# IV. RESULTS

Approaches performed during this thesis according to the aims and objectives of the study:

*(4.1) Cross-reactivity analysis of a large panel of human mAbs with horse leukocytes:*

*Equine leukocytes:*
- PBMC
- Act. PBMC
- Mo-DC
- Mo-MΦ

Platelets
Equine cell lines

- One colour flow cytometry analysis (screening)
- Two colour flow cytometry analysis (confirmation)
- Analysis with equine cell lines, eCAS & EqT8888 (cross-check)
- Immunoprecipitation of the detected CD molecules (MW)

*(4.2) Cross-reactivity analysis of mAbs (positive with horse only) against wild equid leukocytes:*

*Leukocytes of:*
- Somali wild ass (*Equus africanus somalicus*)
- Grevy's zebra (*Equus grevyi*)
- Hartmann's mountain zebra (*Equus zebra hartmannae*)

One colour flow cytometry

*(4.3) Cloning and sequence analysis of CD28 from horse and zoo animal species:*

Horse (*Equus caballus*)
Somali wild ass (*Equus africanus somalicus*)
Grevy's zebra (*Equus grevyi*)
Hartmann's mountain zebra (*Equus zebra hartmannae*)
Greater one-horned rhinoceros (*Rhinoceros unicornis*)
Asian elephant (*Elephas maximus*)
European bison *(Bison bonasus*)
African buffalo (*Syncerus caffer*)
Nubian giraffe (*Giraffa camelopardalis*)

-RT-PCR of CD28
-Cloning and sequence analysis

*(4.4) Anti-human polyclonal antibodies as another tool to analyze cross-reactivity:*

-Anti-human CD28 polyclonal Ab
- equine PBMC → Flow cytometry; Immunoprecipitation
- EqCD28 expression → Western blot

-Anti-human CD25 polyclonal Ab → equine PBMC → Flow cytometry; Immunoprecipitation

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

A panel of 534 monoclonal antibodies (mAbs) against various CD and MHC molecules consisting of 379 mAbs (Table 16) submitted to the HLDA8 animal homologues section (including 4 isotype controls) plus further (non-HLDA8) 155 mAbs (Table 17) were analyzed for reactivity with horse (*Equus caballus*) cells. A few of the submitted mAbs were previously reported to react and their characterization was extended here. The analysis was based on screening the whole mAbs panel by single colour flow cytometry, comparing the staining pattern of horse leukocytes with previously reported staining pattern for human leukocytes. MAbs defined positive in this screening were further analyzed. In particular, mAbs that detected only lymphocyte populations by two colour flow cytometry. The equine leukocyte cell lines, eCAS and EqT8888 were used to obtain further information using one colour flow cytometry again. Finally, immunoprecipitation (IP) was used to add information about the molecular nature of the detected CD molecules.

Each mAb was tested with leukocytes from different blood samples (n≥3). According to their flow cytometry staining pattern, mAbs were categorised in different classes. Only mAbs that stained consistently comparable to human leukocytes were finally assigned "++" in this study. In case of a slight doubt remaining it was termed "(++)". In cases of a questionable staining pattern, i.e. that was variable between different animals, data were termed "?". MAbs with flow cytometric staining, not consistent with human staining pattern, were termed "+". These results might still be due to homologous staining. MAbs that stained weakly were designated "W", whereas mAbs with clear alternate expression pattern from that expected from humans were termed alternate "A". This pattern was less likely to reflect homologous staining. Additionally, in a number of cases, mAbs (regardless of the isotype) that reacted weakly with a minor subpopulation of lymphocytes were termed as weak and alternate "W/A". MAbs that were not tested on the appropriate target cells were termed "NA". Finally, mAbs not showing any staining of equine leukocytes were termed negative "–". Percentages of reactive mAbs are summarised in figure 1.

Analysis of cross-reactivity of human mAbs revealed a clear positive reactivity "++" for 31 mAbs. 15 mAbs defining 12 CD molecules were newly added to the equine immune tool-box filling some substantial gaps in the list of available reagents.

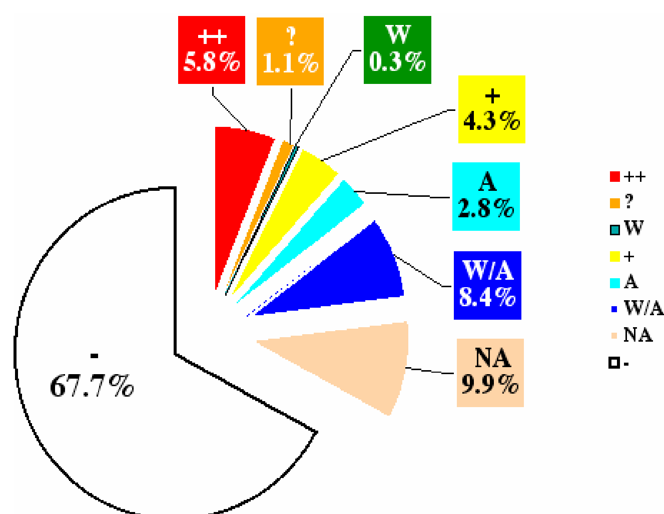


**Fig. 1 Percentages of reactive and non reactive human mAbs.**
Analysis of human mAbs for cross-reactivity with equine leukocytes indicated 31 mAbs (~5.8%) as clear positive reactivity "++", 2 mAbs (~0.3%) with weak positive reactivity "W", 23 mAbs (~4.3%) with positive staining that not consistent with human but possibly still homologous "+", 15 mAbs (~2.8%) with alternate expression pattern "A" from that expected from human immunology and less likely 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%) and in 53 cases (~9.9%), more appropriate target cells, such as thymocytes or bone marrow stem cells, were not available for screening "NA".

### 4.1.1 Single colour flow cytometry:

For analysis of CD markers known to be expressed in resting human cells, equine leukocytes were isolated from citrated fresh blood samples collected from healthy horses of different breed and sex. Leukocytes were isolated by gradient centrifugation through Biocoll solution (ρ 1,090). For analysis of CD markers known to be expressed in activated PBMC only, PBMC were isolated from leukocytes by gradient centrifugation through Biocoll solution (ρ 1,077). Activation of leukocytes, T cells, and B cells was performed as described (3.2.1). For analysis of mAbs detecting markers expressed on dendritic cells (DC) or macrophages (MΦ), monocytes were isolated via adherence and differentiated toward DC and MΦ as described (3.2.1).

For flow cytometric analysis of equine peripheral blood leukocytes, major populations were identified using linear FSC and SSC. For lymphocytes (L), monocytes (M) and neutrophilic granulocytes (G), populations were identified. Additional gates for basophilic granulocytes (B) and eosinophilic granulocytes (E) were drawn, using the settings for human cells in order to include any relevant subpopulation (Fig. 2a). Accordingly, markers of special interest for basophils detection (e.g. CD203) were analyzed at gates G + B and markers of relevance for eosinophils at gates G + E. For acquisition and first analysis, wide area gates were drawn and in case of evidence for a positive reaction, narrow gates were used and if necessary back-

gating applied. For the analysis of activated lymphocytes, special emphasis was drawn to a region along the linear axis of PBL, where bigger cells about to divide should appear (Fig. 2a). For the acquisition and analysis of platelets, log-scale scattergrammes were used, where platelets were the predominant population (Fig. 2b).

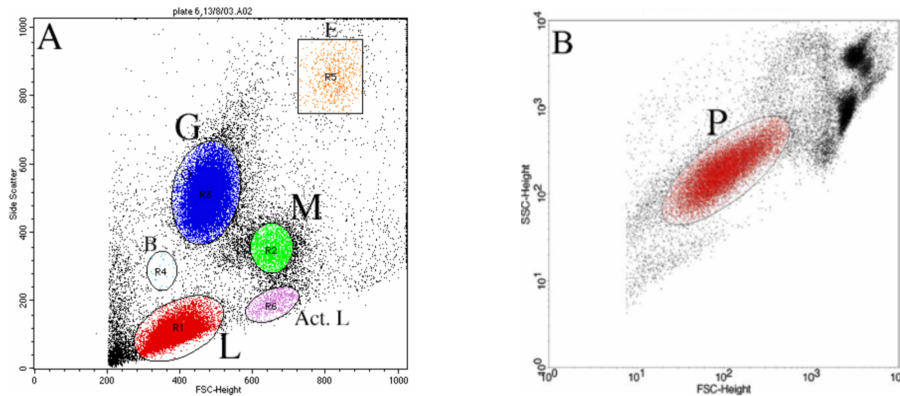


**Fig. 2 Acquisition of equine leukocytes and platelets.**
Leukocyte populations were discriminated using forward (FSC) and side (SSC) scatter (Fig. 2A). By this, lymphocytes (L), monocytes (M), and granulocytes (G) may be determined. In order not to neglect cells additional gates potentially containing basophilic (B) and eosinophilic (E) granulocytes were drawn. Activated lymphocyte population (Act. L) is represented as a region along the linear axis of PBL, where bigger cells about to divide appear. For the detection of platelets (P), log-scale detectors of FSC and SSC were used (Fig. 2B). Displayed are typical examples of the narrow gate setting for analysis. For acquisition, wider gates were used.

Negative cell controls stained with isotypes of muIgG or ratIgG were used for the adjustment of the fluorescence detector's voltage to set a background signal of PBL at $10^0$-$10^1$ (Fig. 3a). Fluorescence compensation is a process in which the green and red fluorescence signals of FITC and PE respectively are adjusted. Positive control mAbs directed against equine CD4 (CVS4) and CD8 (CVS8) were used for the adjustment of spectral overlaps between PE and FITC. Positively stained populations with PE labelled antibody were adjusted to be in the upper left (UL) quadrant (Fig. 3b), while populations stained positively with FITC labelled antibody were adjusted to be at the lower right (LR) quadrant (Fig. 3c).

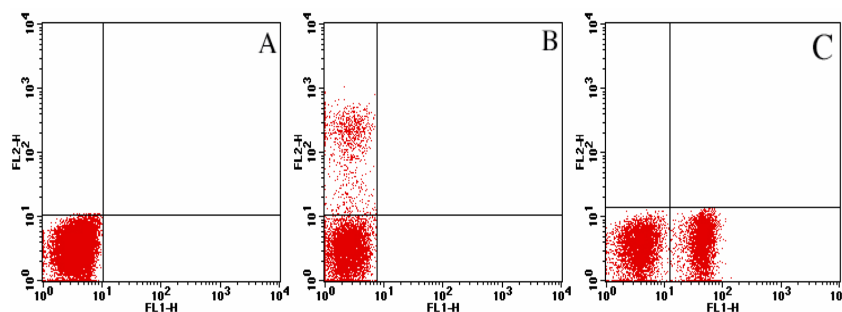


**Fig. 3 Setting of background signal and compensation for FITC and PE:**
Background fluorescence of PBL populations stained with isotype muIgG or ratIgG was adjusted at the lower left quadrant at $10^0$-$10^1$ (a). A PBL population that was positively stained with PE labelled anti-equine CD8 mAb CVS8 was adjusted at the upper left quadrant (b) while populations stained with FITC labelled anti-equine CD4 mAb CVS4 was adjusted to the lower right quadrant (c).

### 4.1.1.1 Dot plots analysis of HLDA8 mAbs:

CD2 or lymphocyte function antigen-2 (LFA-2), is expressed on peripheral T cells and natural killer (NK) cells. The rat IgG2a mAb 39C1.5 (WS no 178) submitted from Beckman-Coulter and directed against human CD2 did stain the vast majority of equine lymphocytes brightly, while possibly a few monocytes were stained weakly and no staining of granulocytes was obtained (Fig. 4) such as reported in humans (Crawford et al., 1999), thereby matching the first criterion for a T cell specific anti-CD2 mAb.

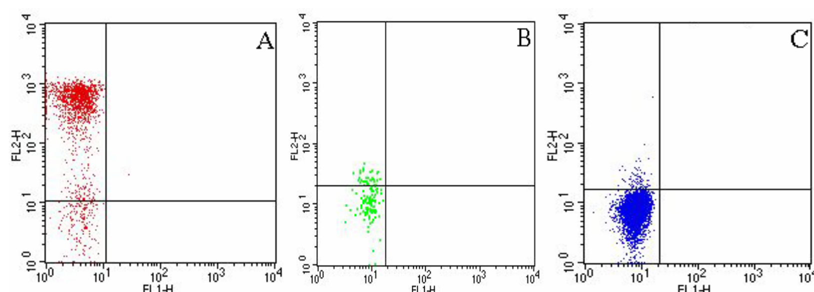


**Fig. 4 Analysis of equine leukocytes using anti-human CD2 clone 39C1.5**.
39C1.5 detects the vast majority of lymphocytes (A), a few monocytes (B) were stained weakly but no staining of granulocytes was detected (C). This mAb is a rat IgG2a isotype and was analyzed by indirect staining using a PE-labelled secondary antibody (FL2).

The mAb HUH73A (WS no 17), submitted from W. Davis, Washington State Univ. (WSU) and directed against CD11a, was reported to cross-react with human, bovine, goat, sheep, camel and lama cells. Nothing was reported for horse cells. Human CD11a is expressed together with CD18 (Integrin $\beta_2$) as a hetero-dimer (also termed leukocyte function antigen, LFA-1) and the main ligands are ICAM-1 (CD54), -2 (CD102), and –3 (CD50). CD11a is expressed in humans on all leukocytes, but not platelets and not on other cells. This staining was in accordance with the obtained results (Fig. 5) and it is most likely that this antibody detected CD11a in horses.

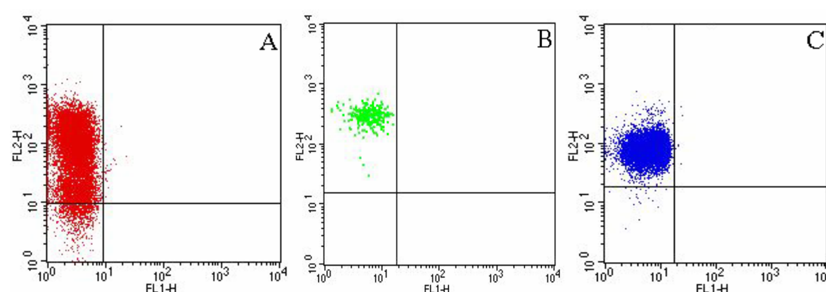


**Fig. 5 Analysis of equine leukocytes using anti-CD11a mAb HUH73A.**
All leukocytes (A, lymphocytes; B, monocytes; C, granulocytes) of all horses tested were stained by this mAb. HUH73A was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

Two mAbs (H20A [WS no 7], and HUH82A [WS no 22]) submitted from W. Davis, WSU, and one mAb from DakoCytomation (MHM23 [WS No 248]) were directed against CD18.

For the mAbs from WSU cross-reactivity with a number of species has been indicated at submission, but only mAb H20A was reported to cross-react with horses. According to the hetero-dimerization with CD11a, CD18 is expressed on all leukocytes and H20A and HUH82A (Fig. 6 and 7, respectively) stained all leukocytes although the expression on lymphocytes was comparatively lower. HUH82A stained cells weaker than H20A. It is likely that both mAbs have a lower affinity for EqCD18. 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).

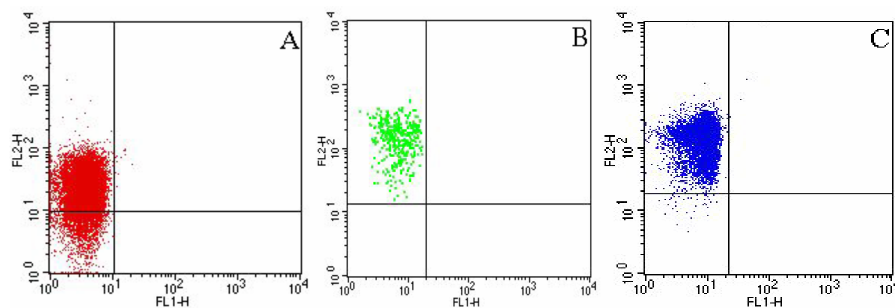


**Fig. 6 Analysis of equine leukocytes using anti-CD18 mAb H20A**.
H20A stained almost all leukocytes of all horses tested (lymphocytes, A), (monocytes, B), and (granulocytes, C). A small population of lymphocytes remained negative (although a clear shift in population occurred). H20A was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

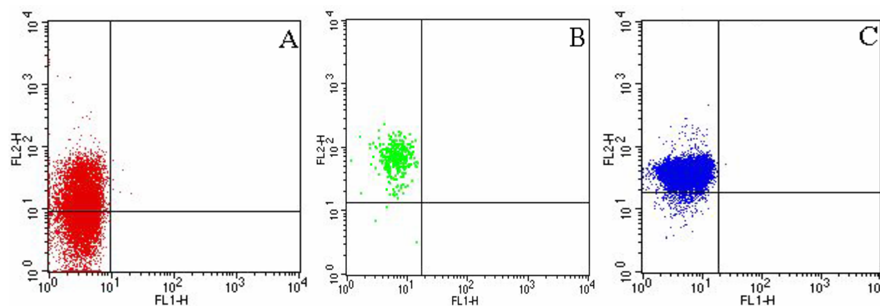


**Fig. 7 Analysis of equine leukocytes using anti-CD18 mAb HUH82A**.
All monocytes (B) and granulocytes (C) were stained with HUH82A. More than one third of lymphocytes (A) were positively stained while the rest remained negative. HUH82A was analyzed by indirect staining using a PE-labelled secondary antibody (FL2).

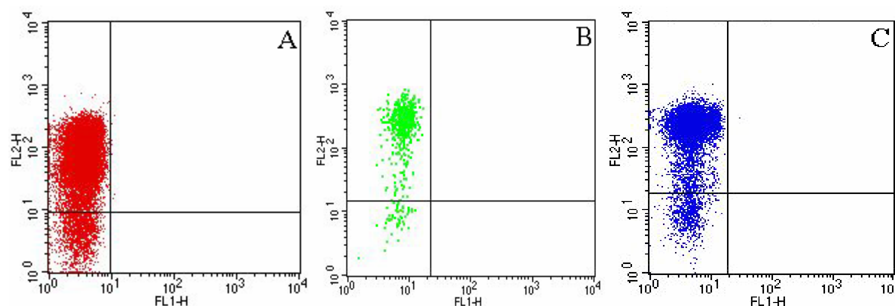


**Fig. 8 Analysis of equine leukocytes using anti-human CD18 mAb MHM23**
MHM23 stained brightly most leukocytes: (lymphocytes, A), (monocytes, B), and (granulocytes, C). However a population of cells always remained negative. MHM23A was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

For the anti-human CD18 mAb BAQ30A (WS no 1), the situation was different. This mAb also reacts with CD18 of various species. However, using this antibody on horses, lymphocytes were negative and only monocytes and granulocytes were positive (Fig. 9). Flow cytometry thus indicated that BAQ30A cross-reacts with the proteolytically truncated form of free (CD11 unassociated) CD18, described before in humans as an activation marker of myeloid cells (Drbal et al., 2001).

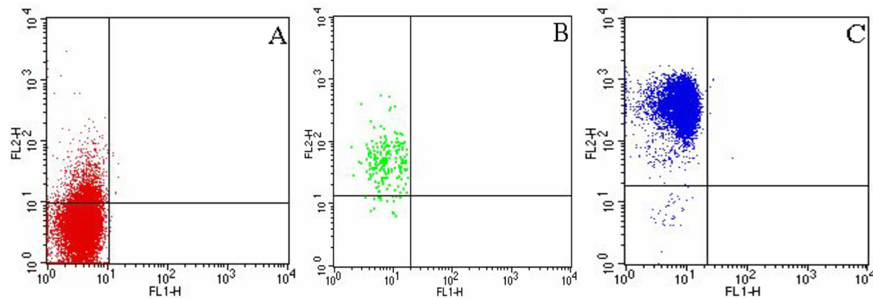


**Fig. 9 Analysis of equine leukocytes using anti-CD18 mAb BAQ30A.**
In this case, monocytes (B) and granulocytes (C) were stained positive whereas lymphocytes (A) remained negative in all cases. BAQ30A was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

Three mAbs (BAG40A [WS no 6], H22A [WS no 8], LT41A [WS no 25]) all submitted by W. Davis, WSU, were directed against CD44. With the exception of mAb LT41A, cross-reactivity with a number of species had been demonstrated already. CD44 is another adhesion molecule, broadly expressed on most cell types (including all leukocytes). CD44 is a receptor for hyaluronan and co-mediates attachment of leukocytes on endothelial cells. For the mAbs BAG40A (WS No 6, Fig. 10) and H22A (WS No 8, Fig. 11) all monocytes and granulocytes and the majority of lymphocytes were positive, but platelets were negative which reflects the human CD44 expression pattern in horses. For LT41A (WS No 25) the staining pattern was slightly different (Fig. 12). Staining intensity was weaker on all leukocytes, but especially lymphocytes, with a proportion of this cell type sometimes remaining negative (although a clear shift in population occurred).

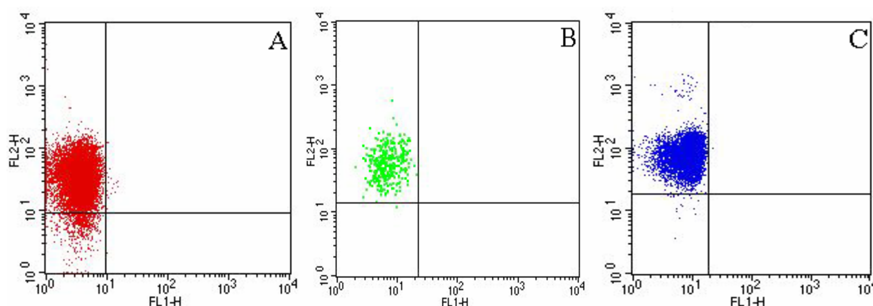


**Fig. 10 Analysis of equine leukocytes using anti-CD44 mAb BAG40A**.
BAG40A stained the vast majority of lymphocytes (A), all monocytes (B) and granulocytes (C). BAG40A was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

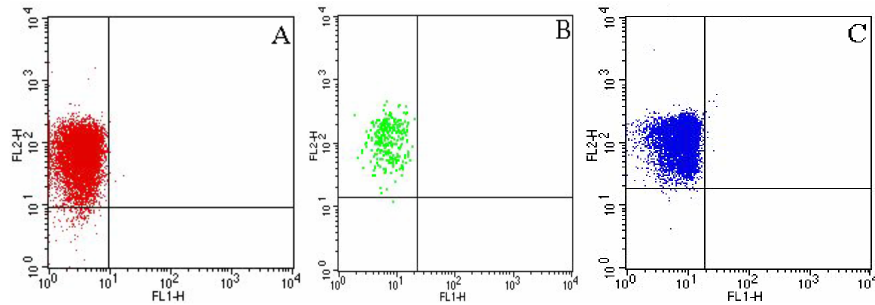


**Fig. 11 Analysis of equine leukocytes using anti-CD44 mAb H22A.**
H22A stained the vast majority of leukocytes: lymphocytes (A), monocytes (B), and granulocytes (C). H22A was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

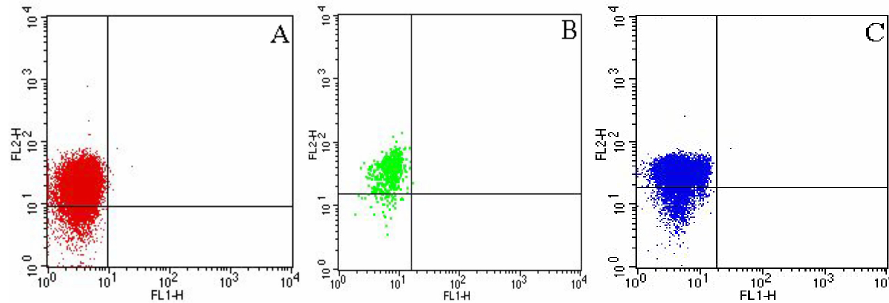


**Fig. 12 Analysis of equine leukocytes using anti-CD44 mAb LT41A.**
LT41A stained all populations less intense than BAG40A or H22A. Nevertheless, a clear shift occurred for lymphocytes (A), monocytes (B) and granulocytes (C). LT41A was analyzed by indirect staining using a PE-labelled secondary antibody (Fl-2).

IgM mAb DH16A (WS No 4, Fig. 13), directed against CD45, submitted by W Davis, reacted positively with all monocytes and granulocytes, and with the majority of lymphocytes allowing for the assumption that DH16A detected at least some CD45 isoforms in horses.

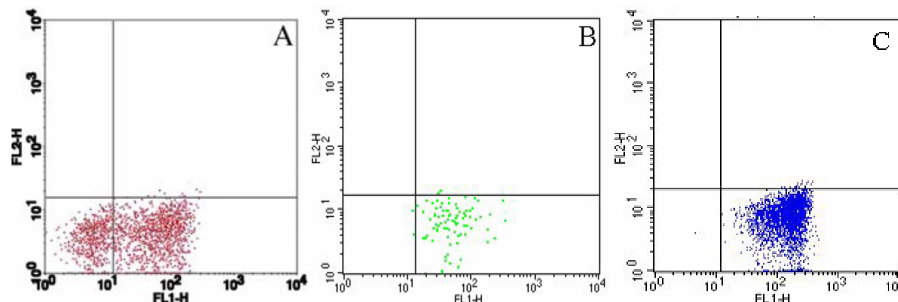


**Fig. 13 Analysis of equine leukocytes using anti-human CD45 mAb DH16A.**
DH16A stained all monocytes (B) and all granulocytes (C) but a population of lymphocytes always remained negative. This mAb is a mouse IgM isotype and analysis was performed using a FITC-labelled secondary anti-IgM specific antibody (FL-1).

The mAb HP2/1 (WS No 207) submitted from Beckman-Coulter, is specific for CD49d and showed a staining pattern for equine cells, which was in accordance to the basic descriptions in Leukocyte typing V-VII[1]. The majority of lymphocytes, granulocytes, and all monocytes

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

were positive (Fig. 14). Granulocyte staining was not in accordance with human data, but has been described on other species (rodents).

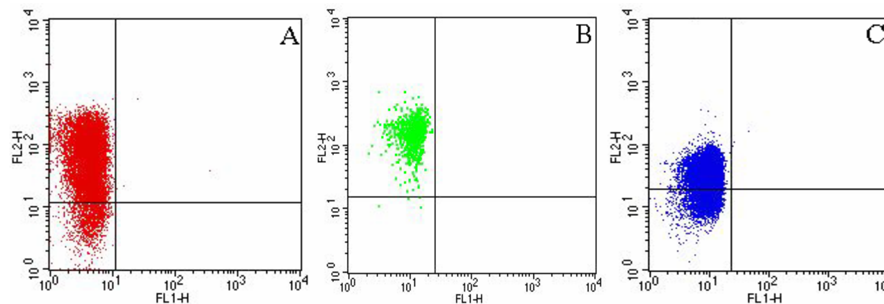


**Fig. 14 Analysis of equine leukocytes using anti-human CD49d mAb HP2/1.**
HP2/1 stained all lymphocytes (A), all monocytes (B), but also some granulocytes (C). Analysis was performed as indirect staining using a PE-labelled secondary antibody (FL-2).

CD91 is a member of the LDL receptor family, synthesised as a single chain molecule but processed into 515 and 85 kDa α and β chains. CD91 binds to α2-macroglobulin. The mAb A2MRα-2 (WS No 318) submitted from Dako-Cytomation, is specific for human CD91 and labelled the majority of equine monocytes but no granulocytes or lymphocytes (Fig. 15). It may therefore be assumed that this mAb detects EqCD91.

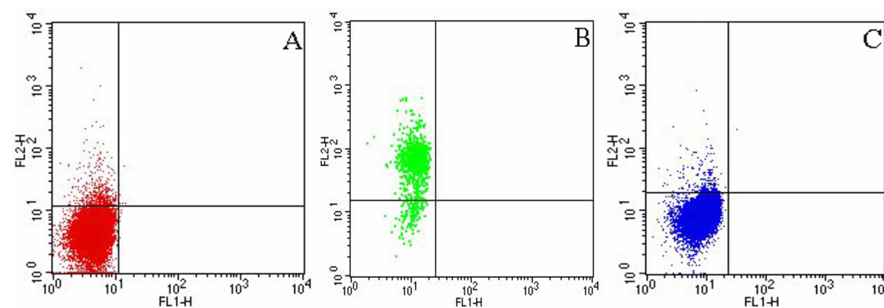


**Fig. 15 Analysis of equine leukocytes using anti-human CD91 mAb A2MRα2.**
A2MRα2 stained the vast majority of monocytes (B) but not lymphocytes or granulocytes. A2MRα2 was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

CD163 is another myeloid marker, expressed only by monocytes/macrophages, and upregulated during activation of cells (Hogger et al., 1998; Philippidis et al., 2004). The mAb Ber-MAC3 (WS No 330), submitted from Dako-Cytomation, is specific for human CD163 and stained the majority of monocytes (Fig. 16) assuming that Ber-Mac3 detects the equine homologue of CD163.

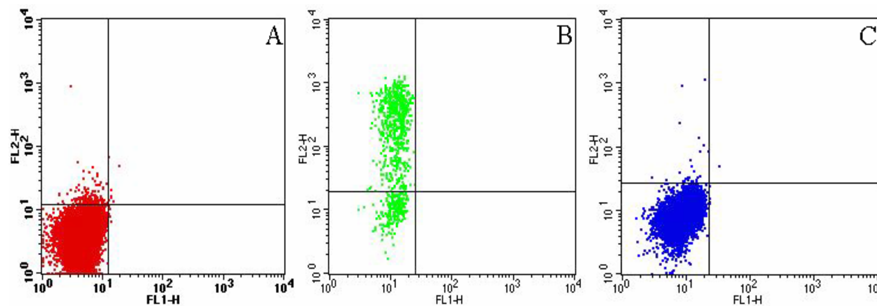


**Fig. 16 Analysis of equine leukocytes using anti-human CD163 mAb Ber-MAC3.**
Ber-MAC3 stained the majority of circulating blood monocytes but no granulocytes or lymphocytes. Ber-MAC3 was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

CD172a, a member of the immunoglobulin superfamily and a member of the signal regulatory protein (SIRP), is a type 1 glycoprotein expressed on $CD34^+$ stem/progenitor cells, macrophages, monocytes, granulocytes and dendritic cells. The anti-human CD172a mAb DH59B stained all equine monocytes and granulocytes (Fig. 17) in a consistent pattern comparable to that of humans supporting the assumption that DH59B detected the equine homologue of CD172a.

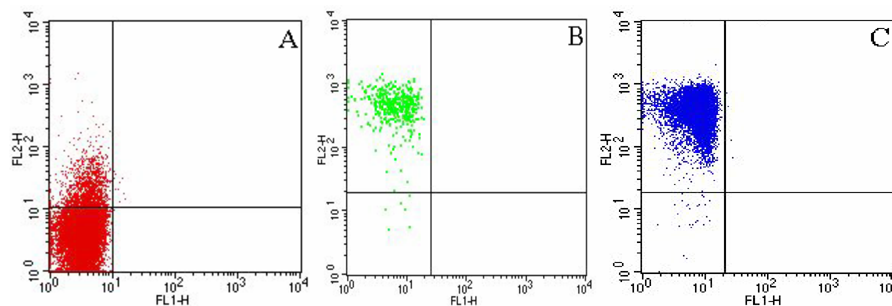


**Fig. 17 Analysis of equine leukocytes using anti-human CD172a mAb DH59B.**
DH59B stained all monocytes (B), all granulocytes (C), and a tiny subpopulation of lymphocytes (A). DH59B was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

The results for antibodies of the HLDA8 cross-reactivity workshop with some reactivity are summarised in tables 6 and 7.

**Legend to tables 6 and 7:**

**Table 6** lists HLDA8 mAbs that cross-reacted with horse (*Equus caballus)* leukocytes in single colour flow cytometry. Information in table 6 includes the [1]workshop number (Works.No), [2]CD number of the detected molecule, [3]source of the mAb identifying the submitting company/scientist, and [4]clone name for each antibody.
All of the listed mAbs are mouse [5]isotypes except 39C1.5 which is of rat origin. Each mAb was tested with leukocytes from different blood samples (n>3). Ranges of the highest and lowest percentages obtained of reactive lymphocytes (Ly)[6], monocytes (Mo)[7], granulocytes (Gran)[8], and platelets[9] are provided. [#]Variable result refers to unstimulated PBMC or regulation of expression on PBL after activation.

"++" indicates a clear reproducible positive result, consistent with described human staining pattern.

"(++)" refers to a clear positive result where minor doubts remain due to lack of direct experience (with mAb or CD molecule).

"W" a weak likely unusable but positive reactivity.

"+" mAbs with flow cytometric staining that was not consistent with human staining pattern. These results might still be due to homologous staining.

"A" mAbs with clear alternate expression pattern from that expected from humans. This pattern was less likely to reflect homologous staining.

"?" is a questionable result in staining, especially due to staining variability between different animals or labs.

"NA" mAbs which were not tested on appropriate target cells.

"W/A" a combination of "A" and "W" designations.

[∫] 3S3 is no longer available from Serotec.

Δ No-significant staining could be demonstrated.

[*]leukocyte percentage was represented as a single value when highest and lowest percentages are identical as in case of lymphocytes stained by DF1485 directed against CD44.

Staining of platelets was demonstrated for a few mAbs only, detecting platelets markers like CD9 and CD29.

**Table 7** lists mAbs that showed weak and alternate (W/A) staining and mAbs that were not tested on appropriate (NA) target cells.

Negative mAbs are listed within the whole panel list only (Appendix, tables 16 and 17).

**Table 6 Single colour flow cytometry of mAbs submitted to animal cross reactivity section of HLDA8 with horse (*Equus caballus*) leukocytes.**

| Works.No[1] | CD[2] | Source[3] | Clone[4] | Species Ig[5] | Result | Reactivity Ly[6] | Reactivity Mo[7] | Reactivity Gran[8] | Platelets[9] | Comments[#] |
|---|---|---|---|---|---|---|---|---|---|---|
| 178 | CD2 | Coulter | 39C1.5 | ratIgG2a | ++ | 66-81 | 18-21 | 1-5 | | |
| 17 | CD11a | Bill Davis | HUH73A | mIgG1 | ++ | 67-99 | 97-99 | 70-99 | | |
| 1 | CD18 | Bill Davis | BAQ30A | mIgG1 | ++ | 6-8 | 61-87 | 97-99 | | |
| 7 | CD18 | Bill Davis | H20A | mIgG1 | ++ | 60-99 | 95-99 | 94-99 | | |
| 22 | CD18 | Bill Davis | HUH82A | mIgG2a | ++ | 78-99 | 95-99 | 78-99 | | |
| 248 | CD18 | Dako | MHM23 | mIgG1 | ++ | 83-98 | 91-99 | 91-99 | | |
| 6 | CD44 | Bill Davis | BAG40A | mIgG3 | ++ | 81-85 | 88-99 | 94-99 | | |
| 8 | CD44 | Bill Davis | H22A | mIgG2a | ++ | 92-93 | 97-98 | 96-99 | | |
| 25 | CD44 | Bill Davis | LT41A | mIgG2a | ++ | 41-97 | 84-98 | 67-73 | | |
| 4 | CD45 | Bill Davis | DH16A | mIgM | (++) | 65-75 | 73-98 | 97-98 | | |
| 207 | CD49d | Coulter | HP2/1 | mIgG1 | (++) | 74-86 | 87-98 | 40-94 | | |
| 318 | CD91 | Dako | A2MRa-2 | mIgG1 | ++ | 3-6 | 69-77 | 0.8-2 | | |
| 330 | CD163 | Dako | Ber-MAC3 | mIgG1 | ++ | 0.2-1 | 64-81 | 0.1-6 | | |
| 5 | CD172a | Bill Davis | DH59B | mIgG1 | ++ | 4-9 | 93-96 | 98-99 | | |
| 196 | CD21 | Coulter | BL13 | mIgG1 | W | 12-15 | 3-5 | 0.7-2 | | |
| 26 | CD9 | Bill Davis | LT86A | mIgG2a | + | 2-15 | 1-64 | 0.2-53 | 28-65 | variable result |
| 53 | CD9 | Serotec | MM2/57 | mIgG2b | + | 8-20 | 11-59 | 39-88 | 22-90 | variable result |
| 238 | CD9 | Dako | P1/33/2 | mIgG1 | ? | 19-22 | 45-71 | 68-90 | 37-88 | up-regulated |
| 195 | CD18 | Coulter | 7 e4 | mIgG1 | A | 15-30 | Δ | Δ | | |
| 76 | CD27 | Serotec | LT27 | mIgG2a | A | 10-29 | 5-8 | 0.5-1 | | no upregulation |
| 78 | CD29 | Serotec | 12G10 | mIgG1 | A | Δ | 59-62 | Δ | | |
| 79 | CD29 | Serotec | 3S3∫ | mIgG1 | ? | 56-74 | 86-97 | 22-45 | 69-93 | up-regulated |
| 271 | CD35 | Dako | To5 | mIgG1 | A | Δ | Δ | 43-96 | | |
| 353 | CD38 | BD Pharmingen | HIT2 | mIgG1 | ? | 65-77 | 0.9-4 | 0.4 | | |
| 278 | CD44 | Dako | DF1485 | mIgG1 | + | 67* | Δ | Δ | | pos after act. only |
| 101 | CD45RB | Serotec | MEM55 | mIgG1 | A | Δ | 27-85 | Δ | | |
| 15 | CD47 | Bill Davis | HUH69A | mIgG1 | + | 34-68 | 54-92 | 0.6-53 | | variable result |
| 16 | CD47 | Bill Davis | HUH71A | mIgG1 | + | 30-61 | 30-87 | 20-25 | | variable result |
| 287 | CD49d | Dako | P4G9 | mIgG3 | + | 2-51 | 3-74 | 1-14 | | variable result |
| 357 | CD49d | BD Pharmingen | 9F10 | mIgG1 | ? | 64-73 | 92-96 | 48-57 | | up-regulated |
| 108 | CD49e | Serotec | JBS5 | mIgG | + | 1-15 | 71-95 | 1-20 | | |
| 294 | CD56 | Dako | C5.9 | mIgG2b | ? | 9-18 | 0.2-1 | 0.1-1 | | |
| 27 | CD59 | Bill Davis | MUC93A | mIgG1 | + | 6-14 | 6-37-18 | 69-96 | | variable monocytes |
| 361 | CD83 | BD Pharmingen | HB15E | mIgG1 | + | 0.4-12 | 3 | 0.4-76 | | up-regulated |
| 12 | | Bill Davis | GBSP71A | mIgG1 | + | 24-52 | 69-95 | 11-27 | 25-96 | no comparison |
| 33 | | Marion Reid | MIMA-51 | mIgG2b | + | 10-24 | 2-41 | 0.3-17 | | no comparison |
| 34 | | Marion Reid | NBL-1 | mIgG1 | + | 4-16 | 26-85 | 1 | | no comparison |

Legend to table 6: see page 53

**Table 7 single colour flow cytometry of mAbs submitted to animal cross reactivity section of HLDA8 with horse leukocytes (W/A and NA mAbs):**

| Works.No | CD | Source | Clone | Species Ig | Result |
|---|---|---|---|---|---|
| 42 | CD2 | Serotec | LT2 | mIgG2b | **W/A** |
| 177 | CD2 | Coulter | SFCI3Pt2H9 | mIgG1 | **W/A** |
| 183 | CD4 | Coulter | BL4 | mIgG2a | **W/A** |
| 185 | CD5 | Coulter | BL1a | mIgG2a | **W/A** |
| 47 | CD6 | Serotec | MEM-98 | mIgG1 | **W/A** |
| 48 | CD7 | Serotec | LT7 | mIgG2a | **W/A** |
| 186 | CD7 | Coulter | 8H8.1 | mIgG2a | **W/A** |
| 187 | CD7 | Coulter | 3A1E-12H7 | mIgG2b | **W/A** |
| 50 | CD8 | Serotec | LT8 | mIgG1 | **W/A** |
| 54 | CD10 | Serotec | SN5c | mIgG1 | **W/A** |
| 190 | CD10 | Coulter | ALB1 | mIgG1 | **W/A** |
| 191 | CD10 | Coulter | J5 | mIgG2a | **W/A** |
| 55 | CD11a | Serotec | clone 38 | mIgG2a | **W/A** |
| 192 | CD11a | Coulter | 25. Mär | mIgG1 | **W/A** |
| 193 | CD11b | Coulter | BEAR1 | mIgG1 | **W/A** |
| 347 | CD14 | BD Pharmingen | M5E2 | mIgG2a | **W/A** |
| 194 | CD16 | Coulter | 3G8 | mIgG1 | **W/A** |
| 67 | CD20 | Serotec | 2H7 | mIgG2b | **W/A** |
| 349 | CD20 | BD Pharmingen | 2H7 | mIgG2b | **W/A** |
| 69 | CD21 | Serotec | LB21 | mIgG1 | **W/A** |
| 71 | CD22 | Serotec | Mc64-12 | mIgG1 | **W/A** |
| 254 | CD22 | Dako | To15 | mIgG2b | **W/A** |
| 197 | CD23 | Coulter | 9P.25 | mIgG1 | **W/A** |
| 261 | CD29 | Dako | K20 | mIgG2a | **W/A** |
| 83 | CD32 | Serotec | AT10 | mIgG1 | **W/A** |
| 201 | CD32 | Coulter | 2 E1 | mIgG2a | **W/A** |
| 265 | CD32 | Dako | KB61 | mIgG1 | **W/A** |
| 85 | CD35 | Serotec | clone E11 | mIgG1 | **W/A** |
| 355 | CD45 | BD Pharmingen | HI30 | mIgG1 | **W/A** |
| 205 | CD45RA | Coulter | ALB11 | mIgG1 | **W/A** |
| 103 | CD49b | Serotec | 16B4 | mIgG1 | **W/A** |
| 150 | CD120a | Serotec | H398 | mIgG2a | **W/A** |
| 172 | CD1a | Coulter | BL6 | mIgG1 | **NA/-** |
| 173 | CD1a | Coulter | B17.20.9 | mIgG2a | **NA/W** |
| 40 | CD1b | Serotec | MT-101 | mIgG1 | **NA/-** |
| 175 | CD1b | Coulter | 4.A7.6 | mIgG2a | **NA/-** |
| 176 | CD1c | Coulter | L161 | mIgG1 | **NA/-** |
| 41 | CD1d | Serotec | NOR3.2 | mIgG1 | **NA/-** |
| 84 | CD33 | Serotec | WM-53 | mIgG1 | **NA** |
| 266 | CD33 | Dako | WM-54 | mIgG1 | **NA** |
| 202 | CD34 | Coulter | 581 | mIgG1 | **NA** |
| 203 | CD34 | Coulter | IMMU133 | mIgG1 | **NA** |
| 267 | CD34 | Dako | BI-3C5 | mIgG1 | **NA** |
| 268 | CD34 | Dako | BIRMA-K3 | mIgG1 | **NA** |
| 269 | CD34 | Dako | QBEnd | mIgG1 | **NA** |
| 270 | CD34 | Dako | TÜK3 | mIgG3 | **NA** |
| 352 | CD34 | BD Pharmingen | 581 | mIgG1 | **NA** |
| 131 | CD80 | Serotec | MEM-233 | mIgG1 | **NA/-** |
| 213 | CD80 | Coulter | MAB104 | mIgG1 | **NA/-** |
| 315 | CD80 | Dako | 2D10.4 | mIgG1 | **NA/-** |
| 360 | CD80 | BD Pharmingen | BB1 | mIgM | **NA/-** |
| 380 | CD80 | Caltag | MEM-233 | mIgG1 | **NA/-** |
| 214 | CD86 | Coulter | HA5.2B7 | mIgG2b | **NA/-** |
| 317 | CD86 | Dako | BU63 | mIgG1 | **NA/-** |
| 220 | CD206 | Coulter | 3.29B1.10 | mIgG1 | **NA/+** |
| 368 | CD209 | R&D Systems | 120507 | mIgG2b | **NA/-** |
| 334 | CD235a | Dako | JC159 | mIgG1 | **NA** |
| 335 | CD236R | Dako | Ret40f | mIgG1 | **NA** |

Legend to table 7: see page 53

#### 4.1.1.2 Dot plots analysis of non-HLDA8 mAbs:

Two mAbs directed against equine leukocyte markers were included in this study as positive controls and as a basis for the further studies (in particular the double labelling). Both mAbs were distributed by VMRD, Pullman, USA. The mAb HB88A is directed against EqCD2. This mAb stained, as expected, the vast majority of PBL but no other cells (Fig. 18). Likewise the mAb HT23A stained such a population and served as a positive control for equine CD5 (Fig. 19).

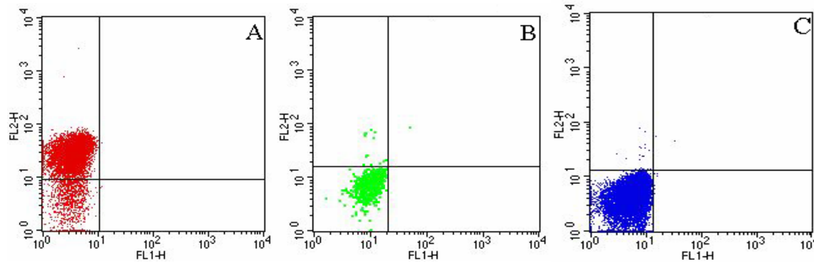


**Fig. 18 Analysis of equine leukocytes with anti-EqCD2 mAb HB88A.**
HB88A stained the vast majority of lymphocytes (A) while no significant staining of monocytes (B) or granulocytes (C) was observed. HB88A was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

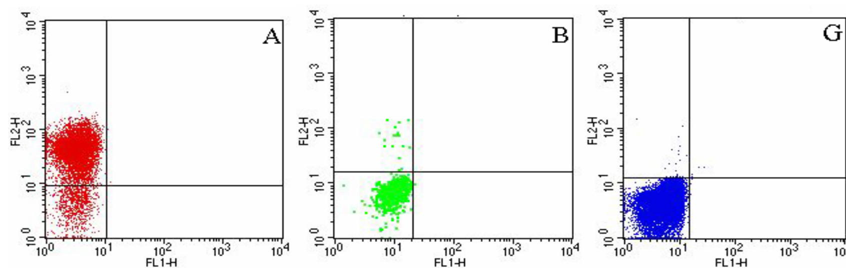


**Fig. 19 Analysis of equine leukocytes with anti-EqCD5 mAb HT23A.**
HT23A stained the vast majority of lympohocytes (A) while no significant staining of monocytes (B) or granulocytes (C) was detected.

The clone M1/70.15.11.5 from Miltenyi Biotec (Bergisch Gladbach, Germany) is a rat IgG2b mAb 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 humans, where, next to myeloid cells, NK cells are also CD11b$^{+}$ (Werfel et al., 1991; Muto et al., 1993; Hogg, 2002a).

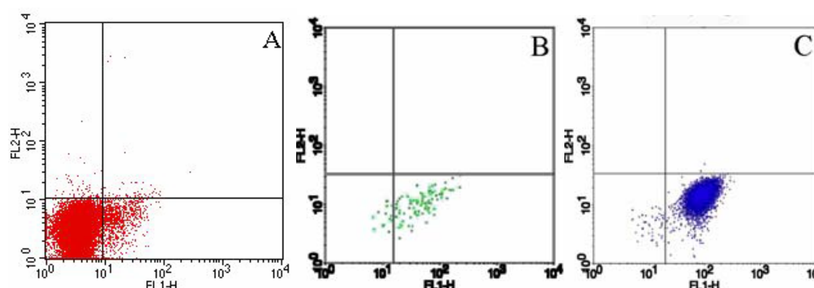


**Fig. 20 Analysis of equine leukocytes with anti-mouse CD11b mAb M1/70.15.11.5.**
M1/70.15.11.5 stained all granulocytes (C), almost all monocytes (B) but only a small population of lymphocytes (A). M1/70.15.11.5 was a directly FITC-conjugated mAb (FL-1).

A significant number of antibodies in this study were directed against human CD14 and four of them (clones 7H3 [big10], big11, big12, and 7D6 [big13]) all from Biometec (Greifswald, Germany) reacted strongly with equine monocytes only (Fig. 21b, 22b, 23b, and 24b respectively). A very small population in the granulocyte gate (c) showed CD14 expression, which could be granulocytes expressing CD14 as described in humans (Haziot et al., 1993; Rodeberg et al., 1997) or represent a small population of monocytes detected in the granulocyte gate. The bright monocyte staining by the four clones supported the assumption that these mAbs detected the equine homologue of CD14.

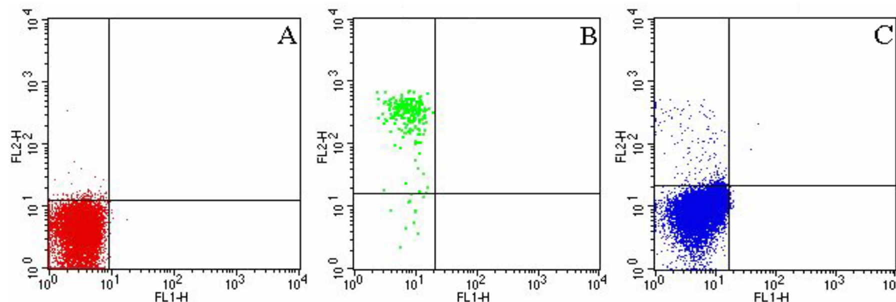


**Fig. 21 Analysis of equine leukocytes with anti-human CD14 mAb big10.**
Big10 detected all equine monocytes (B), very weakly stained a small population of granulocytes (C) but did not stain lymphocytes. Big10 was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

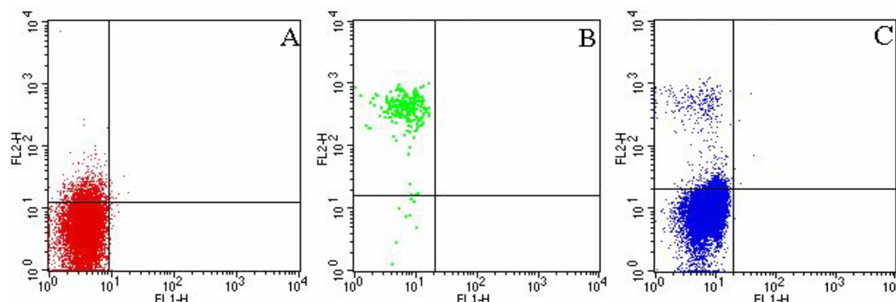


**Fig. 22 Analysis of equine leukocytes with anti-human CD14 mAb big11.**
Big11 stained all equine monocytes (B), very weakly stained a small population of granulocytes (C), but did not stain lymphocytes. Big11 was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

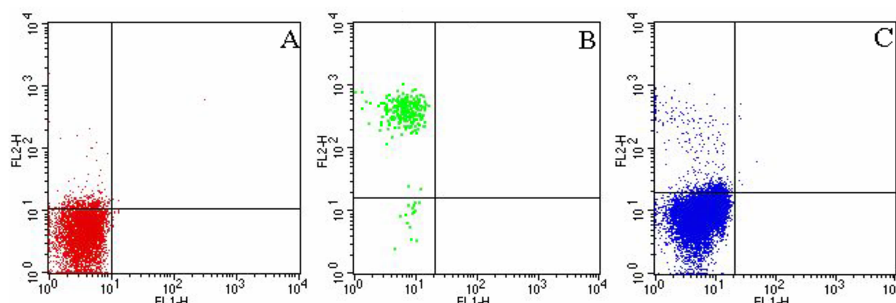


**Fig. 23 Analysis of equine leukocytes with anti-human CD14 mAb big12.**
Big12 stained all equine monocytes (B), very weakly stained a small population of granulocytes (C) and did not stain lymphocytes. Big12 was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

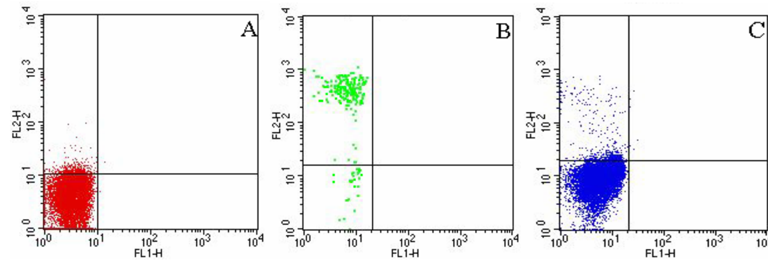


**Fig. 24 Analysis of equine leukocytes with anti-human CD14 mAb big13.**
Big 13 stained all equine monocytes (B); very weak staining of a small population of equine granulocytes (C) was observed while it did not stain lymphocytes. Big 13 was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

Anti-human CD21 mAb B-Ly4 stained a population of lymphocytes brightly and additionally a few cells less intense (Fig. 25a). The positive cells comprised of 9-24.5% of the lymphocytes. This labelling itself, the previous description of reactivity (Lin et al., 2002), and the knowledge of the amount of B-cells expected lead to the result that B-Ly4 most likely stains equine CD21. Detection of 2-3% of granulocytes (Fig. 25c) was in accordance with humans where basophils may express low levels of CD21 (Bacon et al., 1993).

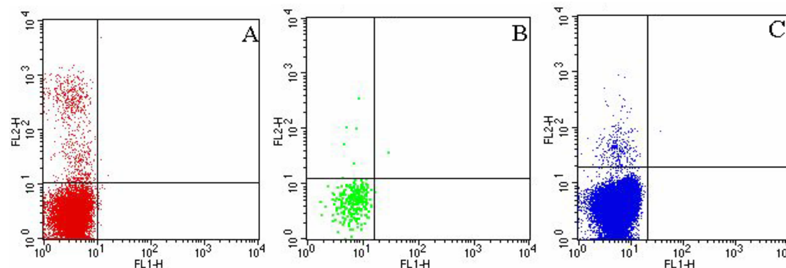


**Fig. 25 Analysis of equine leukocytes with anti-human CD21 mAb B-Ly4.**
B-Ly4 stained a population of 9-24.5% of lymphocytes (A). A population of 2-3% of granulocytes (C) and no monocytes (B) as recorded for humans. B-Ly4 was directly PE-conjugated (FL-2).

CD34 is a haematopoietic stem cell (HSC) marker. The anti-human CD34 mAb AC136 did not stain PBMC of adult horses (data not shown). There was a very limited opportunity to verify the staining 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., 1998). Here, a population of about 17% of the cells (Fig. 26a) were stained using AC136, underlining the potential use of this mAb to detect the equine homologue of CD34.

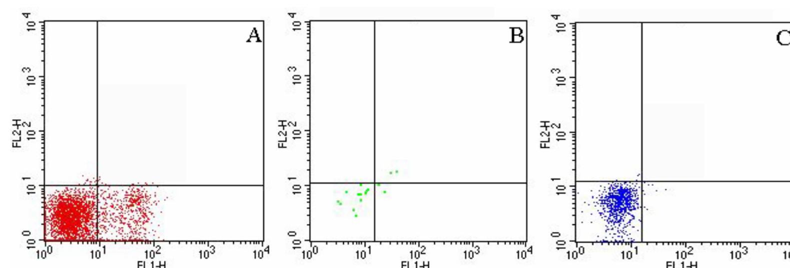


**Fig. 26 Analysis of three days old foal blood leukocytes with anti-human CD34 mAb AC136.**
AC136 stained a population of cells in the lymphocyte gate (a) assumed to be haematopoietic stem cells, while no staining was observed in the monocyte or granulocyte gate. AC136 was directly FITC-conjugated (FL-1).

Anti-human CD68 mAb Ki-M6 did not stain PBM nor LPS activated monocytes in standard flow cytometry (data not shown). However, when intra-cellular staining was applied, Ki-M6 did stain cultured equine monocytes (macrophages) clearly (Fig. 27). This is in accordance with human cells, where CD68 is mainly expressed intracellularly and on cultured cells only (Fukuda, 1991; Saito et al., 1991; Goyert, 2002b).

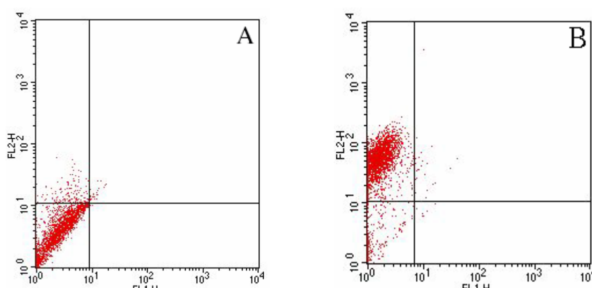


**Fig. 27 Analysis of equine monocytes/macrophages with anti-human CD68 mAb Ki-M6.**
Monocytes were differentiated towards macrophages and stained extra- (A) and intra-cellular (B). All monocytic cells reacted clearly positive when stained inside, whereas extra-cellular staining remained negative (A) with only very few cells weakly stained. Ki-M6 was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

The mAb HB15a (Beckman Coulter) directed against CD83 was also analyzed as part of this study. HB15a repetitively reacted with a population of 14-25% of lymphocytes, which were presumed to be B-lymphocytes (Fig. 28a). Additionally, the mAb did show reactivity in some but not all horses with monocytes (Fig. 28b), but never with granulocytes (Fig. 28c).

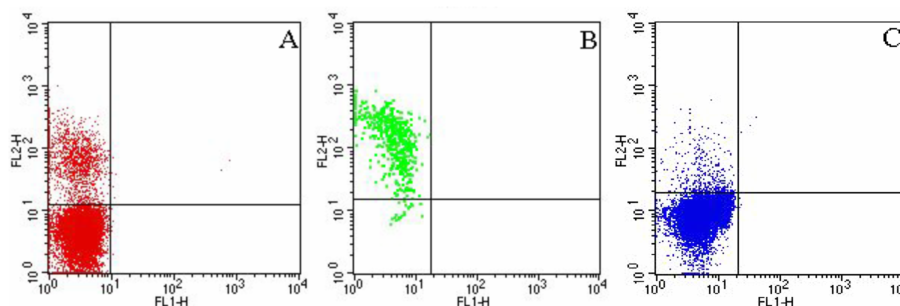


**Fig. 28 Analysis of equine leukocytes with anti-human CD83 mAb HB15a.**
In all horses analyzed, a population of lymphocytes was detected positive, possibly representing B lymphocytes (A). Additionally, in some horses, some or even all monocytes were also stained positive (B) but granulocytes were never detected (C). HB15a was directly PE-conjugated (FL-2).

CD206 is the macrophage mannose receptor (MMR) also termed mannosyl-fucosyl-receptor (MFR). CD206 is an important receptor for pinocytosis and phagocytosis absent on monocytes but up-regulated during differentiation to macrophages (Stahl and Gordon, 1982; Ezekowitz et al., 1990). Additionally CD206 is expressed on immature DC (iDC) (Sallusto and Lanzavecchia, 1994). The anti-human CD206 mAb 3.29B1.10 did not stain horse monocytes, but when differentiated towards dendritic cells by incubation with EqGM-CSF

and EqIL-4 for 48 hours (3.2.1), 3.29B1.10 stained almost all iDC (Fig. 29) giving a staining pattern such as described for human cells (Shepherd et al., 1982).

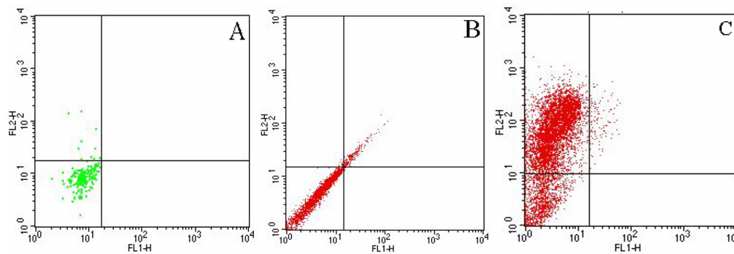

**Fig. 29 Analysis of equine monocytes/dendritic cells with anti-human CD206 (MMR) mAb 3.29B1.10.** Monocytes were differentiated towards dendritic cells by incubation with EqGM-CSF and EqIL-4 (3.2.1). 3.29B1.10 detected mannose receptor on almost all cultured immature dendritic cells (iDC) within 48 hours (C) while not being detected on PBM (A). For staining of iDC, mIgG1 isotype negative control is displayed (B).

The mAb TLR3-7 (Serotec) is directed against the toll like receptor 3 (TLR 3), recently classified CD283 (Zola et al., 2005). This receptor, which detects dsRNA, is located mainly intracellularly, where the reaction of TLR3-7 was detected using differentiated and activated monocyte-derived dendritic cells (Fig. 30). In contrast to CD68, only a population of cells reacted with TLR3-7. I here assumed that this was due to regulated expression and suboptimal conditions in stimulation.

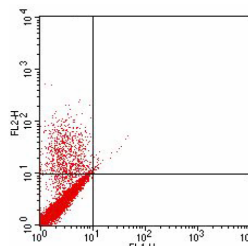

**Fig. 30 Analysis of equine monocytes/dendritic cells with anti-human CD283 (TLR3) mAb TLR3-7.** Monocytes were differentiated towards macrophages and stained intracellular. In contrast to CD68, only a population of cells reacted with TLR3-7 assuming that this was due to regulated expression and suboptimal conditions in stimulation. TLR3-7 was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

The mAb B9.12.1 (Beckman Coulter) directed against the human MHC class I HLA-ABC locus stained all equine leukocytes brightly (Fig. 31). According to the fact that cross-reactivity of anti-MHC mAbs between various species including horses has been described many times before, it is assumed that B9.12.1 detects the equine homologue of MHC class I HLA-ABC locus.

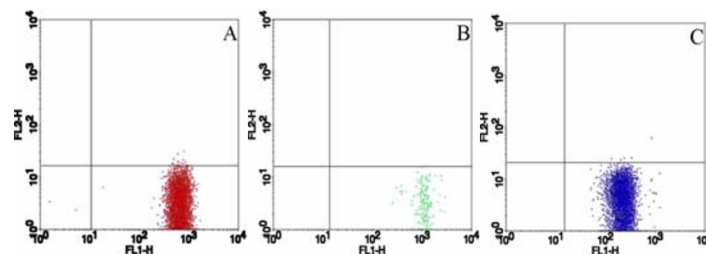

**Fig. 31 Analysis of equine leukocytes using the anti-human MHC class I mAb B9.12.1.** All leukocytes (A, lymphocytes; B, monocytes; C, granulocytes) of all horses tested were brightly stained. B9.12.1 was directly FITC-conjugated (FL-1).

For the more restrictedly expressed MHC class II, the positive control mAb EqT2 (VMRD) and the mAb D-F1 (Serotec) were tested. While EqT2 is designated to react with pan-MHC II, mAb D-F1 is directed against the human HLA-DR only. Accordingly, it was not surprising that this mAb stained monocytes slightly weaker than EqT2 (Fig. 32b and Fig. 33b). Notably: while all monocytes and no granulocytes were stained, a significant population of PBL were stained also (Fig. 32a and Fig. 33a). EqT2 and DF1 lymphocytes staining pattern come in accordance with previous data describing equine MHCII to be expressed by all B and some T cells (Crepaldi et al., 1986; Lunn, 1993). In addition, variation in expression of MHCII antigens on horse lymphocytes, as noticed between EqT2 and DF1, was described before (Crepaldi et al., 1986; Barbis et al., 1994) giving little doubt that both mAbs detected the equine homologue of MHC class II.

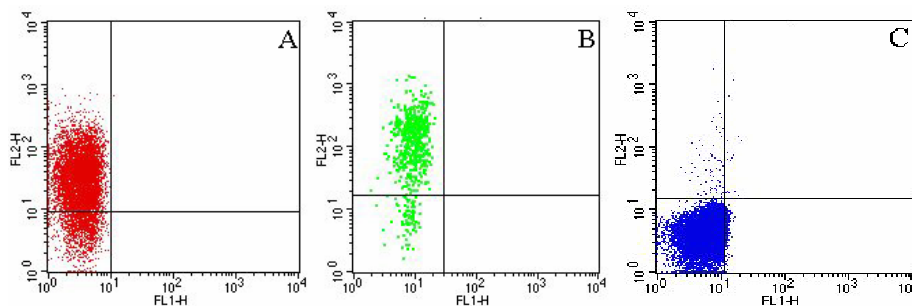


**Fig. 32 Analysis of equine leukocytes with anti-equine MHC class II mAb EqT2**.
EqT2 stained most monocytes (B), most lymphocytes (A) but did not stain granulocytes. EqT2 was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

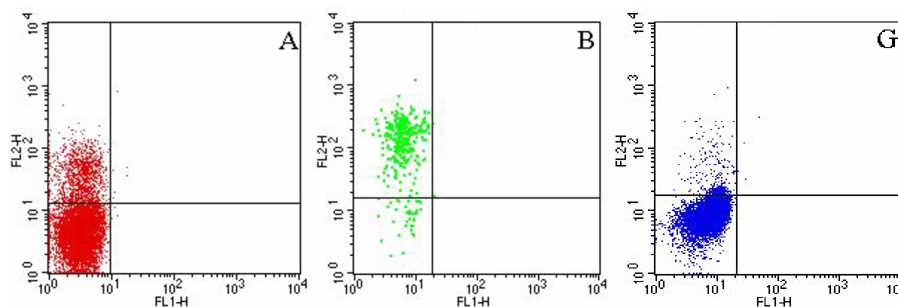


**Fig. 33 Analysis of equine leukocytes with anti-equine MHC class II (HLA-DR) mAb DF1.**
DF1 stained all of monocytes (B), a few lymphocytes but no granulocytes. DF1 was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

**Table 8 Non-HLDA8 mAbs detected positive during the flow cytometric screening**

| Donator | CD No. | Clone | Isotype | Result |
|---|---|---|---|---|
| NatuTec/VMRD | CD2 | HB88A | mIgG1 | ++ |
| NatuTec/VMRD | CD5 | HT23A | mIgG1 | ++ |
| MiltenyiBiotech | CD11b | M1/70.15.11.5 | mIgG2b | ++ |
| Biometec | CD14 | 7H3 (big 10) | mIgG1 | ++ |
| Biometec | CD14 | big 11 | mIgG1 | ++ |
| Biometec | CD14 | big 12 | mIgG1 | ++ |
| Biometec | CD14 | 7D6 (big 13) | mIgG1 | ++ |
| BD | CD21 | B-Ly4 | mIgG1 | ++ |
| MiltenyiBiotech | CD34 | AC136 | mIgG2a | (++) |
| Serotec* | CD68 | Ki-M6 | mIgG1 | ++ |
| Coulter | CD83 | HB15a | mIgG2b | (++) |
| Coulter | CD206 (MMR) | 3.29B1.10 | mIgG1 | ++ |
| Serotec | CD283 (TLR3) | TLR3-7 | mIgG2a | (++) |
| Coulter | HLA- ABC (MHC-I) | B9.12.1 | mIgG2a | ++ |
| NatuTec/VMRD | MHCII | EqT2 | mIgG1 | ++ |
| Serotec* | HLA-DR (MHC-II) | D-F1 | mIgG2a | ++ |
| Serotec | B cells (Canine) | CA2.1D6 | IgG1 | (++) |

**Legend to table 8:**
"++" indicates a clear positive result, consistent with human staining pattern described.
"(++)" refers to a clear positive result where minor doubts remain due to a lack of experience (e.g. with mAb or CD molecule).
"*" these mAbs are not distributed by Serotec anymore.

**Table 9 Non-HLDA8 mAbs that were not detected clearly positive during the flow cytometric screening**

| CD No. | Clone | Isotype | Result |
|---|---|---|---|
| **Donator** | | | |
| **BIOMETEC** | | | |
| CD14 | 7F11 | mIgG2a | **A** |
| CD14 | big16 | mIgG1 | **A** |
| **CHEMICON** | | | |
| CD1b | WM-25 | mIgG1 | **NA/-** |
| CD18 | 68-5A5 | mIgG2a | **W** |
| **COULTER** | | | |
| CD1a | BL-6 | mIgG1 | **NA/-** |
| CD34 | 581 | mIgG1 | **NA** |
| **IMMUNOTOOLS** | | | |
| CD1a | HI149 | IgG1 | **NA/-** |
| CD1a | WM35 | IgG2b | **NA/?** |
| CD3 | MEM-57 | IgG2a | **W/A** |
| CD3 | 16A9 | IgG2a | **W/A** |
| CD7 | 7F3 | IgG2a | **W/A** |
| CD8 | MEM-31 | IgG2a | **W/A** |
| CD10 | 4F9 | IgG2a | + |
| CD11a | MEM-25 | IgG1 | **A** |
| CD16 | MEM-154 | IgG1 | + |
| CD18 | MEM-48 | IgG1 | **W/A** |
| CD20 | MEM-97 | IgG1 | **W/A** |
| CD25 | TB30 | IgG2b | + |
| CD33 | MD33.6 | IgG1 | **NA/-** |
| CD34 | 4H11 | IgG1 | **NA** |
| CD34 | QBEnd10 | IgG1 | **NA** |
| CD35 | E11 | IgG1 | **W/A** |
| CD43 | DFT1 | IgG1 | **A** |
| CD45RA | MEM-56 | IgG2b | + |
| CD45RA | FB-11-13 | IgG1 | **W/A** |
| CD45RO | UCHL1 | IgG2a | **W/A** |
| CD49f | NKI-GoH3 | IgG2a | **W/A** |
| CD103 | 2G5 | IgG2a | + |
| CD122 | MIK-beta1 | IgG2a | **A** |
| HLA-DR (MHC- II) | 1E5 | IgG1 | + |
| MPO | 7.17 | IgG1 | + |
| **MILTENYIBiotech** | | | |
| BDCA-1 (CD1c) | AD5-8E7 | mIgG2a | **NA/-** |
| BDCA-2 (CD303) | AC144 | mIgG1 | **NA/-** |

**Table 9 (continued)**

| CD No. | Clone | Isotype | Result |
|---|---|---|---|
| BDCA-3 (CD141) | AD5-14H12 | mIgG1 | **NA/-** |
| BDCA-3 (CD141) | AD5-5E8.12.3 | mIgG1 | **NA/-** |
| BDCA-4 (CD304) | AD5-17F6 | mIgG1 | **NA/-** |
| CD2 | LT2 | mIgG2b | **A** |
| CD3 | OKT3 | mIgG2a | + |
| CD3 | BW264/56 | mIgG2a | **-** |
| CD4 | M-T466 | mIgG1 | + |
| CD7 | 6B7 | mIgG2a | **W/A** |
| CD19 | SJ25-C1 | Rat IgG2a | **A** |
| CD20 | LT20 | mIgG1 | **A** |
| CD30 | Ki-2 | mIgG1 | **A** |
| CD36 | AC106.3 | mIgG2a | **W/A** |
| CD45R | RA3-6B2 | Rat IgG2a | + |
| CD90.2 | 30-H12 | Rat IgG2b | **W/A** |
| CD123 | AC145 | mIgG2a | **A** |
| CD133/1 | AC133 | mIgG1 | **NA** |
| CD133/2 | AC141 | mIgG2a | **NA** |
| CD133/2 | 293C3 | mIgG1 | **NA** |
| CD138 | B-B4 | mIgG1 | **A** |
| CD205 | NLDC-145 | mIgG2a | **NA/-** |
| CD235a | AC107 | mIgG1 | **NA** |
| TCR alpha/Beta | BW242/412 | mIgG2b | **A** |
| **SEROTEC** | | | |
| CD1a | NA1/34 | mIgG1 | **NA/?** |
| CD1a | O10 | mIgG1 | **NA/-** |
| CD1c | L161 | mIgG1 | **NA/?** |
| CD86 | BU63 | mIgG1 | **NA/-** |
| **VMRD** | | | |
| CD5 | B29A | mIgG2a | **?** |
| CD90 (Thy-1) | DH24A | IgM | + |

**Legend to table 9:**

"W" a weak, likely unusable but positive reactivity.

"+" mAbs with flow cytometric staining that was not consistent with human staining pattern. These results might still be due to homologous staining.

"A" mAbs with clear alternate expression pattern from that expected from humans. This pattern was less likely to reflect homologous staining.

"?" is a questionable result in staining, especially due to staining variability between different animals.

"NA" mAbs which were not tested on appropriate target cells.

Designations may be combined, such as "W/A" and "NA/-"

### 4.1.2 Two-colour flow cytometry of mAbs that cross reacted with horse lymphocytes.

In two-colour flow cytometry, the single-colour protocol was applied first with indirect staining of the mAb to be tested. Isotype mAbs were used to block unspecific reactions and directly conjugated mAbs were used for the second colour, with washing steps between all staining steps. When the two mAbs were of different origin (rat vs. mouse, or IgG vs. IgM), two indirect labellings were performed consecutively, using isotype-specific antisera. Alternatively, two directly conjugated mAbs were used (3.2.3).

MAbs defined positive in the HLDA-8 (4.1.1.1) and non-HLDA-8 (4.1.1.2) screening parts were further analyzed, in particular mAbs that detected only lymphocyte populations. Some mAbs that stained all leukocytes (e.g. CD11a, CD18) or were directed against myeloid markers staining monocytes (CD14, CD91, and CD163) or monocytes and granulocytes (e.g. CD172) were not included in two-colour flow cytometry analysis, because the results would not add to our knowledge. Nevertheless, a few mAbs from the questionable panel were tested by two-colour flow cytometry to check if further analysis could resolve the situation. The results of double-labelling (DL) flow cytometric experiments are summarised in table 10 and additional examples of the dot blot analysis are provided.

To define T cells, mAbs against equine CD2 (clone HB88A) and equine CD5 (clone HT23A) were available and stained the same majority of lymphocytes (Fig. 34).

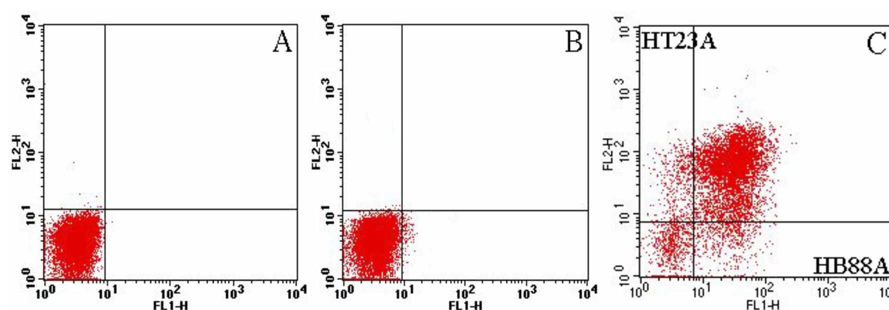


**Fig. 34 Two colour flow cytometry of lymphocytes using anti-equine CD2 mAb HB88A and anti-equine CD5 mAb HT23A.**
Both of HB88A and HT23A stained the same majority of lymphocytes (C). (A) and (B) represent staining of equine lymphocytes using PE- and FITC-conjugated secondary antibodies as negative controls.

Using HB88A and HT23A as controls, it was demonstrated that clone 39C1.5 against human CD2 stained equine T cells specifically (Fig. 35). In addition, 39C1.5 mAb stained all $CD4^+$ positive T cells (Fig. 36a) and almost all $CD8^+$ T Cells with a small sub-population of T cells remaining $CD8^+/CD2^-$ (Fig. 36b). $CD2^-/CD8^+$ cells were described before in humans as NK cells subpopulations (Perussia et al., 1983; Ritz et al., 1988; Nakazawa et al., 1997). The data obtained from two-colour analysis therefore strongly support the notion that 39C1.5 detects

EqCD2. 39C1.5 was used further for DL, since it was available as directly fluorochrome-conjugated mAb.

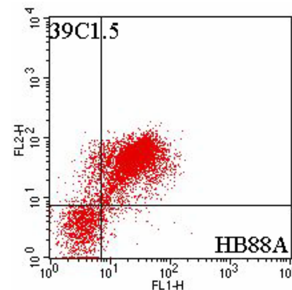


**Fig. 35 Two colour flow cytometry using anti-human CD2 mAb 39C1.5 and anti-equine CD2 mAb HB88A.**
39C1.5 detects the same lymphocytes as HB88A and the staining profile indicates that both mAbs stain the same antigen strongly supporting the notion that 39C1.5 detects EqCD2.

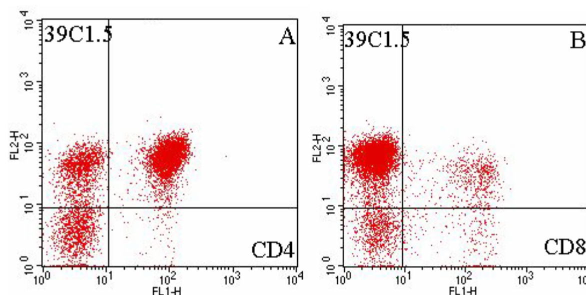


**Fig. 36 Further analysis of anti-equine CD2 mAb 39C1.5 by two colour flow cytometry with anti-equine CD4 (CVS4) and anti-equine CD8 (CVS8) mAbs.**
39C1.5 stained all $CD4^{+}$ positive T cells (A) and almost all $CD8^{+}$ T cells, while a small population was $CD8^{+}/CD2^{-}$ which was assumed to be NK cells (B).

Having defined T cells by CD2 and CD5, allowed for the analysis of the B-cells specific mAbs. Using the CD21 specific B-Ly4, two-colour DL could indeed show that this mAb was B cell-specific (Fig. 37a and b). 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). In addition to B-Ly4, the anti-Canine B cells mAb CA2.1D6 stained non-T cells (Fig. 38a and b).

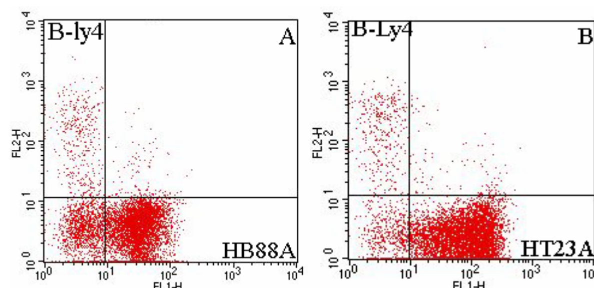


**Fig. 37 Two colour flow cytometric analysis of anti-human CD21 mAb B-Ly4 and anti-equine CD2 mAb HB88A or anti-equine CD5 mAb HT23A.**
B-Ly4 stained a population of lymphocytes while anti-Eq CD2 mAb HB88A (A) or anti-Eq CD5 mAb HT23A (B) stained T cells, supporting that anti-human CD21 mAb B-Ly4 detected the equine homologue of CD21.

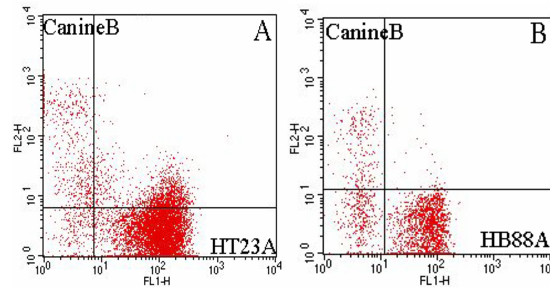


**Fig. 38 Two colour flow cytometry analysis of anti-canine B cell mAb CA2.1D6 with anti-equine CD5 mAb (HT23A) and anti-equine CD2 (HB88A).**
HT23A (A) and HB88A (B) detect T cells showing that anti-canine B cell mAb CA2.1D6 clearly stained B cells.

The CD45RB specific mAb clone DH16A stained most T (Fig. 38a and b) but only few B cells (Fig. 39c). Additional analyses, however, are required to determine which sub-populations were stained and to which extent the staining pattern would be in accordance with naïve or memory lymphocyte subsets such as in humans.

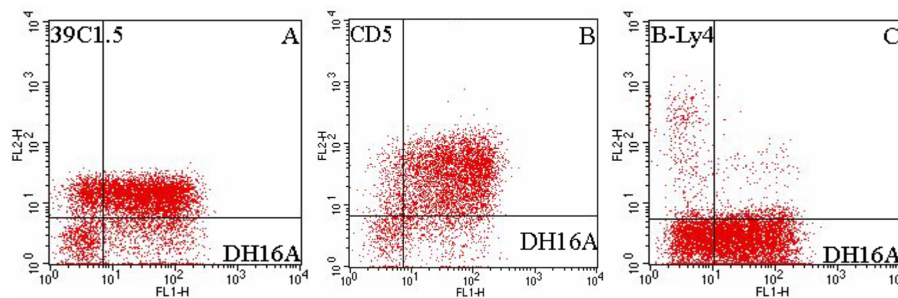


**Fig. 39 Two colour flow cytometry analysis of anti-CD45RB mAb DH16A and anti-human CD2 mAb 39C1.5, anti-equine CD5 HT23A and anti-human CD21- B-ly4.**
39C1.5 (CD2) and HT23A (CD5) detected T cells (A&B) while B-Ly4 stained B cells (C) demonstrating that most (but not all) T cells were positive for DH16A, while only few (but some) B cells were also stained, indicating that anti-CD45RB mAb DH16A detected the equine homologue.

In single colour flow cytometric analysis (Fig. 20) the mAb M1/70.15.11.5 directed against mouse and human CD11b stained only a subpopulation of equine lymphocytes (next to myeloid cells), which were assumed to be NK cells. Two-colour flow cytometry revealed that the CD11b$^{+}$ cells were indeed neither T (Fig. 40a and d) nor B (Fig. 40b) lymphocytes but co-stained weakly with CD8 (Fig. 40c) a feature that also known for human NK cells.

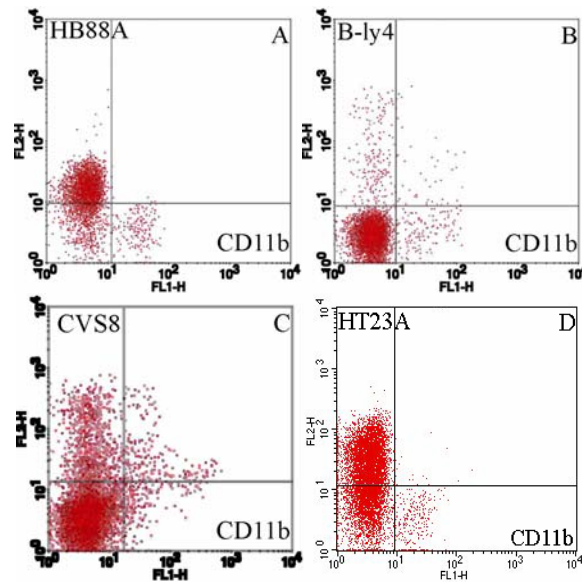


**Fig. 40 Two colour flow cytometry, analysing the expression of CD11b on equine lymphoid cells.**
CD11b was detected using the clone M1/70.15.11.5, directed against mouse CD11b. This marker was not expressed on T cells (A&D) or B cells (B) but on a population of CD8$^{+}$ cells (C), which likely resemble NK cells.

The human CD83-specific mAb HB15a detected B cells only (Fig. 41), and this result supports the previous designation as positively cross-reacting.

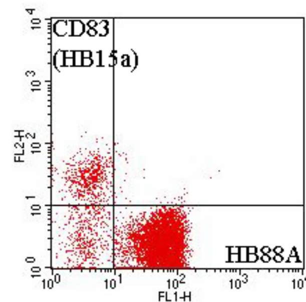


**Fig. 41 Two colour flow cytometry of anti-human CD83 mAb HB15a with anti-equine CD2 mAb HB88A.**
HB88A stained T cells resolving that CD83 clearly stains B cells.

**Table 10: Two colour flow cytometry results.**

| Test mAb CD (clone) | Reference mAb CD (clone) | Result | Conclusion* |
|---|---|---|---|
| **T cell section** | | | |
| CD2 (HB88A) | CD5 (HT23A) | T cells only | positive |
| CD2 (39C1.5) | CD2 (HB88A) | T cells only | positive |
| CD2 (39C1.5) | CD4 (CVS4) | CD4 pos. and neg. cells | positive |
| CD2 (39C1.5) | CD8 (CVS8) | CD8 pos. and neg. cells | positive |
| CD3 (OKT3) | CD2 (39C1.5) | subpopulations of T and B cells | alternate |
| **pan-leukocyte section** | | | |
| CD11a (HUH73A) | CD5 (HT23A) | all lymphocytes stained | positive |
| CD18 (H20A) | CD5 (HT23A) | all lymphocytes stained | positive |
| CD18 (HUH82A) | CD5 (HT23A) | all lymphocytes stained | positive |
| CD44 (BAG40A) | CD5 (HT23A) | all lymphocytes stained | positive |
| CD44 (H22A) | CD5 (HT23A) | all lymphocytes stained | positive |
| CD45R | CD5 (HT23A) | all lymphocytes stained | positive\|questionable |
| CD45R | CD21 (B-Ly4) | all lymphocytes stained | positive\|questionable |
| CD45RA | CD5 (HT23A) | T cells variably positive | questionable |
| CD45RA | CD21 (B-Ly4) | B cells weakly | questionable |
| CD45RB (DH16A) | CD2 (39C1.5) | most T cells positive | positive |
| CD45RB (DH16A) | CD5 (HT23A) | most T cells positive | positive |
| CD45RB (DH16A) | CD21 (B-Ly4) | most B cells negative | positive |
| CD49d (HP2/1) | CD5 (HT23A) | most leukocytes stained | positive |
| **B Cell section** | | | |
| CD10 (4F9) | CD21 (B-Ly4) | most B cells weak plus some T cells | alternate |
| CD19 (LT19) | CD5 (HT23A) | subpopulation of B and T cells | alternate |
| CD20 (LT20) | CD2 (HB88A) | subpopulation of T cells | alternate |
| CD21 (B-Ly4) | CD2 (HB88A) | non-T cells only | positive |
| CD21 (B-Ly4) | CD5 (HT23A) | non-T cells only | positive |
| CD21 (BL13) | CD21 (B-Ly4) | subpopulation of B cells | alternate |
| CD83 (HB15A) | CD2 (HB88A) | non-T cells only | positive |
| Canine B (CA2.1D6) | CD2 (HB88A) | non-T cells only | positive |
| Canine B (CA2.1D6) | CD5 (HT23A) | non-T cells only | positive |
| **miscellaneous** | | | |
| CD11b (M1-70.15.11.5) | CD2 (HB88A) | no-T cells positive | positive |
| CD11b (M1-70.15.11.5) | CD5 (HT23A) | no-T cells positive | Positive |
| CD11b (M1-70.15.11.5) | CD8 (CVS8) | some $CD8^+$ cells/$CD11b^+$ | Positive |
| CD11b (M1-70.15.11.5) | CD21 (B-Ly4) | no B cells positive | Positive |

The nomenclature was used according to the reports on the single colour flow cytometric staining;
"positive" indicates a result consistent with human staining pattern described (in other tables [6&8] used as "++")
"alternate" refers to an expression pattern different from that expected from humans
"questionable" refers to a staining that cannot be further designated at present.
*The conclusion refers to the test mAb clone.

**4.1.3 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 that were detected to cross-react and directed primarily against human leukocyte surface antigens using flow cytometry as described above. eCAS was supposed to be a myeloid cell line derived from bone marrow of a 10 year old warm blood 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.

A panel of forty mAbs (Table 11), included all mAbs identified in previous parts (4.1.1.1, 4.1.1.2, and 4.1.2) to be positive with horse leukocytes, was analyzed for reactivity with eCAS cells. This panel included mAbs that were specific myeloid cell markers like CD14, CD68, CD163, CD172a, and CD206 (MMR). In addition, mAbs directed against the progenitor stem cell marker, CD34, were included.

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). Further three 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 also that eCAS (unlike PBMC) showed non-specific binding when mouse IgG2a mAbs were applied. For this reason, five results remain questionable (mAbs HUH82A [CD18], H22A [CD44], LT41A [CD18], H42A [MHCII], and H58A [MHCI]). Surprisingly, none of the myeloid-specific markers, including CD14 or MHCII were detected positive on eCAS cells.

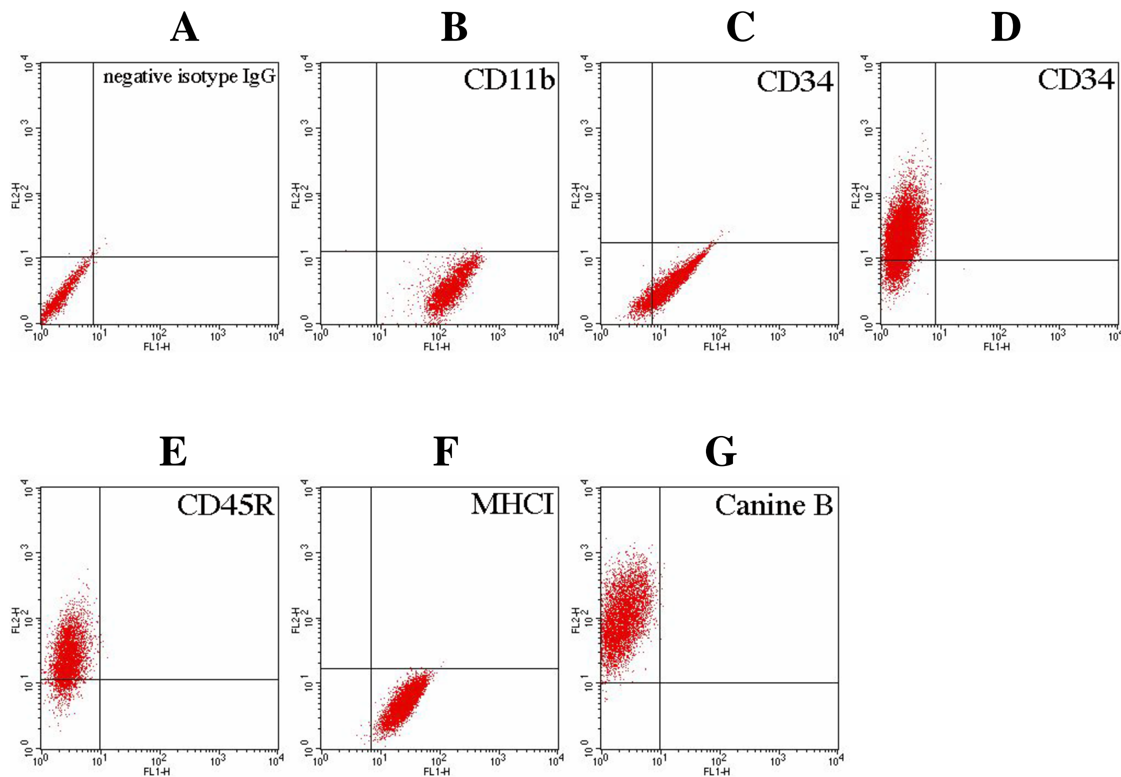


**Fig. 42 Analysis of eCAS cells by flow cytometry, using cross-reactive mAbs.**
eCAS cells expressed only few of the antigens detected by the mAbs used in this study. For IgG2a mAbs, a certain unspecific binding was detected and the setting adopted to this problem (A). The cells reacted positive using mAbs against CD11b clone M1/70.15.11.5 (B), CD34, clone 4H11(C), CD34, clone AC136 (D), CD45R, clone RA3-6B2 (E), anti-MHCI, clone B9.12.1(F) and anti-Canine B cells, clone CA2.1D6 (G). FL-1 refers to FITC and FL-2 refers to PE label (for further details see table 11).

**Table 11 Analysis of eCAS using HLDA-8 and other commercially available mAbs**

| WsNo. | Donator | CD No., specificity | Clone | Ig | Result * |
|---|---|---|---|---|---|
| | VMRD | EqCD2 | HB88A | mIgG1 | - |
| 7 | Coulter | huCD2 | 39C1.5 | ratIgG2a | - |
| | VMRD | EqCD5 | HT23A | mIgG1 | - |
| 17 | Bill Davis | CD11a | HUH73A | mIgG1 | - |
| | MiltenyiBiotech | muCD11b | M1/70.15.11.5 | mIgG2b | ++ |
| | Biometec | huCD14 | big 11 | mIgG1 | - |
| | Biometec | huCD14 | 7H3 (big 10) | mIgG1 | - |
| | Biometec | huCD14 | big 12 | mIgG1 | - |
| | Biometec | huCD14 | 7D6 (big 13) | mIgG1 | - |
| 1 | Bill Davis | CD18 | BAQ30A | mIgG1 | - |
| 7 | Bill Davis | CD18 | H20A | mIgG1 | - |
| 22 | Bill Davis | CD18 | HUH82A | mIgG2a | ? |
| 248 | Dako | huCD18 | MHM23 | mIgG1 | - |
| | BD Pharmingen | huCD21 | B-Ly4 | mIgG1 | W/? |
| 203 | Coulter | huCD34 | IMMU133 | mIgG1 | - |
| 267 | Dako | huCD34 | BI-3C5 | mIgG1 | - |
| 268 | Dako | huCD34 | BIRMA-K3 | mIgG1 | - |
| 270 | Dako | huCD34 | TÜK3 | mIgG3 | - |
| 202 | Coulter | huCD34 | 581 | mIgG1 | W/? |
| | ImmunoTools | huCD34 | 4H11 | mIgG1 | ++ |
| | ImmunoTools | huCD34 | QBEnd10 | mIgG1 | W/? |
| | MiltenyiBiotech | huCD34 | AC136 | mIgG2a | ++ |
| 6 | Bill Davis | CD44 | BAG40A | mIgG3 | - |
| 8 | Bill Davis | CD44 | H22A | mIgG2a | ? |
| 25 | Bill Davis | CD44 | LT41A | mIgG2a | ? |
| 4 | Bill Davis | CD45RB | DH16A | mIgM | - |
| | MiltenyiBiotech | muCD45R (B220) | RA3-6B2 | ratIgG2a | ++ |
| 207 | Coulter | huCD49d | HP2/1 | mIgG1 | - |
| | Serotec | huCD68 | Ki-M6 | mIgG1 | - |
| | Coulter | huCD83 | HB15a | mIgG2b | - |
| 318 | Dako | huCD91 | A2MRa-2 | mIgG1 | - |
| 330 | Dako | huCD163 | Ber-MAC3 | mIgG1 | - |
| 5 | Bill Davis | CD172a | DH59B | mIgG1 | - |
| | Coulter | huCD 206 | 3.29B1.10 | mIgG1 | - |
| | Coulter | huHLA- ABC | B9.12.1 | mIgG2a | ++ |
| | VMRD | EqMHCII | EqT2 | mIgG1 | - |
| | VMRD | EqMHCI | H58A | mIgG2a | ? |
| | Serotec | huHLA-DR | D-F1 | mIgG2a | ? |
| | VMRD | EqMHCII | H42A | mIgG2a | ? |
| | Serotec | B cells (Canine) | CA2.1D6 | mIgG1 | ++ |

**Legend to table 11:**

* The results were classified according to the schedule of the HLDA8 section of this study. "++" is strong positive, "W" is a weak, "?" a questionable staining, "-" is a negative result.

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 that reacted positively with equine PBMC using flow cytometry were analyzed for their reactivity with EqT8888 cells (Table 12). This panel included mAbs directed against lymphoid cell markers like CD2, CD4, CD5, CD8 and CD21. Additionally, three mAbs that are specific for the human CD34 antigen were also tested on these cells. Eleven of the mAbs showed a staining pattern that was considered specific and designated 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). The results 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 (Hormanski et al 1992). CD44 was also expressed, which is not surprising as variants of CD44 have important functions in metastizing tumours, such as lymphomas (Drillenburg and Pals, 2000). EqT8888 cells were also labelled by mAbs which identified CD21 and CD83 (Fig. 43b and e). Both markers were clearly expressed, although weakly and B-Ly4 (CD21) in most cases stained a population of the cells only, nevertheless suggesting a B cell origin of this cell line.

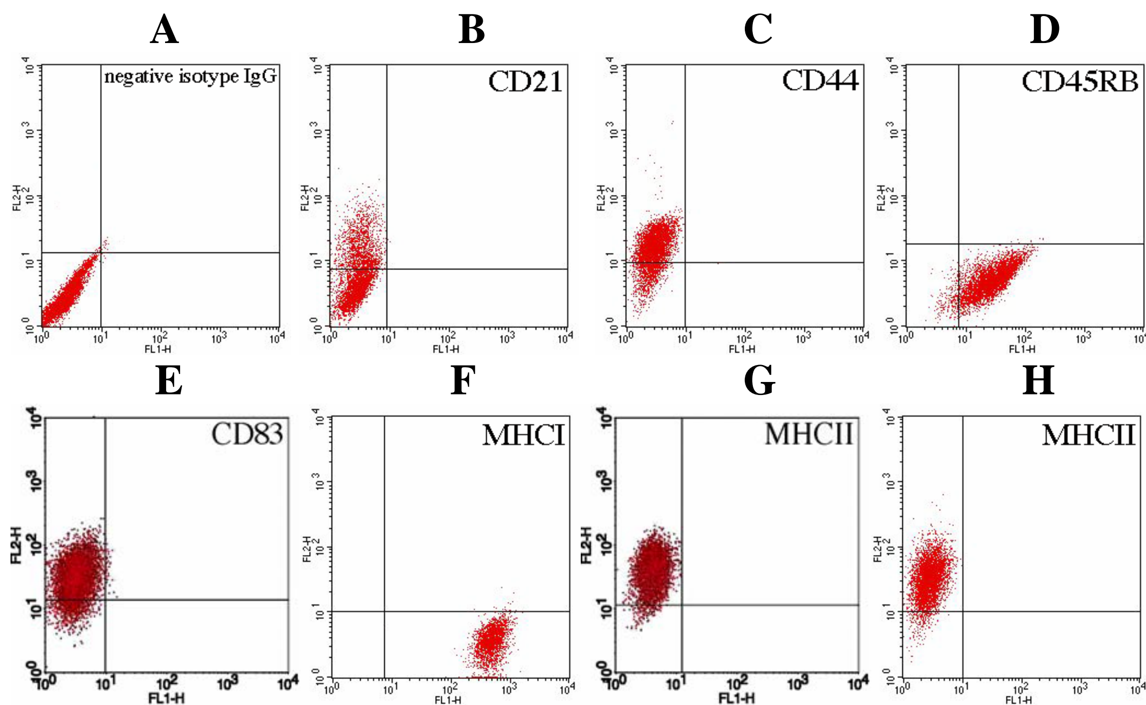


**Fig. 43 Analysis of EqT8888 cells by flow cytometry, using mAbs cross reactive with equine leukocytes.** EqT8888 cells express some of the antigens detected by the mAbs used in this study. The negative control is displayed in (A). At least a population of the cells reacted constantly positive using mAbs against human CD21 clone B-Ly4 (B). Additionally, EqT8888 reacted positive using mAbs against CD44 with clone H22A (C) displayed as example, CD45RB, clone DH16A (D), CD83, clone HB15a (E), MHC class I, clone B9.12.1 (F), MHC class II, clones EqT2 (G) and DF1(H). FL-1 refers to FITC and FL-2 refers to PE as fluorochromes.

**Table 12 Analysis of EqT8888 using HLDA8 and other commercially available mAbs**

| WsNo. | Donator | CD No., specificity | Clone | Ig | Result* |
|---|---|---|---|---|---|
| | VMRD | EqCD2 | HB88A | mIgG1 | - |
| 178 | Coulter | huCD2 | 39C1.5 | rat IgG2a | - |
| | VMRD | EqCD5 | HT23A | mIgG1 | - |
| 17 | Bill Davis | CD11a | HUH73A | mIgG1 | - |
| | MiltenyiBiotech | muCD11b | M1/70.15.11.5 | mIgG2b | - |
| | Biometec | huCD14 | big 11 | mIgG1 | - |
| 1 | Bill Davis | CD18 | BAQ30A | mIgG1 | - |
| 7 | Bill Davis | CD18 | H20A | mIgG1 | - |
| 22 | Bill Davis | CD18 | HUH82A | mIgG2a | - |
| 248 | Dako | huCD18 | MHM23 | mIgG1 | - |
| | BD | huCD21 | B-Ly4 | mIgG1 | ++ |
| | ImmunoTools | huCD34 | 4H11 | mIgG1 | - |
| | Coulter | huCD34 | 581 | mIgG1 | - |
| | MiltenyiBiotech | huCD34 | AC136 | mIgG2a | - |
| 6 | Bill Davis | CD44 | BAG40A | mIgG3 | ++ |
| 8 | Bill Davis | CD44 | H22A | mIgG2a | ++ |
| 25 | Bill Davis | CD44 | LT41A | mIgG2a | ++ |
| | MiltenyiBiotech | muCD45R | RA3-6B2 | ratIgG2a | - |
| 4 | Bill Davis | CD45RB | DH16A | mIgM | ++ |
| 207 | Coulter | huCD49d | HP2/1 | mIgG1 | W/? |
| | Coulter | huCD83 | HB15a | mIgG2b | ++ |
| 318 | Dako | huCD91 | A2MRa-2 | mIgG1 | - |
| 330 | Dako | huCD163 | Ber-MAC3 | mIgG1 | - |
| 5 | Bill Davis | CD172a | DH59B | mIgG1 | - |
| | Coulter | huHLA- ABC | B9.12.1 | mIgG2a | ++ |
| | VMRD | EqMHCI | H58A | mIgG2a | ++ |
| | VMRD | EqMHCII | EqT2 | mIgG1 | ++ |
| | VMRD | EqMHCII | H42A | mIgG2a | ++ |
| | Serotec | huHLA-DR | D-F1 | mIgG2a | ++ |
| | Serotec | canine B | CA2.1D6 | mIgG1 | ? |

**Legend to table 12:**
*The results were classified according to the schedule of the HLDA8 section of this study.
"++" is strong positive, "W" is a weak, "?" a questionable staining, "-" is a negative result.

### 4.1.4 Estimation of the molecular weight of candidate CD molecules using immunoprecipitation

Leukocyte expression pattern of equine CD molecules detected by cross-reacting human mAbs was analyzed using one and two colour flow cytometry (4.1.1 and 4.1.2). Immunoprecipitation was used here to add valuable information about the molecules detected. Surface molecules from equine leukocytes or equine cell lines were biotinylated prior to precipitation with candidate mAbs to determine the molecular weight (MW) of the corresponding molecules in a western blot using streptavidin-AP and chemilumenescence detection (3.2.17). Separation of immunoprecipitated proteins was performed under reducing conditions. The mAb B9.12.1 specific against MHCI, the highly expressed and most

conserved antigen, was used to optimise the technique in first applications. 22 out of 27 mAbs (Table 13) precipitated the corresponding molecules with a MW in accordance with its human homologue. Positive mAbs precipitating the right molecular weight were directed against CD2, CD5, CD11a, CD11b, CD14, CD18, CD21, CD34, CD44, CD83, CD91, CD172a, MHCI, and MHCII. In the cases of mAbs against CD21 (B-Ly4) and CD34 (AC136), IP was followed by western blot reapplying the same mAbs to enhance the signal. MAbs which were expressed internally like CD68 and mAbs of IgM class could not be included into this approach. Five mAbs (Table 13) directed against CD34 (4H11), CD45R (RA3-6B2), CD49d (HP2/1), CD163 (Ber-MAC3), and CD206 (3.29B1.10) failed to immuno-precipitate the corresponding CD molecule. This could be due to low affinity of these mAbs to target antigens (as in case of HP2/1, Ber-MAC3, and 3.29B1.10) or low affinity of rat mAbs Fc portion to Protein G (as in case of RA3-6B2) or to the low expression level of antigen (as for CD34). Results of immunoprecipitation (IP) are summarized in table 13 and additional examples of the immuno-blots are provided.

*Immunoprecipitation of T cell markers, CD2 and CD5:*

CD2 (LFA-2) is a single chain type I transmembrane molecule consisting of 351 amino acids. This co-stimulatory molecule is an approximately 60 kDa glycoprotein member of the Ig superfamily principally expressed by T and NK cells (Meuer et al., 1984; Zarling et al., 1981). Anti-human CD2 mAb 39C1.5 and the reference anti-equine CD2 mAb HB88A precipitated a protein of approximately 65 kDa (Fig. 44) from surface biotinylated equine PBMC leaving little doubt that anti-human mAb 39C1.5 reacted with the equine homologue of CD2. The reference anti-equine CD5 mAb HT23A stained equine T cells in one and two colour flow cytometry (Fig. 19 and 34). HT23A was reported to precipitate equine CD5 as a 69 kDa protein (Crump et al., 1988; Kydd et al., 1994). The same approximate molecular weight was determined here (Fig. 44).

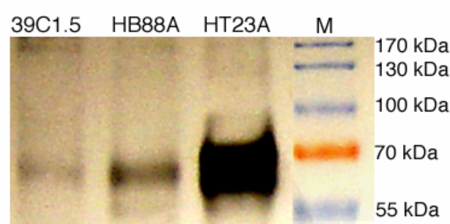


**Fig. 44 Immunoprecipitation of T cell markers, CD2 and CD5.**
Anti-human CD2 clone 39C1.5 and anti-equine CD2 clone HB88A immunoprecipitated a band of approximately 65 kDa from surface biotinylated equine PBMC. Anti-equine CD5 clone HT23A immunoprecipitated a band of approximately 69 kDa.

*Immunoprecipitation of the B cell markers, CD21 and CD83:*

Human CD21, the complement receptor type 2 (CR2), has an approximate molecular weight of 145 kDa under non-reducing conditions and 110 kDa under reducing conditions (Timens, 2002). B-Ly4 immunopreciptated equine CD21 as a band of approximately 125 kDa (Fig. 45a) from EqT8888 cells. A lower band of approximately 95 kDa molecular weight was co-precipitated. The co-precipitated protein could resemble CD19 which is a 95 kDa glycoprotein non-covalently linked to CD21 on the surface of B cells (Tedder et al., 1994; Tedder et al., 1997; Sato and Tedder, 2002). Anti-human CD83 clone HB15a immunoprecipitated a band of approximately 45 kDa (Fig. 45b) from surface biotinylated EqT8888 cells. The obtained molecular weight is analogues to that of human CD83.

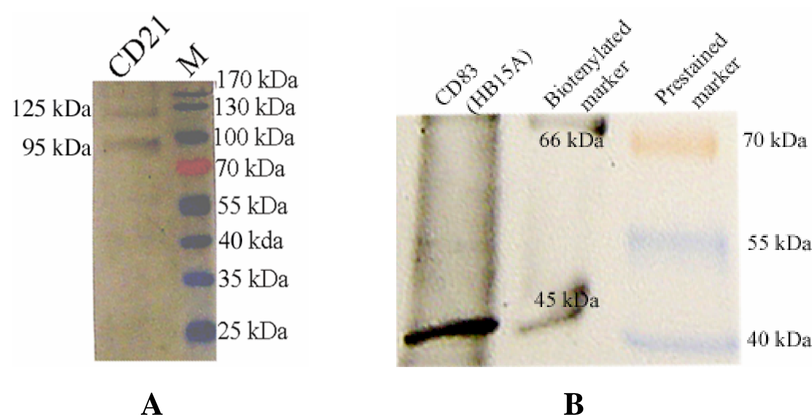


**Fig. 45 Immunoprecipitation of B cell markers, CD21 and CD83.**
Immunoprecipitation of CD21 from biotinylated EqT8888 cells (Fig. 45a) using anti-human CD21 mAb (B-Ly4) followed by membrane blotting and incubation with the same mAb. The equine homologue of CD21 was immunoprecipitated under reducing conditions as a band of approximately 125 kDa with a lower band of approximately 95 kDa co-precipitated. Anti-human CD83 clone HB15a immunoprecipitated a band of approximately 45 kDa (Fig. 45b) from biotinylated equine T8888 cell line. A biotinylated molecular weight marker was used besides the prestained one to confirm the molecular weight of CD83.

*Immunoprecipitation of myeloid cell markers, CD14, CD91 and CD172a:*

Four mAbs directed against human CD14 (clones 7H3 [big10], big11, big12, and 7D6 [big13]) reacted strongly with equine monocytes in flow cytometry (Fig. 21b, 22b, 23b, and 24b respectively). All recognize the similar conformational epitope of aa9-13 and 39-44 (Schütt et al., 1995; http://www.biometec.de). Clone 7H3 (big10) was selected to precipitate equine CD14. A single protein of approximately 55 kDa (Fig. 46a) was precipitated. The obtained molecular weight was analogous to that of human CD14, clearly indicating the detection of the equine homologue.

The myeloid marker CD91 in humans has a β subunit of approximately 85 kDa. The extracellular region binds non-covalently to the 515 kDa α-subunit (Moestrup, 2002). The

mAb A2MRα-2 against human CD91 imunoprecipitated a protein of approximately 100 kDa (Fig. 46b) from surface biotinylated equine PBMC likely representing the β-chain of equine CD91.

Signal-regulatory proteins (SIRP) are glycosylated, Ig-like cell surface receptors expressed on myeloid and neural cells. Human CD172a (SIRPα protein) is approximately 110 kDa in molecular weight (Seiffert et al., 1999; Seiffert et al., 2001; Simmons and Vernon-Wilson, 2002). The human mAb clone DH59B precipitated equine CD172a as a band of approximately 110 kDa (Fig. 46c).

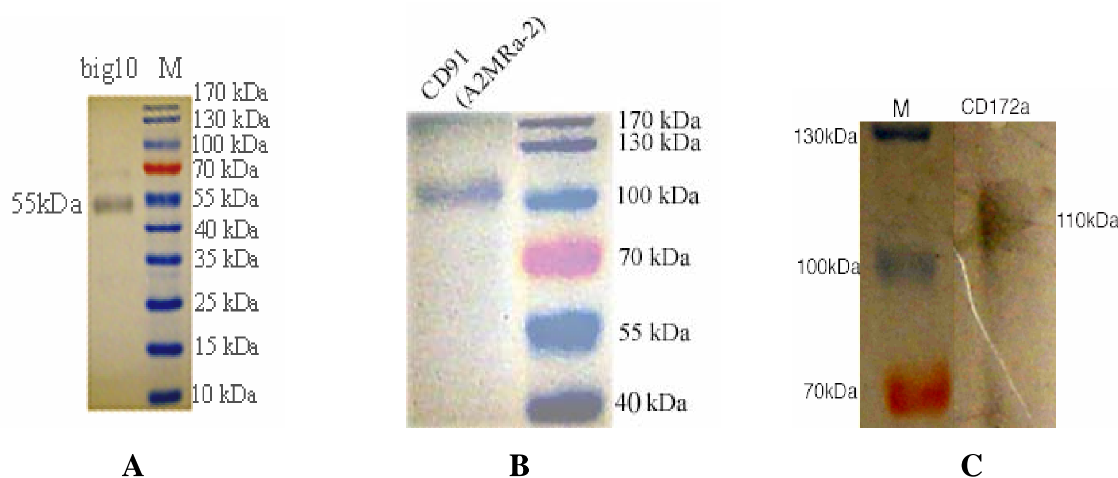


**Fig. 46 Immunoprecipitation of myeloid cell markers, CD14, CD91, and CD172a.**
Anti-human CD14 clone 7H3 (big10) immunoprecipitated a single protein of approximately 55kDa from surface biotinylated equine monocytes demonstrating the detection of the equine homologue of CD14. In fig. 46b, anti-human CD91 mAb clone A2MRα-2 imunoprecipitated a protein of approximately 100 kDa from biotinylated equine PBMC indicating immunoprecipitation of the β-chain of CD91 equine homologue. Fig. 46c represents immunoprecipitation of equine CD172a from biotinylated equine leukocytes (using mAb DH59B) as a protein of approximately 110 kDa molecular weight.

*Immunoprecipitation of leukocyte integrins, CD11a, CD11b, CD18, and CD44.*

The CD11a/CD18 (LFA-1) antigen is a member of the β2 integrin family and functions as a cellular adhesion molecule (Kishimoto et al., 1987). MAbs, H20A, HUH82A, and MHM23 stained all leukocytes clearly in flow cytometry and precipitated two polypeptides of approximately 180 and 100 kDa (Fig. 47a) from biotinylated equine leukocytes under reducing conditions. The 180 kDa polypeptide indicates co-immunoprecipitation of equine LFA-1 α-subunit (CD11a) and the 100 kDa polypeptide represents the β-subunit of equine LFA-1 (CD18). Using mAb BAQ30A, lymphocytes were negative and both monocytes and granulocytes were positive in flow cytometry (Fig. 9). BAQ30A predominantly immunoprecipitated one polypeptide of approximately 90 kDa (Fig. 47a) indicating precipitation of the truncated, CD11a free form, of CD18, as described before for human

CD18 (Drbal et al. 2001). One mAb against LFA-1 α-subunit (CD11a) predominantly immunoprecipitated one polypeptide of about 180 kDa representing equine CD11a.

CD11b (MAC-1) is a 170 kDa integrin α-chain protein expressed as a non-covalently linked heterodimer with CD18 (Hogg, 2002a). Anti-human CD11b clone M1/70.15.11.5 clearly immunoprecipitated two proteins from biotinylated equine leukocytes (Fig. 47b). The upper band of approximately 170 kDa represented CD11b while the lower one, 100 kDa, indicated co-immunoprecipitation of equine CD18.

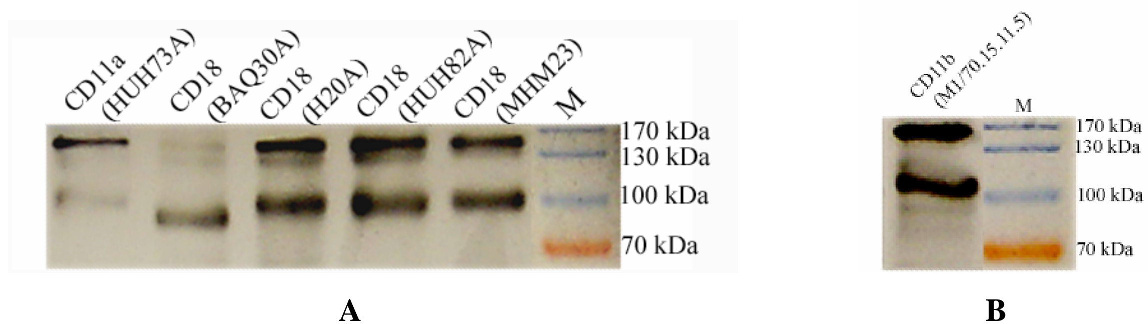


**Fig. 47 Immunoprecipitation of leukocyte integrins, CD11a, CD11b, and CD18.**
MAbs MHM23A against human CD18, HUH82A against bovine CD18 and H20A against human CD18 cross-reacted with equine LFA-1 (CD11a/CD18 heterodimer), immunoprecipitating two polypeptides of about 180 and 100 kDa from biotinylated equine leukocytes under reducing conditions. 180 kDa polypeptide represented the α-subunit of equine LFA-1 (CD11a) and a 100 kDa polypeptide represented the β-subunit of equine LFA-1 (CD18). MAb BAQ30A, against bovine CD18, immunoprecipitated clearly one polypeptide of 90 kDa. MAb HUH73A, against LFA-1 α-subunit (CD11a), predominantly immunoprecipitated one polypeptide of about180 kDa representing equine CD11a. In fig. 47b, anti-human CD11b clone M1/70.15.11.5 immunoprecipitated two proteins from biotinylated equine leukocytes. The upper band of approximately 170 kDa representing CD11b and the lower one of about 100 kDa represented equine CD18.

CD44, a glycosylated transmembrane protein of 65-100 kDa, is widely expressed on the surface of most cell types (Stoll et al., 1989). Three mAbs (BAG40A [WS no 6], H22A [WS no 8], LT41A [WS no 25]) were directed against CD44. All of the three clones precipitated a molecule of approximately 100 kDa from biotinylated equine leukocytes (Fig. 48).

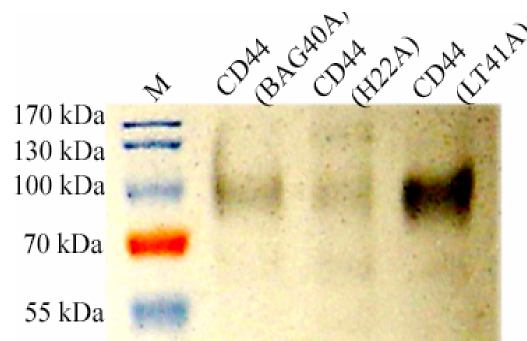


**Fig.48 Immunoprecipitation of equine CD44.**
IP of glycosylated CD44 from surface biotinylated equine leukocytes with three mAbs against CD44 (BAG40A, H22A and LT41A) resulted in one single band of approximately 100 kDa.

*Immunoprecipitation of CD34:*

CD34 is a surface glycophosphoprotein expressed on early hematopoietic 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 HSC (Civin, et al., 1996; Handgretinger, et al., 1999; Burt, 1999). Human CD34 is a heavily glycosylated type I transmembrane protein of approximately 116 kDa molecular weight (Krause et al., 1993). Although the relative mobility of mouse CD34 varies slightly depending on the cell type in which it is expressed, the approximate molecular weight of mouse CD34 is 110 kDa (Krause et al., 1994). AC136 precipitated a molecule of approximately 100 kDa from biotinylated eCAS cells (Fig. 49) underlining the potential use of this mAb to detect the equine homologue of CD34. Immunoprecipitated CD34 was membrane blotted and incubated with the same mAb (AC136) followed by incubation with AP-conjugated secondary antibody to enhance signal resolution.

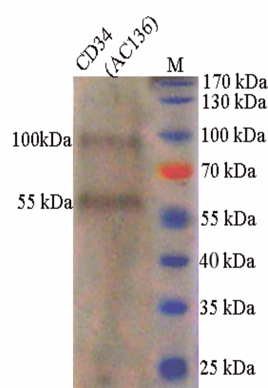


**Fig.49 Immunoprecipitation of CD34.**
Equine CD34 was immunoprecipitated using anti-human CD34 clone AC136. Immunopreciptated proteins were blotted and incubated with the same mAb (AC136) followed by incubation with AP-conjugated secondary antibody to enhance signal resolution. CD34 was precipitated as a band of approximately 100 kDa indicating detection of CD34 equine homologue. One lower band of approximately 55 kDa resembled the Ig heavy chain of AC136 detected with AP-conjugated anti-mouse.

*Immunoprecipitaion of equine MHCI and MHCII:*

EqMHC class I molecules are composed of a 44 kDa transmembrane heavy chain and a 14kDa non-covalently linked light chain termed beta 2 microglobulin ($\beta_2$M) (Antczak, 1992; Kydd et al., 1994). EqMHC class II molecules are composed of two non-covalently linked polypeptide chains: $\alpha$ (31-34 kDa), and $\beta$ (26-28 kDa) (Kydd et al., 1994). Anti-MHCI mAbs B9.12.1 and H58A (Fig. 50a) precipitated two bands of approximately 45 kDa (representing the MHCI heavy chain) and 12 kDa (representing $\beta_2$M) from EqT8888 cells. Anti-MHCII

mAbs EqT2 and H42A (Fig. 50b) precipitated alpha (approximately 33 kDa) and beta (approximately 28 kDa) polypeptides of equine MHCII from EqT8888.

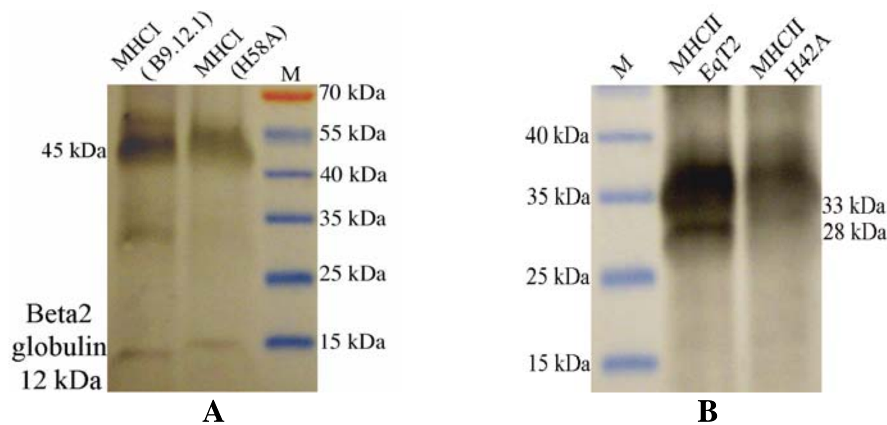


**Fig. 50 Immunoprecipitation of equine MHCI and MHCII.**
Immunoprecipitation of MHCI from biotinylated EqT8888 cell line using two clones B9.12.and H58A. Both mAbs precipitated the glycosylated polypeptide of MHCI (approximately 45kDa) and the non-covalently attached $\beta_2$ globulin which was 12 kDa (Fig. 50a). Immunoprecipitation of MHCII from biotinylated EqT8888 cell line (Fig. 50b) using clones EqT2 and H42A. Both mAbs immunoprecipitated alpha (approximately 33 kDa) and beta (approximately 28 kDa) polypeptides.

**Table 13 Immunoprecipitation of candidate CD molecules from biotinylated horse leukocytes and equine cell lines.**

| WS | Target CD | Clone | IP | Approx. Mwt. | Cells used | Comment |
|---|---|---|---|---|---|---|
| 178 | huCD2 | 39C1.5 | + | 65 | equine PBMC | |
| | EqCD2 | HB88A | + | 65 | equine PBMC | |
| | EqCD5 | HT23A | + | 69 | equine PBMC | |
| 17 | huCD11a | HUH73A | + | 180 | equine leukocytes | |
| | muCD11b | M1/70.15.11.5 | + | 170 | equine leukocytes | |
| | huCD14 | 7H3(big10) | + | 55 | Monocytes | |
| 1 | CD18 | BAQ30A | + | 90 | equine leukocytes | Truncated CD11 free form of EqCD18 |
| 7 | CD18 | H20A | + | 100 | equine leukocytes | |
| 22 | CD18 | HUH82A | + | 100 | equine leukocytes | |
| 248 | huCD18 | MHM23 | + | 100 | equine leukocytes | |
| | huCD21 | B-Ly4 | + | 100 | EqT8888 | detected by IP/WB |
| | CD34 | AC136 | + | 100 | eCAS | detected by IP/WB |
| | CD34 | 4H11 | - | - | eCAS | |
| 6 | CD44 | BAG40A | + | 100 | equine leukocytes | |
| 8 | CD44 | H22A | + | 100 | equine leukocytes | |
| 25 | CD44 | LT41A | + | 100 | equine leukocytes | |
| | huCD49d | HP2/1 | - | - | equine leukocytes | |
| | huCD83 | HB15a | + | 45 | equine PBMC | |
| | huCD91 | A2MRa-2 | + | 100 | Monocytes | |
| | huCD163 | Ber-MAC3 | - | - | equine monocytes | |
| 5 | CD172a | DH59B | + | 110 | equine leukocytes | |
| | huCD206(MMR) | 3.29B1.10 | - | - | equine MΦ | |
| | huHLA-ABC(MHCI) | B9.12.1 | + | 45 + 12 | EqT8888 | |
| | EqMHCI | H58A | + | 45 + 12 | EqT8888 | |
| | EqMHCII | EqT2 | + | 33 + 28 | EqT8888 | |
| | EqMHCII | H42A | + | 33 + 28 | EqT8888 | |

**Legend to table 12**

"+" indicates immunoprecipitation of the candidate equine homologue.

"-" indicates that the mAb failed to immunoprecipitate the candidate equine CD molecule.

"Approx. Mwt." indicates the obtained approximate molecular weight in kDa

"WS" workshop number in HLDA8.

"IP" immunoprecipitation result.