4.2 Cross-reactivity analysis of mAbs (defined positive with horse leukocytes) with wild equids using single colour flow cytometry

Domestic and wild equids belong to the family *Equida*e, Genus *Equus* where six subgenera emerged (Groves, 2002): *Equus*, *Asinus*, *Hemionus*, *Hippotigris*, *Quagga*, and *Dolichohippus*. The equid specialist group of IUCN recognised the African wild ass (*Equus africanus*) as a critically endangered species, the Grevy's zebra (*Equus grevyi*) and the mountain zebra (*Equus zebra*) as endangered (Moehlman, 2002). Trying to establish a leukocyte immune tool-box for wild equids, the principle of mAbs cross-reactivity analysis was applied here with the horse close relatives, selected wild equids. Most mAbs identified to react positive with horse leukocytes (4.1) were analyzed for cross-reactivity with wild equid leukocytes using one colour flow cytometry. Fresh blood samples ($n \geq 1$) were collected from Somali wild ass, Grevy's zebra, and Hartmann's mountain zebra. Leukocyte separation, immunofluorescence staining, and flow cytometric analyses were performed using the same protocols used for domestic horse (see 3.2.1, 3.2.3 and 3.2.4). Most but not all mAbs were found positive. 13 mAbs clearly cross reacted with all three species. Positive mAbs were directed against huCD2 (39C1.5), EqCD4 (CVS4), huCD5 (HT23A), huCD11a (HUH73A), huCD11b (M1/70.15.11.5), huCD18 (H20A and MHM23), huCD49d (HP2/1), huCD163 (Ber-MAC3), huCD172a (DH59B), huMHCI (B9.12.1), EqMHCII (EqT2) and anti-canine B cells (CA2.1D6). Eight mAbs against EqCD2 (HB88A), EqCD8 (CVS8), CD18 (BAQ30A and HUH82A), CD44 (BAG40A, H22A, and LT41A), and huCD91 (A2MRa-2) clearly cross-reacted only with Somali wild ass leukocytes. 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 with zebra species only and most of them were positive, except clone big11 which tested negative with Hartmann's zebra cells. Anti-huCD206 could be tested with Hartmann's zebra MΦ only but remained negative. Results of cross-reactivity with wild equids are summarized in table 14 and additional examples of dot blot analysis are provided.

Two different clones, 39C1.5 and HB88A, against human and equine CD2 were analyzed here. While 39C1.5 cross reacted with all three animal species (Fig. 51) HB88A, the reference mAb generated against horse CD2, cross-reacted only with Somali wild ass leukocytes (Fig. 52). Knowledge from two colour DL analysis of horse PBL indicated that both clones react with different epitopes of CD2 as both of them stained all horse PBL at the same time.

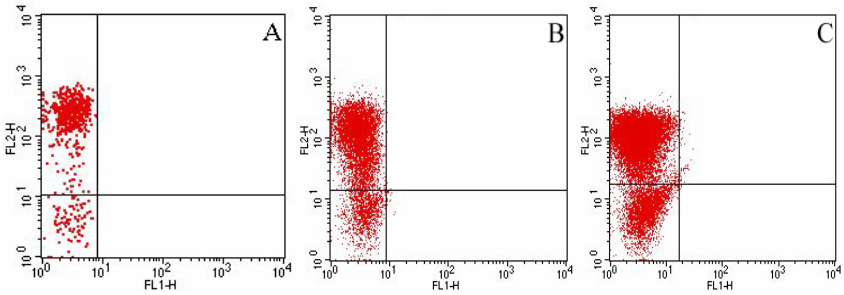

Fig. 51 Analysis of Somali wild ass (A), Grevy's zebra (B), and Hartmann's mountain zebra (C) PBL using anti-human CD2 clone 39C1.5.
39C1.5 detected the vast majority of lymphocytes of the three species. No staining of monocytes or granulocytes was detected (not shown). This mAb is a rat IgG2a isotype and was analyzed by indirect staining using a PE-labelled secondary antibody (FL2).

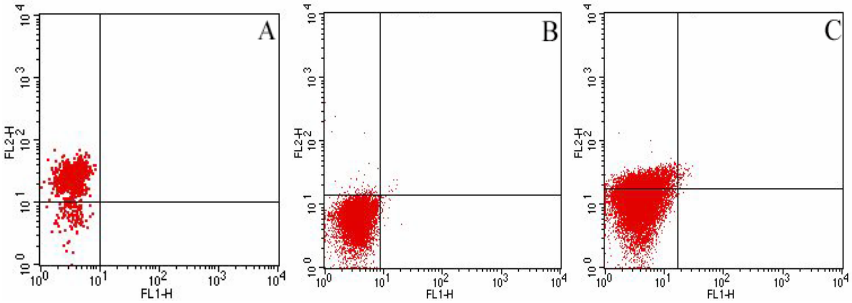

Fig. 52 Analysis of Somali wild ass (A), Grevy's zebra (B), and Hartmann's mountain zebra (C) PBL using anti-Equine CD2 clone HB88A.
HB88A stained the vast majority of Somali wild ass lymphocytes while no staining of Grevy's zebra lymphocytes (B) was detected. For Hartmann's mountain zebra it is likely a population shift but not clear positive staining (C). No staining pattern was observed for monocytes or granulocytes (not shown). HB88A was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

Three further markers of lymhocytes, CD4, CD5 and CD8 were analyzed. Clone CVS4 directed against equine CD4 reacted positive with PBL of the three species (Fig. 53). In case of CD5, clone HT23A detected the majority of lymphocytes in all three species, but the staining of Hartmann's zebra was rather weak (Fig. 54). For CD8 the situation was different, clone CVS8 detected only PBL of Somali wild ass, while no reactivity with zebra's PBL was obtained (Fig. 55).

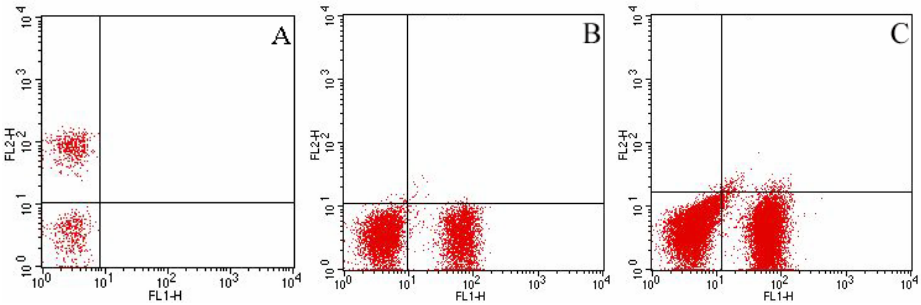

Fig. 53 Analysis of Somali wild ass (A), Grevy's zebra (B), and Hartmann's mountain zebra (C) PBL with anti-Equine CD4 clone CVS4.
CVS4 stained a population of lymphocytes of the three species clearly. No staining pattern was observed for monocytes or granulocytes (not shown). CVS4 was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2) in case of Somali wild ass, while in Hartmann's mountain zebra and Grevy's zebra it was stained using a FITC-labelled secondary antibody (FL-1).

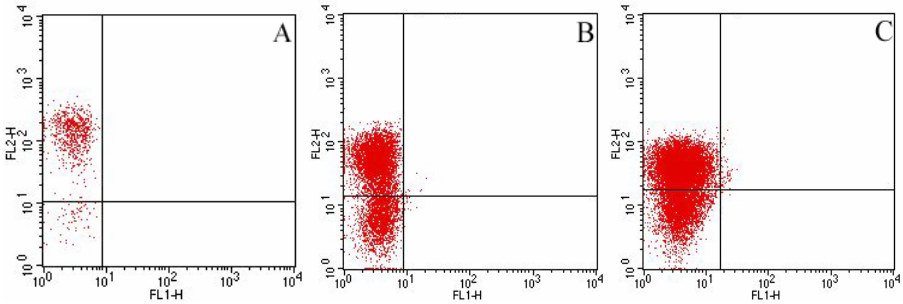

Fig. 54 Analysis of Somali wild ass (A), Grevy's zebra (B), and Hartmann's mountain zebra (C) PBL with anti-Equine CD5 clone HT23A.
HT23A stained the vast majority of Somali wild ass lymphocytes and most PBL of Grevy's and Hartmann's mountain zebras. No staining pattern was observed for monocytes or granulocytes (not shown). HT23A was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2)

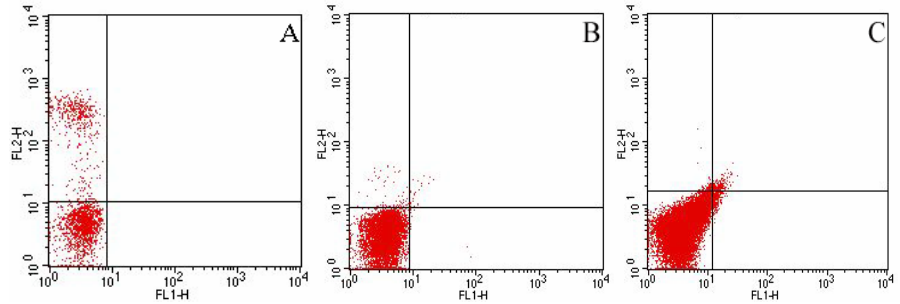

Fig. 55 Analysis of Somali wild ass (A), Grevy's zebra (B), and Hartmann's mountain zebra (C) PBL with anti-Equine CD8 clone CVS8.
CVS8 detected CD8 on Somali wild ass lymphocytes while Grevy's and Hartmann's zebra showed no cross-reactivity with CVS8. CVS8 was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

CD11a was expressed by almost all leukocytes of Somali wild ass as detected by HUH73A (Fig. 56). The analogous staining was observed with Grevy's and Hartmann's zebras (data not shown, table 14).

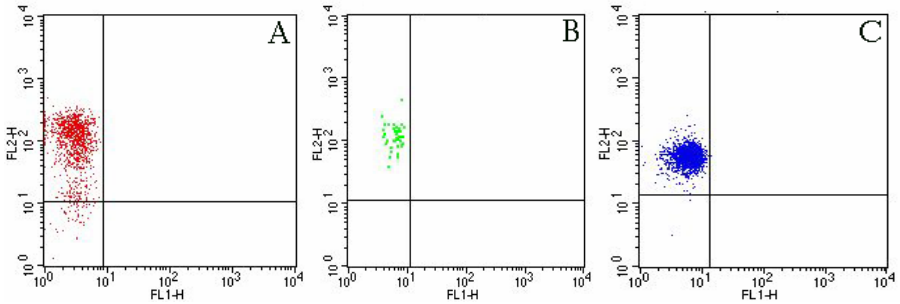

Fig. 56 Analysis of Somali wild ass leukocytes with anti-human CD11a clone HUH73A.
HUH73A stained all leukocytes of Somail wild ass as seen for lymphocytes (A), monocytes (B), and granulocytes (C). HUH73A was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

Anti-human CD11b clone M1/70.15.11.5 stained all monocytes and granulocytes of the three tested species. Additionally, a small population of lymphocytes, assumed to be NK cells, was stained (Fig. 57, table 14).

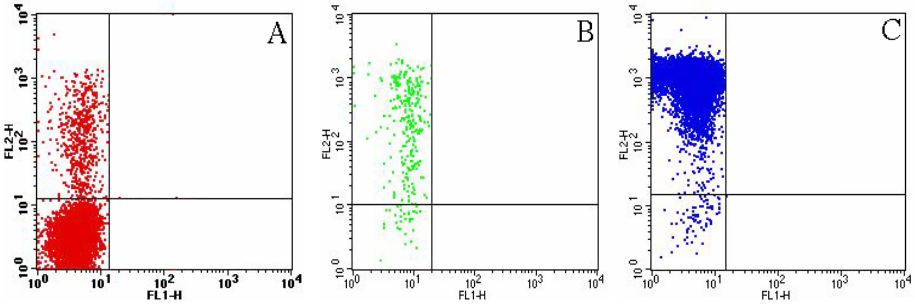

Fig. 57 Analysis of Grevy's zebra leukocytes with anti-human CD11b clone M1/70.15.11.5.
M1/70.15.11.5 stained all of monocytes and granulocytes of Grevy's zebra (B and C). A population of Lymphocytes assumed to be NK cells was stained (A). M1/70.15.11.5 was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

Four mAbs were directed against human CD14 (clones 7H3 [big10], big11, big12, and 7D6 [big13]). These clones stained horse monocytes brightly (Fig. 21- 24). All clones detected most of Grevy's zebra monocytes (Fig. 58a, b, c, and d respectively) indicating a conservation of this epitope in Grevy's zebra. In case of Hartmann's mountain zebra all clones except clone big 11 (Fig. 59b) stained monocytes which could be also due to a low affinity of this clone. Somali wild ass was not tested.

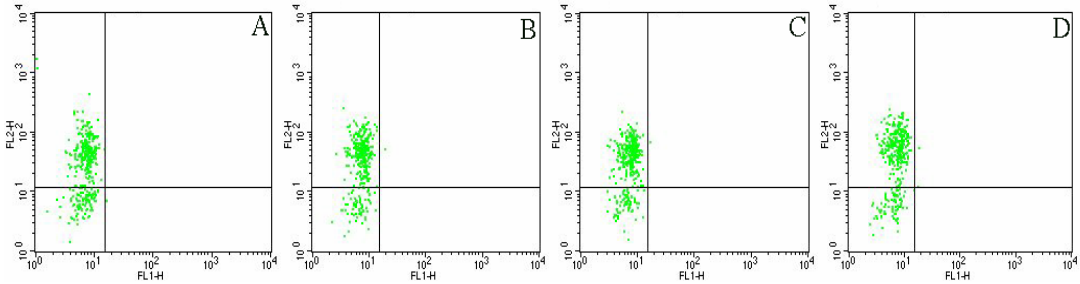

Fig. 58 Analysis of Grevy's zebra monocytes with anti-human CD14 clones big10 (A), big11 (B), big 12 (C), and big 13 (D).
All anti-human CD14 clones detected Grevy's zebra monocytes. Big10, 11, 12 and big13 were analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

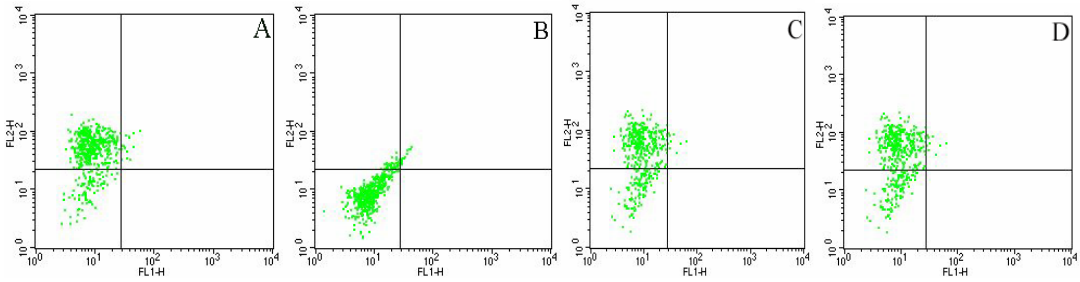

Fig. 59 Analysis of Hartmann's mountain zebra monocytes with anti-human CD14 clones big10 (A), big11 (B), big 12 (C), and big 13 (D).
All clones detected Hartmann's mountain zebra monocytes except clone big11 (B). All clones were analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

BAQ30A previously precipitated the truncated, CD11a un-associated form of CD18 in horse (Fig. 47a) with positive staining of monocytes and granulocytes while lymphocytes were not

stained in flow cytometry (Fig. 9). The same staining pattern was obtained with Somali wild ass (Fig. 60). In case of both zebra sp. granulocytes were stained positive (Fig. 60F and I) but monocytes showed little to no staining and lymphocytes were not stained (Fig. 60D and G). The staining pattern for Grevy's and Hartmann's zebras had to be classified weak or negative respectively (Table 14).

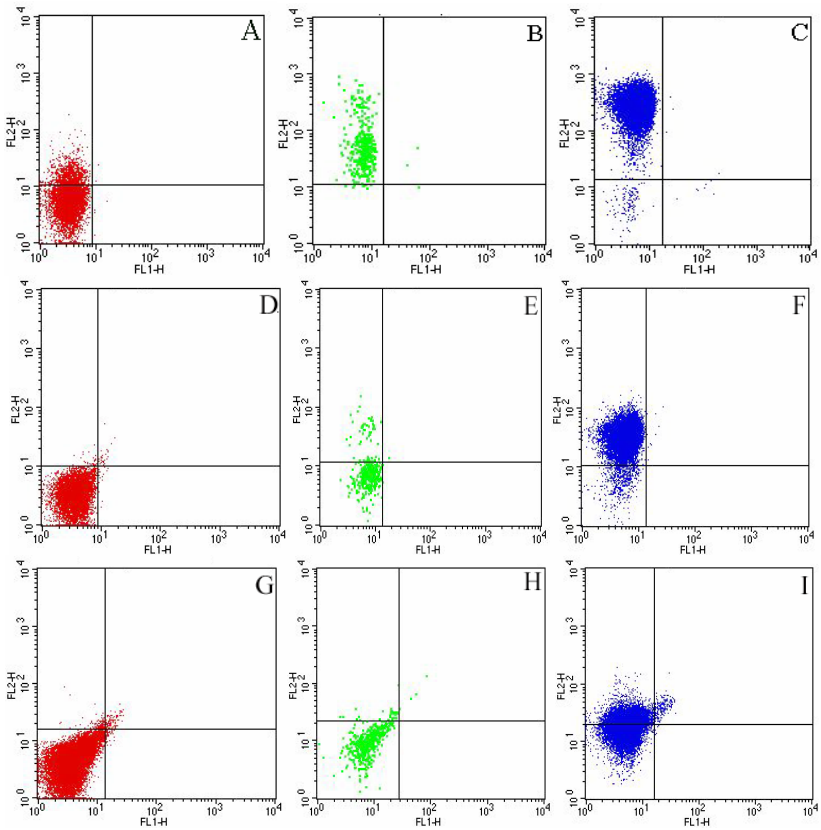

Fig. 60 Analysis of Somali wild ass (A-C), Grevy's zebra (D-F), and Hartmann's mountain zebra (G-I) leukocytes with anti-CD18 clone BAQ30A.
BAQ30A detected all granulocytes (C) and monocytes (B) and possibly a few lymphocytes (A) of Somali wild ass. Granulocytes of Grevy's zebra were stained clearly (F) while in case of hartmann's mountain zebra granulocytes (I) show a population shift only. Weak (E) and negative (H) staining of monocytes was obtained in zebras. No staining of lymphocytes (D and G) was detected in these two species. BAQ30A was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

Anti-CD18 clone H20A stained all leukocytes of the three species (Fig. 61 and table 14) showing a staining pattern like that obtained for horse.

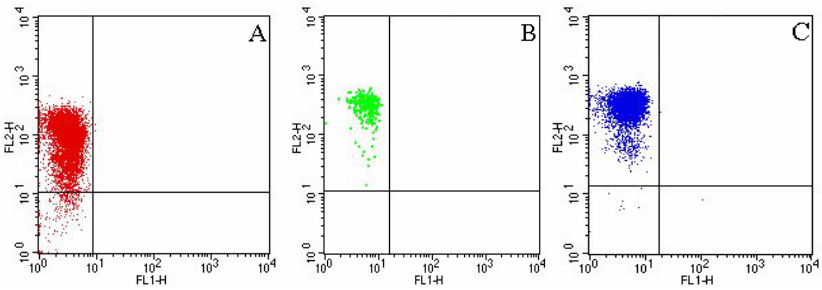

Fig. 61 Analysis of Somali wild ass leukocytes with anti-CD18 clone H20A.
H20A stained all lymphocytes (A), monocytes (B), and granulocytes (C) of Somali wild ass and the other two species (data not shown). H20A was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

Anti-CD18 clone HUH82A stained all leukocytes of Somali wild ass clearly (Fig. 62a, b, and c). Staining pattern of Grevy's zebra was to be considered negative and staining pattern for Hartmann's zebra leukocytes was classified negative alike (Table 14).

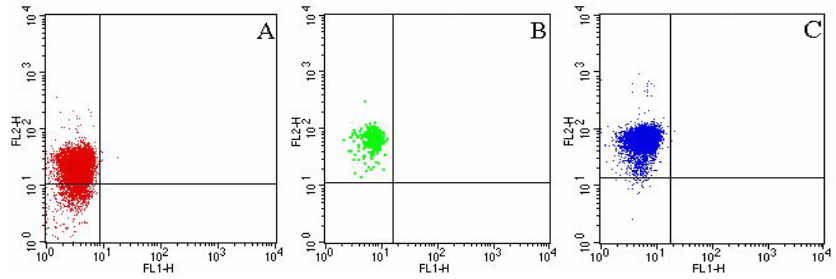

Fig. 62 Analysis of Somali wild ass leukocytes with anti-CD18 clone HUH82A.
HUH82A stained all leukocytes of Somali wild ass (A-C) clearly. HUH82A was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2). (A) lymphocytes, (B) monocytes, (C) granulocytes.

In case of anti-human CD18 clone MHM23 all of the three species leukocytes (Fig. 63, table 14) were clearly stained positive giving no doubt that MHM23 stained CD18 of the three species.

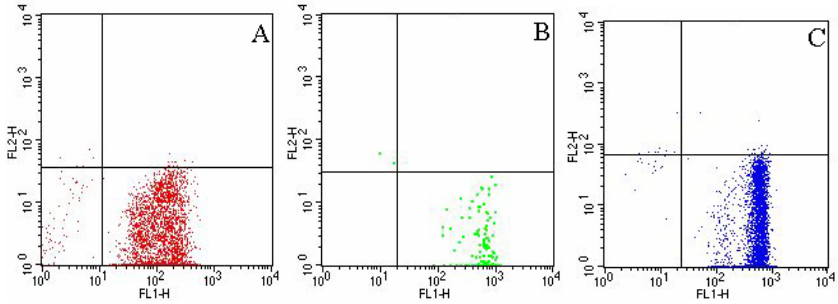

Fig. 63 Analysis of Somali wild ass leukocytes with anti-human CD18 clone MHM23.
MHM23 stained all leukocytes of Somali wild ass clearly (A-C) and the other two species (not shown). MHM23 was analyzed by indirect staining using a FITC-labelled secondary antibody (FL-1). (A) lymphocytes, (B) monocytes and (C) granulocytes.

The B cell marker CD21 clone B-Ly4 clearly detected a population of lymphocytes in Grevy's and Hartmann's zebra lymphocytes (Fig. 64b and c). Staining pattern obtained with both zebras was similar to that obtained for horses. B-Ly4 stained Somali wild ass lymphocytes weakly (Fig. 64a).

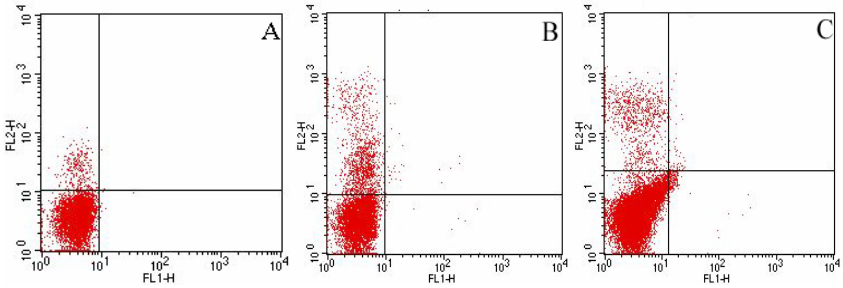

Fig. 64 Analysis of Somali wild ass (A), Grevy's zebra (B), and Hartmann's mountain zebra (C) PBL with anti-human CD21 clone B-Ly4.
B-Ly4 stained clearly a population of Grevy's and Hartmann's mountain zebra lymphocytes (B and C) while a weak staining of Somali wild ass lymphocytes (A) was obtained. No staining of monocytes or granulocytes was observed (not shown). B-Ly4 was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

The anti-canine B cell mAb reacted with a population of lymphocytes of the three animal species (Fig. 65) which was assumed to be B cells. This staining pattern was similar to that for horses.

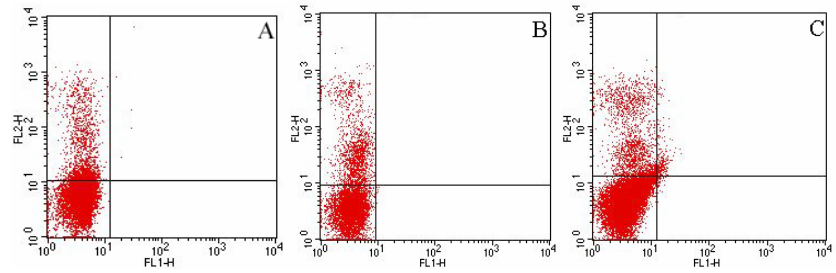

Fig. 65 Analysis of Somali wild ass (A), Grevy's zebra (B), and Hartmann's mountain zebra (C) PBL with anti-canine B cell clone CA2.1D6.
CA2.1D6 stained a population of Somali wild ass (A), Grevy's zebra (B) and Hartmann's mountain zebra (C) lymphocytes. Positively stained populations were assumed to be B cells. CA2.1D6 was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

Three clones against CD44, BAG40A (Fig. 66A-C), H22A (D-F) and LT41A (G-I), stained Somali wild ass leukocytes positively with a staining pattern similar to that obtained for horses.

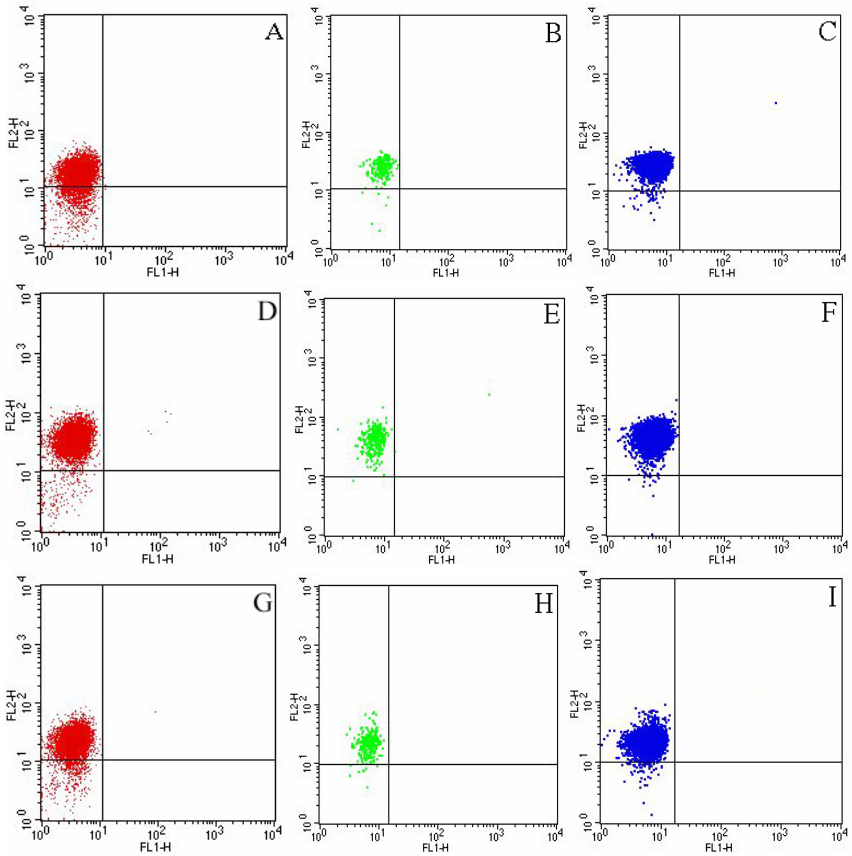

Fig. 66 Analysis of anti-CD44 mAb clones BAG40A (A-C), H22A (D-F), and LT41A (G-I) with Somali wild ass leukocytes.
BAG40A, H22A and LT41A stained all lymphocytes (A, D and G), all monocytes (B, E and H), and all granulocytes (C, F and I). Levels of expression were not high but almost all cells were stained. BAG40A, H22A and LT41A were analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

Grevy's zebra leukocytes were weakly stained with anti-CD44 clones BAG40A, H22A, and LT41A (Table 14). The obtained staining pattern was to some extent similar to that obtained with horses (Fig.10-12), but without further repetition impossible to judge if it would be of value or not. None of anti-CD44 mAbs cross-reacted with Hartmann's mountain zebra leukocytes indicating the failure to detect a non-conserved epitope in Hartmann's zebra.

Anti-human CD49d mAb clone HP2/1 stained all monocytes, the majority of lymphocytes and a population of granulocytes of the three animal species (Fig. 67, table 14). A staining pattern, which in analogy to horses, was considered positive.

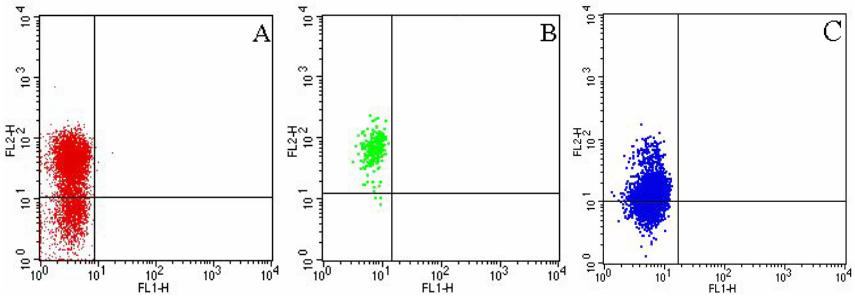

Fig. 67 Analysis of Somali wild ass (A-C) leukocytes with anti-human CD49d clone HP2/1.
HP2/1 stained all monocytes of Somali wild ass (B), a population of granulocytes (C), and the majority of lymphocytes (A). HP2/1 was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

Anti-human CD91 clone A2MRα-2 stained monocytes of Somali wild ass weakly (Fig. 68a) while it did not stain monocytes of Grevy's or Hartmann's zebras (Fig. 68b and c). No lymphocytes or granulocytes were stained by CD91 in all cases (data not shown).

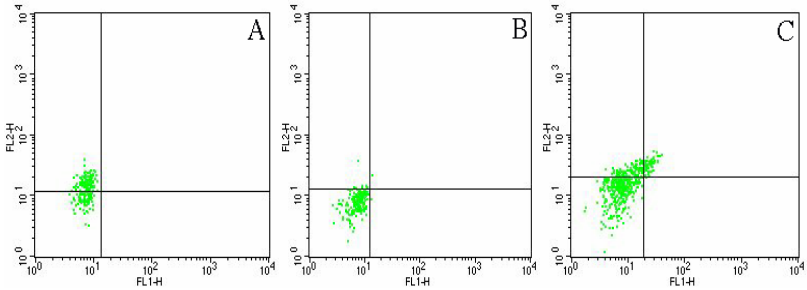

Fig. 68 Analysis of Somali wild ass (A), Grevy's zebra (B), and Hartmann's mountain zebra (C) monocytes with anti-human CD91 clone A2MRα-2.
This mAb stained Somali wild ass monocytes (A) which appear as a population shift while in case of Grevy's and Hartmann's mountain zebras no staining of monocytes (B and C) was detectable. A2MRα-2 was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

CD163 is another myeloid marker, expressed only by monocyte/macrophages, and upregulated during activation of cells. The mAb Ber-MAC3 stained most monocytes of the three animal species (Fig. 69) giving no doubt that Ber-MAC3 detects CD163 in the three animal species.

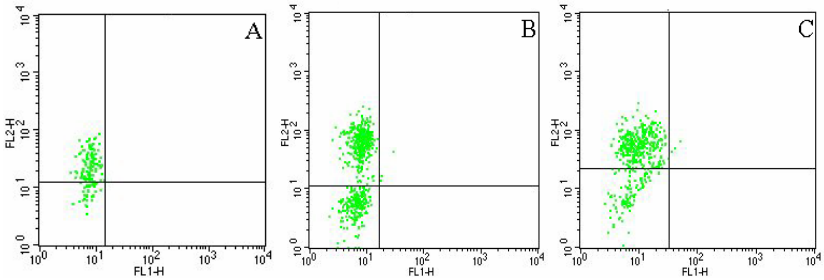

Fig. 69 Analysis of Somali wild ass (A), Grevy's zebra (B), and Hartmann's mountain zebra (C) monocytes with anti-human CD163 clone Ber-MAC3.
Ber-MAC3 stained the majority of monocytes of the three species. No staining of lymphocytes and granulocytes was found (not shown). Ber-MAC3 was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

Anti-human CD172a clone DH59B stained most monocytes and granulocytes (Fig. 70) of Somali wild ass (A-B), Gravy's zebra (C-D) and Hartmann's zebra (E-F), a pattern obtained before with horses (Fig. 17).

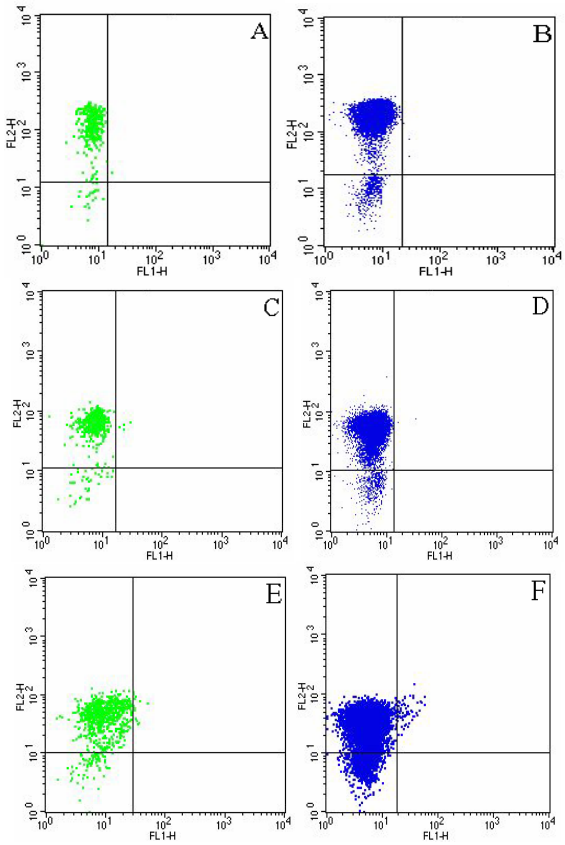

Fig. 70 Analysis of Somali wild ass (A and B), Grevy's zebra (C and D), and Hartmann's mountain zebra (E and F) monocytes (A, C, E) and granulocytes (B, D, F) with anti-human CD172a clone DH59B.
DH59B stained most of monocytes and granulocytes of the three animal species. No staining of lymphocytes was observed (not shown). DH59B was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

Anti-human MHCI (HLA-ABC) clone B9.12.1 stained all leukocytes of the three animal species brightly (Fig. 71, table 14) as obtained before with horses (Fig. 31).

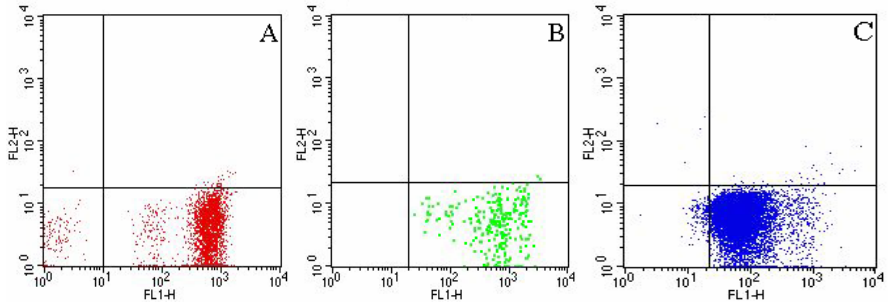

Fig. 71 Analysis of Somali wild ass (A-C) leukocytes with anti-human MHCI (HLA-ABC) clone B9.12.1.
B9.12.1 stained all leukocytes of the three animal species (partially not shown). B9.12.1 was analyzed by indirect staining using a FITC-labelled secondary antibody (FL-1). (A) lymphocytes, (B) monocytes, (C) granulocytes.

The mAb EqT2 directed against equine MHCII stained most monocytes (Fig. 72B, E, and H). A lymphocyte population of varying percentages between different species (or samples) was stained as described before for equine MHCII (Crepaldi et al., 1986; Barbis et al., 1994). No or little staining of granulocytes was obtained where likely a few monocytes were stained.

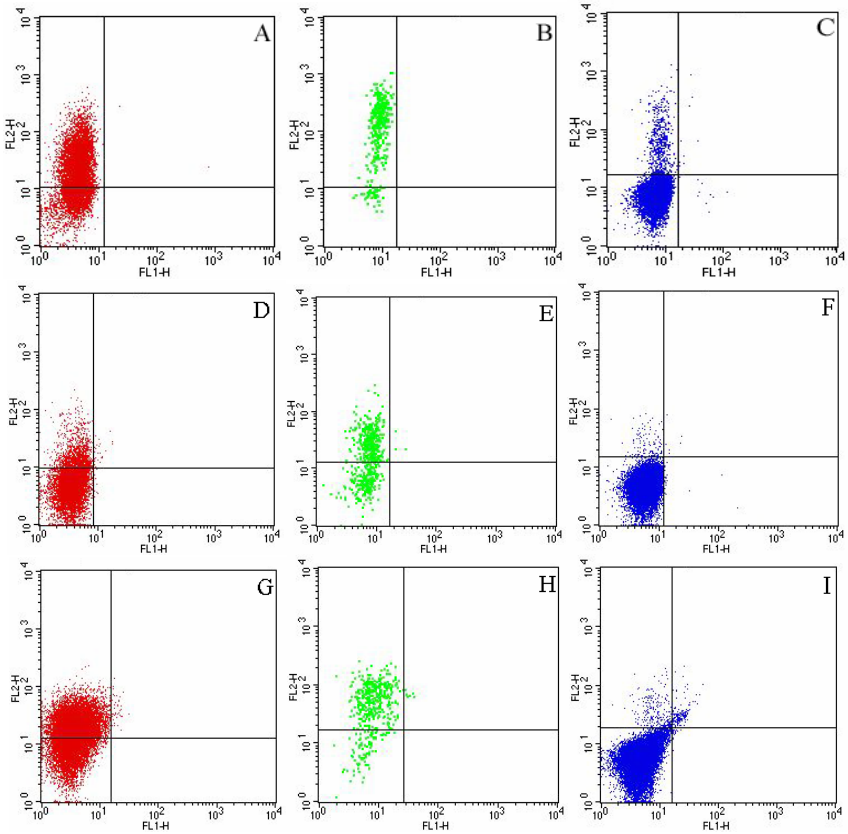

Fig. 72 Analysis of Somali wild ass (A-C), Grevy's zebra (D-F), and Hartmann's mountain zebra (G-I) leukocytes with anti-equine MHCII clone EqT2.
EqT2 stained most monocytes of Somali wild ass (B), Grevy's zebra (E), and Hartmann's mountain zebra (H). Many of Somali wild ass lymphocytes (A), Grevy's zebra lymphocytes (E) and Hartmann's mountain zebra lymphocytes (G) were MHCII^{+}. Very few granulocytes in particular of Somali wild ass (C) were MHCII^{+}. EqT2 was analyzed by indirect staining using a PE-labelled secondary antibody (FL-2).

Table 14 Analysis of horse cross reactive mAbs with Somali wild ass, Grevy's zebra, and Hartmann's mountain zebra leukocytes

WS	Source	CD	Clone	Somali wild ass	Grevy's zebra	Hartmann's zebra	Comment
178	Coulter	huCD2	39C1.5	++	++	++	
	Natutec/VMRD	EqCD2	HB88A	++	-	?	
	Serotec	EqCD4	CVS4	++	++	++	
	Natutec/VMRD	EqCD5	HT23A	++	++	(++)	
	Serotec	EqCD8	CVS8	++	-	-	
17	Bill Davis	huCD11a	HUH73A	++	++	++	
	MACS	huCD11b	M1/70.15.11.5	++	++	++	
	Biometec	huCD14	7H3 (big10)	NT	++	++	
	Biometec	huCD14	big11	NT	++	-	
	Biometec	huCD14	big12	NT	++	++	
	Biometec	huCD14	7D6 (big13)	NT	++	++	
1	Bill Davis	CD18	BAQ30A	++	W	-	
7	Bill Davis	huCD18	H20A	++	++	++	
22	Bill Davis	huCD18	HUH82A	++	W	-	
248	DAKO	huCD18	MHM23	++	++	++	
	BD	huCD21	B-Ly4	W	++	++	weak staining of PBL
6	Bill Davis	CD44	BAG40A	++	W	-	population shift
8	Bill Davis	CD44	H22A	++	W	-	population shift
25	Bill Davis	CD44	LT41A	++	W	-	population shift
	Coulter	huCD49d	HP2/1	++	++	++	
	DAKO	huCD91	A2MRa-2	++	-	-	
	DAKO	HuCD163	Ber-MAC3	++	++	++	
5	Bill Davis	CD172a	DH59B	++	++	++	
	Coulter	huCD206(MMR)	3.29B1.10	NT	NT	-	Staining of cultured cells only
	Coulter	huHLA-ABC(MHCI)	B9.12.1	++	++	++	
	Natutec/VMRD	EqMHCII	EqT2	++	++	++	
	Serotec	B cells (Canine)	CA2.1D6	++	++	++	

Legend to table 12:

"++" 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 repetition.
"?" questionable result in staining, especially due to staining variability between different animals.
"W" weak, likely unusable but positive reactivity.
"NT" not tested.
"-" clear negative staining.
"WS" HLDA8 workshop number.
"CD" Cluster of differentiation.