V. DISCUSSION

MAbs specific to equine leukocyte antigens are important tools to study equine leukocyte biology and characterise immunologically important molecules. The rational of the present study was to provide domestic and wild equine immunology tool-box with antibodies to leukocyte markers, filling gaps of available reagents or rather establish a leukocyte tool-box for some members of the family *Equidae*. To achieve the target, a panel of well-defined commercially available anti-human (and anti-animal) leukocyte mAbs was analyzed for (cross)-reactivity with equid leukocytes using one- and two- colour flow cytometry. A few of the submitted mAbs were previously reported to react with horse cells and their characterization was extended here. In parallel, CD28 was assessed through cloning and sequence analysis of cDNA from horse, other members of family *Equidae*, and some wild life species representing different evolutionary relations to horses. Moreover, equine CD28 was expressed for further analysis. In addition, two polyclonal antibodies against human CD25 and CD28 were analyzed for cross reactivity with equine leukocytes.

5.1 Cross-reactivity analysis of a large panel of mAbs with horse leukocytes

It is generally accepted that well characterised monoclonal antibodies cross-reacting with leukocytes from other species are of considerable interest. Before accepting that a mAb reacts with the homologues antigen, the expression pattern and possibly the approximate molecular weight of the detected antigen need to be compared. At this study, 534 mAbs were analyzed for reactivity with horse leukocytes. The analysis resulted in 31 mAbs (~5.8%) with clear positive "++" reactivity, 2 mAbs (~0.3%) with weak positive "W" reactivity, 23 mAbs (~4.3%) with positive "+", but possibly still homologous staining, 15 mAbs (~2.8%) with a clear alternate expression pattern "A" from that expected from human immunology, thus unlikely to reflect homologous staining. 6 mAbs (~1.1%) with questionable "?" staining, 45 mAbs (~8.4%) with weak-positive reactivity and alternate expression pattern "W/A", and 356 negative "-" mAbs (~67.7%). In 53 cases (~9.9%), more appropriate target cells, such as thymocytes or bone marrow stem cells, were not available "NA" for screening.

Positive mAbs "++" were directed against CD2 (clones 39C1.5 and HB88A), CD5 (clone HT23A), CD11a (clone HUH73A), CD11b (clone M1/70.15.11.5), CD14 (clones 7H3, big 11, big 12, and 7D6), CD18 (clones BAQ30A, H20A, HUH82A, and MHM23), CD21 (clone B- Ly4), CD34 (clone AC136), CD44 (clones BAG40A, H22A, LT41A, and DH16A), CD45(clone DH16A), CD49d (clone HP2/1), CD68 (clone Ki-M6), CD83 (clone HB15a), CD91 (clone A2MRa2), CD163 (clone Ber-MAC3), CD172a (clone DH59B), CD206 (clone

3.29B1.10), CD283 (clone TLR3-7), MHCI (clone B9.12.1), MHCII (clones EqT2 and D-F1), and a canine B cell molecule (clone CA2.1D6).

Cross-reactivity analysis was based on screening the whole mAb panel by single colour flow cytometry as a method of choice, comparing the staining pattern of horse leukocytes with human leukocytes (from literature). Most mAbs defined positive in screening were further analyzed. In particular mAbs that detected only lymphocyte populations were analyzed using two colour flow cytometry. Some of the additional flow cytometry analyses of the HLDA8 workshop mAbs were carried out in collaboration with the Animal Health Trust (Dr. Julia Kydd) and the Univ. Colorado (Prof. Paul Lunn). The equine leukocyte cell lines, eCAS and EqT8888 were used to analyze the mAbs in a cross-check step, using flow cytometry again, with regard to the mAbs of the HLDA8 workshop in collaboration with Dr. J. Kydd and her team. Finally, immunoprecipitation was used to complement and add valuable information about the molecular nature of the detected CD molecules.

Leukocytes and PBMC were isolated using Biocoll (Ficoll) solution. Although this method might activate some cells (esp. monocytes) slightly, it was assumed to be the method of choice, as it has not been linked to the down-regulation of markers. The alternate method of direct staining of Abs in peripheral blood was not considered a suitable alternative, since more than 100 mAbs were tested in parallel. Erythrocyte lyses to some extend damages leukocytes, if isotonic balance cannot be restored immediately, which was considered the greater risk. Using flow cytometry, none of the negative control antibodies used in this study showed non-specific staining on horse PBMC or platelets. Previous reports have shown that non-specific staining by primary antibodies is almost exclusively due to mouse IgG2a antibody (Steinbach and Thiele, 1994), and indeed the only unspecific staining occurred when IgG2a was applied to eCAS cells.

In immunoprecipitation, surface molecules from equine leukocytes and equine cell lines were biotinylated prior to precipitation with candidate mAbs and protein-G sepharose to determine the molecular weight of the corresponding molecules in a western blot assay, using streptavidin-AP and chemilumenescence detection. Despite the advantage of unstained protein markers over the pre-stained ones for more precise estimation of molecular weights in SDS-PAGE, pre-stained markers were used in this study to suit membrane blotting and chemilumenecence detection. MAbs which are expressed internally like CD68 could not be included into this approach where radioisotopes would be required for metabolic labelling of internal antigens and mAbs of IgM class could not be linked to protein G sepharose. As the

abundance of antigen in the original sample was a crucial factor for immunoprecipitation, CD antigens were immunoprecipitated from equine cell lines where possible.

Lymphocyte markers:

The mAbs HB88A and HT23A were developed against equine **CD2** and **CD5** respectively (Crump et al., 1988; Tumas et al., 1994). Both mAbs were included as positive controls, especially as a basis for the double labelling (DL). Here, both mAbs were used to define T cells and stained the same majority of lymphocytes, namely T lymphocytes. In immunoprecipitation, HB88A precipitated equine CD2 as a protein of approximately 65 kDa and HT23A precipitated equine CD5 as a protein of approximately 69 kDa which is in accordance with the previous data (Crump et al., 1988; Tumas et al., 1994). The rat IgG2a mAb 39C1.5 directed against human CD2 did stain the vast majority of equine lymphocytes and possibly a few monocytes. $CD2^+$ monocytes were reported in humans to be circulating dendritic cells (Crawford et al., 1999), thus this finding would not oppose a T cell specific mAb. Using HB88A, it was demonstrated that clone 39C1.5 stained equine T cells specifically (Fig. 35). In addition, it stained all $CD4^+$ positive cells (Fig. 36a) and almost all $CD8^+$ cells with a small sub-population of T cells remaining $CD8^+/CD2^-$. Such a subpopulation was described before in humans as a very small subpopulation of NK cells (Perussia et al., 1983; Ritz et al., 1988; Nakazawa et al., 1997). Detection of the same population of cells by 39C1.5 and HB88A indicated that each mAb detected a different epitope. The data obtained from two-colour analysis thus strongly support the notion that 39C1.5 detects EqCD2. Additionally, 39C1.5 precipitated a protein of approximately 65 kDa leaving no doubt that anti-human mAb 39C1.5 reacts with the equine homologue of CD2. 39C1.5 was further used for double labelling, since it was available as a directly fluorochrome conjugated mAb.

The mAb B-Ly4 directed against human **CD21** was designated, using flow cytometry, to cross-react with equine cells before and reacted with a population of lymphocytes (Lin et al., 2002). In contrast to the mAb BL13 with its very weak staining, B-Ly4 stains a population of lymphocytes brightly and additionally fewer cells less intense (Fig. 25). The positive cells comprised of 9-24.5% of the lymphocytes. This labelling itself, the previous description of reactivity and the knowledge on the amount of B-cells expected, lead to the conclusion that B-Ly4 stains equine CD21. Detection of 2-3% of horse granulocytes by B-Ly4 was not surprising and is in accordance with humans where basophils may express low levels of CD21 (Bacon et al., 1993). Two-colour double labelling, using HB88A and HT23A, could show that

B-Ly4 was B cell-specific (Fig. 37). Two colour flow cytometry revealed a negligible population of T cells weakly co-stained with B-Ly4 (Fig. 37). Such a population was described in humans as a small sub-population of T cells expressing low levels of CD21 (Fischer et al., 1991; Braun et al., 1998).

Human CD21 has an approximate unreduced molecular weight of 145 kDa (Timens, 2002). Here, B-Ly4 immunoprecipitated equine CD21 under reducing conditions as a band of approximately 125 kDa (Fig. 45). Given the fact that nothing is known about the molecular structure of equine CD21, the potential difference to human CD21 was not considered contradictory to the antigen detected being equine CD21. Cell surface molecules of B cells include CD19, a 95 kDa glycoprotein which forms a complex with CD21, CD18, and Leu-13 (Tuveson et al., 1991; Tedder et al., 1994; Tedder et al., 1997; Sato and Tedder, 2002). Using B-Ly4, a band of approximately 95 kDa was co-precipitated, which could resemble CD19 (Fig. 45a). The obtained results provide no doubt that B-Ly4 detects the equine homologue of CD21.

The anti-Canine B cell mAb, **CA2.1D6** was indicated to cross-react from canine to equine B cells. The mAb also stained non-T cells as shown in two colour flow cytometry (Fig. 38). In this case, however, the mAb was classified as conditionally positive "(++)", as no further information about the antigen in dogs was available. Both B-Ly4 and CA2.1D6 detected the same population of cells, but slightly differed in staining profile and intensity, thus indicating that CA2.1D6 is not CD21-specific.

The mAb HB15a (Beckman Coulter) directed against **CD83** was also analyzed. It reacted with a population of 14-25% of lymphocytes, which were presumed to be B-lymphocytes. However, the mAb did show reactivity in some but not all horses with monocytes (Fig. 28). The data on CD83 expression in humans is slightly complex. In blood, only a population of DC stains positive. The marker has therefore been suggested uniquely for mature DC. However, CD83 was originally cloned from activated B cells and was also detected in LCs, which are certainly the hallmark cells for immature myeloid DC (Zhou et al., 1992; Kozlow et al., 1993). Additionally, CD83 may at least on MoDC be induced during maturation and accordingly may appear on myeloid cells (Zhou and Tedder, 1995). In two colour flow cytometry, HB15A detected B lymphocytes only (Fig. 41), and additionally, immunoprecipitated a band of approximately 45 kDa (Fig. 45b) which is analogous to human CD83 (Zhou and Tedder, 1995). Therefore, the staining of CD83 was considered "++".

Myeloid cell markers:

As a key receptor for lipopolysaccharide (LPS) on the surface of monocytes and macrophages, **CD14** is involved in non-specific host defence mechanisms against gram-negative bacteria. The 39-44 amino acid motif forms an essential part of the LPS-binding site of human CD14 (Stelter et al., 1997). A significant number of antibodies at this study were directed against human CD14 and four of them (clones 7H3, big11, big12, and 7D6) recognize the similar conformational epitope of aa 9-13 and 39-44 (Schütt et al., 1995; http://www.biometec.de). The four clones reacted strongly with equine monocytes in flow cytometry (Fig. 21-24). A very small population in the granulocyte gate showed CD14 expression, which could be granulocytes expressing CD14 as described in humans (Haziot et al., 1993; Rodeberg et al., 1997) or likely a small population of monocytes detected in the granulocyte gate. The bright monocyte staining by the four clones supports the assumtion that these mAbs detect the equine homologue of CD14. Additionally, clone 7H3 (big10) precipitated equine CD14 from monocytes as a single protein of approximately 55 kDa (Fig. 46). The obtained molecular weight was analogous to humans CD14 (Goyert, 2002a) supporting the notion that these mAbs detect equine CD14. In addition, comparison of sequences between human and equine CD14 indicated conservation of the aa9-13 and 39-44 LPS binding region in horses (data not shown).

CD68, a member of the lysosomal associated membrane proteins (lamp) family of lysosomal/plasma membrane shuttling proteins, is localised primarily in endosomal/lysosomal compartments of human macrophages (Saito et al., 1991; Holness and Simmons, 1993) with only low and variable surface expression (Fukuda, 1991; Goyert, 2002b). The mAb Ki-M6 against human CD68 has been suggested in an immunohistochemistry (IHC) study to detect equine CD68 (Siedek et al., 2000). Here, Ki-M6 when intracellular staining was applied did stain cultured equine monocytes (macrophages) clearly whereas on extra-cellular staining only very few cells were weakly stained (Fig. 27). This was in accordance with human cells, where CD68 is mainly expressed intracellularly and in cultured cells only. Using Ki-M6 as positive control, all HLDA-8 and non-HLDA-8 anti-CD68 mAbs were analyzed by intracellular staining, but Ki-M6 remained the only mAb with positive staining pattern.

The mAb A2MRα-2 is specific for human **CD91** and labelled the majority of equine monocytes but no granulocytes or lymphocytes (Fig. 15). A staining pattern which was in accordance with CD91 description in humans (Herz et al., 1990; Moestrup, 2002). Additionally, A2MRα-2 imunoprecipitated a protein of approximately 100 kDa (Fig. 46), a

molecular weight which was close to the reported size of the β-chain of human CD91 (Herz et al., 1990; Moestrup, 2002) supporting the notion that A2MRα-2 detects the equine homologue of CD91.

CD163 is another myeloid marker, expressed only by monocytes/macrophages, and up-regulated during activation of cells (Hogger, 1998; Philippidis et al., 2004). The mAb Ber-MAC3 is specific for human CD163 and stained the majority of equine monocytes (Fig. 16) analogous to human CD163 (Backe et al., 1991; Sulahian et al., 2000). Ber-MAC3 failed to precipitate CD163 from equine monocytes which may be explained by a low affinity for the recognised antigen. Nevertheless, depending on the clear staining pattern of monocytes it was assumed that Ber-Mac3 detects the equine homologue of CD163.

Human **CD172a** (SIRPα protein), approximately 110 kDa, inhibits cell signaling by recruiting phosphatases to the cell membrane. The mAb DH59B was originally described to cluster at SWC3, a swine antigen (Lunney et al., 1994). SWC3 was later found to be identical to SIRP-1 or CD172 (Alvarez et al., 2000). SIRP-1/CD172 is expressed on the same cell types in rats (OX41) and humans (Lunney et al., 1994, Adams et al., 1998; Veillette et al., 1998; Alvarez et al., 2000). At this study, DH59B detected a molecule that is intensely expressed on all equine monocytes and granulocytes (Fig. 17) and was therefore identical to the pattern expected for CD172a. Additionally, DH59B precipitated CD172a as a band of approximately 110 kDa (Fig. 46c). The fussy band may be due to the variable glycosylation as observed in rat CD172a (Sano et al., 1990; van den Nieuwenhof et al., 2001). Flow cytometry analysis and immunoprecipitation results are also in accordance with previous data obtained for DH59B reacting with a 96 kDa equine cell surface molecule (Tumas et al., 1994). The obtained data give no doubt that DH59B detects the equine homologue of CD172. However, my data are not sufficient to distinguish between CD172a and CD172b and the antibody itself has not been further characterised not to recognize CD172b (W. Davis, pers. comm.).

CD206, the macrophage mannose receptor (MMR) also termed mannosyl-fucosyl-receptor (MFR), has an approximate molecular weight of 162 kDa (Ramkumar et al., 2002) in humans and is absent on monocytes but up-regulated during differentiation to MΦ (Stahl and Gordon, 1982; Ezekowitz et al., 1990). Additionally, CD206 is expressed on immature dendritic cells (iDC) (Sallusto and Lanzavecchia, 1994). The anti-human CD206 mAb 3.29B1.10 did not stain horse monocytes (Fig. 29). To further analyze the mAb with equine cells, monocytes were differentiated towards dendritic cells by incubation with EqGM-CSF and EqIL-4 for 48 hours. 3.29B1.10 then stained almost all cells (Fig. 29) giving a staining pattern like that

described for human (Shepherd et al., 1982) supporting the notion that 3.29B1.10 detects the equine homologue of CD206. High molecular weight antigens are often more difficult to address and analyze by SDS-PAGE. Thus my attempt to immunoprecipitate CD206 using 3.29B1.10 may have failed according to these difficulties. In addition, low affinity of 3.29B1.10 might be another reason for the negative result.

Panleukocyte molecules:

Leukocyte integrins are a family of adhesion molecules sharing a common β-subunit (CD18) non-covalently associated with a distinct α-subunit with approximate molecular weights of 180 kDa (CD11a), 170 kDa (CD11b) or 150 kDa (CD11c), respectively (Kürzinger et al., 1981; Sanchez-Madrid et al., 1983; Hildreth and August, 1985; Springer et al., 1985). The mAb HUH73A, directed against CD11a, was reported to cross-react with human, bovine, goat, sheep, camel and lama cells, but nothing was known for horse cells. **CD11a** is expressed on all leukocytes, but not on platelets or on non-haematopoietic cells. This staining was in accordance with the obtained results (Fig. 5). In addition, HUH73A immunoprecipitated only equine CD11a as a single polypeptide of approximately 180 kDa from leukocytes, supporting the notion that HUH73A detected equine CD11a. Three mAbs (H20A, HUH82A, and MHM23) were directed against **CD18** and complement the tool-box here. For the mAbs HUH82A and H20A, cross-reactivity with a number of species has been indicated at submission, but only mAb H20A was reported to cross-react with horses (Kydd et al., 1994; Lunn et al., 1998, Zhang, et al., 1998). According to the hetero-dimerization with CD11a, CD18 is expressed on all leukocytes and H20A and HUH82A stained all leukocytes. It is likely that both mAbs have lower affinity to EqCD18 but there is no rational doubt that the mAbs detect CD18 on horses. The obtained results are in accordance with the previous reports for equine LFA-1 where its expression on lymphocyte was reported as a broad distribution while LFA-1 expression on granulocytes was quite homogenous (Zhang et al., 1998). The mAb MHM23 was also specific for human CD18 and was positive on equine leukocytes, staining slightly more intensely than the previously described ones and should alike react with equine CD18, although in some cases a sub-population of lymphocytes remained negative (Fig. 8). In contrast to mAbs directed against the LFA-1 α chain, all three mAbs precipitated two polypeptides of approximately 180 and 100 kDa (Fig. 47a) from equine leukocytes. The 180 kDa polypeptide indicated co-immunoprecipitation of the α-subunit (CD11a) while the 100 kDa polypeptide represented the β-subunit (CD18) of equine LFA-1. This profile was in

accordance with the previously reported data for equine LFA-1, where β-chain specific mAbs detected both α and β-subunits (Kydd et al., 1994; Lunn et al., 1998; Zhang, et al., 1998).

Although the mAb BAQ30A is directed against human CD18 alike, the situation was different. Using this mAb, lymphocytes were negative and both monocytes and granulocytes were positive when screened (Fig. 9). This staining pattern was observed previously for mAb CZ3.1 which failed to label lymphocytes of certain horses in two colour flow cytometry, but labelled granulocytes from all horses tested (Kydd et al., 1994; Zhang et al., 1998). Hogarth and McKenzie (1992) referred to this phenomenon in mouse to polymorphism of the LFA-1 molecule. Here, immunoprecipitation clearly added information. BAQ30A precipitated one protein of approximately 90 kDa (Fig. 47a), indicating a leukocyte antigen with a lower molecular weight which, at the same time, was not associated to LFA-1 α chain (CD11a). Drbal et al., (2001) described a proteolytically truncated, CD11a free form, of human CD18 as a marker of myeloid cells. This marker was detected on monocytes and neutrophils but not on lymphocytes, a pattern similar to that obtained with BAQ30A.

The clone M1/70.15.11.5 is directed against mouse and human **CD11b**. This mAb also stained equine monocytes and granulocytes and a small population of lymphocytes weakly (Fig. 20). Such staining was in accordance with human cells, where, next to myeloid cells, NK cells are also $CD11b^+$ (Werfel et al., 1991; Muto et al., 1993; Hogg, 2002a). Two-colour flow cytometry revealed that the $CD11b^+$ cells were indeed neither T nor B lymphocytes but co-stained weakly with CD8 (Fig. 40) a feature also known for human NK cells (Perussia et al., 1983; Werfel et al., 1991; Hogg, 2002a). Additionally, M1/70.15.11.5 precipitated two proteins from biotinylated equine leukocytes (Fig. 47b). The upper band, approximately 170 kDa, represented CD11b while the lower one, approximately 100 kDa, indicated co-precipitation of equine CD18. The obtained molecular weights were analogous to human Mac-1, CD11b/CD18 (Hogg, 2002a) leaving no doubt that M1/70.15.11.5 detected the equine homologue of CD11b.

CD44, a glycosylated transmembrane protein is widely expressed on the surface of most cell types including leukocytes (Stoll et al., 1989). CD44 mediates adhesion of leukocytes, is involved in T lymphocytes activation (Horejsi, 1991), leukocyte attachment to and rolling on endothelial cells, and homing (Miyake et al., 1990). Three mAbs (BAG40A, H22A, and LT41A) were directed against CD44. BAG40A and H22A were reported to detect equine CD44 (Tavernor et al., 1993; Kydd et al., 1994) and to cross-react with a number of species. For the mAbs BAG40A (Fig. 10) and H22A (Fig. 11) all monocytes and granulocytes and the

majority of lymphocytes were positive, but platelets remained negative which reflects the human CD44 expression pattern. For LT41A (WS No 25) the staining pattern was slightly different (Fig. 12). Staining intensity was weaker on all leukocytes. This weak expression pattern of CD44 by lymphocytes was described before for BAG40A and H22A where all T lymphocytes, but only about 35-70% of the B cells appeared to be positive suggesting that these mAbs label B lymphocytes with lower intensity (Kydd et al., 1994). BAG40A, H22A, and LT41A all precipitated a molecule of approximately 100 kDa (Fig. 48) which is in accordance with previous reports for equine CD44 (Kydd et al., 1994; Lunn et al., 1998). Thus I concluded that all mAbs detect EqCD44.

For **CD45** cassette exons occur, which are variably expressed on haematopoietic cells (Hermiston et al., 2003; Lynch, 2004). The three variable exons are also termed A (4), B (5), and C (6) and the existing isoforms in humans (in other species there may be more) may be distinguished by mAbs against CD45RA, the 220 kDa isoform (detecting all isoforms with exon 4), CD45RB, the 200 kDa isoform (isoforms with exon 5) and RO, the 180 kDa isoform (isoform without exons 4-6) (Janeway, 1992; Lynch, 2004). The mouse IgM mAb DH16A reacted positively with all monocytes and granulocytes (Fig. 13). In addition, DH16A stained some T but no B cells in two colour flow cytometry (Fig. 39). DH16A has been tested recently on human CD45RBC transfectants and is specific for CD45RB (W. Davis and V. Horejsi, pers. comm.). Considering all information available, there was no reason to assume that DH16A did not detect at least some CD45 isoforms in horses. There are, however, a few questions that need to be addressed. Therefore DH16A was classified as a restricted positive "(++)". At present DH16A was termed αCD45 – this is however not correct and should be changed into CD45RB. For horses, however, there is no detailed knowledge on the CD45 gene. Thus, it was not possible to say if DH16A detects all “B” isoforms. One of the main purposes of αCD45 antibodies in humans is to distinguish differentiation and activation states of lymphocytes, in that resting/naive T cells and activated/memory T cells express different variants of CD45. For this application DH16A cannot be recommended until further experiments in horses are performed.

The **CD49** gene family members (CD49a-f) are also known as the α1-α6 integrins that non-covalently associate with CD29 (β1-integrin) to form the very late antigen 1-6 (VLA 1-6). The expression of the CD49 genes differs significantly between the various leukocyte subpopulations (Tanaka, 2002). The mAb HP2/1 is specific for human CD49d and showed a staining pattern for equine cells, which was not quite in accordance but similar to the

descriptions at Leukocyte typing V-VII[1]. The majority of the lymphocytes and granulocytes, and all monocytes were positive (Fig. 14). Human eosinophils and basophils are known to express CD49d and this antigen is weakly expressed on rat neutrophils. The present finding of a significant amount of equine granulocytes, including most neutrophils is therefore not contradictory to a detection of CD49d (Fig. 14). The inability of HP2/1 to precipitate the equine homologue of CD49d could be referred to low affinity. However, HP2/1 was classified as restricted positive "(++)" until a final decision can be made after further analysis of the EqCD49 family (e.g. expression cloning).

Non-lineage restricted molecules:

CD34 is a surface glycophosphoprotein expressed on early haematopoietic stem and progenitor cells (Katz et al., 1985; Andrews et al., 1986) and has been accepted as one of the most important molecules on human stem cells (Civin et al., 1996; Burt, 1999; Handgretinger et al., 1999). Human CD34 is a heavily glycosylated type I transmembrane protein of approximately 116 kDa molecular weight (Krause et al., 1993; Krause et al., 1996) and the relative mobility of mouse CD34 varies slightly depending on the cell type in which it is expressed (Krause et al., 1994). Four mAbs against CD34 (AC136, 581, 4H11 and QBEnd10) were analyzed at this study and none of them stained adult horses leukocytes (data not shown). Analyses of anti-human CD34 mAbs with eCAS cell line revealed a staining pattern of 4H11 and QBEnd10 but particularly AC136 stained eCAS cells (Fig. 42). In addition, AC136 immunoprecipitated a molecule of approximately 100 kDa from eCAS (Fig. 49). There was only a very limited opportunity to verify the staining of AC136 using peripheral blood from a three day old foal as a potential source of stem cells such as described in humans (Geissler et al., 1986; de la Sella et al., 1996; Li et al., 1998a). Here also, a population of about 17% of the cells were stained using AC136 (Fig. 26), underlining the potential use of this mAb which was assigned as restricted positive "(++)" according to the unresolved problem of a well defined positive control.

The toll like receptor 3 (TLR3), recently classified **CD283** (Zola et al., 2005), detects dsRNA mainly intracellularily (Sen and Sarkar, 2005). TLR3 recognizes viral dsRNA and its synthetic mimetic polyinosinic-polycytidylic acid (poly I:C). TLR3 expression is commonly considered to be restricted to dendritic cells, NK cells, and fibroblasts. The mAb TLR3-7 was directed against TLR 3 and detected it in differentiated, activated equine monocyte-derived

[1] basic data on expression pattern was obtained from the CD Guides of Leukocyte typing V-VII if not stated otherwise.

dendritic cells when intracellular staining was performed (Fig. 30). In contrast to CD68, only a population of cells reacted with TLR3-7, assuming that this was due to regulated expression and suboptimal conditions in stimulation. The result remains conditionally positive only "(++)" although many TLRs (including TLR-3) are expressed by some cells only but the situation in horses has not investigated further to my knowledge.

EqMHC class I molecules are composed of a 44 kDa transmembrane heavy chain and a 14 kDa non-covalently linked light chain termed beta 2 microglobulin (β_2M) (Antczak, 1992; Kydd et al., 1994). The mAb B9.12.1 directed against the human MHC class I HLA-ABC locus reacted with all equine leukocytes (Fig. 31) and precipitated two bands of approximately 45 kDa (representing the MHCI heavy chain) and 12 kDa (representing β_2M) from surface biotinylated EqT8888 (Fig. 50) which was in accordance with the data reported for equine MHCI. Therefore B9.12.1 was classified as "++".

EqMHC class II molecules are composed of two non-covalently linked polypeptide chains: α (31-34 kDa), and β (26-28 kDa) (Kydd et al., 1994). Two mAbs directed against MHCII were designated at this study "++": the positive control mAb EqT2 and the mAb D-F1. While EqT2 was designated to react with pan-MHC II, mAb D-F1 was directed against the human HLA-DR only. Accordingly, it was not surprising that this mAb stained slightly weaker than EqT2. Notably, while all monocytes and no granulocytes were stained, a significant population of both resting and activated PBL were stained alike (Fig. 32 and 33). It has been described earlier that equine lymphocytes (all B and some T cells) are MHC class II positive (Crepaldi et al., 1986; Lunn, 1993). EqT2 (Fig. 50) also precipitated alpha (approximately 33 kDa) and beta (approximately 28 kDa) polypeptides of equine MHCII from surface biotinylated EqT8888. The phenomenon of cross-reactivity of anti-MHC mAbs is not new and many more mAbs cross-react alike (e.g. Crepaldi et al., 1986; Monos et al., 1989). Equine specific mAbs and cross-reacting anti-human mAbs open the gate to the analysis of MHC II subclasses and expression of MHC II by subpopulations of lymphocytes.

Two mAbs at this study displayed a **weak positive "W" reactivity**. Clone BL13 directed against human CD21 stained a small population (< 20% of PBL) of equine lymphocytes weakly (data not shown) and also demonstrated a positive reaction against several other species in HLDA8 workshop (Saalmüller et al., 2005). Existing mAbs specific for equine B cells, detect only 10-24% of PBL and in rare cases B cells comprise as little as 4% of the cells (Tumas et al., 1994). Comparable to B-Ly4, BL13 was designated as weak (W) in its reactivity and thus there was no reason to continue with this clone. In addition, BL13 was

reported before to recognize either different molecules or homologues expressed at different levels on horse B cells (Merant et al., 2003). The mAb 68-5A5 most likely stains the equine homologue of CD18 weakly. Again, there were already some anti-CD18 mAbs with better staining, so this mAb was not further analyzed.

A number of antibodies showed **positive, but compared to human different staining "+"** (n=23), clearly **alternate staining "A"** (n=15), or **questionable "?"** (n=6) staining that was not further considered (Tables 6 and 9). These mAbs were generally not investigated using immunoprecipitation or cell lines. With mAbs directed against CD3 (OKT3), CD4 (M-T466) and CD10 (4F9), for example, a clear staining was observed, but this was not interpreted as homologous or alternate. These mAbs were later also included into double labelling, but did not perform as expected in case of homologous staining. Three mAbs directed against CD9 showed a positive but variable staining pattern in the first screening but for the clone P1/33/2 (selected as the most promising one), repeated analysis at partner labs at CSU and AHT did not generate sufficient data to agree to a conclusion. MAbs 7F11 and big16 against human CD14 did not stain equine monocytes but instead a population of lymphocytes and anti-CD25 clone TB30 failed to detect any up regulation after lymphocytes activation. One antibody against CD18 (clone 7e4, Coulter) clearly reacted with a subpopulation of equine PBL, but not with any other cell type.

Of the two mAbs specific for human CD29 reacting with equine cells, 12G10 clearly showed an alternate expression pattern and only stained a subpopulation of lymphocytes (but no other cells). The second mAb, 3S3, showed an expression pattern similar to that described for humans during first staining, although not all equine PBL were positive. This mAb was further analyzed, but no consistent staining pattern was achieved and neutrophil data ranged from negative to positive. Although this might also be in accordance with the pattern of expression of CD29 in humans, where a weak to negative staining of neutrophils is described, 3S3 still requires further investigation and its specificity in horses remains questionable "?".

CD35 is the complement receptor 1 (CR 1) specific for C3b/C4b. In humans, this marker is expressed on myeloid cells (both neutrophils and monocytes) and some T and B cells. The mAb To5 stained the majority of equine granulocytes but no other leukocyte populations. The staining pattern was therefore designated alternate ("A" tabl 6) since the possibility that CD35 is differentially expressed in equids cannot be excluded. From the fact that complement receptors are widely used and important for immune-regulation, mainly at the innate level of immunity, it was however considered unlikely.

CD44 has been discussed already. The mAb DF1485 against human CD44 was positive after activation only – a pattern not observed with other mAbs. In theory, and according to alternate splicing, it could be possible that DF1485 in horses detected a variant of CD44 that is activation dependent. In humans, however, there is no indication that expression of the antigen recognised by DF1485 is dependent upon lymphocyte activation. Therefore, it was assumed that DF1485 detected an equine antigen other than CD44, but the matter remains unresolved at present. The rat mAb RA3-6B2 is designated to react against mouse CD45R (Miltenyi Biotec) and is described to be specific for the mouse B cell B220-antigen, the 220kDa large ABC-isoform of CD45 (Marvel and Mayer, 1988; Hathcock et al., 1992; Rodig et al., 2005). CD45 is a marker expressed in various isoforms and B220 is a particular enigmatic variant. On the one hand used as a valuable B cell marker for mice, it is also expressed on some mouse T cells (Marvel and Mayer, 1988). In humans, B220 is expressed by only a subset of B cells that do not express the memory B-cell marker CD27 (Rodig et al., 2005). It must be mentioned in this context that isoforms of CD45 may occur in parallel. For example, the CD45R/B220/ABC isoform is specific for mouse B cells, but those cells also express other isoforms, such as CD45RA or RC, which are not exclusive for mouse B cells. It was not possible to obtain much additional information on this mAb but it seems to be specific for the ABC form. The reaction with horse leukocytes was clearly positive, but in contrast to the mAb DH16A, RA3-6B2 recognised all equine leukocytes. Therefore, the mAb had to be designated "+" until further studies are performed to resolve the nature of equine CD45. The mAbs HUH69A and HUH71A were originally submitted as “pan-Leukocyte” markers and later designated CD47. CD47 is expressed on many cell types, including all leukocytes. Neither mAbs perfectly matched this criterion. They clearly stained all types of equine cells – but the intensity was weak and the percentage variable. All monocytes were stained, but only majority of lymphocytes and granulocytes. The available data were insufficient to conclude that this mAb identifies equine CD47 and accordingly was classified as "+".

The CD49 gene-family has been discussed above. Three further mAbs (JBS5, P4G9, and 9F10) showed specific staining, but none of them reacted analogous to human staining in further analysis (Table 1). The mAb P4G9 showed variable staining of leukocytes and compared with HP2/1 there were significantly fewer positive cells. This pattern of staining was not identical to CD49d but may be explained at least partially by a very low affinity of this mAb. The mAb 9F10, showed a staining comparable to HP2/1 and was submitted to a second round of analysis (data not shown). In further analysis, the data could not be

confirmed, as it failed to detect positive granulocytes. As mentioned, neutrophils of humans are not positive and rat (and equine) neutrophils stain only weakly. Therefore, 9F10 remained questionable "?" until further investigation is performed. The mAb C5.9 directed against CD56 sparked my interest when only a small population of lymphocytes (9-18%) stained positive in first screening. This would be in accordance with human CD56, which is a valuable marker of NK cells. However, during further analysis, C5.9 stained all equine granulocytes in one lab. As this issue could not be resolved subsequently, the specificity of this mAb remained questionable. Myeloperoxidase (MPO) is a heme-enzyme present in the granules of granulocytes and has been demonstrated to participate in the oxygen dependent microbicidal activity of these cells (Nauseef et al., 1983). When internal staining was applied, mAb 7.17 stained all neutrophils but also a subpopulation of lymphocytes. Therefore, the data were not in accordance with humans. An analogous problem occured with the mAb DH24A which detects CD90 in dogs (Cobbold and Metcalfe, 1994) but reacts clearly "+" with equine granulocytes only. DH24A may thus be a valuable marker for equine granulocytes but most likely does not stain the equine CD90, which would be expected on T cells and their precursors (Table 9).

The mAb B29A distributed from VMRD was generated using PBL from multiple species for immunization and according to its detection of IgM-pos cells was presumed to detect equine B- cells (Tumas et al., 1994). The staining, however, was weak and it remained unclear if B29A detects all equine B cells as indicated before (Kydd et al., 1994; Tumas et al., 1994). It can also be excluded that B29A detect CD5 as in cattle. Three mAbs (clones GBSP71A, MIMA-51, and NBL-1) were submitted to the workshop without any designation and reacted with some equine cells. The specificity of the mAbs remained unclear. A few mAbs also reacted with equine platelets (Table 6) but all of them were from the alternate/questionable group and none of them was a specific marker for human platelets. A number of mAbs (n=45) reacted weakly with a subpopulation of lymphocytes (≤8%), resulting in an alternate staining. These mAbs were of various isotypes. It is likely that the binding is “specific” – as two mAbs of the same clone (2H7, WS.No. 67 and 349) that were not tested in parallel produced a similar pattern. All mAbs with such staining were designated **weak/alternate "W/A"** and shall not be discussed further.

A number of mAbs (n=53) could not be tested on the most appropriate equine target cells (e.g. progenitor stem cells) during screening, as these were unavailable (Table 7) and this group of mAbs was designated as **"N/A"**. Eleven mAbs were directed against CD1 proteins. CD1,

comprises a gene-family (CD1a-e) of which at least three members are expressed in all species (CD1a-c). Equine thymocytes were not available to test CD1 mAbs. The mAbs of this part of the study were analyzed on MoDC after the screening and all except one, B1.20.9 (NA/W), failed to show reactivity (NA/-). The expression pattern of EqCD1 mRNA and staining on EqMoDC was not analyzed in parallel and the expression of CD1 on DC is variable and not all DC express CD1.

Three mAbs were directed against CD33 (Tables 7 and 9), which is a marker of myeloid progenitors but also of some monocytes in humans. Equine monocytes were negative when these mAbs were tested, while myeloid progenitors were not available.

A number of mAbs (n=7) were directed against human CD80 or CD86 also known as B7 molecules (Table 7). Both molecules are expressed on subsets of human PBMC (esp. some B cells and monocytes) and especially DC, but were negative on equine leukocytes, activated PBMC, and MoDC. A positive control mAb was directed against CD86 (clone IT2.2; Hammond et al., 1999; Lin et al., 2002). A mAb directed against CD209 (DC-Sign) was retested on MoDC but did not react either. Two mAbs were directed against CD235a or CD236R, which are expressed on erythrocytes. These mAbs were not analyzed on erythrocytes and were therefore assigned to the (N/A) group. The remaining mAbs (n=356) did not show any staining **"-"** during screening and were not considered further.

In summary, 534 mAbs (including four isotype controls) were analyzed for their cross-reactivity against equine leukocytes. Although just a few antibodies were positive on equine cells (31/534; approx. 5.8%), the approach may be considered useful, especially as their specificity has been defined in this study. Of the positive mAbs, 15 mAbs defining 12 CD molecules were newly added to the equine immune tool-box. These mAbs were directed against CD11b, CD14, CD18, CD34, CD45RB, CD49d, CD68, CD83, CD91, CD163, CD172a, CD206 and CD283.

5.2 Flow cytometric analysis of human mAbs using eCAS and EqT8888 equine cell lines

The equine leukocyte cell lines, eCAS and EqT8888, were used to analyze mAbs in a cross-check step using flow cytometry. **eCAS** was supposed to be a myeloid cell line derived from bone marrow of a 10-year-old horse (Werners et al., 2004). eCAS cells have been attributed macrophage characteristics but it is well known that cell lines can change their characteristics during the first 20-50 passages. For example, the most differentiated human macrophage cell line available, MonoMac 6, retains many characteristics of progenitor cells and is not positive

for many myeloid markers (Ziegler-Heitbrock et al., 1988). A panel of forty mAbs (Table 11) mostly defined positive with horse leukocytes, was analyzed here for reactivity with eCAS cells. This panel included mAbs specific to myeloid markers like CD14, CD68, CD163, CD172a, and CD206 (MMR). Six mAbs directed against CD11b, CD34, CD45R, MHC class I, and canine B cell marker showed a staining pattern that was considered specific and designated positive (Table 11 and Fig. 42). Three further mAbs specific for human CD21 (B-Ly4) and CD34 (581 and QBEnd10) reacted weakly with the eCAS cell line and were designated weak/questionable (Table 11). It should be mentioned that eCAS (unlike PBMC) showed non-specific binding when mouse IgG2a mAbs were applied. For this reason, results of five mAbs which were also analyzed by AHT remained questionable. Besides, the staining of eCAS cells using a mAb specific for canine B cells was surprising, although the nature of the canine antigen detected by CA2.1D6 is unknown (Fig. 42).

The staining of CD34 was also a surprise, since CD34 is a stem cell marker and eCAS cells were attributed macrophage characteristics (Werners et al., 2004). More surprisingly, no myeloid-specific markers, including CD14, and MHCII were detected positive on eCAS cells although eCAS have been reported to be of myeloid origin and to express CD14 as detected by an anti-peptide antiserum (Werners et al., 2004). However the expected molecular weight of CD14 is 50-55 kDa (Goyert, 2002a), and western blot analyses in the previous publication resulted in the detection of a protein with a molecular weight of about 160 kDa. This discrepancy between the original description and my findings is hardly to be explained. I just assume here that the cell line had changed its characteristics since the earlier experiments (e.g. by reverse differentiation). The results should not be interpreted that eCAS cells are of B cell origin, but they also did not display macrophage characteristics and seem to resemble early progenitor cells.

EqT8888 is a lymphoid cell line derived from a lymph node of a 2-year-old Arabian horse with an anaplastic undifferentiated lymphosarcoma (Hormanski et al., 1992). Twenty seven mAbs were analyzed for reactivity with EqT8888 cells (Table 12). This panel included mAbs directed against lymphoid cell markers like CD2, CD4, CD5, CD8 and CD21. Eleven mAbs showed a staining pattern that was considered specific and designated truly positive. Positive mAbs were directed against CD21, the canine B cell marker, CD44, CD45RB, CD83, MHC class I, and class II (Table 12 and Fig. 43). Staining of EqT8888 cells with these mAbs confirmed that EqT8888 cells are negative for the equine T cell markers CD2 and CD5, but positive for both of equine MHC class I and class II. CD44 was also expressed, which is not

surprising as variants of CD44 have important functions in metastizing tumours, such as the lymphoma from which the cell line derived (Drillenburg and Pals, 2000). EqT8888 cells were also labelled by mAbs which identified CD21 and CD83 (Fig. 42). Both markers were clearly expressed, although weakly and B-Ly4 (CD21) in most cases stained a population of the cells only, however suggesting a B cell origin of this cell line. In Hodgkin's disease for example, both CD21 and MHC class II molecules have been described on so called Reed-Sternberg cells, which are, however, deficient in antigen-presentation (Bosshart and Jarrett, 1998; Nakamura, et al. 1999).

In conclusion, a broad panel of mAbs predominantly specific for human leukocyte surface antigens but cross-reactive with equine PBMC were applied on equine cell lines (EqT8888 and eCAS). A limited number of these mAbs reacted positively with the equine lines. Based on these data eCAS may represent a very early stage of a myeloid cell line and EqT8888 may be a kind of B cell lymphoma. However, definitive classification requires further analysis.

5.3 Preliminary analysis of cross-reactivity of mAbs (defined positive with horse leukocytes) with wild equids leukocytes using single colour flow cytometry

Domestic and wild equids belong to the family *Equida*e, Genus *Equus* where six subgenera exist (Groves, 2002): *Equus*, which contains horses; *Asinus*, which contains the true asses and is represented by the African wild ass; *Hemionus*, which contains Asian wild asses; onager and kiangs; *Hippotigris*, which contains the mountain zebras; *Quagga*, which contains the Plains zebras; and *Dolichohippus*, which contains the Grevy's zebra. Attempting to establish a leukocyte immune tool-box for the Somali wild ass (*Equus africanus somalicus*), Grevy's zebra (*Equus grevyi*), and Hartmann's mountain zebra (*Equus zebra hartmannae*), the principle of analyzing mAbs cross-reactivity was applied here using most mAbs defined positive with horse leukocytes. Due to the limitation in the number of blood samples ($n \geq 1$) to be analyzed, this attempt has to be considered a preliminary analysis. 22 mAbs spanning 16 different CD antigens (Table 14) were analyzed with the three species. The majority (13/20), but not all mAbs cross-reacted with the three species. Eight mAbs clearly cross-reacted with Somali wild ass leukocytes only. One mAb against huCD21 (B-Ly4) clearly cross-reacted with both zebra species but weakly stained Somali wild ass leukocytes. Anti-huCD14 mAbs were tested against zebra species only and were positive except clone big11 using Hartmann's zebra monocytes.

Cross-reactivity of the majority of mAbs with all three wild equids was not surprising since they belong to the same genus, *Equus*, and evolutionary conservation of the detected epitopes

was expected. However, while 20 out of 22 mAbs cross reacted with Somali wild ass leukocytes, only 13 out of 22 mAbs cross-reacted with leukocytes of zebras. The higher rate of mAbs cross-reacting with Somali wild ass leukocytes indicates a higher degree of conservation of the antigenic determinantes. Exemplarily, anti-human CD2 mAb 39C1.5 cross-reacted with lymphocytes of the three species (Fig. 51), while the reference mAb HB88A against equine CD2 cross-reacted only with Somali wild ass leukocytes (Fig. 52) indicating non-conservation of the latter epitope in both zebras.

Interestingly, a gradual decline in staining intensity from "++" in Somali wild ass via "W" in Grevy's zebra to "-" in Hartmann's zebra was observed in five cases (anti-CD18 clones BAQ30A and HUH82A; anti-CD44 clones BAG40A, H22A, and LT41A) (Fig. 60, 62, 66 and table 14). This gradual difference could reflect different affinities, possibly linked to the pattern of evolutionary conservation and mutations in genus *Equus*. Genetic approaches were used before (George and Ryder, 1986; Oakenfull and Clegg, 1998; Oakenfull et al., 2000) and discussed controversially the relation of zebras/asses and horses. My data of both genetic analysis of CD28 and mAb cross-reactivity between equids show that Somali wild ass seem immunologically more closely related to horses than zebras.

In summary, analysis of mAbs for cross-reactivity with wild equids species reflected antigenic variation between these species with some but little diversity observed between them which is in accordance with the evolutionary conservation in the genus *Equus*. Cross-reactive mAbs analyzed at this part of the study may be considered the basis to establish an immune tool-box for these wild equids but require re-analysis to confirm consistency of the obtained results.

5.4 Cloning and sequence analysis of CD28 from horses and zoo animal species

According to the paradigm of costimulation, (Lafferty and Cunningham, 1975), a T cell needs two signals to become fully activated. The "second signal" is provided by the engagement of a so called co-stimulatory receptor. The first to be discovered and still the most prominent of these co-stimulatory receptors is CD28. In horses, a unique T cell surface molecule was suggested to be the orthologue of human CD28. Although this had similar biochemical characteristics to CD28, the number of equine PBL expressing this molecule differed considerably from human lymphocytes and lacking *in vitro* expressed equine CD28 made it difficult to designate this antigen (Kydd et al., 1994; Byrne et al., 1997; Lunn et al., 1998). Unfortunately, none of the anti-human CD28 mAbs analyzed at this study was defined positive according to the human criteria. The importance of CD28 and this inability to detect a

mAb increased the interest to analyze the mRNA of this protein in horses and different zoo animal species.

The predicted CD28 precursor protein, with typical features of an integral membrane protein, was shown to be encoded by ~220 aa residues in the different animal species. The observed slight differences in the number of the coding amino acid residues were due to insertions or deletions of nucleotide triplets in the CD28 cDNA at various places. The mature protein (Fig. 75) was supposed to comprise of an extra-cellular domain with homology to an IgV-like domain (Aruffo and Seed, 1987), a hydrophobic membrane-spanning domain, and a cytoplasmic tail. Comparison of CD28 aa sequences revealed a good degree of interspecies conservation with aa identity ranging from 68-99% and significant stretches of identical amino acid composition. Higher degrees of conservation were observed between members of *Artiodactyla* and *Perisodactyla*, clustering members of each order in a mono-phylitic clade (Fig. 76). Comparison of the CD28 extra-cellular domains, revealed 18 aa mismatches between equids and humans (Fig. 75) which could be the reason for the inability of six human CD28 specific mAbs to stain horse leukocytes. The hexapeptide "MYPPPY" motif in the extra-cellular domain is critical for CD28 ligand binding (Peach et al., 1994; Wang et al., 2002; Lühder et al., 2003) and was conserved in the analyzed species except for members of the order *Artiodactyla* where the first methionine was replaced by leucine. The first methionine in cattle was not essential for binding CD86 (Parsons et al., 1996), whereas substitution of the last proline or tyrosine with alanine abolished binding to CD86 in humans (Peach et al., 1994), demonstrating a critical role for this motif for CD28 binding to CD86. Conservation of the "MYPPPY" motif and the adjacent aa residue in humans, horses and other wild life animals rather suggests the existence of some cross-reactive Ab.

While mAbs that provide co-stimulation to T cells were designated "classical" mAbs, those capable of inducing full activation of primary resting T cells in the absence of TCR ligation (signal 1) were designated "superagonistic mAbs" where the aa residues 78-82 at the C"D motif form the superagonistic epitope (Tacke et al., 1997; Lühder et al., 2003; Margulies, 2003). The C"D motif was conserved in each of the analyzed orders but not between most of them (Fig. 75) which suggests not only an evolutionary selection process but would also explain the lack of cross-reactivity in this area.

The cytoplasmic domain (Fig. 75, blue arrow) which is both necessary and sufficient for association with phosphatidylinositol 3'-kinase (Stein et al., 1994), was highly conserved in all species. The "YMNM" motif was 100% conserved and is a potential binding site for interaction with signaling proteins such as the *src*-homology 2 (SH2) domains of the p85

subunit of the phosphoinositide3-hydroxy kinase (PI 3-kinase) (Songyang et al., 1993; June et al., 1994). Conservation of the "YMNM" motif was reported before for domestic cattles, chickens, and humans (Parsons et al., 1996).

5.5 Anti-human polyclonal antibodies used as another tool to analyze cross-reactivity

Due to their specificity, monoclonal antibodies are excellent primary antibodies in various assays. However, when not available, polyclonal antibodies can provide a substitute. Inability of some human mAbs to cross-react with equine leukocytes could be due to tiny mismatches in the target epitope (i.e. sequential dependent epitopes) or due to changes in the three-dimentional conformation. Such differences were assumed when equine CD28 cDNA sequence was analyzed. Accordingly an alternative approach using Abs of polyclonal specificity was included here. CD28 and CD25 were chosen due to their importance in the biology of T cell activation. Affinity purified anti-human polyclonal Abs raised against the recombinant human extra-cellular part of each CD molecule, were used to minimize the risk of false negative signals.

The anti-human **CD28** polyclonal antibody (Table 1) stained a small population of equine resting PBL (Fig. 77) in one colour flow cytometry. This staining pattern itself was in accordance with the data reported for EqWC4 (the proposed orthologue of equine CD28) (Byrne et al., 1997) but again not in accordance with human data.

EqWC4 was originally described by Byrne et al. (1997) expressed extra-cellularly as one molecule of approximately 46 kDa, detected in immunoprecipitation from equine PBMC. At this study, *in vitro* expression of equine CD28 was performed in insect cells (3.2.16) and indicated its existence as two proteins of approximately 57 and 46 kDa, using the reference anti-V5 mAb directed against the C-terminal tagged V5 epitope of the expressed recombinant protein (Fig. 78). This result was in accordance with the proposed *in vivo* expression of EqWC4, detected as two proteins of approximately 46 and 52 kDa when analyzed under reducing conditions from cell lystaes (Byrne et al., 1997). The anti-human CD28 polyclonal antibody alike detected these two bands in immunoprecipitation from PBMC and WB from insect cells, indicating cross-reactivity with the equine homologue of CD28. Immunoaffinity purification and WB of EqWC4 from equine thymocyte lysates, under non-reducing conditions, had previously revealed additional bands of approximately 52 and 92 kDa. While the 52 kDa protein was explained as an intracellular precursor to the 46 kDa molecule (since

it was not immunoprecipitated from surface labelled PBMC) the 92 kDa band was explained as an intracellular dimer (Byrne et al., 1997).

Human CD28 is encoded by a single copy gene but transcribed as a series of at least 4 distinct mRNA species of 1.3-, 1.5-, 3.5-, and 3.7-kb. Utilization of an alternate non-consensus polyadenylation signal leads to generation of the longer transcript of 3.7 kb and 1.7 kb respectively. The internal splicing of 252 bp from exon 2 (encoding for an extra-cellular domain) leads to the size differences within splice variant pairs (i.e 1.3/1.5 versus 3.5/3.7). The deletion occurs in the extracellular domain and does not affect surface expression of the splice variant (Lee et al., 1990; Hanawa et al., 2002). Indeed, I have seen additional bands during the RT-PCR amplification of CD28 but since the band at the expected size of CD28 ORF provided the full-length sequence did not attempt to sequence other amplicons. The description of the detected proteins of 57 and 46 kDa in horses and insect cells however can not be explained with CD28 splice variants, since the huCD28 splice variant protein has an approximate MW of 22 kDa (Hanawa et al., 2002).

Thus, the most likely explanation seems to be that the 92 kDa EqCD28 represented the homodimer CD28 form *in vivo* as described before (Byrne et al., 1997). The two other forms detected require further analysis. A co-precipitation of a non-related protein can be excluded since two bands also appear during the analysis of transfectants. Although slightly speculative, another explanation could be that the approx. 57 kDa variant represent a precursor protein as discussed before (Byrne et al., 1997) or a differentially glycosylated form occurring mainly intracellularly but also expressed on the surface of T cells since, where it was detected here by immunoprecipitation (Fig. 77). Such an intracellular variant is known for huCTLA-4 which also binds CD80/86 - but as a negative regulator of T-cell activation (Leung et al., 1995).

In conclusion, the anti-human CD28 polyclonal Ab likely detected a protein to be EqCD28 on a small population of lymphocytes in flow cytometry and precipitated two proteins of 57 and 46 kDa from horse lymphocytes. Cloning and expression of EqCD28 in insect cells supports this notion. Additional analyses of possible equine CD28 variants and analysis of the surface expressed protein (i.e. by protein sequencing), could produce further information about the nature of equine CD28.

Stimulation of T cells corresponds to high expression levels of the IL-2Rα, **CD25**, a 55 kDa glycoprotein also known as the low-affinity interleukin-2 receptor alpha chain. Its expression on activated T and B cells is dramatically up-regulated, but human CD25 is also expressed by

a 5-10% subpopulation of non-activated $CD4^+$ T cells, some of which act as regulatory T cells (Waldmann, 1986; Waldmann, 1991; Jonuleit et al., 2001; Dieckmann et al., 2001; Sugamura, 2002).

Flow cytometric analysis of equine CD25 expression levels at different time intervals after PHA activation, using the goat anti-human IL-2Rα polyclonal antibody (Table 1), revealed a clear staining and upregulation of equine CD25 (Fig. 79). This labelling and the upregulation upon activation lead to the notion that this Ab most likely stains equine CD25. Staining of a small population of lymphocytes at zero hour (Fig. 79a) was not regarded contradictory to the previous data.

To further characterize equine CD25, immunoprecipitaion was performed 48 hours post-activation to use the highest expression level of CD25. The antibody immunoprecipitated a molecule of approximately 55 kDa molecular weight (Fig. 80) which is analogous to human CD25 (Sharon et al., 1986; Sugamura, 2002) and confirms the detection of equine CD25 by this polyclonal Ab.

The obtained results using highly purified human polyclonals support this approach in the analysis of equine leukocytes when their cross-reactivity can be confirmed. Background signals may be eliminated or neglected according to their intensity.

While the immune systems of humans, herbivores (horse), ruminants (cattle, sheep, goat), carnivores (cat, dog) and omnivores (pig) are broadly similar, this study underlines that differences of immmunological importance exist between the species at the molecular level. However, nucleotide and amino acid sequences are more similar between animal species than by comparison with humans (Steinbach et al., 2002; Steinbach et al., 2005; results 4.3). Therefore, it might be useful in the future to test other anti-species mAbs designated against swine, ruminants or other species on equine leukocytes.