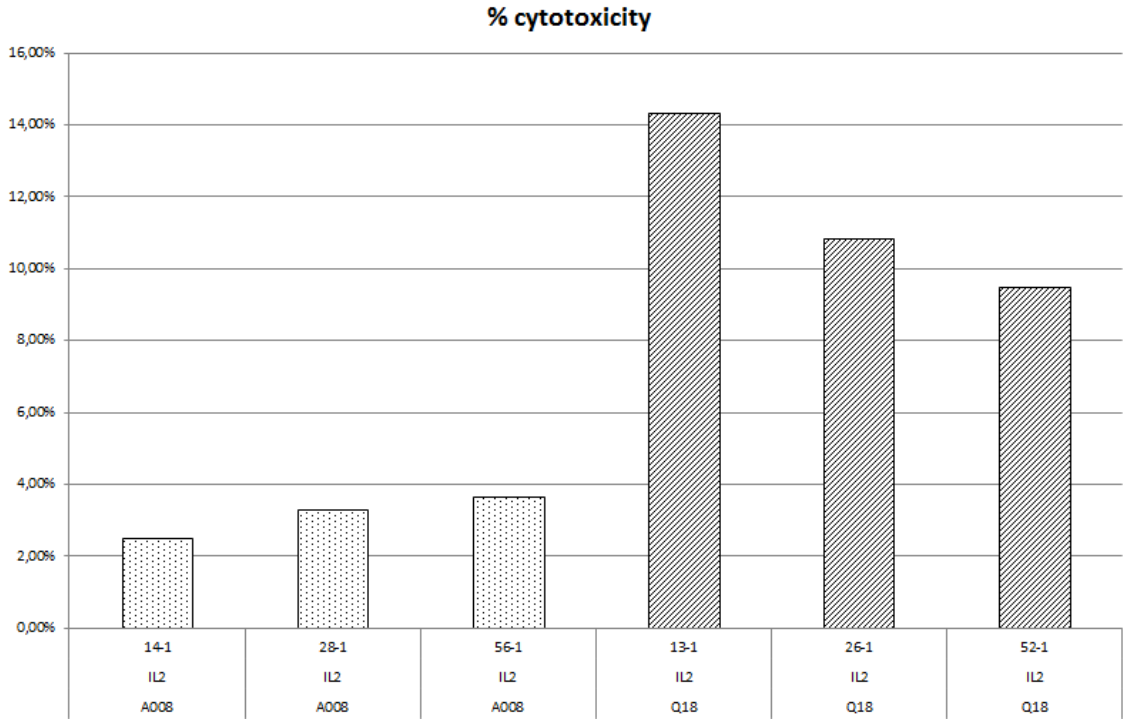


Figure 14: Unreliability of the assay shown with horse A008 and Q18: influence of order of the measurement of the samples as cytotoxicity increases within the waiting time between samples

Only the first-measured E:T ratio is used for the final analysis of the cytotoxicity (see Figure 15). Overall, the foals show a higher LAK activity than the mares, whereas the cytotoxic activity is for each individual horse highly specific.

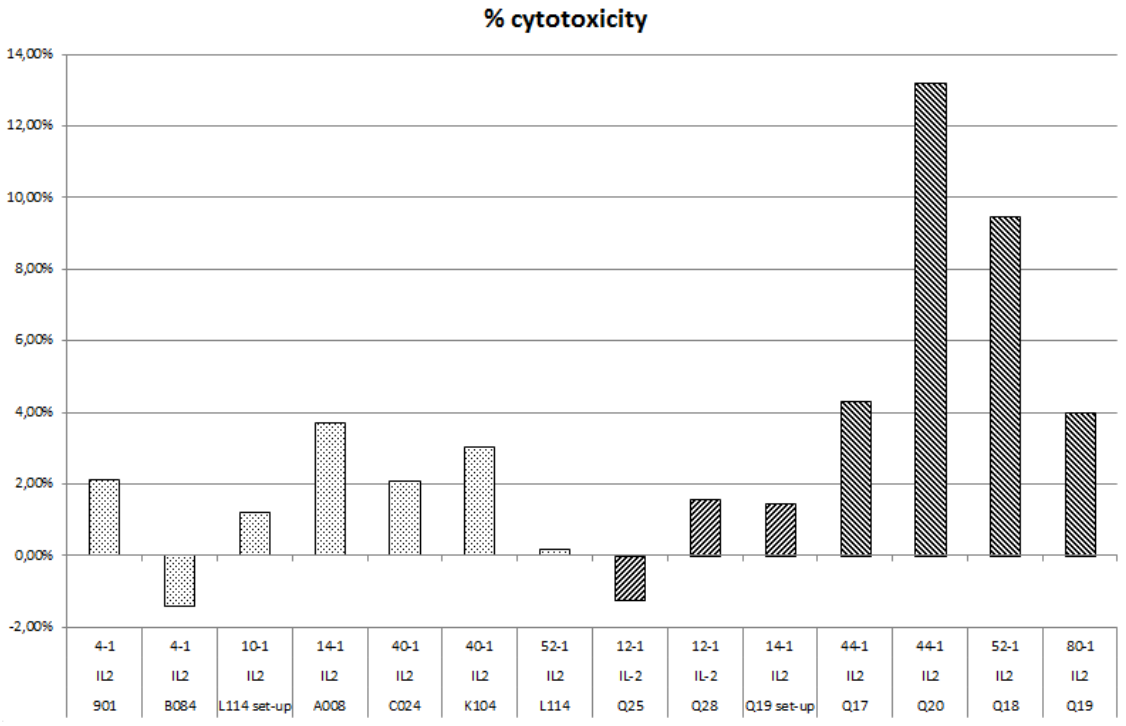


Figure 15: Final analysis of cytotoxicity by E:T ratio by first-measured sample: foals show a higher cytotoxicity in all cases compared to adult horses

4.3 Gene expression assay

The RNA yield for all horses ranges from 15.888 ng/µL to 132.707 ng/µL. Appendix 12 shows the RNA yields from blood samples of foals and adult mares.

A ThreeWayANOVA test (factor A: foal or adult; factor B: mare-foal pair; factor C: treatment) is used to examine any statistically significant differences between those factors.
For all mRNA expression profiles, there is a statistically significant difference between the treatment groups. Between foals and adult horses there is only a statistically significant difference for the IL-2Rα mRNA expression. A statistically significant difference between the foal-mare pairs occurs only at the perforin mRNA expression.

A TwoWayANOVA test (factor A: age group; factor B: treatment) is used to determine if there is a difference in the age group and the treatment itself, and at which age group the difference occurs. Table 17 shows that there is, in all cases, a statistically significant difference for the factor "treatment", as the P-value for each cytokine is less than 0.050. A statistically significant difference occurs only for IL-2Rα for the factor "age group" and "age group x treatment". The age group "6 weeks to 6 months" shows a statistically significant difference ($P < 0.001$) compared to the group "adult" within the IL-2 treatment.

Table 17: Results of TwoWayANOVA about different age groups and treatments (media, 300 U recombinant equine IL-2): statistically significant difference only for IL-2Rα noted by asterisks (*)

Source of variation	Granzyme B	IFNγ	IL-2Rα	Perforin	TNFα
Age group	0.512	0.052	0.036*	0.955	0.462
Treatment			< 0.001*		
Age group x treatment	0.512	0.052	0.036*	0.955	0.462

Even if there is no statistically significant difference in the age group and treatment the (see Table 17), Figure 16 shows a trend between those two parameters, which suggests a decrease in the mRNA expression with age.

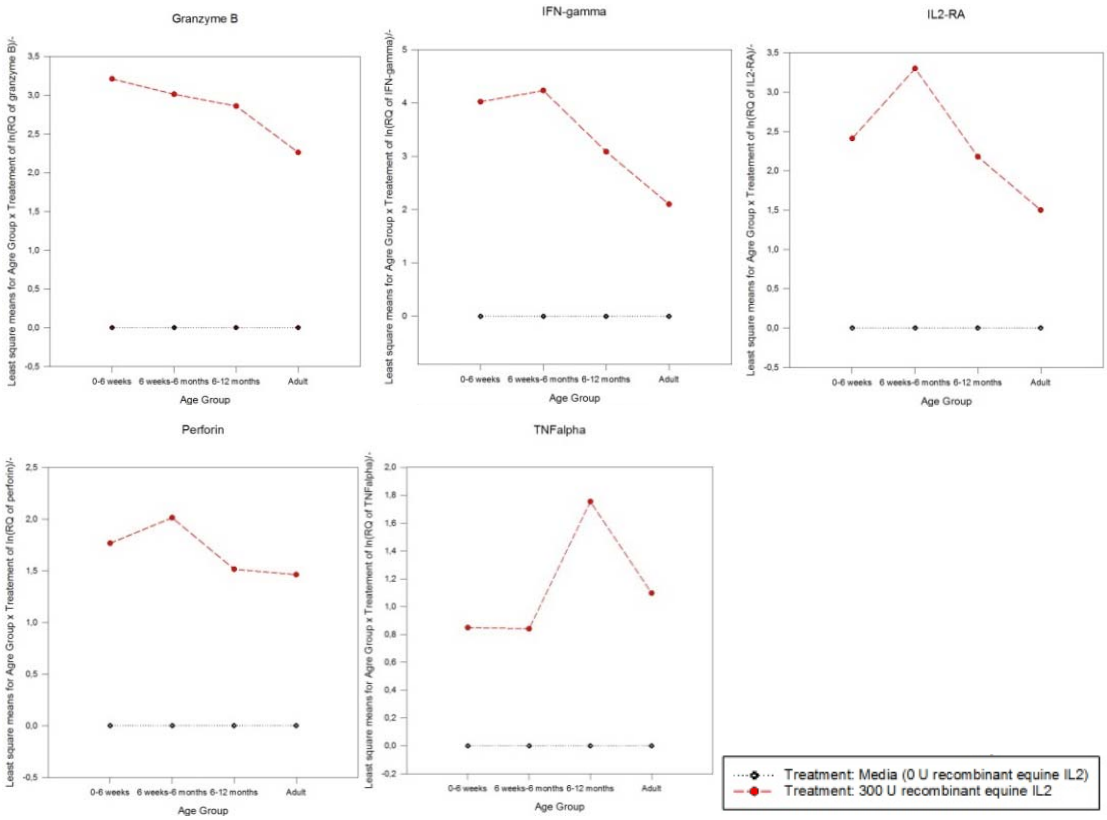


Figure 16: Least Square Means for Day x Treatment about different age groups and treatments of foals and adult horses (as a representation of variance among sample groups): no statistically significant difference, but decreasing trend over age

Table 18 and Figure 17 show the result of the Pearson-Correlation analysis. All pairs of variables have positive correlation coefficients and P value below 0.050.

Table 18: Results of the Pearson-Correlation analysis: statistical significance and positive correlation of all pairs of variables

		ln(RQ of IFNγ)	ln(RQ of IL-2Rα)	ln(RQ of perforin)	ln(RQ of TNFα)
ln(RQ of granzyme B)	Correlation coefficient	0.876	0.855	0.585	0.638
	P value	$7.734*10^{-16}$	$1.064*10^{-14}$	$1.29*10^{-5}$	$1.07*10^{-6}$
	Number of samples	47	48	48	48
ln(RQ of IFNγ)	Correlation coefficient	-	0.944	0.649	0.614
	P value	-	$3.048*10^{-23}$	$8.21*10^{-7}$	$4.49*10^{-6}$
	Number of samples	-	47	47	47
ln(RQ of IL-2Rα)	Correlation coefficient	-	-	0.681	0.709
	P value	-	-	$1.02*10^{-7}$	$1.69*10^{-8}$
	Number of samples	-	-	48	48
ln(RQ of perforin)	Correlation coefficient	-	-	-	0.726
	P value	-	-	-	$5.30*10^{-9}$
	Number of samples	-	-	-	48

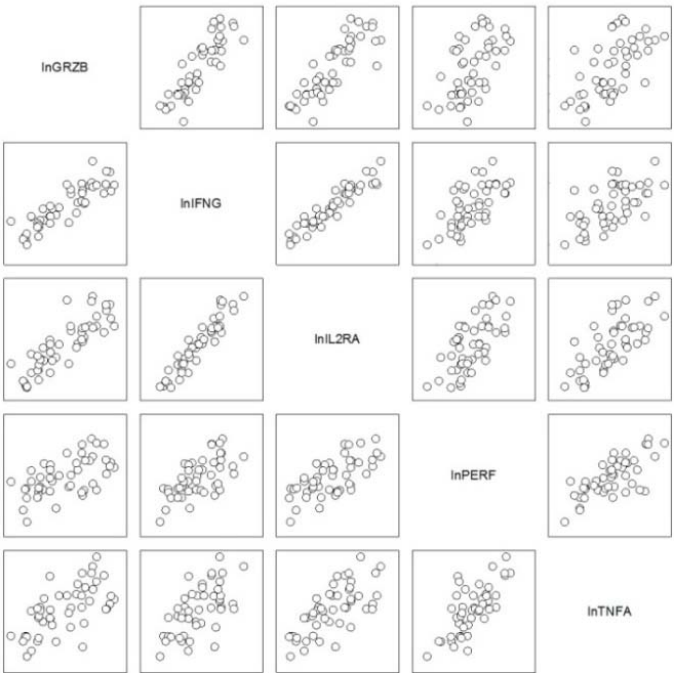


Figure 17: Pearson-Correlation analysis: all pairs of variables have positive correlation coefficients

5 Discussion

5.1 Validation of the IL-2 stimulation assay

5.1.1 Optimal amount of recombinant equine IL-2

The optimal amount of recombinant equine IL-2 is determined by the measurement of granzyme B, IFNγ, IL-2Rα, perforin and TNFα by qPCR, and 300 U (equivalent to 900 ng) recombinant equine IL-2 is found to produce a statistically significant effect both in the foal and the adult horse sample set. Thus, 300 U recombinant equine IL-2 is chosen as the optimal amount of recombinant equine IL-2 for all further experiments.

5.1.2 Optimal incubation time

The statistical analysis of the untreated media samples shows no differences over the incubation period of five days both in the foal and the adult horse sample set. This result is expected as there is no increased amount of IL-2 responsible for the induced gene expression [14].

A statistically significant difference between the untreated media sample and the IL-2 stimulated sample is seen for all mRNA expression profiles (except for perforin) both in the foal and the adult horse sample set. The cDNA for perforin failed to amplify during the course of the qPCR reaction indicating low or no expression of the mRNA in both the adult and foal samples.

In terms of the adult PBMC cultures, four days of incubation is determined the optimal incubation period with recombinant equine IL-2. By comparison, the foal sample set appears heterogeneous in the response to IL-2, with a peak of mRNA gene expression seen at two and four days of incubation. Nonetheless, this peak is specific for each individual foal. To be consistent with the data gained from adult horses, four days of incubation are chosen as the optimal incubation period with recombinant equine IL-2. This time period is also consistent with other reports indicating maximal induction of LAK cytotoxic activity after four days of culture [48].

5.2 LAK assay

The results obtained from the flow cytometric analysis of LAK cell activity are confounded by the delay in measuring target cell killing using the flow cytometer. The difference between measuring the first and the last sample (about three hours) consistently led to the detection of a higher cytotoxicity in the later samples that are analyzed. Thus, it is not possible to utilize all of the data collected for this purpose. However, it is possible to focus only on those samples and E:T ratios that had similar incubation times. Using this approach it is possible to show that the foals' PBMC cultures exhibited more cytotoxic activity than those from the adult horses. This concurs with the results of the gene expression assay as the foals also exhibit a higher gene expression profile than the adult horses. Nevertheless, these results should be interpreted with caution since the E:T ratio studied here is 14:1, while according to literature the E:T ratio should be at least 20:1 [30, 48].

5.3 Gene expression assay

Overall there is no statistically significant difference between the age groups in gene expression in the LAK cell cultures. An exception to this is the IL-2Rα mRNA expression, as there is a statistically significant difference between the age group "6 weeks to 6 months" and "adult". There is also a trend in the data for granzyme B and IFNγ mRNA expression to decrease with foal age. It is also noted that foals appear to have a greater variability within their gene expression and that this variability decreased with the foal's age. This observation is also supported by the results obtained from the LAK assay. A reason for this could be that the immune system of the foal is not fully developed and responds less precisely to stimulation with IL-2 compared to the immune system of an adult horse. Furthermore, it could be demonstrated that there is no relationship between the foal and their mare in context of gene expression. This indicates that maternal factors may not contribute to the differences seen in foal responses to IL-2.

The expression of granzyme B, IFNγ and TNFα in the IL-2 stimulated cultures are highly correlated. This result is expected as those cytokines are all involved in LAK cell function. The correlation between IFNγ and IL-2Rα supports the evidence that foals are able to respond to IL-2 by producing similar amounts of IFNγ to those seen in adult horses. Since prior work has shown that foals are thought to be deficient in their ability to produce IFNγ in response to mitogen stimulation, the current results indicate that IFNγ production in the foal is likely limited through the insufficient activation of T cells and their subsequent reduced expression of the high-affinity IL-2 receptors following their activation. This is also consistent with reports of defective accessory cell function and impaired T-cell receptor signaling in neonatal PBMC [20]. The use of a high dose of IL-2 likely triggers gene expression through the low affinity IL-2 receptor, thus bypassing the need for initial activation of the cells. While it is initially presumed that foals would have decreased LAK responses compared to adults, the results showed increased mRNA expression of granzyme B, IFNγ, and TNFα by foal PBMC in response to the high dose of IL-2. These unexpected results can be explained as follows: *in vivo* foals show a failure of T cell activation that appears until four months of age [22]. This leads to less production of IL-2, and thus of IFNγ and other inflammatory- and cytotoxic-related cytokines. In the presence of higher concentrations of IL-2 the foals mounted a vigorous response. However, it is also shown that the individual response among the foals is quite variable with some foals exhibiting responses more similar to those of adult PBMC, while others are less so. Overall, it could be shown that a high dose of IL-2 could induce LAK activity as well as the mRNA expression of related genes. It should also be noted that there is no relationship between foal and mare in terms of IL-2-induced gene expression.

5.4 Conclusion

Despite the unreliability of the flow cytometric LAK assay, the core of the research problem – to gain a better understanding about LAK activity in foals, and the relationship of this activity to the ability of those same cells to produce IFNγ – could still be evaluated partially and qualitatively. Throughout the course of the flow cytometric assay, LAK killing of target cells in the samples continued during the measurement process of the flow cytometer, leading to variable results between replicate samples measured at the same time point and an overall statistical effect based on incubation time, rather than based on E:T ratio as expected. Although no statistically significant results could be reported based on these data, an overall trend suggests that stimulated PBMC cultures from neonatal foals demonstrate robust LAK activity on a scale similar to those of adult horses, although the cytotoxicity for each specific horse used in this study is highly variable, even at the same E:T ratio.

As expected, an increased expression of LAK activity-associated genes, including perforin and granzyme B occurred. Further, it is shown that LAK cells of foals are able to produce IFNγ when stimulated with recombinant equine IL-2 *in vitro*, an effect not predicted by the hypothesis. Thus, the susceptibility of foals to *R. equi* does not appear to be due to a deficiency in this innate immune response. Rather, these findings give insight into an alternative possible mechanism for the increased susceptibility of foals as it seems likely that the dysregulation of IFNγ expression is linked to the insufficient activation of T cells leading to reduced production of IL-2, since the foals' PBMC responded well to the IL-2 in the cultures.

Overall, based on the data from the flow cytometric and gene expression assays, it seems that the non-specific immunity declines with age whereas the specific immunity improves. More importantly, these conclusions suggest that there is much more to be discovered about the production of IL-2 in foals and its relation to LAK activity, overall immune function, and susceptibility to *R. equi* and other pathogens.

6 Future prospects

6.1 Comparison of methods to measure LAK activity

While it is not the original intention of this research project to compare methods for assessing LAK function, circumstances allowed us to do so.

The traditional method of measuring LAK activity is the chromium release assay, however, a huge disadvantage of this method is the use of radioactive reagents. Because of this, flow cytometry has begun to replace chromium release as the preferred method of measuring LAK activity. The main advantage of this method is the lack of radioactivity, which makes it possible to measure LAK activity in a standard laboratory without any special safety concerns. The main disadvantage of this is that there is still no standard protocol available that works for every tumor target cell line. Thus, any flow cytometry protocol for the measurement of LAK activity must be first tested and validated in order to develop an effective staining method. Gating the fluorescence readings is also time-consuming, as it also has to be custom-fit to each sample set. Precise gating is very important as it influences the interpretation of results. Finally, because LAK activity continues over time while samples are being measured by the flow cytometer, flow rate and speed of the flow cytometer itself is of critical importance. To improve the accuracy of the assay, a flow cytometer with a fast flow rate and autosampler should be used. Additionally, flow cytometers are expensive pieces of equipment and require advanced training to operate effectively.

The determination of LAK activity through gene expression is a promising alternative method as this allows the measurement of more samples in a shorter period of time due to no longer needing to cultivate target tumor cells, a costly and time-consuming process. A significant advantage of RNA isolation from whole blood samples the possibility of direct stimulation of *in vivo* samples, either on whole blood with heparinized blood collection tubes or directly on isolated PBMCs themselves, which allows for simulation of *in vitro* responses without putting research animals at risk for side effects, as well as allowing for testing of multiple stimulations and doses. Furthermore, the expression of multiple genes can be determined within a single sample, allowing for analysis of a broad range of reactions to the same stimulation across multiple subject animals and time points. However, the gene expression assay is only an indirect method of measuring LAK activity and there is no validated gene that is in direct correlation with LAK activity yet – this presents another possible avenue for further research. While equipment costs of this method are also high, qPCR machines are widely available in most laboratories.

6.2 Possible improvements of methods

Since there are reduced yields of RNA from the heparinized blood collection tubes at the longer incubation times, alternative methods of blood collection should be considered, as after three days of incubation the blood started clotting. To circumvent this problem, additional heparin (or other anticoagulants) could be added to the blood samples to avoid clotting over the course of the incubation period.

The sample set of only six horses per group is very small and should be increased in further studies to reduce the variance in results and improve statistical power. A further limitation of this study is that the flow cytometric method of detecting LAK activity is not stable enough to give proper results, as the killing activity of the LAK cells continued outside of the CO_2 incubator while the samples are being analyzed. This could be overcome by using a flow cytometer with a faster flow rate and auto-sampler to reduce the waiting time between the samples. A further possibility is to stop the killing activity of LAK cells by fixing the EqT8888/LAK cell mix after staining with PE-Annexin V with para-formaldehyde. It may also be worthwhile to test and validate other commercially available apoptosis detection kits, such as a photometric lactate dehydrogenase (LDH) cytotoxicity assay kit. This latter approach can avoid some of the limitations of the flow-based methods owing to quicker sample preparation and analyses.

6.3 Possible future projects

It is shown in the gene expression assay that foals are able to produce the same amount of IFNγ as adult horses. Relating to the increased susceptibility of young foals to *R. equi*, the next hypothesis would be that progressor foals (foals which exhibit clinical symptoms and signs of pneumonia following exposure to *R. equi*) have a low IFNγ mRNA expression level compared to regressor foals (foals which remain healthy and exhibit no clinical symptoms following exposure to *R. equi*), which show a high IFNγ mRNA expression level. In relation to LAK activity it would be expected that progressor foals have a lower LAK activity and IFNγ mRNA expression than regressor foals.

The role of regulatory T cells in the LAK cell-mediated immunity of foals could be addressed, as regulatory T cells suppress the immune response. Another possibility is to examine the gene expression (via qPCR) of the transcription factor FoxP3 (forkhead box P3), as it stimulates the expression of proteins needed for regulatory T cells. In foals a higher gene expression of FoxP3 would be expected compared to adult horses.

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AKT	PKB = Protein kinase B
ANOVA	analysis of variance
APAF-1	apoptotic protease activating factor-1
BAX	Bcl-2-associated X protein
Bcl-2	B cell lymphoma 2
BID	BH3 (Bcl-2 homology domain 3) interacting-domain death agonist
CAD	caspase-activated DNase
CD	cluster of differentiation
cDNA	complementary DNA
CCD camera	charge-coupled device camera
CFSE	5(6)-Carboxyfluorescein diacetate N-succinimidyl ester
CIS	cytokine-inducible SH2 (Src Homology 2)-containing protein
CO_2	carbon dioxid
CT	threshold cycle
CTL	cytotoxic T lymphocytes
DED	death effector domain
DISC	death inducing signaling complex
DNA	deoxyribonucleic acid
DR	death receptor
E:T ratio	effector to target ratio
ERK	extracellular signal-regulated kinases; MAPK = mitogen-activated protein kinase
FACS analysis	fluorescence-activated cell scanning analysis
FADD	FAS associated death domain
FAS-L	first apoptosis signal-ligand
FAS-R	first apoptosis signal-receptor
FoxP3	forkhead box P3
GDP	guanosine diphosphate
GRB2	growth factor receptor binding protein 2
GTP	rat sarcoma-guanosine-5'-triphosphate
ICAD	inhibitor of caspase-activated DNase
IFNγ	interferon gamma
IL	interleukin
IL-2R	IL-2 receptor
JAK	Janus kinase

LAK cell	lymphokine-activated killer cell
LDH	lactate dehydrogenase
MEK	MAPKK = mitogen-activated protein kinase kinase
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NK cell	natural killer cell
p70S6K	p70S6 kinase; S6K1 =Ribosomal protein S6 kinase beta-1
PBMC	peripheral blood mononuclear cells
PE	R-phycoerythrin
PI-3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PMA	Phorbol 12-Myristate 13-Acetate
qPCR	quantitative real-time polymerase chain reaction
RAF	rat fibrosarcoma; MAPKKK = mitogen-activated protein kinase kinase kinase
RAS	rat sarcoma
RHEB	Ras homolog enriched in brain
RNA	ribonucleic acid
S6K	ribosomal S6 kinase
SHC	src homology 2 domain containing transforming protein 1
SOCS	suppressor of cytokine signaling proteins
SOS	son of sevenless
src	sarcoma
STAT	signal transducer and activator of transcription
T_h1 cell	Type 1 helper T cell
TNF	tumor necrosis factor
TRAAD	TNF receptor-associated death domain
TRAIL	TNF-related apoptosis-inducing ligand
βGus	beta-Glucuronidase

List of symbols

% $CFSE^{+}AV^{+}_{media\ release}$	apoptotic target cells + effector cells in well	%
% $CFSE^{+}AV^{+}_{target\ in\ well}$	apoptotic target cells + IL-2-stimulated effector cells in well	%
% $CVSE^{+}AV^{+}_{total\ release}$	heat killed target cells in well	%
% cytotoxicity	LAK cell cytotoxicity	%
A	amount	-
c	cell concentration	cells/mL
C	calibrator	-
CT	threshold cycle	-
ED_{50}	effective dose 50	ng/mL
m	mass	µg; ng
N	number of cells per	cells/mL
R_E	ratio of effector to target cells	-
RQ	relative quantification (fold change of amplification)	-
T	number of target cells /cells	-
U	units	-
V	volume	mL; µL
β	mass concentration	µg/mL
ΔCT	difference in number of cycles between gene of interest and endogenous control	-
ΔΔCT	cycle difference between ΔCT and calibrator value for gene of interest	-

Appendix 1

Table 19 shows the horses which are used in this project, their date of birth and for which part of the project they are used.

Table 19: Horses used in this project with date of birth

Horse ID	Date of birth	Use
F01	04/12/2017	gene expression assay
F02	04/03/2017	
F04	04/03/2017	
F05	04/20/2017	
F06	04/16/2017	
F09	04/19/2017	
F12	01/31/2017	gene expression assay
F13	01/30/2017	
F16	01/29/2017	
F17	01/22/2017	
F18	01/22/2017	
F19	01/28/2017	
Q15	22/06/2016	(set-up) gene expression assay
Q17	28/06/2016	(set-up) gene expression assay and LAK assay
Q18	28/06/2016	
Q19	01/07/2016	
Q20	01/07/2016	
Q25		LAK assay
Q28		
Q29	07/09/2016	gene expression assay
J06		set-up gene expression assay
J10		
N123		
O121		
A04		gene expression assay
A05		
A12		
A18		
901		set-up gene expression assay and LAK assay
A008		LAK assay
B084		
K104		
L114		(set-up) gene expression assay and LAK assay
C024		gene expression assay and LAK assay

Appendix 2

IL-2 Dose Determination

1. Dissolve 100 µg of recombinant equine IL-2 in 10 mL 1 % fetal bovine serum (FBS) phosphate-buffered saline (PBS) to obtain a stock with a mass concentration of 10 µg/mL.
2. Invert gently all Monoject™ Blood Collection Tubes with green stoppers, which are containing whole blood until homogenized.
3. Separate samples into treatment groups based on amount of IL-2: 0 U ("media"), 30 U (= 90 ng), 100 U (= 300 ng), 300 U (= 900 ng), 1000 U (= 3000 ng)
4. Invert the tube immediately before adding the IL-2.
5. Add to each tube the adequate amount of the IL-2 stock.

Some calculations have to be done in advance (see Equation 1, Equation 2, Equation 3).
First, the mass of one unit is calculated with the help of Equation 1:

$$m_{U=1} = ED_{50} * V_{bs}$$

Equation 1

$m_{Unit=1}$ ..... mass of one unit /ng
ED_{50} ..... effective dose 50 /(ng/mL)
V_{bs} ..... volume of blood sample /mL

Applied:

$$\mathrm{m_{U=1}} = 1\ \mathrm{ng/mL} * 3\ \mathrm{mL} = 3\ \mathrm{ng}$$

Then the mass of recombinant equine IL-2 which should be added to the blood sample can be calculated by Equation 2.

$$m_{IL-2} = \frac{U_{wanted} * m_{U=1}}{U}$$

Equation 2

m_{IL-2} ..... mass of IL-2 added to blood sample /ng
U_{wanted} ..... units wanted to be tested /-
$m_{Unit=1}$ ..... mass of one unit /ng
U ..... one unit /-

Applied for 30 U (all further calculations are performed analogous)

$$m_{IL-2} = \frac{30\ U * 3\ ng}{1\ U} = 90\ ng$$

The adequate amount of IL-2 can be calculated with help of Equation 3.

$$V_{Stock} = \frac{m_{IL-2}}{\beta_{stock}}$$

Equation 3

V_{Stock} ..... volume of added to blood sample /µL
m_{IL-2} ..... mass of IL-2 added to blood sample /µg
β_{stock} ..... mass concentration of recombinant equine IL-2 stock /(µg/mL)

Applied for 30 U (all further calculations are performed analogous)

$$V_{Stock} = \frac{90\ ng}{10\ ng/\mu L} = 9\ \mu L$$

6. Invert the tube three times to homogenize.
7. After all samples have been stimulated, invert all tubes simultaneously five times to homogenize.
8. Put all tubes in an incubator at 37 °C for one to five days.
9. Transfer each sample from a Monoject™ Blood Collection Tubes with green stoppers with to a Tempus™ Blood RNA Tube, which is a closed evacuated plastic tube for the collection, processing and transportation of venous whole blood specimens to stabilize RNA without pretreatment of the blood sample prior to purification for gene expression profiling [54].
10. Shake each tube vigorously for 10 seconds immediately after the blood is transferred.
11. Incubate all Tempus™ Blood RNA Tubes for 15 minutes at room temperature, followed by storage at -20 °C until ready for RNA isolation.

Appendix 3

RNA isolation from whole blood samples

1. Turn on the centrifuge and prepare the following settings:
   a. Temperature: 4 °C
   b. Speed: 3000 x g
   c. Time: 30 minutes
2. Wipe down benchtop, gloves, pipettes, tip boxes, etc. with 70 % Ethanol, followed by RNase *AWAY*® Reagent.
3. Transfer each entire sample from the Tempus™ Blood RNA Tube to a 50 mL labeled conical tube.
4. Add 3 mL non-sterile 1 X PBS to the sample.
5. Vortex each sample vigorously for 15-30 seconds to ensure proper mixing and thorough washing of the sample. Frothing of the sample at this step is normal.
6. Centrifuge at 3000 x g for 30 minutes at 4 °C. After the centrifugation, handle the tubes carefully so as not to dislodge the clear RNA pellet from bottom of tube.
7. Pour gently to discard supernatant.
8. Stand tubes upside-down on absorbent paper for two minutes to drain residual supernatant.
9. Blot any remaining liquid from the tube rims by using clean absorbent paper.
10. Add 600 µL PureLink™ Viral Lysis Buffer to the pellet and vortex briefly to suspend it.
11. Transfer the lysate to an iPrep™ Sample Tube.
12. Power on, load and configure the iPrep™ machine in the “No-DNase mode” with a total elution volume of 100 µL.

Appendix 4

RNA isolation from PBMC's

The following procedure is employed for RNA isolation:

1. Add 5 mL warm cRPMI with 2.5 % FES to labeled 15 mL tubes. The media composition, which has a shelf-life of two weeks, is as follows:
   a. 96.4 mL RPMI 1640 medium without L-Glutamine
   b. 2.5 mL fetal equine serum
   c. 1 mL PSG
   d. 100 µL 2-mercaptoethanol
2. Thaw the vial quickly in a 37 °C water bath until the last ice crystal is beginning to melt. Immediately transfer the cell suspension drop by drop to the labeled 15 mL tube containing the warm cRPMI.
3. Spin the cells down at 500 x g for five minutes.
4. Pour off the supernatant.
5. Resuspend the cells in 10 mL cRPMI with 2.5 % FES.
6. Count the cells with the Beckman Coulter Vi-CELL XR in a 1:10 dilution of 100 µL resuspended cells and 900 µL cRPMI.
7. Pull off the appropriate volume of cells (to plate at $3*10^6$ cells/mL).
8. Spin the cells down at 500 x g for five minutes.
9. Pour off the supernatant.
10. Resuspend the cells in 4 mL cRPMI with 2.5 % FES.
11. Transfer 1 mL of the re-suspended cells to each of four replicate wells in a 24-well flat bottom plate.
12. Stimulate two wells with 300 U recombinant equine IL-2 to generate LAK cells. Thereby, the mass of one unit is calculated with the help of Equation 1. Then the mass of recombinant equine IL-2, which should be added to the blood sample, can be calculated by Equation 2 and the adequate amount of IL-2 with help of Equation 3.
13. Incubate the cells at 37 °C in 5 % CO_2 in air for four days.
14. Remove $1.5*10^6$ cells after four days from each well. Scrape the bottom of the well thoroughly to free up any adhered cells. Transfer the cells to a labeled 1.5 mL microcentrifuge tube.
15. Centrifuge at 1,500 x g for two minutes.
16. Remove the supernatant from each tube by flicking into a waste container and rake the pellet to break up the cell pellet.
17. Add 1 mL of TRIzol® reagent to each tube and vortex to re-suspend the cells.

18. Store lysed cells at -80 °C until ready for RNA isolation.
19. Wipe down the working environment with RNase *AWAY*® Reagent. Filtered tips are used for the actual RNA extraction process and the unfiltered tips are used for transferring TRIzol® reagent into microcentrifuge tubes.
20. Remove the samples from the -80 °C freezer.
21. Start the microcentrifuge on “Fast Cool” at 4 °C.
22. Transfer each of the samples into a labeled 1.5 mL Eppendorf tube.
23. Make sure that the tops on the Eppendorf tubes are secure and vortex quickly to homogenize the sample.
24. Add 200 µL of chloroform to each sample and mix vigorously for 15 to 20 seconds.
25. Let the samples sit for five minutes at room temperature.
26. Centrifuge at 12,000 x g for 15 minutes at 4 °C in the microcentrifuge.
27. Transfer 420 µL of the upper aqueous phase into the fresh Eppendorf tubes.
28. Add 320 µL of pure isopropanol to each sample.
29. Pulse vortex each sample.
30. Incubate at -20 °C for at least 30 minutes (or overnight, if necessary).
31. Centrifuge at 20,000 x g for ten minutes at 4 °C.
32. Set the water bath to 60 °C for later use.
33. Decant the supernatants by upending tubes and pressing against a wipe-all. Blot, but do not delay. The tubes should not sit for more than five minutes and be sure not to rattle the tubes.
34. Add 800 µL of 75 % ethanol and pulse vortex.
35. Centrifuge at 15,000 x g for five minutes at 4 °C.
36. Remove the majority of the supernatant (~ 700 µL) from each tube.
37. Centrifuge at 15,000 x g for five minutes at 4 °C.
38. Remove as much of the supernatant as possible, leaving the pellet untouched. Repeat centrifuge step, if needed.
39. Place the tubes in a rack on its side directly in the path of airflow in the hood for rapid drying (at least five minutes).
40. Add 60 µL of RNase-free water into each tube by pipetting up and down to help to dissolve the pellet.
41. Place the tube in 60 °C water bath for ten minutes.
42. Pulse vortex the tube.
43. Store at -80 °C.

Appendix 5

RNA Quantitation

1. Place the RNA samples in a bucket of ice.
2. Wipe down the benchtop, pipettes, tip boxes and the Epoch™ Microplate with 70 % Ethanol, followed by RNase *AWAY*® Reagent.
3. Power on the Epoch™ Microplate Spectrophotometer.
4. Prepare a blank by adding 2 µL of 1 M TRIS (pH = 8.0) for samples from Tempus™ Blood RNA Tubes, or 2 µL of RNase-free water for samples processed with TRIzol® reagent to microspots A2 and A3 of the microwell plate.
5. Open the program *Gen5* and select “Nucleic Acid Quantification”. Make sure that microspots A2 and A3 are marked as “blank” and the rest of the microspots are “empty”.
6. Place the microplate on the tray and press “Read”.
7. Once the blank has been read and both wells are read as similar, approve the read. If one or both of the blanks are not accepted, wipe off the plate with 70 % Ethanol and try again.
8. Wipe the plate off with 70 % Ethanol and load the spots with 2 µL of the (gently vortexed) samples each.
9. When ready, change the wells on the program *Gen5* to “sample”. Any empty spots should be marked as “empty”.
10. Inset the microplate into the spectrophotometer and start the read.
11. Approve the batch, when the read is finished.
12. Continue with measuring the samples (see step 8).
13. When all measurements are finished, end the batch and all data are automatically transferred into an *MS Office Excel* sheet.
14. Enter sample names into the generated *Excel* sheet and save it.

Appendix 6

Synthesis of first strand cDNA

The following information about the procedure is taken from the laboratory standard protocol:

1. Input the data from spectrophotometer onto the calculation program (Spec Calculator). The total volume of RNA and RNase-free water should equal 41.5 µL. The total amount of RNA in the sample for synthesis of first strand cDNA should be 1 µg. The volume of RNA could be calculated with the help of Equation 4.

$$V_{RT\ RNA} = \frac{m_{RT\ RNA}}{\beta_{spectrophotometer\ RNA}}$$

Equation 4

$V_{RT\ RNA}$ ..... volume of purified RNA sample used for synthesis of first strand cDNA /µL
$m_{RT\ RNA}$ ..... mass of RNA used for synthesis of first strand cDNA /ng
$\beta_{spectrophotometer\ RNA}$ ..... mass concentration of RNA measured with the spectrophotometer /(ng/µL)

Applied for Q18 Media D1 (all further calculations are performed analogous)

$$V_{RT\ RNA} = \frac{1000\ ng}{49.623\ ng/\mu L} = 20.152\ \mu L$$

The volume of RNase-free water could be gained with the help of Equation 5.

$$V_{RNase-free\ H_2O} = V_{total} - V_{RT\ RNA}$$

Equation 5

$V_{RNase\text{-}free\ H_2O}$ ..... volume of RNase-free water used for synthesis of first strand cDNA /µL
V_{total} ..... volume of RNase-free water and purified RNA sample /µL
$V_{RT\ RNA}$ ..... volume of purified RNA sample used for reverse transcription /µL

Applied for Q18 Media D1 (all further calculations are performed analogous)

$$V_{RNase-free\ H_2O} = 41.5\ \mu L - 20.152\ \mu L = 21.348\ \mu L$$

2. Remove the RNA samples from the freezer and thaw on ice.
3. Remove the reagents that are required for Master Mix preparation from the freezer and thaw on ice.
4. Wipe the benchtop, instruments and gloves down with RNase *AWAY* Reagent.
5. Label the PCR tubes.

6. Add RNase-free water to the labeled PCR tubes, following Equation 5.
7. Label a 1.5 mL Eppendorf microcentrifuge tube for the Master Mix preparation. When making Master Mix, prepare enough for two extra samples. Prepare the Master Mix as follows and add these amounts of reagents for each sample:
    a. Add 16 µL of 5 X Avian Myeloblastosis Virus (AMV) buffer.
    b. Add 16 µL of $MgCl_2$.
    c. Add 4 µL of dNTP.
    d. Add 1 µL of RNasin.
    e. Add 1 µL of Oligo dT primer.
    f. Add 0.5 µL of AMV Reverse Transcriptase.
8. Vortex the Master Mix.
9. Add 38.5 µL of Master Mix to each PCR tube.
10. Add appropriate amount of RNA to each PCR tube (see Equation 4).
11. Vortex the tube, which contains the mixture of RNase-free water, Master Mix and RNA.
12. Place samples into the thermal cycler and start the Reverse Transcription protocol (15 minutes at 42 °C, then five minutes at 95 °C and then storage at 4 °C) to gain a single stranded cDNA.

Appendix 7

Quantitative real-time PCR

The following protocol is based on [48] Liu et. al. (2011) and is slightly modified.

The pipetting of the 10 µL reaction volume is done with an ep*Motion*® 5070 pipetting robot.

1. All primer/probe sets are designed in intron-spanning region and fail to amplify genomic DNA and reverse transcription negative RNA samples. This step is confirmed by prior research.
2. Prepare PCR primer-probe master mixes in reagent reservoirs at a 1:10 dilution of primer-probe sets to PCR master mix.
3. Put a 384 well plate in the robot.
4. Run the primer-probe-loading program on the ep*Motion*® 5070 platform. This program loads 5.5 µL of the primer-probe master mixes as prepared in step 2 into columns on the 384-well plate. All reactions are performed in duplicate.
5. Dilute the single stranded cDNA with an equal volume (80 µL) of RNase-free water in the fume hood.
6. Transfer 80 µL of the diluted cDNA into a new 96 well plate.
7. Once the pipetting of the primer/probe master mix is finished, remove the reagent reservoirs and place the 96 well plate with the diluted cDNA in the pipetting robot.
8. Run the cDNA-loading program on the ep*Motion*® 5070 platform. This program loads 4.5 µL of the diluted cDNA (equivalent to approximately 30 ng) as prepared in step 5 into rows on the 384-well plate.
9. Once the pipetting of the cDNA is finished, seal the 384 well plate with an optical adhesive film.
10. Centrifuge the sealed plate at 300 x g for three minutes.
11. Put the sealed 384 well plate in the quantitative real-time PCR machine and start the quantitative real-time program.
12. PCR reactions are incubated for two minutes at 50 °C, then for ten minutes at 95 °C, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds.
13. The program *LinRegPCR* is used to validate PCR data, including proper amplification efficiency (between 1.8 and 2.2) and correlation coefficients ($R^2 > 0.99$).

14. The ΔΔCT-method is for the relative quantitation of the changes in gene expression (see Equation 6). The housekeeping gene Beta-glucuronidase (β-Gus) is selected for a characteristic lack in variability between samples of varying types (animal age, stimulation, incubation period, and so on). The threshold cycle (CT) is the number of cycles that is required to reach the amplification threshold.

$$\Delta CT = CT_{gene\ of\ interest} - CT_{endogenous\ control}$$

Equation 6

ΔCT ..... difference in number of cycles between gene of interest and endogenous control /-
$CT_{gene\ of\ interest}$ ..... threshold cycle for gene of interest /-
$CT_{endogenous\ control}$ ... threshold cycle for endogenous control /-

Applied for Q18 D1 media, for IFNγ (all further calculations are performed analogous)

$$\Delta CT = 32.044 - 23.648 = 8.396$$

15. The average of the media control sample of each horse is used to calculate the calibrator for each target gene (see Equation 9). The calibrator is the control sample used for comparison between treated and untreated sample.

$$\Delta\Delta CT = \Delta CT - C$$

Equation 7

ΔΔCT ..... cycle difference between ΔCT and calibrator value for gene of interest /-
ΔCT ..... difference in number of cycles between gene of interest and endogenous control /-
C ..... calibrator /-

Applied for Q18 D1 media, for IFNγ (all further calculations are performed analogous)

$$\Delta\Delta CT = 8.396 - 8.032 = 0.364$$

16. Relative quantity (RQ) is calculated as $2^{-\Delta\Delta CT}$ (see Equation 10).

$$\mathrm{RQ} = 2^{-\Delta\Delta CT}$$

Equation 8

RQ ..... relative quantification (fold change of amplification) /-
ΔΔCT ..... cycle difference between ΔCT and calibrator value for gene of interest /-

Applied for Q18 D1 media, for IFNγ (all further calculations are performed analogous)

$$\mathrm{RQ} = 2^{-0.364} = 0.777$$

Appendix 8

EqT8888 target cells

The following points are important while handling the EqT8888 cells.

1. The EqT8888 cells that are used as target cells in the LAK assay are maintained in suspension in RPMI-1640 media supplemented with 10 % FBS at 37 °C and 5 % CO_2 in air. The media composition is as followed:
   a. 44 mL RPMI 1640 medium with Glutamax
   b. 50 mL AIM V® Medium
   c. 500 µL MEM Non-Essential Amino Acids Solution
   d. 500 µL Sodium Pyruvate
   e. 100 µL Gentamycin
   f. 100 µL 2-mercaptoethanol
   g. 10 mL FBS
2. The cells are monitored daily under the microscope.
3. The cells are split as needed, e.g. when the color of the medium changes to orange or yellow, which is a sign that the nutrition ingredients have been consumed. If a cell concentration of $1.5*10^6$ cells/mL is used, the cells need to be split every other day. Furthermore, the cells are split the day before the LAK assay. This is necessary to ensure that the cells are in the log-phase of growth prior to CFSE staining. To passage the cells, the following steps are taken:
   a. Warm media to 37 °C in sterile 37 °C water bath.
   b. Transfer gently the entire content of the cell culture flask with a 10 mL serological pipette into a 50 mL tube.
   c. Centrifuge at 100 x g for five min at room temperature.
   d. Pour supernatant out, but do not turn the tube upside-down.
   e. Resuspend the cell pellet in 10 mL fresh media by knocking gently at the bottom of the tube.
   f. Count the cells with the Beckman Coulter Vi-CELL XR in a 1:10 dilution of 100 µL resuspended cells and 900 µL media.
   g. Split and transfer the cells into new flask and fill up with the appropriate amount of media. The total volume of a T75 flask is 40 mL. With the help of Equation 9 the volume to transfer from the 10 mL cell stock into one new flask is calculated.

$$V_{transfer} = \frac{N_{cell\ wanted} * V_{flask}}{N_{Vi-CELL}}$$

Equation 9

$V_{transfer}$ ..... volume to transfer from counted stock into one new flask /mL
$N_{cells\ wanted}$ ..... desired number of cells per mL in new flask /cells/mL
V_{flask} ..... total volume of new flask /mL
$N_{Vi\text{-}CELL}$ ..... number of cells per mL from Vi-CELL counter /cells/mL

Applied (all further calculations are performed analogous)

$$V_{transfer} = \frac{1.5 * 10^5 cells/mL * 40\ mL}{8.51 * 10^6\ cells/mL} = 0.705\ mL$$

4. The cultivation media with 10 % DMSO is used as freezing media for the EqT8888 cells. Approximately 40*10^6 cells are frozen in 1 mL freezing media. The number of vials, which can be gained from the left-over cells in the counted 10 mL stock, can be calculated by using Equation 10. The computer-labeled vials are put into a freezing container at -80 °C overnight. On the next day, the vials are transferred into the liquid nitrogen tank.

$$A_{vials} = \frac{N_{Vi-CELL} * V_{cs} - A_{flasks} * N_{cell\ wanted} * V_{flask}}{N_{cells\ per\ vial} * V_{vial}}$$

Equation 10

A_{vials} ..... amount of freezing vials /-
$N_{Vi\text{-}CELL}$ ..... number of cells per mL from Vi-CELL counter /(cells/mL)
V_{cs} ..... volume of counted stock /mL
A_{flaks} ..... amount of flaks used for further cultivation /-
$V_{transfer}$..... volume to transfer from counted stock into new flask /mL
$N_{cells\ wanted}$ ..... desired number of cells per mL in new flask /(cells/mL)
V_{flask} ..... total volume of new flask /mL

Applied (all further calculations are performed analogous)

$$A_{vials} = \frac{8.51 * 10^6\ cells/mL * 10\ mL - 2 * 1.5 * 10^5\ cells/mL * 40\ mL}{40 * 10^6\ cells/mL * 1\ mL} = \sim 2\ vials$$

Appendix 9

PBMC isolation

The following information about the procedure is taken from the laboratory standard protocol:

1. Set up tubes with 10 mL room-temperature Ficoll-Paque Plus.
2. Transfer slowly the top layer of blood (plasma) to the tubes by overlaying the layer on top of the Ficoll-Paque Plus.
3. Spin for 30 minutes at 500 x g at room temperature with slow brake at the end.
4. Transfer slowly cell layer (“fluffy” layer) to a new 50 mL tube. Do not disturb or pipette up the pellet containing red blood cells.
5. Top off the tube with 40 mL warm PBS and invert gently to mix.
6. Spin for ten minutes at 500 x g at room temperature with fast brake at the end.
7. Dump the supernatant.
8. Flick and rake the pellets.
9. Top off to 40 mL with warm PBS.
10. Spin for ten minutes at 300 x g at room temperature with fast brake at the end.
11. Dump the supernatant.
12. Flick and rake the pellets.
13. Top off to 40 mL with warm PBS.
14. Spin for ten minutes at 300 x g at room temperature with fast brake at the end.
15. Dump the supernatant.
16. Flick and rake the pellets.
17. Resuspend the cell pellets in 10 mL PBS (make sure to mix well).
18. Dilute 100 µL cells into 900 µL warm PBS in ViCell counting cups and count.
19. Spin for ten minutes at 500 x g at room temperature with fast brake at the end.
20. Dump the supernatant.
21. Flick and rake the pellets.
22. Resuspend the cell pellets in freeze media.
23. Place the cryo-tubes in the round ethanol freezing containers and in the -80 °C overnight and transfer to liquid nitrogen the next day for long-term storage.

Appendix 10

LAK activity

The following information about the procedure is partly taken from the laboratory standard protocol:

1. Add 5 mL warm cRPMI with 2.5 % FES to labeled 15 mL tubes. The media composition, which has a shelf-life of two weeks, is as follows:
2. 96.4 mL RPMI 1640 medium without L-Glutamine
3. 2.5 mL fetal equine serum
4. 1 mL PSG
5. 100 µL 2-mercaptoethanol
6. Thaw the vial quickly in a 37 °C water bath until the last ice crystal is beginning to melt. Immediately transfer the cell suspension drop by drop to the labeled 15 mL tube containing the warm cRPMI.
7. Spin the cells down at 500 x g for five minutes.
8. Pour off the supernatant.
9. Resuspend the cells in 10 mL cRPMI with 2.5 % FES.
10. Count the cells with the Beckman Coulter Vi-CELL XR in a 1:10 dilution of 100 µL resuspended cells and 900 µL cRPMI.
11. Pull off the appropriate volume of cells (to plate at 3*106 cells/mL).
12. Spin the cells down at 500 x g for five minutes.
13. Pour off the supernatant.
14. Resuspend the cells in 12 mL cRPMI with 2.5 % FES.
15. Transfer 1 mL to each duplicate well in a 24-well flat bottom plate.
16. Stimulate six wells with 300 U recombinant equine IL-2 to generate LAK cells. The mass of one unit is calculated with the help of Equation 1. Then the mass of recombinant equine IL-2, which should be added to the blood sample, can be calculated by Equation 2 and the adequate amount of IL-2 by Equation 3.
17. Incubate the cells at 37 °C in 5 % CO_2 in air for 3.5 days.
18. After 3.5 days the LAK cells are counted using a ViCell-XR instrument. This step and the following steps are done while CFSE staining of the EqT8888 target cells.
19. Transfer each well to a labeled 15 mL tube.
20. Add 9 mL cRPMI with 2.5 % FES.
21. Spin the cells down at 500 x g for five minutes.
22. Pour off the supernatant.
23. Resuspend the cells in 10 mL cRPMI with 2.5 % FES.

24. Count the cells with the Beckman Coulter in a 1:10 dilution of 100 µL resuspended cells and 900 µL cRPMI.
25. Pull off the appropriate volume of cells into a 15 mL tube. Equation 11 shows the calculation of the lowest possible E:T ratio, which will be done to generate three equal distant E:T ratios. It is important to round down the result in every case. In grey color the derivation of the final Equation 11 is shown.

$$N_{Vi-CELL} * V_{cs} = A_{replicate} * (R_E * T + 2 * R_E * T + 4 * R_E * T)$$

$$N_{Vi-CELL} * V_{cs} = A_{replicate} * 7 * R_E * T$$

$$R_{E\ low} = \frac{N_{Vi-CELL} * V_{cs}}{A_{replicate} * 7 * T}$$

Equation 11

$R_{E\ low}$ ..... lowest possible ratio of effector to target cells to make the three highest E:T ratios /-
$N_{Vi\text{-}CELL}$ .... number of cells per mL from Vi-CELL counter /(cells/mL)
V_{cs} ..... volume of counted stock /mL
$A_{replicate}$ .... amount of replicates /-
T ..... number of target cells /cells

Applied for Q18 Media (all further calculations are performed analogous)

$$R_{E\ low} = \frac{0.56 * 10^6\ cells/mL * 10\ mL}{3 * 7 * 3 * 10^4\ cells} = 8.88 \rightarrow 8{:}1$$

With the help of Equation 12 the medium E:T ratio can be calculated.

$$R_{E\ med} = 2 * R_{E\ low}$$

Equation 12

$R_{E\ med}$ ..... medium ratio of effector to target cells to make the three highest E:T ratios /-
$R_{E\ low}$ ..... lowest possible ratio of effector to target cells to make the three highest E:T ratios /-

Applied for Q18 Media (all further calculations are performed analogous)

$$R_{E\ med} = 2 * 8 = 16 \rightarrow 16{:}1$$

With the help of Equation 13 the highest possible E:T ratio can be calculated.

$$R_{E\ high} = 4 * R_{E\ low}$$

Equation 13

$R_{E\ high}$ ..... highest possible ratio of effector to target cells to make the three highest E:T ratios /-
$R_{E\ low}$ ..... lowest possible ratio of effector to target cells to make the three highest E:T ratios /-

Applied for Q18 Media (all further calculations are performed analogous)

$$R_{E\ high} = 4 * 8 = 32 \rightarrow 32{:}1$$

The number of cells needed for the respective E:T ratio can be calculated with the help of Equation 14.

$$N_{cn\ low/med/high} = R_{E\ low/med/high} * A_{replicate} * T$$

Equation 14

$N_{cn\ low/med/high}$ .. number of cells needed for the respective E:T ratio /cells
$R_{E\ low/med/high}$ .. respective ratio of effector to target cells to make the three highest E:T ratios /-
$A_{replicate}$ ..... amount of replicates /-
T ..... number of target cells /cells

Applied for Q18 Media E:T_{low} (all further calculations are performed analogous)

$$N_{cn\ low} = 8 * 3 * 3 * 10^4\ cells = 0.72 * 10^6\ cells$$

With the help of Equation 15 the volume of the counted stock needed for the respective E:T ratio can be calculated.

$$V_{neeeded} = \frac{N_{cn\ low/med/high}}{N_{Vi-CELL}}$$

Equation 15

V_{needed} ..... volume of counted stock needed for the respective E:T ratio /mL
$N_{cn\ low/med/high}$ .. number of cells needed for the respective E:T ratio /cells
$N_{Vi\text{-}CELL}$ ..... number of cells per mL from Vi-CELL counter /(cells/mL)

Applied for Q18 Media E:T_{low} (all further calculations are performed analogous)

$$V_{neeeded} = \frac{0.72 * 10^6\ cells}{0.56 * 10^6\ cells/mL} = 1.286\ mL$$

26. Spin the cells down at 500 x g for five minutes.
27. Resuspend the cells in 300 µL cRPMI with 2.5 % FES.

Appendix 11

LAK cell flow cytometric assay

The following protocol is based on [48] Liu et. al. (2011) and is slightly modified.

1. Label one 50 mL centrifuge tubes as "$CFSE^+$" and add $1x10^7$ EqT8888 cells suspended in 1 mL of 1 x PBS.
2. Turn the light in the fume hood off and add slowly 1 mL of light sensitive 3 µM CFSE (1.8 µL CFSE stock + 29.9982 mL 1 x PBS) to $CFSE^+$ tube.
3. Shake the tube on vortex at low speed (level six out of ten) for eight minutes.
4. Add 2 mL FBS to stop the CFSE reaction. Afterwards, the light in the fume hood can be turned on again.
5. Centrifuge at 300 x g for ten minutes.
6. Pour off supernatant gently, being sure not to disturb the cell pellet.
7. Resuspend the cells with ten mL of 10 % FBS-PBS.
8. Centrifuge at 300 x g for ten minutes.
9. Pour off supernatant gently, being sure not to disturb the cell pellet.
10. Resuspend the cells with 10 mL of 10 % FBS-PBS.
11. Centrifuge at 300 x g for ten minutes.
12. Pour off supernatant gently, being sure not to disturb the cell pellet.
13. Resuspend the cells in each tube with 33.3 mL cRPMI (see Equation 16; concentration: $3*10^5$ cells/mL).

$$V_{cRPMI} = \frac{c_{cells\ have} * V_{have}}{c_{cells\ wanted}}$$

Equation 16

V_{cRPMI} ..... volume of cRPMI needed to reach a defined cell concentration /mL
$c_{cells\ have}$..... available cell concentration /(cells/mL)
V_{have}..... available cell concentration /(cells/mL)
$c_{cells\ wanted}$ ..... wanted cell concentration /(cells/mL)

Applied

$$V_{cRPMI} = \frac{1 * 10^7 cells/mL * 1\ mL}{3 * 10^5\ cells/mL} = 33.333\ mL$$

14. Dispense 100 µL of labeled EqT8888 cells (concentration: $3*10^4$ cells/mL) into a 96 well V-bottom plate.
15. Add to each well of target cell 100 µL volume of LAK cells at different cell concentration to yield the desired effector to target (E:T) ratios.

16. Centrifuge the plate at 200 x g for five minutes.
17. Incubate "$CFSE^+$Live" plate at 37 °C with 5 % CO_2 in air for two hours. Incubate 1 mL of the $CFSE^+$ EqT8888 target cells in a 15 mL tube at 56 °C for two hours.
18. Centrifuge the plate at 500 x g for five minutes.
19. Flick the plate gently to remove the supernatant and pat dry with a paper towel.
20. Resuspend cells with 200 µL of 1 x PBS.
21. Centrifuge the plate at 500 x g for five minutes.
22. Flick the plate gently to remove the supernatant and pat dry with a paper towel.
23. Resuspend cells with 100 µL of 1 x Annexin V binding buffer (1:10 dilution of 10 x Annexin V binding buffer with 10 % FBS-PBS; store at 4 °C).
24. Cover plate with aluminum foil.
    a. Add 5 µL of PE-Annexin V to the one triplicate.
    b. Wait 15 minutes, covered at room temperature.
    c. Transfer cells each to their own FACS flow tube on ice.
    d. Add 400 µL Annexin V binding buffer to each tube on ice.
    e. Read the cells using the BD Accuri™ C6 (settings: 10,000 events, five minutes, sample volume: 400 µL). When the first read is started, start with point 24.a again to ensure constant flow of samples.
25. Target cells are gated using FL1 (CFSE; λ_{ex} = 492 nm) and 3000 gated events are acquired for each sample.
26. The percentage of cytotoxicity is determined as followed (see Equation 17):

$$\%\ cytotoxicity = \frac{\left(\%\ CFSE^+AV^+_{target\ in\ well}\right) - \left(\%\ CFSE^+AV^+_{media\ release}\right)}{\left(\%\ CFSE^+AV^+_{total\ release}\right) - \left(\%\ CFSE^+AV^+_{media\ release}\right)} * 100$$

Equation 17

% cytotoxicity ..... LAK cell cytotoxicity /%
% $CFSE^+AV^+_{target\ in\ well}$ ..... apoptotic target cells + IL-2-stimulated effector cells in well /%
% $CFSE^+AV^+_{media\ release}$ ..... apoptotic target cells + effector cells in well /%
% $CVSE^+AV^+_{total\ release}$ ..... heat killed target cells in well /%

Appendix 12

Table 20 to Table 24 show the detailed RNA yield of all horses and foals used in this study.

Table 20: RNA yield from blood samples of adult horses among different treatments (media, 30 U, 100 U, 300 U, 1000 U recombinant equine IL-2)

Horse ID	Treatment	RNA yield /ng/µL
O121	Media	9.008
	30 U IL-2	11.048
	100 U IL-2	9.279
	300 U IL-2	10.000
	1000 U IL-2	8.897
N123	Media	8.429
	30 U IL-2	11.317
	100 U IL-2	10.435
	300 U IL-2	7.480
	1000 U IL-2	5.560
J06	Media	16.206
	30 U IL-2	16.768
	100 U IL-2	16.985
	300 U IL-2	18.493
	1000 U IL-2	12.135

Table 21: RNA yields from blood sample of foals among different treatments (media, 30 U, 100 U, 300 U, 1000 U recombinant equine IL-2)

Horse ID	Treatment	RNA yield /ng/µL
Q15	Media	28.998
	30 U IL-2	28.619
	100 U IL-2	50.683
	300 U IL-2	22.772
	1000 U IL-2	23.295
Q17	Media	37.362
	30 U IL-2	47.439
	100 U IL-2	36.157
	300 U IL-2	47.654
	1000 U IL-2	33.272
Q18	Media	47.579
	30 U IL-2	15.804
	100 U IL-2	29.572
	300 U IL-2	46.524
	1000 U IL-2	31.329
Q19	Media	4.493
	30 U IL-2	15.362
	100 U IL-2	4.917
	300 U IL-2	14.259
	1000 U IL-2	18.411

Table 22: RNA yields from blood sample of adult horses over time (1-5 days)

Day	Horse ID	Treatment	RNA yield /ng/µL
1	J10	Media	39.597
		300 U IL-2	49.162
	N126	Media	31.287
		300 U IL-2	46.357
	901	Media	32.576
		300 U IL-2	32.864
	L114	Media	32.219
		300 U IL-2	19.795
2	J10	Media	16,181
		300 U IL-2	11.109
	N126	Media	13.969
		300 U IL-2	13.359
	901	Media	19.092
		300 U IL-2	14.109
	L114	Media	10.564
		300 U IL-2	10.885
3	J10	Media	9.060
		300 U IL-2	8.375
	N126	Media	10.597
		300 U IL-2	6.047
	901	Media	6.397
		300 U IL-2	5.884
	L114	Media	8.076
		300 U IL-2	7.800
4	J10	Media	9.139
		300 U IL-2	6.120
	N126	Media	7.634
		300 U IL-2	9.631
	901	Media	9.420
		300 U IL-2	5.123
	L114	Media	5.387
		300 U IL-2	4.959
5	J10	Media	7.466
		300 U IL-2	6.449
	N126	Media	4.878
		300 U IL-2	4.367
	901	Media	7.744
		300 U IL-2	4.791
	L114	Media	9.085
		300 U IL-2	5.787

Table 23: RNA yields from blood sample of foals over time (1-5 days)

Day	Horse ID	Treatment	RNA yield /ng/µL
1	Q17	Media	83.379
		300 U IL-2	61.916
	Q18	Media	57.257
		300 U IL-2	49.623
	Q19	Media	42.935
		300 U IL-2	45.979
	Q20	Media	40.951
		300 U IL-2	41.563
2	Q17	Media	26.540
		300 U IL-2	18.998
	Q18	Media	17.419
		300 U IL-2	15.988
	Q19	Media	15.868
		300 U IL-2	9.851
	Q20	Media	16.904
		300 U IL-2	12.132
3	Q17	Media	12.450
		300 U IL-2	9.684
	Q18	Media	10.361
		300 U IL-2	6.910
	Q19	Media	7.696
		300 U IL-2	8.692
	Q20	Media	8.677
		300 U IL-2	9.268
4	Q17	Media	12.660
		300 U IL-2	1.281
	Q18	Media	6.590
		300 U IL-2	4.801
	Q19	Media	10.121
		300 U IL-2	6.668
	Q20	Media	6.158
		300 U IL-2	6.514
5	Q17	Media	9.773
		300 U IL-2	6.240
	Q18	Media	8.116
		300 U IL-2	7.852
	Q19	Media	6.439
		300 U IL-2	12.061
	Q20	Media	10.305
		300 U IL-2	3.002

Table 24: RNA yields from blood sample of foals and adult mares

Age Group	Horse ID	Treatment	RNA yield /ng/µL
one day to six weeks	F1	Media	62.081
		300 U IL-2	49.079
	F2	Media	46.005
		300 U IL-2	48.793
	F4	Media	67.424
		300 U IL-2	56.819
	F5	Media	26.835
		300 U IL-2	81.191
	F6	Media	48.269
		300 U IL-2	63.202
	F9	Media	37.068
		300 U IL-2	66.929
six weeks to six months	F12	Media	39.471
		300 U IL-2	91.919
	F13	Media	38.021
		300 U IL-2	15.888
	F16	Media	77.978
		300 U IL-2	79.941
	F17	Media	59.600
		300 U IL-2	88.278
	F18	Media	43.610
		300 U IL-2	132.707
	F19	Media	56.018
		300 U IL-2	122.717
six months to one year	Q15	Media	40.143
		300 U IL-2	127.345
	Q17	Media	115.446
		300 U IL-2	79.891
	Q18	Media	67.014
		300 U IL-2	76.579
	Q19	Media	30.740
		300 U IL-2	95.780
	Q20	Media	64.596
		300 U IL-2	49.885
	Q29	Media	64.352
		300 U IL-2	79.581

Continuation of Table 24

adult mares	A4	Media	66.653
		300 U IL-2	39.782
	A5	Media	50.178
		300 U IL-2	42.492
	A12	Media	51.214
		300 U IL-2	125.503
	A18	Media	30.488
		300 U IL-2	30.275
	C24	Media	55.647
		300 U IL-2	70.329
	L114	Media	46.338
		300 U IL-2	109.813