Differences in leukocyte differentiation molecule abundances on domestic sheep (*Ovis aries*) and bighorn sheep (*Ovis canadensis*) neutrophils identified by flow cytometry

Margaret A. Highland^{a,b,*}, David A. Schneider^{a,b}, Stephen N. White^{a,b}, Sally A. Madsen-Bouterse^a, Donald P. Knowles^{a,b}, and William C. Davis^a

^a Department of Veterinary Microbiology and Pathology, College of Veterinary Medicine, Washington State University, Pullman, WA 99164-7040, USA

^b Animal Disease Research Unit, United States Department of Agriculture, Pullman, WA 99164-6630, USA

Email: Margaret A. Highland Maggie.Highland@ARS.USDA.GOV – David A. Schneider David.Schneider1@ARS.USDA.GOV – Stephen N. White Stephen.White@ARS.USDA.GOV - Sally A. Madsen-Bouterse smadsen@vetmed.wsu.edu – Donald P. Knowles Don.Knowles@ARS.USDA.GOV – William C. Davis davisw@vetmed.wsu.edu

* Corresponding author at: Animal Disease Research Unit, United States Department of Agriculture, 3033 ADBF, Washington State University, Pullman, WA 99164-6630, USA. Tel.: +1 509 335 6327; fax +1 509 335 8328. E-mail address: Maggie.Highland@ARS.USDA.GOV (M. Highland).

Abstract

Although both domestic sheep (DS) and bighorn sheep (BHS) are affected by similar respiratory bacterial pathogens, experimental and field data indicate BHS are more susceptible to pneumonia. Cross-reactive monoclonal antibodies (mAbs) for use in flow cytometry (FC) are valuable reagents for interspecies comparative immune system analyses. This study describes cross-reactive mAbs that recognize leukocyte differentiation molecules (LDMs) and major histocompatibility complex antigens on DS and BHS leukocytes. Characterization of multichannel eosinophil autofluorescence in this study permitted cell-type specific gating of granulocytes for evaluating LDMs, specifically on neutrophils, by single-label FC. Evaluation of relative abundances of LDMs by flow cytometry revealed greater CD11a, CD11b, CD18 (β_2 integrins) and CD 172a (SIRP α) on DS neutrophils and greater CD14 (lipopolysaccharide receptor) on BHS neutrophils. Greater CD25 (IL-2) was identified on BHS lymphocytes following Concanavalin A stimulation. While DS and BHS have similar total peripheral blood leukocyte counts, BHS have proportionately more neutrophils.

Keywords

bighorn sheep; domestic sheep; flow cytometry; monoclonal antibody; leukocyte; autofluorescence; eosinophil; neutrophil; leukocyte differentiation molecule; major histocompatibility complex

1. Introduction

Monoclonal antibodies (mAbs) for use in flow cytometry (FC) are valuable for immune system analyses. The current paucity of identified cross-reactive mAbs limits comparative interspecies studies needed to better understand host response differences to infectious agents. For example,

the current paucity of reagents identified for use in domestic sheep (DS; *Ovis aries*) and bighorn sheep (BHS; *Ovis canadensis*) has limited comparative immunology research in these species. The importance of characterizing host response differences to infectious agents in these two ovine species is one of ecologic and socioeconomic importance. Although both DS and BHS are affected by similar respiratory bacterial pathogens, including *Mycoplasma ovipneumoniae* and members of the Pasteurellaceae family, experimental and field data indicate BHS are more susceptible to these agents [1-3]. It has been suggested that this susceptibility difference may at least in part be due to a difference in the response of neutrophils to bacterial agents [4]. The mechanisms driving susceptibility difference are not known. However, the impacts of these differences include reduced BHS species recovery efforts (ecologic impact) and the belief that absolute separation of DS from BHS is necessary. Implementation of absolute separation has become an economic detriment to the DS sheep industry in the Western United States due to ongoing loss of public land grazing permits required for ranchers to maintain operations and pressures placed on private landowners that have DS (socioeconomic impact). The primary objectives of this study were to use FC to 1) identify mAbs, previously developed for use in domestic ruminants (cattle, goats, and DS), that recognize conserved orthologous epitopes of leukocyte differentiation molecules (LDMs) and the major histocompatibility complex (MHC) on both DS and BHS leukocytes, and 2) use relative median immunofluorescence intensity (MFI) as a measure of relative abundance to comparatively evaluate DS and BHS leukocyte LDMs, with specific attention given to neutrophils.

2. Materials and methods

2.1. Animals

Sheep used in this study were housed and maintained at Washington State University (WSU; Pullman, WA, USA) according to the Institutional Animal Care and Use Committee guidelines and Association for Assessment and Accreditation of Laboratory Animal Care and included 13 clinically healthy sheep (4-year-old BHS: 3 rams and 2 ewes; 2-year-old Columbia-Suffolk cross DS: 4 ewes; and 4-year-old Suffolk DS: 2 wethers and 2 ewes).

2.2. Peripheral blood leukocyte isolation and Concavalin A stimulation

Blood was collected by jugular venipuncture into a syringe containing 0.5 M ULTRAPURE™ EDTA (GIBCO, Grand Island, NY, USA), pH adjusted from 8.0 to 7.6 with 6N HCl, at 10 µL per mL blood. Isolations of total leukocytes for immunolabeling and cell sorting and peripheral blood mononuclear cells (PBMC) for Concavalin A (conA; Sigma Chemical Co., St. Louis, MO, USA) stimulation and immunolabeling were performed, at room temperature, on 20 mL blood aliquots, in 50 mL polypropylene conical tubes by first centrifuging (1500 X g for 20 minutes) and disposing of the plasma. For total leukocyte isolations, the buffy coat and red blood cell pellet were resuspended to 10 mL with 1XPBS containing 200 µg/L EDTA (1X PBS-EDTA), and red blood cells were removed by hypotonic lysis by adding 36 mL of heat sterilized double distilled water for 20 seconds with gentle inversion; physiologic osmolarity was immediately restored by adding 4 mL of 10X PBS containing 2 mg/L EDTA (10X PBS-EDTA). Samples were centrifuged (600 X g, 10 minutes), supernatants discarded, pelleted leukocytes resuspended in 1 mL of 1X PBS-EDTA, hypotonic lysis repeated, and the leukocyte pellet was suspended in 1X PBS-EDTA.

PBMC isolation from buffy coats was performed by density gradient centrifugation following the WSU Flow Cytometry Laboratory online protocol, except 1X PBS-EDTA was used as the buffer and residual red blood cells were lysed as described above [5]. Isolated PBMC were stimulated

in 6-well plates (1×10^7 cells/well) for 4 days at 37°C under 5% CO₂, in RPMI medium 1640 (GIBCO, Grand Island, NY, USA), buffered with sodium bicarbonate and HEPES (Fisher Scientific, Waltham, MA, USA), containing 5 µg ConA/mL. Density gradient centrifugation was performed on cultured cells to remove dead cells and debris.

Cell viability and concentration were quantified using Trypan blue dye exclusion and an automated cell viability counter (Cellometer® Auto T4, Nexcelom Bioscience, Lawrence, MA, USA).

2.3. Fluorescence activated cell sorting and fluorescence microscopy

A Becton Dickinson FACSVantage SE (BD Immunocytometry Systems, San Jose, CA, USA) was used for cell sorting the 2 populations of granulocytes observed on FL1-SSC and FL2-SSC dot plots (Figure 1A(b), (c) and (d)). Cell sorting was performed by setting sort gates on a FL2-SSC dot plot. Prolong® Gold antifade reagent with DAPI (Invitrogen, Life Technologies, Grand Island, NY, USA) was used to stain isolated leukocytes and the sorted granulocyte populations for epifluorescence microscopy using an Axio Imager.M1 microscope (Carl Zeiss Microimaging, Thornwood, NY, USA).

2.4. Quantification of leukocyte composition

The composition of total peripheral blood leukocytes (PBMC, neutrophils, and eosinophils) from 3 DS and 3 BHS was analyzed by FC based on intrinsic differences in granularity (SSC) and differing autofluorescence (FL1 and FL2) of neutrophils and eosinophils (Figure 1A). Manual cell counts were performed on blood smears from blood collected from each sheep (DS n=8; BHS n=5) on 3 separate days over the course of several months. Slides were stained with Differential Quik Stain (Polysciences, Inc., Warrington, PA, USA), dry cover slipped, and

examined by light microscopy at 400X magnification. A manual cell counter was used to record PBMC and neutrophils up to a total count of 100 cells.

2.5. Flow cytometry (FC)

Primary mAbs obtained from the WSU Monoclonal Antibody Center were evaluated for cross-reactivity in DS and BHS by immunolabeling total leukocytes from DS (n=3) and BHS (n=3) (Table 1; mAb #17_HUH82A_CD18 not included in initial screening). The mAbs are referenced herein by “mAb#_mAb clone_specificity”. ConA stimulated lymphocytes from these 6 sheep were immunolabeled with a subset of the mAbs directed against activation LDMs (Table 1: #41_GB110A_ACT16; #42_CACT116A_CD25; #43_GB112A_CD25; #44_LCTB2A_CD25; #45_CACT200A_SLAM9; #46_CACT206A_SLAM9). Indirect detection of primary mAb binding was performed using FITC-conjugated goat isotype specific anti-mouse antibodies: anti-IgM(μ) (Caltag Medsystems Ltd., Buckingham, UK), anti-IgG1 and IgG3(γ) (Life Technologies, Invitrogen™; Grand Island, NY, USA), and anti-IgG2a and IgG2b (Southern Biotech, Birmingham, AL, USA). Isolated total leukocytes were indirectly labeled following the guidelines of the WSU Monoclonal Antibody Center online protocol [6]. All reagents and incubations were kept at 4°C. Labeling was performed in V-bottom 96-well plates using 5×10^5 total leukocytes per well or 2.5×10^5 ConA stimulated lymphocytes per well. Each primary mAb was incubated at a final concentration of 5 μ g/mL. Secondary antibodies were titrated for optimization and used at a final dilution of 1:100 (α -IgM), 1:200 (α -IgG1, α -IgG2a, and α -IgG2b), or 1:400 (α -IgG3). Control wells, containing first wash buffer (no 1° Ab) and second step reagents (including 2° Ab), were set up for cells from each animal to control for non-specific binding of the 2° Ab. Isotype controls for analyzing immunolabeled neutrophils included mAbs #41-46 (Table 1).

A subset of the mAbs (#11_HUH73A_CD11a; #12_MM12A_CD11b; #14_CAM36A_CD14, #15_CAM66A_CD14; #16_BAQ30A_CD18; #17_HUH82A_CD18; and #26_DH59B_CD172a) was then used to immunolabel leukocytes isolated from blood collected from 8 DS and 5 BHS on the same day. This mAb subset (except #17_HUH82A_CD18) was also used to immunolabel whole blood from each of the 13 sheep, using the same final concentrations of primary and secondary antibodies described. In brief, 100 μ L of whole blood was diluted 1:1 with 1X PBS-EDTA prior to labeling and erythrocytes were lysed subsequent to labelling using BD FACSTTM Lysing Solution (BD Biosciences; San Jose, CA, USA) following the manufacturer's recommendations. Following all immunolabeling, cells were fixed in 200 μ L of 2% PBS buffered formaldehyde and were left at 4°C until analyzed by FC the following day. A Becton Dickinson FACSort and Cell Quest software (BD Immunocytometry Systems, San Jose, CA, USA) were used to acquire data following immunolabeling.

2.6. Data analysis

FCS Express version 4 software (DeNovo Software, Glendale, CA, USA) was used to analyze data collected from 2.5×10^4 total leukocytes and ConA stimulated leukocytes from each sample. Gating strategies for analyzing total leukocytes and ConA stimulated leukocytes are illustrated in Figure 1A(a) and 1B(a), respectively. The software statistics application was used to determine total number of cells and percentage of immunolabeled cells in each gate based on fluorescent intensity (FL1) relative to the appropriate negative control. Only cells having a fluorescence intensity greater than the MFI of the negative control cells were used to calculate the MFI of immunopositive cells. Percentage data for the initial FC differential cell counts and FC data for ConA stimulated lymphocytes/blasts (DS n=3; BHS n=3) were analyzed by Mantel-Haenszel tests in the

frequency procedure of SAS version 9.2 (SAS Institute, Cary, NC, USA) to calculate P-values as well as odds ratios and 95% confidence intervals for interspecies comparisons. These tests were stratified by animal to minimize the influence of any individual animal results on the overall results for each analysis. Cell data from manual leukocyte counts and FC analyses of neutrophils immunolabeled with a subset of the mAbs (DS n=8; BHS n=5) were analyzed using GraphPad Prism 6 software (GraphPad Software, Inc. La Jolla, CA). Unpaired t tests or Welch's correction for unequal variances, when F-test indicated unequal variance ($P > 0.5$), were performed and two-tailed P values were calculated. Results were considered significant for all analyses at $P \leq 0.05$.

3. Results

3.1. Autofluorescence of sheep eosinophils confirmed by cell sorting and fluorescence microscopy

Prior to identifying cross reactive mAbs for the present study, FC analysis of total peripheral blood leukocytes from DS and BHS identified a distinct subpopulation of granulocytes with higher autofluorescence in the FL1 and FL2 channels (Figure 1A(c), (d)). This subpopulation is also observed on FL3-SSC dot plots (not shown). The subpopulations of granulocytes from 2 DS and 2 BHS were sorted on FL2-SSC dot plots and collected separately (Figure 1A(c), yellow and purple events). Cells collected from the UR quadrant of each sample (purple events) were predominantly eosinophils while those collected from the UL quadrant (yellow events) were >95% neutrophils as evaluated by light and epifluorescence microscopy (Supplementary file 1). Although sheep eosinophils and neutrophils have a similar forward and side scatter, best illustrated by the overlapping purple (eosinophils) and yellow (neutrophils) gated events in the FSC-SSC dot plots in Figure 2A(e), the multichannel autofluorescence characteristic of sheep

eosinophils permits separate analysis of neutrophils and eosinophils in single-immunolabeled leukocyte preparations.

3.2. PBMC and granulocyte composition is significantly different in clinically healthy DS and BHS bloods

FC was used to quantify the composition of total peripheral blood leukocytes from DS and BHS, based on intrinsic differences in granularity and autofluorescence between cell types as described. While the number of total isolated leukocytes was similar between the two species, BHS had a significantly higher proportion of neutrophils as compared to DS, while DS had a significantly higher proportion of PBMC ($P \leq 0.0001$). Viability of the isolated total leukocytes was not significantly different between the two species ($P = 0.69$). Manual cell counts performed on three separate blood draws from each sheep (DS: $n=8$; BHS: $n=5$) supported this finding. Data are summarized in Table 2.

3.3. Comparative analysis of mAbs reactive with DS and BHS leukocytes by FC

Forty-six mAbs, which recognize epitopes of LDMs and of the MHC, were observed to show similar patterns of immunoreactivity on peripheral blood leukocytes isolated from DS and BHS, as evaluated by FL1-SSC dot plots (Table 1, Supplementary file 2). The initial mAb panel analysis was performed on 3 DS and 3 BHS and included all of the mAbs, except one (#17_HUH82A_CD18) which was evaluated in subsequent analyses.

3.4. LDM abundance on DS and BHS neutrophils

The initial mAb panel analysis (45 mAbs) revealed interspecies differences in MFIs for neutrophils labeled with mAbs directed against CD14 (#14_CAM36A_CD14; #15_CAM66A_CD14), CD11a (#11_HUH73A_CD11a), CD11b (#12_MM12A_CD11b), CD18 (#16_BAQ30A_CD18), and CD172a (#26_DH59B_CD172a) (Supplementary file 3). In brief,

immunolabeled DS neutrophils had a greater MFI when immunolabeled with mAbs directed against CD11a, CD11b, CD18, and CD172a and BHS neutrophils had a greater MFI with each anti-CD14 mAb.

To further investigate the observed interspecies differences, these 6 mAbs, as well as a second anti-CD 18 mAb (#17_HUH82A_CD18), were used to immunolabel leukocytes isolated on the same day from 8 DS and 5 BHS. Labeling with mAbs directed against CD11a, CD11b, and CD172a resulted in significantly greater MFIs on immunopositive DS neutrophils as compared to BHS ($P = 0.0004$, 0.0489 , and <0.0001 , respectively). The MFI of immunopositive neutrophils for each anti-CD14 mAb (#14_CAM36A_CD14; #15_CAM66A_CD14) was again significantly higher in BHS ($P = 0.0018$ and 0.0038 , respectively), with a similar BHS:DS average MFI ratio for each mAb (5.72 and 5.03 , respectively). Although DS neutrophils again exhibited a greater average MFI for each mAb directed against CD18 (#16_BAQ30A_CD18; #17_HUH82A_CD18), with a similar DS:BHS MFI ratio for each anti-CD18 mAb (1.15 and 1.23 , respectively), the difference was not significant for either ($P = 0.0656$ and 0.0650 , respectively). Results are summarized in Table 3A.

In order to determine if the observed differences in interspecies cell surface abundances of these LDMs are due to cellular changes occurring during the leukocyte isolation procedure (ie. erythrocyte lysis) or in whole blood over time, samples of whole blood were immunolabeled at 1 and 5 hours post collection from each of the 13 sheep. At 1 hour, similar significant interspecies differences were observed with greater CD14 on immunopositive BHS neutrophils (#14_CAM36A_CD14; $P = 0.0010$ and #15_CAM66A_CD14; $P = 0.0002$) and greater CD11a (#11_HUH73A_CD11a; $P = 0.0048$), CD11b (#12_MM12A_CD11b; $P = 0.0005$), and CD172a (#26_DH59B_CD172a; $P < 0.0001$) on immunopositive DS neutrophils. At 5 hours, the MFIs

for each anti-CD14 antibody remained significantly greater on BHS neutrophils ($P = 0.0349$ and 0.0003) and the MFIs for CD11a, CD11b, and CD172a remained significantly greater in DS ($P = 0.0092$, 0.0028 , and 0.0002 , respectively). Although the MFI of the anti-CD11a mAb significantly decreased on neutrophils between 1 and 5 hours for both DS and BHS ($P = 0.0033$ and 0.0397 , respectively), the MFI of DS neutrophils remained significantly greater than BHS neutrophils at both time points. While the MFI of anti-CD18 (#16_BAQ30A_CD18) was higher on DS neutrophils labeled in whole blood at both 1 and 5 hours post blood collection, only the 1 hour time point was significant (1 hour: $P = 0.0217$; 5 hours: $P = 0.4703$).

Additional analyses were performed to evaluate potential age-associated differences in neutrophils isolated from the 2 and 4 year old DS immunolabeled with the subset of 6 mAb. Only anti-CD11a exhibited a significant difference, with a higher MFI observed on neutrophils isolated (post-erythrolysis immunolabeling) from the 2 year old sheep ($P = 0.0133$); however, both 2 and 4 year old DS neutrophils exhibited significantly higher MFIs than BHS neutrophils ($P = 0.0012$ and 0.0133 , respectively). There were no significant age-associated differences in the DS neutrophils immunolabeled in whole blood at either 1 or 5 hours post blood collection ($P > 0.5$ for each analysis).

3.4. ConA stimulated lymphocytes/blasts

Viability of ConA stimulated lymphocytes/blasts was not significantly different between DS and BHS ($P = 0.0570$) following gradient clean-up and prior to immunolabeling with mAbs directed against T cell activation LDMs (#41_GB110A_ACT116, #42_CACT116A_CD25, #43_GB112A_CD25, #44_LCTB2_CD25, #45_CACT200A_SLAMF9, and #46_CACT206A_SLAMF9). Stimulated lymphocytes/blasts for each animal were separated into 2 gates (gate-1 and gate-2) using the same FSC-SSC parameters for each animal with the higher

FSC gate (gate-2) predicted to include the majority of the lymphoblast cells (Figure 1B). DS had a statistically higher proportion of cells in gate-2 ($P < 0.0001$). However, the means and standard deviations of distribution percentages and calculated odds ratios of DS and BHS cells in gate-1 (DS = $79.1\% \pm 7.3$; BHS = $83.5\% \pm 2.1$; OR = 1.33, 95% CI = 1.30-1.37) and gate-2 (DS = $20.9\% \pm 7.3$; BHS = $16.5\% \pm 2.1$; OR = 1.25, 95% CI = 1.22-1.28) indicate that the gate distribution differences were small. The 3 anti-CD25 mAbs exhibited significantly higher MFIs ($P < 0.0001$) for immunopositive BHS gate-2 cells as compared to DS gate-2 cells, with similar BHS:DS MFI ratios (1.80, 1.75, and 1.75). Significantly greater MFIs were also observed for BHS gate-2 cells immunolabeled with anti-ACT16 ($P < 0.0001$). While the MFI of one of the anti-SLAMF9 mAb (#46_CACT206A_SLAMF9) was observed to be significantly greater in BHS gate-2 cells as compared to DS gate-2 cells ($P < 0.0001$) the MFIs of the second anti-SLAMF9 mAb (#45_CACT200A_SLAMF9) was not significantly different between DS and BHS ($P = 0.268$). Results are summarized in Table 3B.

4. Discussion

Based on the comparable and expected immunoreactivity patterns of the mAbs evaluated in this study, we conclude that we have identified a set of reagents for use in interspecies comparative analyses of DS and BHS immune systems. Characterization of eosinophil multichannel (FL1, FL2, and FL3) autofluorescence in this study permitted cell specific single-label FC analysis of neutrophils. Similar intrinsic differences in autofluorescence between neutrophils and eosinophils have been previously described in rodents, humans, and non-human primates [7-9]. We have also identified, by cell sorting and fluorescence microscopy, differential autofluorescence between eosinophils and neutrophils in peripheral blood leukocytes from cattle, horses, and goats (data not shown).

The interspecies comparative analyses performed in this study on immunolabeled neutrophils and ConA stimulated lymphocytes revealed differences between DS and BHS in labeling intensities of molecules that play critical roles in host response to bacterial infection, including the β_2 integrin subunits, CD18, CD11a, and CD11b; CD14, a pathogen recognition receptor that binds LPS as a multimeric complex (CD14/TLR4/MD2); CD172a (also known as SIRP α), an inflammation regulatory protein; and CD25, a subunit of the receptor for IL-2.

We interpret the observed differences to be an interspecies difference in cell surface abundance of protein antigens rather than a difference in affinity, particularly for CD18, CD11b, and CD14 since these proteins have been characterized to exhibit 99% interspecies identity at the amino acid sequence level [10-12]. This is further supported by the observation that each anti-CD18 mAb (BAQ30A and HUH82A) exhibited a similar DS:BHS neutrophil MFI ratio (1.15 and 1.23, respectively) and each anti-CD14 mAb (CAM36A and CAM66A) exhibited a similar BHS:DS neutrophil MFI ratio (5.72 and 5.03, respectively). Specificity of mAbs BAQ30A and MM12A specifically for sheep CD18 and CD11b antigens, respectively, has been previously described [13, 14].

Previous reports, and results repeated in our laboratory (manuscript in preparation), indicate that BHS neutrophils have a heightened sensitivity, as compared to DS neutrophils, to the leukocytolytic effects of leukotoxin A (LktA), a primary virulence factor secreted by pathogenic strains of Pasteurellaceae (*Mannheimia haemolytica* and *Bibersteinia trehalosi*) [4, 17-19].

Heterodimeric β_2 integrins, CD11a/CD18 and CD11b/CD18, function as receptors for LktA to elicit leukocytotoxic effects, particularly through toxin interaction with the CD18 subunit [15, 16]. It has been implied that this heightened *in vitro* sensitivity may reflect an exacerbated and detrimental *in vivo* pulmonary inflammatory response to LktA-bearing Pasteurellaceae bacterial

infections in BHS. Therefore, identifying either a similar or higher abundance of the LktA receptor subunits, CD18, CD11a, and CD11b, on isolated DS neutrophils in this study is perhaps unexpected and lends to question whether there is an alternative pathway by which LktA elicits neutrophil leukocytotoxicity.

The higher abundance of CD14 on BHS neutrophils as compared to DS may be one such pathway, as LktA is reported to complex with LPS at an LktA to LPS molar ratio of 1:60 [20]. CD14 is a pattern recognition receptor that plays a vital role in host innate immune response to Gram-negative bacteria, such as members of the Pasteurellaceae family. CD14 binds to lipopolysaccharide (LPS), a cell-wall component of Gram-negative bacteria, and acts through toll-like receptor 4 (TLR4) signaling to activate transcription factor NF κ B, which in turn activates transcription of genes and subsequent release of cytokines involved in host defense against bacterial infection. Although this signaling pathway is vital for host response to infection, an exacerbated response can result in undesirable consequences such as systemic shock. LPS-induced proinflammatory CD14/TLR4 signal pathways have also been shown to increase Mac-1 (CD11b/CD18) on the surface of human neutrophils [21]. Additionally, LPS binding to CD14 has been reported to aid in the delivery of LPS-exotoxin complexes to the surface of target cells [22]. The role of CD14 in LktA induced cytolysis has been previously investigated using CD14 transfected human embryonic kidney (HEK) cells with and without coexpression of LFA-1 (CD11a/CD18) or Mac-1 (CD11b/CD18) [12]. While it was concluded that CD14 does not enhance LktA-induced cytotoxicity, the conclusion of that study was limited by the lack of toll-like receptor 4 (TLR4) coexpression, as formation of a LPS/CD14/TLR4/MD2 complex is required for the proinflammatory cell signal transduction induced by LPS binding. Therefore, further research is needed to investigate the role of CD14 in LktA induced *in vitro*

cytotoxicity and relevance to *in vivo* host response to acute infection with LktA-bearing Pasteurellaceae and other Gram-negative bacteria.

Cross-reactivity of the anti-human mAb DH59B (CD172a) in DS has been previously described [23]. CD172a, also known as signal regulatory protein α (SIRP α), is a plasma membrane protein expressed mainly by myeloid cells and is involved in downregulation of myeloid cell activity. CD172a serves as a regulatory protein to suppress inflammatory processes such as phagocytosis and migration of neutrophils, important processes in regulating exacerbation of the immune response and subsequent host tissue damage. Since neutrophils are acute responders to bacterial infections, the higher abundance of CD172a identified in this study on DS neutrophils as compared to BHS is of interest considering the reported increased susceptibility of BHS to bacterial pneumonia.

We observed changes in abundances (MFIs) of CD18 and CD11a on neutrophils labelled in whole blood at 1 and 5 hours post-collection with mAbs BAQ30A and HUH73A, respectively. This indicates that there are time-dependent cellular changes occurring following blood collection, independent of erythrocyte lysis performed to isolate leukocytes for use in *in vitro* experiments, suggesting that time may be of essence in repeat analyses in order to maximize test-retest reliability.

We also observed differences in ConA stimulated lymphocytes/blasts, with BHS exhibiting a higher MFI with each of the anti-CD25 mAbs (CACT116A, GB112A, and LCTB2A). Since the MFI was proportionately similar for each of the three CD25 antibodies, the higher MFI is interpreted as a higher abundance of CD25 antigen on activated BHS lymphocytes/blasts as compared to DS, since it would be unlikely that 3 mAbs would exhibit similar interspecies affinity differences. CD25 is part of the heterodimeric protein receptor for IL-2, a cytokine that

acts in promoting the differentiation of lymphocytes (T cells) into effector T cells and memory T cells, thus serving a pivotal role in humoral immunity. Identification of these cross-reactive mAbs and others (GB110A, CACT200, CACT206) that recognize lymphocyte activation molecules and the initial analyses performed in this study provide reagents (mAbs) and an opportunity for future lymphocyte stimulation studies.

In conclusion, this study describes cross-reactive mAbs that recognize LDM and MHC antigens on DS and BHS leukocytes. Characterization of eosinophil autofluorescence allowed for comparative evaluation of DS and BHS neutrophils by single-label FC. Interspecies comparative analyses revealed significant differences in LDMs on neutrophils that play critical roles in host response to bacterial infection, including greater abundances of CD11a, CD11b, CD18 (β_2 integrins) and CD 172a (SIRP α) on DS PMN, and greater CD14 (lipopolysaccharide receptor) on BHS neutrophils.

Conflict of interest

The mAbs used in the present study were developed by WCD or associates in the course of their studies on the immune response to pathogens in ruminants and made available through the non-profit service center of Washington State University, College of Veterinary Medicine, Monoclonal Antibody Center. WCD claims there were no conflicts of interest in collaborating in the use of the mAbs in the present study. The authors declare no conflicts interest.

Abbreviations

DS: domestic sheep; BHS: bighorn sheep; FC: flow cytometry; PBMC: peripheral blood mononuclear cells; LDM: leukocyte differentiation molecule; CD: complex of differentiation;

MHC: major histocompatibility complex; ConA: Concavalin A; Ab: antibody; mAb: monoclonal antibody; MFI: relative median fluorescence intensity

Acknowledgments

We would like to thank Dr. Siddra A. Hines, Dr. Rohanna Dassanayake, Victoria Mack, and James Reynolds for advice and technical assistance and Dr. Charles Frevert for technical editing and critical review of the manuscript. This work was supported by a fellowship training grant from Zoetis (previously Pfizer) Animal Health-Morris Animal Foundation (145-02-13K-2540-0992) and ADRU-ARS-USDA CRIS Project funding ((2090-32000-031-00D).

References

- [1] T.E. Besser, E.F. Cassirer, K.A. Potter, K. Lahmers, J.L. Oaks, S. Shanthalingam, S. Srikumaran, W.J. Foreyt, Epizootic pneumonia of bighorn sheep following experimental exposure to *Mycoplasma ovipneumoniae*, *PLoS One* 9(10) (2014) e110039.
- [2] R.P. Dassanayake, S. Shanthalingam, C.N. Herndon, P.K. Lawrence, E. Frances Cassirer, K.A. Potter, W.J. Foreyt, K.D. Clinkenbeard, S. Srikumaran, *Mannheimia haemolytica* serotype A1 exhibits differential pathogenicity in two related species, *Ovis canadensis* and *Ovis aries*, *Vet Microbiol* 133(4) (2009) 366-71.
- [3] W.J. Foreyt, D.A. Jessup, Fatal pneumonia of bighorn sheep following association with domestic sheep, *J Wildl Dis* 18(2) (1982) 163-8.
- [4] R.M. Silflow, W.J. Foreyt, R.W. Leid, *Pasteurella haemolytica* cytotoxin-dependent killing of neutrophils from bighorn and domestic sheep, *J Wildl Dis* 29(1) (1993) 30-5.

- 364 [5] W.C. Davis, Washington State Univeristy, Flow Cytometry Laboratory, Isolation of Cells
 365 from Peripheral Blood. <[http://vmp.vetmed.wsu.edu/resources/flow-cytometry-laboratory/cell-](http://vmp.vetmed.wsu.edu/resources/flow-cytometry-laboratory/cell-isolation)
 366 isolation>, (accessed 12/30/2015).
- 367 [6] W.C. Davis, Washington State Univeristy, Flow Cytometry Laboratory, Labeling Cells with
 368 Monoclonal Antibodies (mAbs) for Flow Cytometry.
 369 <<http://vmp.vetmed.wsu.edu/resources/flow-cytometry-laboratory/labeling-cells>> (accessed
 370 12/30/2015).
- 371 [7] D.A. Dorward, C.D. Lucas, A.L. Alessandri, J.A. Marwick, F. Rossi, I. Dransfield, C.
 372 Haslett, K. Dhaliwal, A.G. Rossi, Technical advance: autofluorescence-based sorting: rapid and
 373 nonperturbing isolation of ultrapure neutrophils to determine cytokine production, *J Leukoc Biol*
 374 94(1) (2013) 193-202.
- 375 [8] D.E. Fuerst, J.R. Jannach, Autofluorescence of eosiniphils: a bone marrow study, *Nature* 205
 376 (1965) 1333-1334.
- 377 [9] L. Zhang, M.P. Soares, Y. Guan, S. Matheravidathu, R. Wnek, K.E. Johnson, A. Meisher,
 378 S.A. Iliff, J.S. Mudgett, M.S. Springer, B.L. Daugherty, Functional expression and
 379 characterization of macaque C-C chemokine receptor 3 (CCR3) and generation of potent
 380 antagonistic anti-macaque CCR3 monoclonal antibodies, *J Biol Chem* 277(37) (2002) 33799-
 381 810.
- 382 [10] W. Liu, K.A. Brayton, J. Lagerquist, W.J. Foreyt, S. Srikumaran, Cloning and comparison
 383 of bighorn sheep CD18 with that of domestic sheep, goats, cattle, humans and mice, *Vet*
 384 *Immunol Immunopathol* 110(1-2) (2006) 11-6.
- 385 [11] P.K. Lawrence, S. Srikumaran, CD11b of *Ovis canadensis* and *Ovis aries*: molecular
 386 cloning and characterization, *Vet Immunol Immunopathol* 119(3-4) (2007) 287-98.

- 387 [12] R.P. Dassanayake, P.K. Lawrence, D.P. Knowles, W.C. Davis, W.J. Foreyt, S. Srikumaran,
 388 Co-expression of ovine LPS receptor CD14 with Mannheimia haemolytica leukotoxin receptor
 389 LFA-1 or Mac-1 does not enhance leukotoxin-induced cytotoxicity, Vet Immunol Immunopathol
 390 141(1-2) (2011) 84-91.
- 391 [13] V.K. Gupta, I. McConnell, J. Hopkins, Reactivity of the CD11/CD18 workshop monoclonal
 392 antibodies in the sheep, Vet Immunol Immunopathol 39(1-3) (1993) 93-102.
- 393 [14] R. Brodersen, F. Bijlsma, K. Gori, K.T. Jensen, W. Chen, J. Dominguez, K. Haverson, P.F.
 394 Moore, A. Saalmuller, D. Sachs, W.J. Slierendrecht, C. Stokes, O. Vainio, F. Zuckermann, B.
 395 Aasted, Analysis of the immunological cross reactivities of 213 well characterized monoclonal
 396 antibodies with specificities against various leucocyte surface antigens of human and 11 animal
 397 species, Vet Immunol Immunopathol 64(1) (1998) 1-13.
- 398 [15] R.P. Dassanayake, S.K. Maheswaran, S. Srikumaran, Monomeric expression of bovine
 399 beta2-integrin subunits reveals their role in Mannheimia haemolytica leukotoxin-induced
 400 biological effects, Infect Immun 75(10) (2007) 5004-10.
- 401 [16] R.S. Gopinath, T.C. Ambagala, M.S. Deshpande, R.O. Donis, S. Srikumaran, Mannheimia
 402 (Pasteurella) haemolytica leukotoxin binding domain lies within amino acids 1 to 291 of bovine
 403 CD18, Infect Immun 73(9) (2005) 6179-82.
- 404 [17] W.J. Foreyt, R.M. Silflow, Attempted protection of bighorn sheep (*Ovis canadensis*) from
 405 pneumonia using a nonlethal cytotoxic strain of Pasteurella haemolytica, biotype A, serotype, J
 406 Wildl Dis 32(2) (1996) 315-21.
- 407 [18] S.J. Sweeney, R.M. Silflow, W.J. Foreyt, Comparative leukotoxicities of Pasteurella
 408 haemolytica isolates from domestic sheep and free-ranging bighorn sheep (*Ovis canadensis*), J
 409 Wildl Dis 30(4) (1994) 523-8.

- 410 [19] R.M. Silflow, W.J. Foreyt, Susceptibility of phagocytes from elk, deer, bighorn sheep, and
411 domestic sheep to *Pasteurella haemolytica* cytotoxins, *J Wildl Dis* 30(4) (1994) 529-35.
- 412 [20] J. Li, K.D. Clinkenbeard, Lipopolysaccharide complexes with *Pasteurella haemolytica*
413 leukotoxin, *Infection and immunity* 67(6) (1999) 2920-7.
- 414 [21] E.B. Lynam, S.I. Simon, Y.P. Rochon, L.A. Sklar, Lipopolysaccharide enhances
415 CD11b/CD18 function but inhibits neutrophil aggregation, *Blood* 83(11) (1994) 3303-11.
- 416 [22] L.E. Mansson, P. Kjall, S. Pellett, G. Nagy, R.A. Welch, F. Backhed, T. Frisan, A. Richter-
417 Dahlfors, Role of the lipopolysaccharide-CD14 complex for the activity of hemolysin from
418 uropathogenic *Escherichia coli*, *Infect Immun* 75(2) (2007) 997-1004.
- 419 [23] P.J. Griebel, G. Entrican, M. Rocchi, T. Beskorwayne, W.C. Davis, Cross-reactivity of
420 mAbs to human CD antigens with sheep leukocytes, *Vet Immunol Immunopathol* 119(1-2)
421 (2007) 115-22.
- 422 [24] J. Naessens, C.J. Howard, J. Hopkins, Nomenclature and characterization of leukocyte
423 differentiation antigens in ruminants, *Immunology today* 18(8) (1997) 365-8.
- 424 [25] C.R. Mackay, W.R. Hein, M.H. Brown, P. Matzinger, Unusual expression of CD2 in sheep:
425 implications for T cell interactions, *Eur J Immunol* 18(11) (1988) 1681-8.
- 426 [26] M.F. Beya, M. Miyasaka, L. Dudler, T. Ezaki, Z. Trnka, Studies on the differentiation of T
427 lymphocytes in sheep. II. Two monoclonal antibodies that recognize all ovine T lymphocytes,
428 *Immunology* 57(1) (1986) 115-21.
- 429 [27] T. Ezaki, M. Miyasaka, M.F. Beya, L. Dudler, Z. Trnka, A murine anti-sheep T8
430 monoclonal antibody, ST8, that defines the cytotoxic T-lymphocyte population, *International*
431 *Archives of Allergy and Applied Immunology* 82(2) (1987) 168-177.

- 432 [28] P. Vilmos, E. Kurucz, I. Ocsosvski, G. Keresztes, I. Ando, Phylogenetically conserved
 433 epitopes of leukocyte antigens, *Vet Immunol Immunopathol* 52(4) (1996) 415-26.
- 434 [29] C.J. Howard, W.I. Morrison, A. Bensaid, W. Davis, L. Eskra, J. Gerdes, M. Hadam, D.
 435 Hurley, W. Leibold, J.J. Letesson, et al., Summary of workshop findings for leukocyte antigens
 436 of cattle, *Vet Immunol Immunopathol* 27(1-3) (1991) 21-7.
- 437 [30] A. Saalmuller, J.K. Lunney, C. Daubengerger, W. Davis, U. Fischer, T.W. Gobel, P.
 438 Griebel, E. Hollemweguer, T. Lasco, R. Meister, H.J. Schuberth, K. Sestak, P. Sopp, F.
 439 Steinbach, W. Xiao-Wei, B. Aasted, Summary of the animal homologue section of HLDA8,
 440 *Cellular immunology* 236(1-2) (2005) 51-8.
- 441 [31] J. Naessens, J. Hopkins, Introduction and summary of workshop findings, *Veterinary*
 442 *Immunology and Immunopathology* 52 (1996) 213-235.
- 443 [32] C.R. Mackay, W.L. Marston, L. Dudler, O. Spertini, T.F. Tedder, W.R. Hein, Tissue-
 444 specific migration pathways by phenotypically distinct subpopulations of memory T cells, *Eur J*
 445 *Immunol* 22(4) (1992) 887-95.
- 446 [33] K.T. Park, S. Burnett, W.C. Davis, Development and characterization of a monoclonal
 447 antibody specific for bovine CD209, *Vet Immunol Immunopathol* 163(3-4) (2015) 216-20.
- 448 [34] W.C. Davis, S. Marusic, H.A. Lewin, G.A. Splitter, L.E. Perryman, T.C. McGuire, J.R.
 449 Gorham, The development and analysis of species specific and cross reactive monoclonal
 450 antibodies to leukocyte differentiation antigens and antigens of the major histocompatibility
 451 complex for use in the study of the immune system in cattle and other species, *Vet Immunol*
 452 *Immunopathol* 15(4) (1987) 337-76.
- 453 [35] W.C. Davis, L.E. Perry, T.C. McGuire, The identification and analysis of major functional
 454 populations of differentiated cells, in: N.J. Stern, H.R. Gamble (Eds.), *Hybridoma Technology in*

- 455 Agricultural and Veterinary Research, Rowman and Allanheld, Totowa, New Jersey, 1984, pp.
456 121-150.
- 457 [36] C.R. Mackay, M.F. Beya, P. Matzinger, Gamma/delta T cells express a unique surface
458 molecule appearing late during thymic development, *Eur J Immunol* 19(8) (1989) 1477-83.
- 459 [37] W.C. Davis, L.R. Heirman, M.J. Hamilton, S.M. Parish, G.M. Barrington, A. Loftis, M.
460 Rogers, Flow cytometric analysis of an immunodeficiency disorder affecting juvenile llamas,
461 *Vet Immunol Immunopathol* 74(1-2) (2000) 103-20.
- 462 [38] P.J. Griebel, W.C. Davis, J.D. Reynolds, An analysis of the growth and differentiation of B
463 cells isolated from follicles of the ileal Peyer's patch of sheep, *Immunology* 75(4) (1992) 601-7.
- 464 [39] N.D. MacHugh, P.G. Taracha, P.G. Toye, Reactivity of workshop antibodies on L cell and
465 COS cell transfectants expressing bovine CD antigens, *Veterinary Immunology and*
466 *Immunopathology* 39(1) (1993) 61-67.
- 467 [40] K.T. Park, K.S. Seo, N.A. Godwin, B.J. Van Wie, M.Y. Gulbahar, Y.H. Park, W.C. Davis,
468 Characterization and expression of monoclonal antibody-defined molecules on resting and
469 activated bovine alphabeta, gammadelta T and NK cells, *Vet Immunol Immunopathol* (2015).

Table 1

Summary of monoclonal antibodies reactive with domestic sheep and bighorn sheep leukocytes

mAb#	mAb clone	Specificity (LDM/MHC)	Ig isotype	Reference
1	TH97A	CD1b	G2a	[35]
2	MUC2A	CD2	G2a	[35]
3	GC1A	CD4	G2a	[29]
4	GC50A	CD4	M	[35]
5	17D	CD4	G1	[36]
6	ST1A	CD5	G2a	[37]
7	ST8	CD8	M	[38]
8	CACT80C	CD8 α	G1	[39]
9	7C2B	CD8 α	G2a	Davis, WSU*
10	BAT82A	CD8 β	G1	[40]
11	HUH73A	CD11a	G1	[41]
12	MM12A	CD11b	G1	[29]
13	BAQ153A	CD11c	M	[22]
14	CAM36A	CD14	G1	[29]
15	CAM66A	CD14	M	Davis, WSU*
16	BAQ30A	CD18	G1	[22]
17	HUH82A	CD18	G2a	[41]
18	BAQ15A	CD21	M	[42]
19	GB25A	CD21	G1	[29]
20	FW4-101	CD29	G1	[39]
21	DH16A	CD45B	M	[39]

22	ILA116A	CD45R0	G3	[42]
23	TH17A	CD47	M	[39]
24	FW3-218	CD49d	G2b	[43]
25	DU1-29	CD62L	G1	[29]
26	DH59B	CD172a (SIRP- α)	G1	[34]
27	209MD26A	CD209 (DC-SIGN)	G2a	[44]
28	H58A	MHC CL I	G2a	[45]
29	H42A	MHC CL II	G2a	[45]
30	TH14B	MHC CL II BoLA-DR	G2a	[46]
31	TH81A5	MHC CL II BoLA-DQ	G2a	[45]
32	CACTB6A	TCR1-N6 (γ chain predicted)	M	[39]
33	86D	TCR1 (γ chain predicted)	G1	[47]
34	B7A1	WC1-N1	M	[45]
35	BAQ4A	WC1-N2	G1	[40]
36	CACTB7A	WC1-N3	G1	[42]
37	BAQ89A	WC1-N4	G1	[40]
38	GB54A	WC1-N25	G2a	[29]
39	GB45A	WC1-N26	G1	[48]
40	BAQ44A	B cell (B-B2)	M	[49]
41	GB110A	ACT16	M	[42]
42	CACT116A	CD25	G1	[50]
43	GB112A	CD25	G1	Davis, WSU*
44	LCTB2A	CD25	G3	[42]
45	CACT200A	SLAM9	G1	[51]
46	CACT206A	SLAM9	G2a	[51]

mAb = monoclonal antibody; CD = complex of differentiation; LDM = leukocyte differentiation molecule; MHC = major histocompatibility complex

Table 2

Summary of domestic sheep and bighorn sheep peripheral blood leukocyte composition

(A)	Species	Total (x10 ⁶ /mL)	%			
			Viability	PBMC	PMN	Eosinophils
	DS	5.3 ±1.88	95.6 ± 1.3	62.1 ±4.5**	35.0 ±4.3	2.5 ±1.3
	BHS	4.8 ±1.97	95.5 ± 1.2	19.8 ±4.9	74.9 ± 6.0**	2.9 ± 3.3*
(B)	DS			66.3 ± 2.0*	31.7 ± 2.0	
	BHS			42.3 ± 4.5	57.7 ± 2.6*	

DS = domestic sheep; BHS = bighorn sheep; PBMC = peripheral blood mononuclear cells. PMN = neutrophils. Mean values are indicated ± standard deviations of the mean. A. Viability and flow cytometric analysis of leukocytes isolated from peripheral blood (BHS n=3; DS n=3). Percentage data analyzed by Mantel-Haenszel tests and total counts by two-tailed unpaired t test. Asterisk (*) is adjacent to the higher value for interspecies comparisons that have statistical significance ($P \leq 0.05$). A double asterisk (**) indicates a large difference ($P \leq 0.05$, OR >2). B. Manual cell counts of 100 total PBMC and neutrophils (eosinophils excluded from analysis) performed on whole blood collected at 3 separate time points for each sheep (BHS n=5; DS n=8). The mean of the triplicate samples from each sheep were used to analyze the data by unpaired t tests; two-tailed P-values ≤ 0.05 were considered significant (*).

Table 3

Immunoreactivity of monoclonal antibodies with peripheral blood neutrophils and Concavalin A stimulated lymphocytes from domestic sheep and bighorn sheep

	LDM	mAb clone	IP (% $\pm$ SD)		MFI $\pm$ SD)	
			DS	BHS	DS	BHS
A	CD11a	HUH73A	99.3 $\pm$ 1.0	99.6 $\pm$ 0.2	446.7 $\pm$ 36.9.0*	324.3 $\pm$ 51.6
	CD11b	MM12a	98.5 $\pm$ 1.2	98.9 $\pm$ 1.0	671.6 $\pm$ 234.7*	415.9 $\pm$ 128.5
	CD14	CAM36A	85.5 $\pm$ 4.5	99.4 $\pm$ 0.2*	25.3 $\pm$ 5.1	144.7 $\pm$ 44.6*
	CD14	CAM66A	77.4 $\pm$ 7.3	99.0 $\pm$ 0.2*	18.5 $\pm$ 3.9	93.1 $\pm$ 23.1*
	CD18	BAQ30A	99.7 $\pm$ 0.2	99.8 $\pm$ 0.1	961.7 $\pm$ 153.1	839.4 $\pm$ 49.67
	CD18	HUH82A	99.7 $\pm$ 0.1	99.8 $\pm$ 0.1	833.72 $\pm$ 154.9	679.3 $\pm$ 77.6
	CD172a	DH59B	98.2 $\pm$ 1.6	99.3 $\pm$ 0.4	444.2 $\pm$ 48.0*	206.56 $\pm$ 42.5
B	ACT16	GB110A	32.6 $\pm$ 13.8	40.1 $\pm$ 8.3*	29.0 $\pm$ 10.2	78.7 $\pm$ 11.7*
	CD25	CACT116A	58.8 $\pm$ 2.6	68.4 $\pm$ 3.3*	164.3 $\pm$ 53.0	295.7 $\pm$ 57.8*
	CD25	GB112A	56.6 $\pm$ 3.1	67.8 $\pm$ 3.2*	165.9 $\pm$ 57.9	289.5 $\pm$ 47.5*
	CD25	LCTB2A	54.7 $\pm$ 3.5	61.1 $\pm$ 3.2*	85.8 $\pm$ 26.5	150.0 $\pm$ 32.7
	SLAMF9	CACT200A	67.2 $\pm$ 5.9	66.3 $\pm$ 8.2	301.5 $\pm$ 192.1	594.1 $\pm$ 179.4
	SLAMF9	CACT206A	51.7 $\pm$ 11.1	66.6 $\pm$ 7.5*	99.6 $\pm$ 88.8	355.0 $\pm$ 109.8*

IP = immunopositive; MFI = median fluorescence intensity; SD = standard deviation; LDM = leukocyte differentiation molecule; mAb = monoclonal antibody; DS = domestic sheep; BHS = bighorn sheep. MFI calculated only for IP neutrophils and ConA stimulated lymphocytes (cells with fluorescence intensity > negative control MFI). Mean values are indicated $\pm$ standard deviations of the mean. (A) Analyses of DS (n=8) and BHS (n=5) neutrophils. Data analyzed by unpaired two-tailed t tests or unpaired t test with Welch's correction for unequal variances; P-values ≤ 0.05 were considered significant (*) (DS n=8; BHS n=5). (B) Concavalin A stimulated lymphoblast analyses. Data analyzed by Mantel-Haenszel tests. An asterisk (*) is placed adjacent to the higher value for interspecies comparisons that have statistical significance (P ≤ 0.05); a double asterisk (**) indicates a large difference (P ≤ 0.05 , OR >2); no large differences were identified.

Figure 1 Gating criteria used for analysis of indirect FITC-labeled leukocyte mAb reagents and cell sorting

(A) Representative dot plots from negative control samples of 1 domestic sheep (top row) and 1 bighorn sheep (bottom row) are shown. (a) FSC-SSC dot plots illustrate the criteria used for gating PBMC (orange gate is predicted to contain lymphocytes; blue gate predicted to contain monocytes and larger sized lymphocytes); red events represent total granulocytes. (b) FL1-SSC dot plots of events gated on FSC-SSC dot plots; this represents the gating strategy used to evaluate immunoreactivity of each of the 45 monoclonal antibodies illustrated in Additional file 2. (c) FL2-SSC dot plots illustrate the gating criteria used to: separately gate PMN (yellow events, open arrow) and eosinophils (purple events, solid arrow) for analyzing FITC immunolabeling specifically of PMN in the FL1 channel (d); sort granulocyte subpopulations (open and closed arrows); and to quantify relative percentages of each population of leukocytes. Black events (white star) are total PBMC. (e) FSC-SSC illustrates the overlap of eosinophils (purple) and PMN (yellow).

(B) Representative dot plots of Concavalin A cultured lymphocytes from 1 domestic sheep (top row) and 1 bighorn sheep (bottom row) are shown. FSC-SSC dot plots illustrate the criteria used for gating larger lymphocytes (blue events in Gate 2) from smaller lymphocytes (orange events in Gate 1). Gate 2 is predicted to include the majority of the activated lymphocytes (lymphoblasts). Following this gating strategy, immunoreactivity of indirect FITC-labeled cells was analyzed in the FL1 channel (SSC-FL1 dot plots).

Figure 1

Figure 1