4.4 Anti-human polyclonal antibodies as another tool to analyze horse (*Equus caballus)* leukocytes

Specificity of monoclonal antibodies for a defined antigenic epitope is an advantage. In cases where the detected antigen is not highly conserved, anti-human monoclonal antibodies failed to cross-react with the target equine epitope. Such a phenomenon was likely observed for CD28. Despite the homology between human and equine CD28 protein, there were 18 mismatching aa residues in the extra-cellular domain, which could be a reason for the failure of all mAbs tested (Tables 16 and 17) to cross react with CD28 of horse or in fact any of animal species included at the HLDA8 workshop (Saalmüller et al., 2005).

As CD28 (and CD25) were very important targets of the present study, an alternative approach was used. Anti-human polyclonal Abs (R&D systems) raised against the recombinant extra-cellular part of the human protein and affinity purified, were applied since none of the submitted mAbs against human CD28 or CD25 were positive.

4.4.1 Analysis of anti-human CD28 polyclonal Ab

The anti-human CD28 polyclonal antibody (Table 1) stained a small population of equine resting PBL (Fig. 77a) in one colour flow cytometry. This staining pattern was in accordance with the data reported for EqWC4 (the proposed orthologue of equine CD28) but not in accordance with human data, where CD28 is expressed on most lymphocytes (Byrne et al., 1997). To obtain more information about the molecular nature of the detected molecule, immunoprecipitation was performed. While human CD28 is expressed as a homodimeric protein of 44 kDa, anti-human CD28 polyclonal antibody immunoprecipitated two proteins of approximately 57 and 46 kDa from surface biotinylated equine PBL (Fig. 77b).

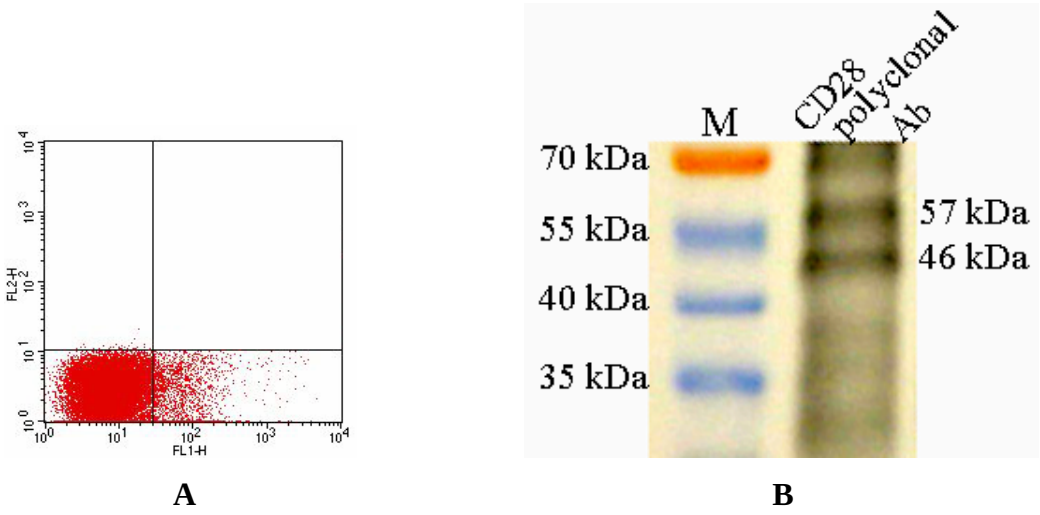

Fig. 77 Flow cytometric analysis and immunoprecipitation of equine CD28 from equine PBL using anti-human polyclonal Ab.
A) Anti-human CD28 polyclonal Ab from R&D systems stained a small population of equine PBL clearly with a fluorescence adjusted to eliminate the slightly higher background signals (not shown). Anti-human CD28 polyclonal antibody was analyzed using a FITC-conjugated anti-goat secondary antibody (FL-1). B) Anti-human CD28 polyclonal Ab immunoprecipitated two proteins of approximately 57 and 46 kDa from surface biotinylated equine PBL.

In vitro expression of equine CD28:

To provide more insight for the nature of equine CD28, stable insect cell transfectants expressing equine CD28 were produced (3.2.16) and used for further analysis.

Cell pellets of CD28 transfected and negative control high five cells were electrophoresed in 8% SDS-polyacrylamide gel. Separated cellular proteins were then blotted onto nitrocellulose membrane before probing with reference anti-V5 mAb directed against the C-terminal tagged V5 epitope of the expressed recombinant protein. The reference anti-V5 epitope mAb did not detect any protein in the non-transfected high five insect cells (Fig. 78a), while detecting two faint bands of approximately 57 and 46 kDa (indicated by two arrows, Fig. 78a). Anti-human CD28 polyclonal antibody alike detected two bands of the same molecular weights as those detected with anti-V5 mAb (Fig.78b).

The obtained results indicated expression of equine CD28 as two proteins of about 57 and 46 kDa approximate molecular weights and that equine CD28 was expressed on a small population of equine lymphocytes only.

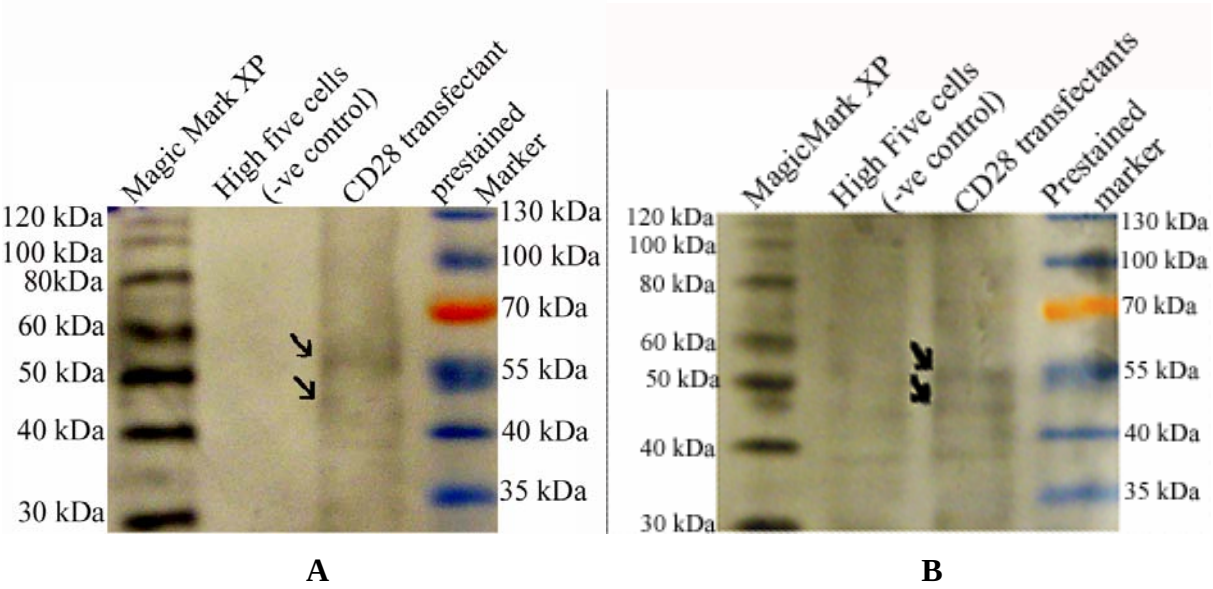

Fig.78 Western blot of recombinant CD28 with anti-V5 mAb and anti-human CD28 polyclonal Ab.
The anti-V5 epitope mAb did not detect any protein in the non-transfected high five insect cells (Fig. 78a), while detecting two faint bands of 56 and 46 kDa approximate molecular weight (indicated by two arrows). Anti-human CD28 polyclonal antibody detected two bands of the same molecular weights as those detected with anti-V5 (Fig. 78b). Faint bands of recombinant CD28 are likely due to limited amount of transfectant per lane and antibodies concentration as suggested by the manufacturer. Using higher concentrations, however, non-specific bands were obtained in addition (data not shown).

4.4.2 Analysis of anti-human CD25 polyclonal Ab.

Full stimulation of lymphocytes produces high levels of IL-2 (from T cells) corresponding with high expression levels of the IL-2Rα (CD25). CD25, a 55 kDa glycoprotein, also known as the low-affinity interleukin-2 receptor alpha chain, is expressed on activated T and B cells with expression levels dramatically upregulated upon activation with antigen or mitogen. Human CD25 is also expressed by 5-10% subset of non-activated $CD4^+$ T cells, some of which act as regulatory T cells (Waldmann, 1986; Waldmann, 1991; Jonuleit et al., 2001; Dieckmann et al., 2001; Sugamura, 2002).

The goat anti-human IL-2Rα polyclonal antibody was tested for cross-reactivity with equine PBL in a time-kinetic flow cytometric study to monitor the presumed upregulation of equine CD25 expression upon activation. Equine PBL were activated with PHA and tested at zero hour (before activation), 12 hr, 24 hr, and 48 hr after activation. Non-activated cells always show a small population of lymphocytes clearly stained (Fig. 79a). After 12 hours activation, the expression profile of CD25 dramatically changes to about 80% positive PBL (Fig. 79b). After 24 hours and 48 hours activation, 95 and 99% of PBL were stained brightly (Fig. 79c and d, respectively), supporting the assumption that the goat anti-human IL-2Rα polyclonal antibody detected equine CD25. The few cells stained at zero hours could represent resting T

cells expressing CD25 as described for humans (Sakaguchi et al., 1995) or pre-activated cells (from the period from blood collection to PBMC isolation).

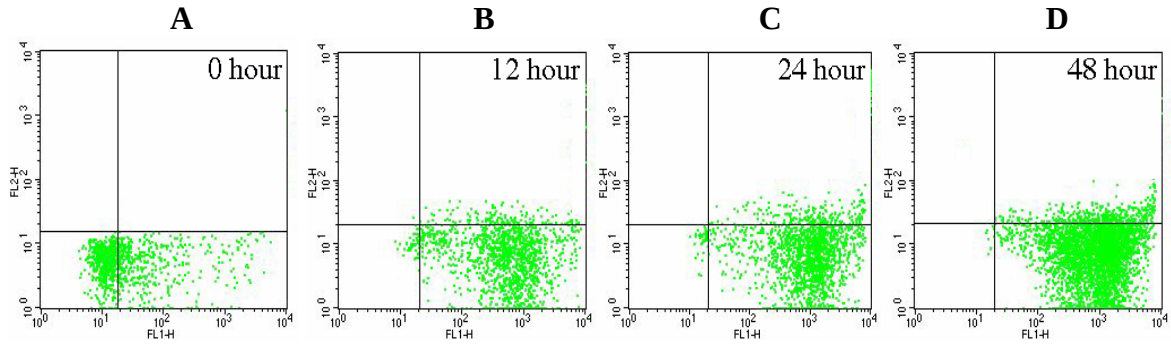

Fig. 79 Expression of IL-2Rα (CD25) by PHA activated equine PBL.
The goat anti-human IL-2Rα polyclonal antibody reacted with a small population of resting PBL at zero hours. Expression of CD25 was dramatically increased within 12 hours (B) where about 80% of PBL were stained. After 24 and 48 hours of activation, almost all PBL expressed CD25 (C and D). Anti-human IL-2Rα polyclonal antibody was analyzed by indirect staining using a FITC-labelled anti-goat secondary antibody (FL-1).

To further characterize the detected molecule, immunoprecipitation was performed on surface biotinylated, 48 hr activated, equine lymphocytes using anti-human CD25 polyclonal antibody. 48 hours activation time was selected to get the opportunity of the highest expression level of CD25. Anti-human polyclonal antibody immunoprecipitated a molecule of approximately 55 kDa (Fig. 80) which was analogous to that known for human CD25.

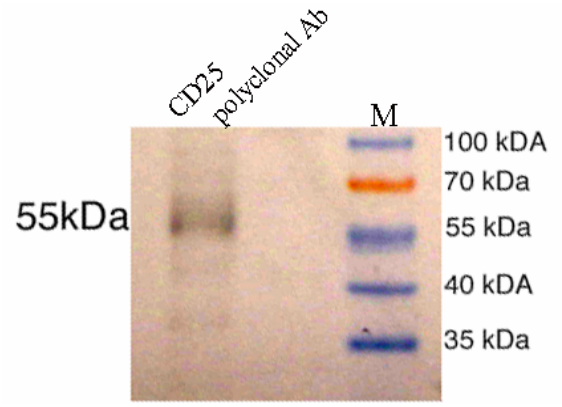

Fig. 80 Immunoprecipitation of equine IL-2Rα from PHA activated equine PBL.
Equine CD25 was immunoprecipitated from 48 hour PHA activated equine lymphocytes as a protein band of approximately 55 kDa.