

The US Veterinary Immune Reagent Network – update on reagents for the horse.

Bettina Wagner¹, Susanna Babasyan¹, Esther Kabithé¹, Julie Hillegas¹, Joanna LaBresh², Dannielle Tompkins³, Cynthia Baldwin³

¹ Cornell University, Ithaca, NY; ² Kingfisher Biotech, St. Paul, MN; ³ University of Massachusetts, Amherst, MA

The US Veterinary Immune Reagent Network (US-VIRN, www.vetimm.org) aims to develop new tools for ruminants, swine, horse, poultry and aquaculture species to improve immunological research in infectious diseases and animal health, and to contribute to new vaccine development strategies and food safety. For the horse, several new reagents were developed including recombinant cytokines and chemokines, and monoclonal antibodies (mAbs) to cytokines and cell surface molecules. Recombinant cytokines and chemokines were produced by Kingfisher Biotech in a yeast system. Equine recombinant IL-1 β , IL-2, IL-4, IL-6, IL-13, IL-17A, GM-CSF, CCL2, CCL3, CCL5, CCL11, CXCL9 and CXCL10 were produced and can be obtained from Kingfisher (www.kingfisherbiotech.com). Monoclonal antibodies (mAbs) to cytokines and cell surface markers were produced at Cornell University. For cell surface molecules, recombinant proteins were expressed in mammalian IgG or IL-4 fusion protein system (Fig. 1) and used for immunization of mice. Fully characterized mAbs with proven specificity to the native protein were developed to equine CD14 (Fig. 2) and CD23 (Fig. 3) and to equine IL-10 (Figs. 4+5), IL-4 (Figs 5+6), IL-2 (Fig. 7), and IFN- α (Table 1). These mAbs can be obtained from Cornell University (<http://www.cctec.cornell.edu/> or bw73@cornell.edu). Additional mAbs that are either in the production or characterization process include reagents to IL-1 β , IL-6, IL-13, GM-CSF, CCL2, CCL3, CCL5, CCL11, and CD25, CD28, CD40, Fc ϵ RI α , TCR α , TCR δ , and TCR γ .

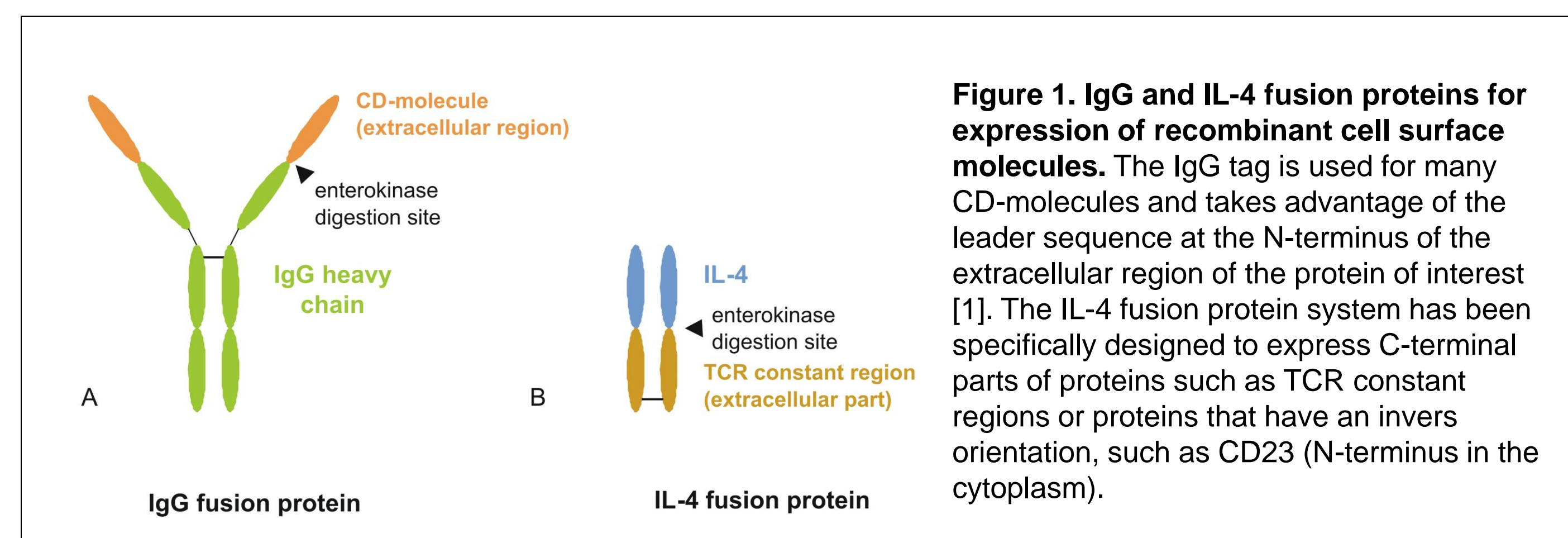


Figure 1: IgG and IL-4 fusion proteins for expression of recombinant cell surface molecules. The IgG tag is used for many CD-molecules and takes advantage of the leader sequence at the N-terminus of the protein of interest [1]. The IL-4 fusion protein system has been specifically designed to express C-terminal parts of proteins such as TCR constant regions or proteins that have an inverse orientation, such as CD23 (N-terminus in the cytoplasm).

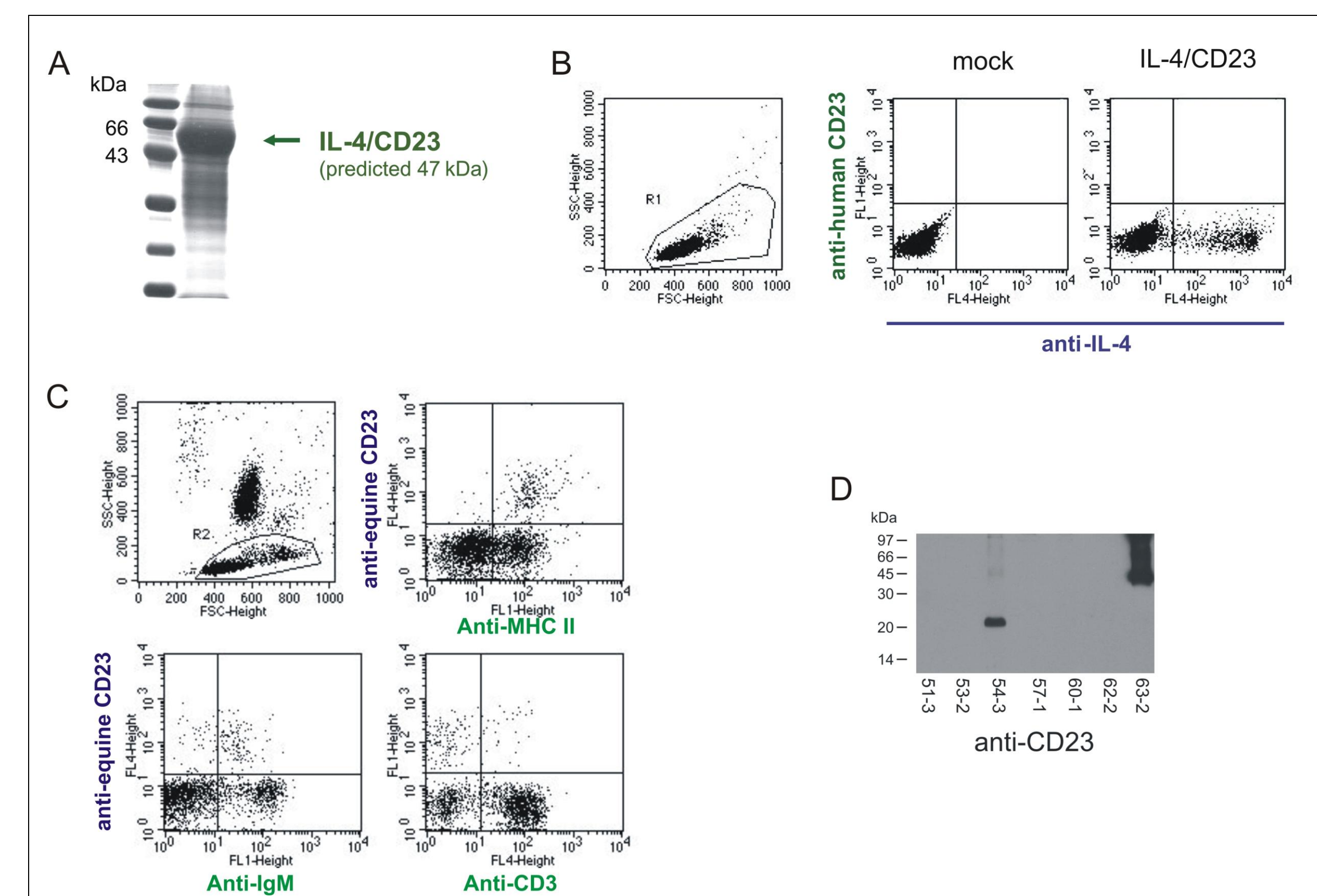


Figure 3: Monoclonal antibodies to equine CD23. (A) Recombinant mammalian expressed IL-4/CD23 fusion protein. The protein was purified and used for immunization (B) Flow cytometric analysis of an IL-4/CD23 transfectant for screening of an anti-human CD23 antibody that was previously described to cross-react with equine PBMC. The transfectant used for screening purposes is at an intermediate stage and not yet stable. Only around 30% of the cells produced the recombinant IL-4/CD23 fusion protein as indicated by the anti-IL-4 staining. The anti-human CD23 mAb did not detect the recombinant protein, while all anti-equine CD23 mAbs detected the recombinant protein (not shown). (C) Phenotyping of CD23+ cells in equine PBMC by flow cytometric analysis. The anti-equine CD23 mAbs (here shown for clone 53-2) recognized a MHCII+ population in equine PBMC. Most of these cells were also IgM+ (B-cells), while CD23 was only found on a very few equine T-cells (CD3+). (D) Western blot using all seven mAbs to equine CD23 and the recombinant equine CD23 protein. Only clones anti-CD23 54-3 and 63-2 detect the protein using this method. The upper protein (47kDa) detected by clone 63-2 corresponds to the complete IL-4/CD23 protein, the lower band (21kDa) detected by clone 54-3 is likely to be a degraded fragment of the recombinant protein. When used for flow cytometric analysis, all seven anti-equine CD23 mAb clones resulted in identical images as that shown in C for clone 51-3. The data suggested that the seven mAbs detected at least three different epitopes of the native equine CD23

Table 1: Applications for anti-equine cytokine mAbs (the numbers refer to the mAb clones that work and are used for a particular method).

	Anti-IL-2	Anti-IL-4	Anti-IL-10	Anti-IFN- α	reference
ELISA & Multiplex assay	134	13G7 25	292-2 165-2	29B 240-2	[2, 3, 4] [5]
Flow cytometry (PBMC)	134	13G7 25	165-2 292-1 292-2	ongoing	[2, 3]
Western blot (rec. protein)	134	25	weak	no	

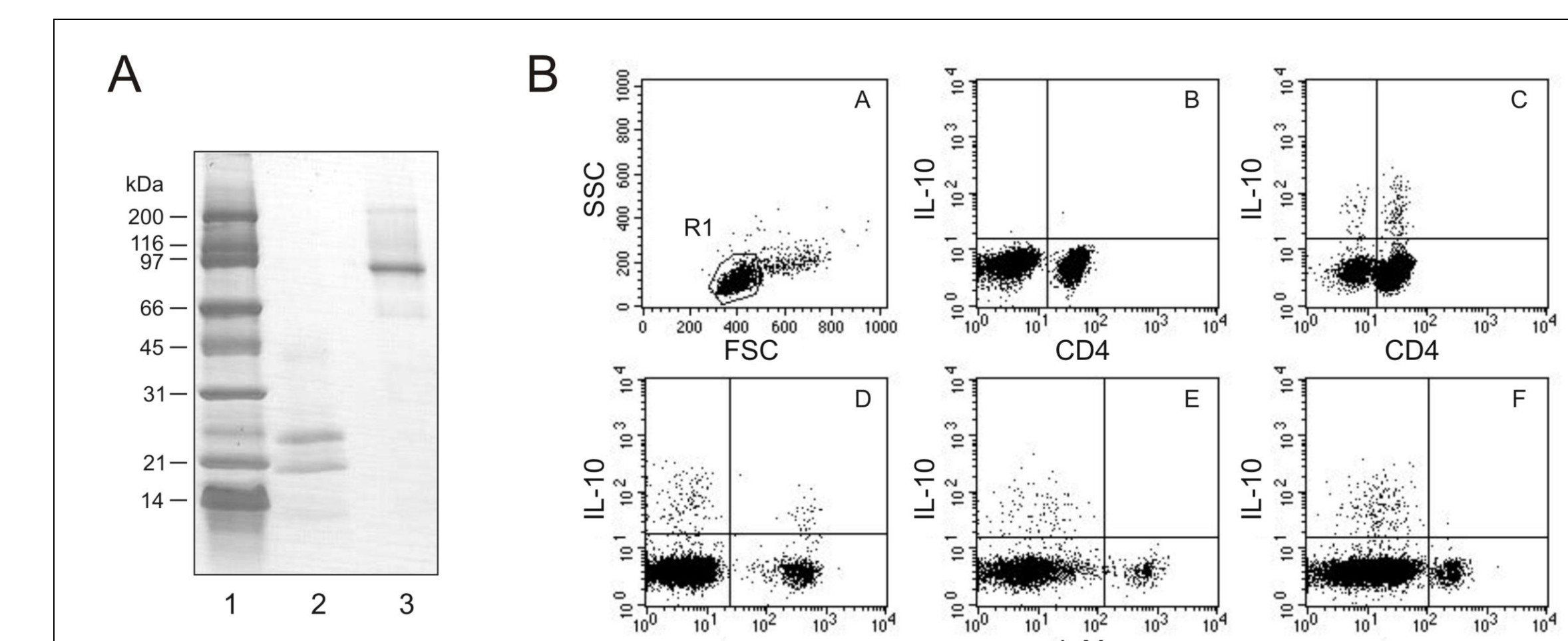


Figure 4: Monoclonal antibodies to equine IL-10 [3]. (A) SDS-PAGE of equine IL-10. The gel was stained by Coomassie. Equine IL-10 was expressed as a mammalian IgG fusion protein. The fusion protein was purified and IL-10 was removed from the IgG part by enzymatic digestion. The gel shows the recombinant IL-10 in two different glycosylation forms (lane 2) and the remaining IgG part after digestion (lane 3). (B) Intracellular staining of equine PBMC using monoclonal antibodies to equine cytokines and flow cytometric analysis. The PBMC were stimulated with PMA and ionomycin or kept in medium for control. The incubation (4 hours) was performed in the presence of the secretion blocker Brefeldin A. After incubation, the cells were fixed in formalin buffer and intracellular staining was performed in a buffer containing saponin [2, 4]. The cells were also staining with various cell surface makers. All mAbs were purified and directly conjugated to Alexa 488 or 647 dyes. Both, CD4+ and CD8+ equine T-cells produced IL-10.

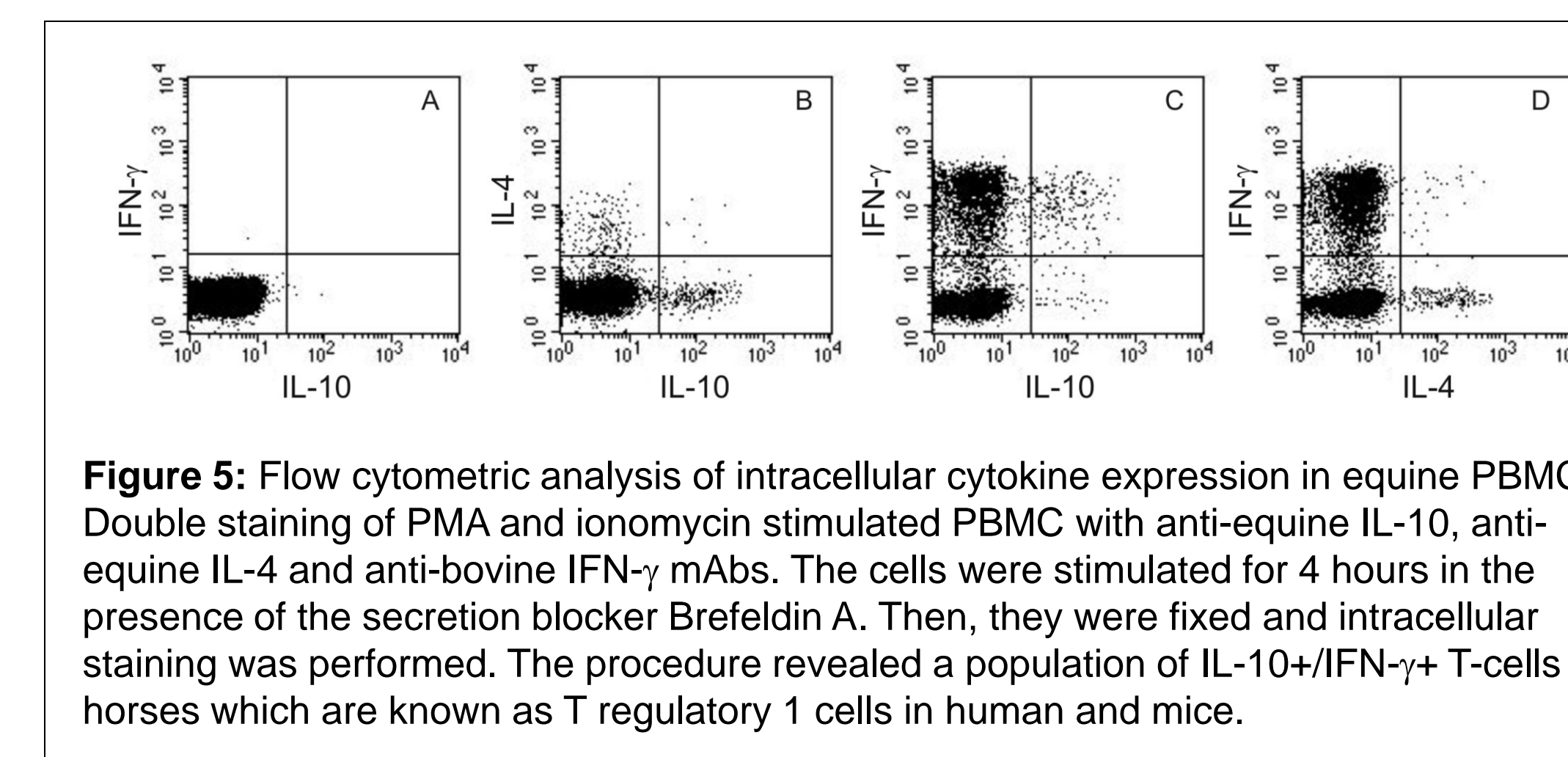


Figure 5: Flow cytometric analysis of intracellular cytokine expression in equine PBMC. Double staining of PMA and ionomycin stimulated PBMC with anti-equine IL-10, anti-equine IL-4 and anti-bovine IFN- γ mAbs. The cells were stimulated for 4 hours in the presence of the secretion blocker Brefeldin A. Then, they were fixed and intracellular staining was performed. The procedure revealed a population of IL-10+/IFN- γ + T-cells in horses which are known as T regulatory 1 cells in human and mice.

References

- Wagner B, Robeson J, McCracken M, Wattring E and Antczak DF. 2005. Horse cytokine/IgG1 fusion proteins – mammalian expression of biologically active cytokines and a system to verify antibody specificity to equine cytokines. *Vet. Immunol. Immunopathol.*, 105: 1-14.
- Wagner B, Hillegas JM and Antczak DF. 2006. A monoclonal antibody to equine interleukin 4. *Vet. Immunol. Immunopathol.*, 110: 363-367.
- Wagner B, Hillegas JM, Brinker DR, Horohov DW and Antczak DF. 2008a. Characterization of monoclonal antibodies to equine interleukin-10 and detection of T regulatory 1 cells in horses. *Vet. Immunol. Immunopathol.*, 122: 57-64.
- Wagner B, Hillegas JM, Flaminio MJB and Wattring E. 2008b. Monoclonal antibodies to equine interferon- α (IFN- α): New tools to neutralize IFN activity and to detect secreted IFN- α . *Vet. Immunol. Immunopathol.*, 125: 315-325.
- Wagner B and Freer H. 2009. Development of a bead-based multiplex assay for simultaneous quantification of cytokines in horses. *Vet. Immunol. Immunopathol.*, 127: 242-248.

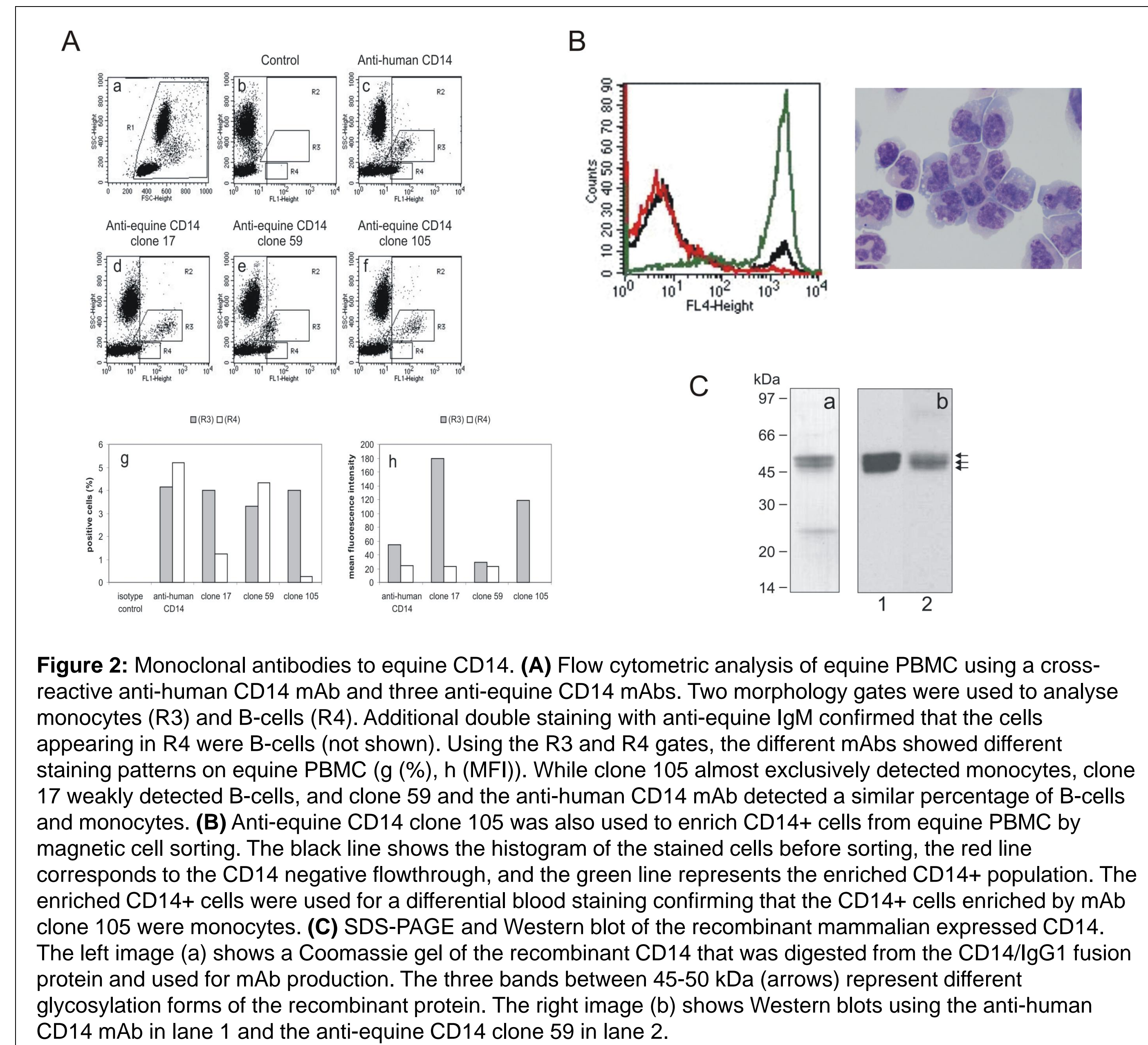


Figure 2: Monoclonal antibodies to equine CD14. (A) Flow cytometric analysis of equine PBMC using a cross-reactive anti-human CD14 mAb and three anti-equine CD14 mAbs. Two morphology gates were used to analyse monocytes (R3) and B-cells (R4). Additional double staining with anti-equine IgM confirmed that the cells appearing in R4 were B-cells (not shown). Using the R3 and R4 gates, the different mAbs showed different staining patterns on equine PBMC (g (%), h (MFI)). While clone 105 almost exclusively detected monocytes, clone 17 weakly detected B-cells, and clone 59 and the anti-human CD14 mAb detected a similar percentage of B-cells and monocytes. (B) Anti-equine CD14 clone 105 was also used to enrich CD14+ cells from equine PBMC by magnetic cell sorting. The black line shows the histogram of the stained cells before sorting, the red line corresponds to the CD14 negative flowthrough, and the green line represents the enriched CD14+ population. The enriched CD14+ cells were used for a differential blood staining confirming that the CD14+ cells enriched by mAb clone 105 were monocytes. (C) SDS-PAGE and Western blot of the recombinant mammalian expressed CD14. The left image (a) shows a Coomassie gel of the recombinant CD14 that was digested from the CD14/IgG1 fusion protein and used for mAb production. The three bands between 45-50 kDa (arrows) represent different glycosylation forms of the recombinant protein. The right image (b) shows Western blots using the anti-human CD14 mAb in lane 1 and the anti-equine CD14 clone 59 in lane 2.

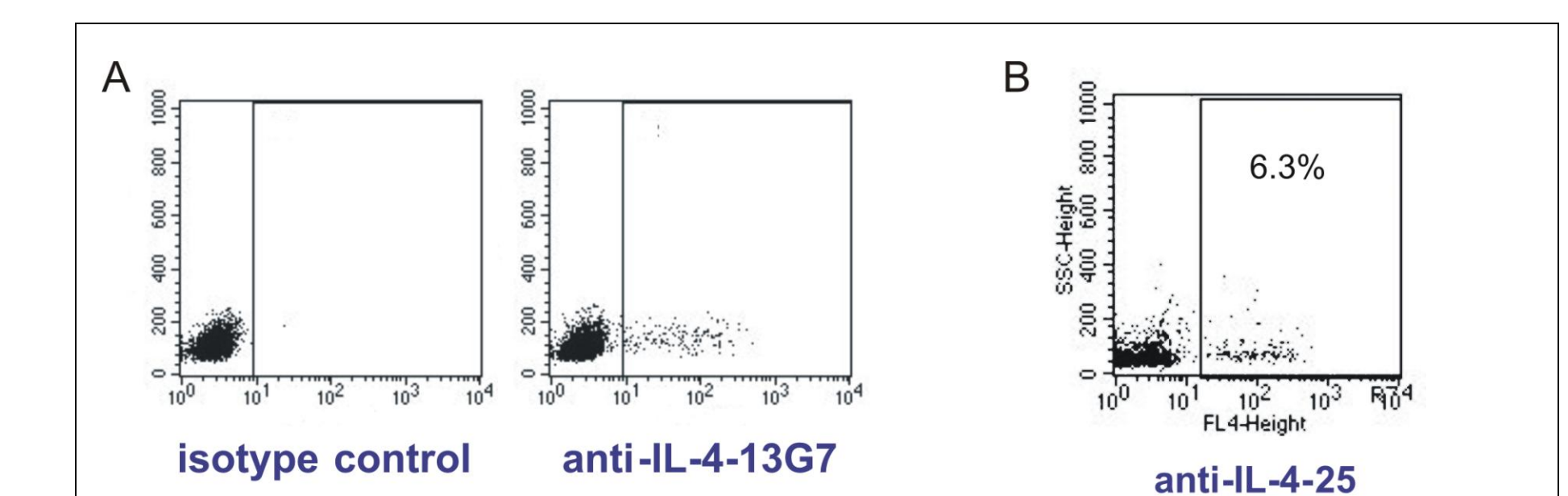


Figure 6: Monoclonal antibodies to equine IL-4. (A) The first mAb to equine IL-4 (clone 13G7) was developed using a IgG fusion protein derived recombinant equine IL-4 protein [2]. Anti-IL-4 13G7 worked well in ELISA and also detected IL-4 producing cells in PMA stimulated equine PBMC. (B) By using the IL-4 fusion protein system, additional mAbs to equine IL-4 were developed. These mAbs (clones 25, 26, 38-1) also detected IL-4 production in PBMC stimulated with PMA. (C) In an ELISA using the clone 13G7 as the coating antibody and the new mAbs for detection, clones 25, 38-1 (and 26 – not shown).

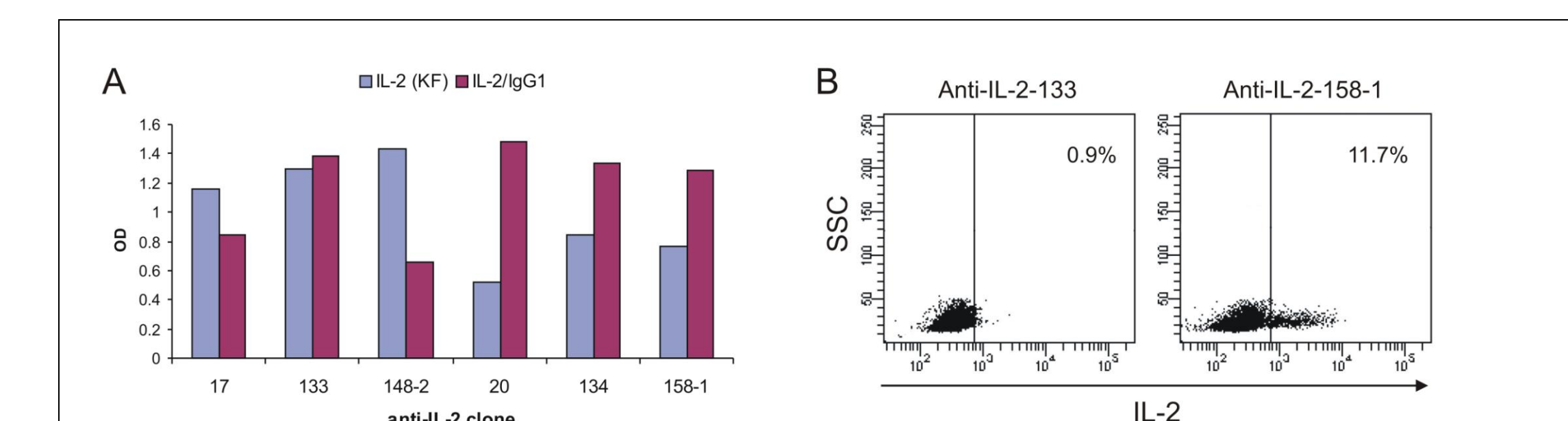


Figure 7: Monoclonal antibodies to equine IL-2. These were the first mAbs developed at Cornell to a recombinant yeast protein. (A) The initial screening of the mAbs was performed by ELISA using the recombinant yeast expressed IL-2 and mammalian expressed equine IL-2/IgG1 fusion protein. Note the differences in signal intensity of the mAbs if the reactivity to both recombinant IL-2 proteins is compared. Anti-IL-2 clones 20, 134 and 158-1 showed a higher signal on the mammalian expressed protein. (B) Flow cytometric analysis of PMA stimulated equine PBMC after staining with the anti-IL-2 mAbs. Clones 158-1 (also 20 and 134) detected a positive populations in these cells, while the remaining three clones 133 (also 17 and 148-2) did not.

