

Flow Cytometry Baseline on Peripheral Leukocyte Cell Profiles for Dairy Goat Kids

¹Darcy M. Fletcher, ¹Megan B. Vogt, ¹Hannah E. Pirner, ²Ann M. Hess, ³Richard A. Bowen, ¹Marcela Henao-Tamayo and ¹Torsten M. Eckstein

¹Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Campus Delivery 1682, Colorado State University, Fort Collins, CO 80523, USA

²Department of Statistics, College of Natural Sciences,

Campus Delivery 1877, Colorado State University, Fort Collins, CO 80523, USA

³Department of Biomedical Sciences, College of Veterinary Medicine and Biomedical Sciences, Campus Delivery 1683, Colorado State University, Fort Collins, CO 80523, USA

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ABSTRACT

Small ruminants, like goats, would make excellent animal models for not only infectious diseases in large ruminants but also analogous diseases in humans, such as human tuberculosis, Crohn's disease, melioidosis and brucellosis. The main disadvantage for the small ruminant model is the lack of sufficient baseline data on normal, healthy goat kids. Furthermore, most reagents (antibodies and the like) were not developed for goats or sheep. It is important to demonstrate that available resources, especially from the bovine system, cross-react with the caprine and/or ovine system. Finally, potential breed differences have to be evaluated before goat or sheep studies are compared. In this study, leukocyte cell populations were defined in twenty-six dairy goat kids via flow cytometry. We report no significant differences between three breeds of dairy goat kids and demonstrate the effective use of various antibodies for caprine immune cell markers. No breed-specific differences were detected for any leukocyte cell population or for markers specific for various antigen-presenting cells or T cell populations. Interestingly, however, statistical significant differences were found for leukocyte cell populations for the two different time points two weeks apart presented in this study.

Keywords: Goat, Peripheral Blood Leukocytes, Cell Surface Marker, Flow Cytometry

1. INTRODUCTION

Animal models are key elements in evaluating pathogenicity and virulence of various bacterial and viral agents (Horvat, 2009; Ross *et al.*, 2012; Hibiya *et al.*, 2011; Munson *et al.*, 2012; McConnell *et al.*, 2013; Uzal and McClane, 2012). While the mouse model is a sufficient animal model for most human infectious diseases (Yi and Li, 2012; Hviid *et al.*, 2010; Williams *et al.*, 2009; Drescher and Sosnowska, 2008; Groschup and Buschmann, 2008; Zhang and Moss, 2012; Shi *et al.*, 2011), increasing attempts are being made to use

animal models more applicable to zoonotic and animal diseases (Kahn, 2012). The mouse model has distinct advantages over other animal models (financial restrictions, availability of various reagents, presence of various mouse strains), but, in the case of infectious diseases, often lacks correlation to the original host and its immune responses. This correlation is even more important for chronic and recurrent infectious diseases present in humans and animals such as tuberculosis, melioidosis and brucellosis (Padilla-Carlin *et al.*, 2008; Dharmadhikari and Nardell, 2008; Kahl-McDonagh *et al.*, 2006; Soffler *et al.*, 2012).

Corresponding Author: Torsten M. Eckstein, Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Campus Delivery 1682, Colorado State University, Fort Collins, CO 80523, USA Tel: (970) 491-2015 Fax: (970) 491-1815

Considering the amount of financial, space and material resources expended on large animals (horses, cattle), goats serve as excellent animal models for infectious diseases found in ruminants and humans. Over the last decade many reagents were developed for use in ruminants. Although they were designed primarily for use in cattle, most of them exhibit cross-reactivity with closely related ruminants, such as sheep and goat (Mosaad *et al.*, 2006).

Unlike with mouse strains, individual goats do not always react in a manner similar to each other. Furthermore, goats were bred for different purposes, such as fiber, meat and dairy. Many different breeds exist within these groups and it is not known yet how their immune system is organized and might react to certain pathogens. It is also not known if data from various goat breeds can easily be compared or if their healthy immune systems fundamentally differ. Although many studies focus on the immune system of goats, only a couple of publications studied the cellular immune system in goat kids a few weeks old. Neither publication focused on potential breed differences but instead detailed new techniques to characterize various cell populations. With the trend of using small ruminants as key animal models for ruminant infectious diseases, it is important to discover and evaluate any potential breed differences to allow for better breed selection for animal studies and comparison of various studies and their conclusions using different breeds. Recently, flow cytometry has played a stronger role in describing various cell populations of animals in models other than the mouse model.

Here, we provide the flow cytometry baseline results on dairy goat kids for various leukocyte cell populations as well as baseline data on several T cell populations and antigen-presenting cell markers.

2. MATERIALS AND METHODS

2.1. Animals

Twenty-six goat kids aged two to five days were purchased from CCI/Juniper Valley Products (Canon City, Colorado) and transferred the same day to our campus. The goat kids were housed on Colorado State University Foothills Campus in accordance with CSU animal ethics regulations (#11-3120A). All goats were housed in the same barn until the age of seven weeks. Goats were fully milk fed (three times a day) with whole cow milk purchased from Walmart. The goats were less than 6 weeks old at the time of the blood draw. By breed, there were fourteen Alpines, three Saanens, seven

Anglo-Nubians, one LaMancha and one Toggenburg. The LaMancha and Toggenburg were not considered in the comparative analyses because there is only a single goat for those breeds; however, data obtained for the two animals will still be presented. The fourteen Alpines were further divided into the following sub-breeds: Alpine-Chamoise (seven goats), Alpine-Cou Blanc (four goats), Alpine-Sundgau (two goats) and Alpine Broken (one goat). The three Saanens were identified as two Saanen-mix and one Saanen-Sable. The Anglo-Nubian breed was not further divided.

2.2. Blood Draw and White Blood Cell Preparation

Two blood draws were performed due to the young age of the goat kids and the amount of blood needed to perform all anticipated cell marker-staining experiments. The first blood draw was performed at age 3.5 weeks, while the second blood draw was performed at age 5.5 weeks. For each blood draw four milliliters of blood were obtained aseptically from the jugular vein into an EDTA solution for immediate analyses. Each sample was transferred to a separate 50 mL polypropylene conical tube. Samples were treated with Gey's solution (155mM NH₄Cl, 1mM KHCO₃) to lyse the red blood cells. A volume of Gey's solution equal to that of the blood was added to each sample. After five minutes incubation, Phosphate Buffered Saline (PBS) was added in equal volume to Gey's solution. White blood cells were pelleted via centrifugation for 5 min at 259g at 4°C. The supernatant was discarded and pellets washed twice in 10 mL PBS each, centrifuging and discarding the supernatant of each wash. After the second wash, clean pellets were resuspended in complemented RPMI media (RPMI media, 8.7% of Fetal Bovine Serum, 1M HEPES Buffer, 50× MEM without L-glutamine, 100mM Sodium Pyruvate, 200 mM L-glutamine solution, Penicillin (10,000 units mL⁻¹)/Streptomycin (10,000 ug mL⁻¹) solution) (cRPMI). Pellets containing a visible red blood cell layer were treated with an additional 5 mL Gey's solution for 1-2 min before adding 10 mL PBS. Pellets were reformed via centrifugation under the aforementioned conditions and then resuspended in cRPMI media.

2.3. Antibodies for Cell Markers

Conjugated antibodies for flow cytometry cell surface marker staining were used for the following cell markers: CD1, CD4, CD8, CD335, MHC class II, WC1 and CD14 (**Table 1**).

Table 1. Conjugated monoclonal antibodies for various cell surface markers used in this study, their primary reactivity, isotype, fluorophore and the cell phenotypes they primarily represent

mAb	Reactivity	Clone	Iso-type	Fluoro-phore	Cell phenotype	Company	cat.No
CD1	ovine	20.27	IgG1	A647	Antigen Presenting (lipids)	AbD Serotec	MCA2212A647
CD4	Ovine	44.38	IgG2a	A647	T Helper Lymphocyte	AbD Serotec	MCA2213A647
CD8	Bovine	CC63	IgG2a	FITC	Cytotoxic T Lymphocyte	AbD Serotec	MCA 837F
CD335	Bovine	AKS1	IgG1	A488	Natural Killer cells	AbD Serotec	MCA 2365A488
MHCII	Ovine	34.68	IgG2a	FITC	Antigen Presenting (lipids)	AbD Serotec	MCA2226F
WC1	Bovine	CC15	IgG2a	FITC	$\gamma\delta$ T Lymphocyte	AbD Serotec	MCA 838F
CD14	Human	M532	IgG2a	PE	Monocyte	BioLegend	301806

2.4. Staining of Cell Markers

Whole lysed blood was stained with monoclonal antibodies to identify cell surface markers. Cells (5×10^6 cells mL^{-1}) were incubated for 20 min at 4°C with 10 μL of each monoclonal antibody. Following the incubation, the cells were washed three times with 200 μL PBS, centrifuged at 260 g and resuspended in 200 μL PBS.

2.5. Flow Cytometry

Samples were analyzed via a flow cytometer (FACSCantoII, Becton Dickinson, USA) equipped with BD FACSDiva software (Becton Dickinson, USA). A minimum of 1500 events was collected per sample. Profiles were analyzed with FlowJo (FlowJo, USA). Four panels were analyzed: CD4/CD8, CD4/WC1, CD1/CD14 and MHCII/CD14. Panels CD4/CD8 and CD4/WC1 were performed on the first blood draw at the age of 3.5 weeks, while CD14/CD1 and CD14/MHC class II were measured on the second blood draw at the age of 5.5 weeks. Originally, CD335 was also included. Since no measurable amounts of CD335 positive cells were detected, this data set was not included in the overall analyses.

The fluorescence in each sample was determined using a BD FACSCanto II (BD Bioscience) and the acquired data was analyzed using the FlowJo software (TreeStar, Ashland, OR). Gate strategy included selection of cell populations according to their side/forward scatter profile. Thereafter, the fluorescence of positive cells was compared to their corresponding isotype-matched control and the percentage of positive cells and intensity of fluorescence was recorded as percentage of positive cells and Mean Fluorescence Channel (MFC), respectively.

The gating strategy to identify granulocytes, monocytes and lymphocytes is presented in each figure. Briefly, Forward Scatter (FSC-A) represents the cell size in a linear pattern, while the Side Scatter (SSC-A) represents the granulation of each cell in a linear pattern. Granulocytes have strong granulation with a variable cell size, while lymphocytes and monocytes have less

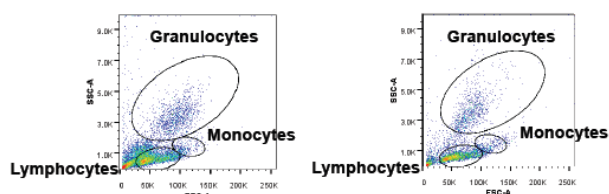
granulation. Lymphocytes are smaller in size than monocytes. Cell population according to their size and granulation are encircled. The individual gating procedure for each panel is described in each figure specifically. Some cell marker analyses were also back-gated to detect the location of the positive cells within the original plot. When analyzing cells for the marker CD14, positive cells were back gated to identify the cell type they originated from. The back gating approach resulted in two CD14+ cell populations: one associated with monocytes and one associate with granulocytes.

2.6. Statistical analyses. Statistical analysis was done using SAS 9.3 (SAS, Cary, NC). For the antibody data, one-way ANOVA Ftests were done to test for differences between breeds for each antibody separately. A Benjamini-Hochberg adjustment was used to account for multiple testing (Benjamini and Hochberg, 1995). For the granulocyte, lymphocyte and monocyte data, one-way ANOVA F-tests were done to test for differences between breeds for each week and draw separately. A Benjamini-Hochberg adjustment was again used to account for multiple testing (Benjamini and Hochberg, 1995). In addition, paired t-tests were done to test for differences between panels and time points.

3. RESULTS

3.1. Gating Results at the Age of 3.5 and 5.5 Weeks

The goat whole lysed blood cell samples were analyzed via flow cytometry according to their SSC-A and FSC-A profiles to determine presence and abundance of the three major cell populations: Lymphocytes, monocytes and granulocytes. Accordingly, granulocytes (variable sizes, high granulation) were identified as SSC-A^{high}/FSC-A^{variable} whereas lymphocytes (small size, low granulation) were identified as SSC-A^{low}/FSC-A^{low} and monocytes (medium size, low granulation) as SSC-A^{low}/FSC-A^{medium} (Fig. 1).



Tag #	Breed	3.5 weeks			5.5 weeks		
		Lymphocytes	Mono-cytes	Granulo-cytes	Lymphocytes	Mono-cytes	Granulo-cytes
6578	Alpine	46.4	3.38	12.8	44.9	2.88	11
6583	Alpine	42.2	2.31	8.6	60	7.59	10.2
6593	Alpine	39.9	2.76	19	53.6	5.97	16.7
6617	Alpine	46.6	5.49	23.2	41.4	2.83	6.1
6618	Alpine	20.9	0.87	12.4	60.6	5.07	7.45
6621	Alpine	49.6	5.43	7.69	55.1	4.26	15.6
6629	Alpine	56.2	5.09	14.7	40.7	2.17	9.38
6630	Alpine	52.7	2.93	7.9	30.3	2.09	3.65
6631	Alpine	49.7	5.02	10.9	51.5	5.12	16.3
6632	Alpine	55.7	6.63	12.3	64.7	2.77	5.23
6670	Alpine	27.1	2.95	26.4	52.8	2.95	4.97
6673	Alpine	61.2	8.38	9.79	49.5	2.29	5.91
6677	Alpine	64.3	4.34	7.76	53.2	3.05	2.86
6678	Alpine	44.4	3.61	17.8	63.4	3.47	9.06
Average	Alpine	46.921	4.228	13.66	51.55	3.751	8.886
6616	Anglo-Nubian	51.8	2.3	8.94	17	0.78	3.61
6622	Anglo-Nubian	39.3	2.38	13.5	41.2	4.12	15
6623	Anglo-Nubian	45.1	2.37	14	31.8	2.56	23.9
6625	Anglo-Nubian	60.2	4.15	9.03	36.3	3.22	16
6679	Anglo-Nubian	15	1.49	33.1	52.6	0.67	3.62
6682	Anglo-Nubian	53.8	3.67	5.82	62	1.84	6.51
6686	Anglo-Nubian	40.4	2.75	21.8	36.4	2.23	11.6
Average	Anglo-Nubian	43.657	2.73	15.17	39.61	2.203	11.46
6620	Saanen	59.3	2.87	14.7	50.5	3.76	9.25
6624	Saanen	33.4	2.5	24.3	9.59	0.53	0.53
6671	Saanen	32.3	2.58	27	56.3	3.97	10.6
Average	Saanen	41.667	2.65	22.00	38.797	2.753	6.79
6619	La Mancha	36.5	1.94	7.4	32.9	4.64	19.1
6627	Toggenburg	59.7	4.03	5.21	35.6	5.53	24.6

Fig. 1. Flow cytometry plots of goat #6593 (as one example for the gating strategy) at age 3.5 weeks (left plot) and at the age of 5.5 weeks (right plot). The two plots demonstrate the strategy of gating for all goats included in this study. Forward scatter represents the cell size in an exponential pattern, while side scatter represents the granulation of each cell in a linear pattern. Granulocytes have strong granulation, while lymphocytes and monocytes have less granulation. Lymphocytes are smaller in size than monocytes. Cell population according to their size and granulation are encircled. The table below the plots shows the amounts of lymphocytes, monocytes and granulocytes obtained at each time points as well as the averages for each breed

Figure 1 presents the data obtained within the second set (panel CD4/CD8) of week 3.5 in comparison with results obtained in week 5.5 of age. Statistical analyses of the various breeds at each time point and panel did not reveal any significant breed-associated differences (**Table 2**).

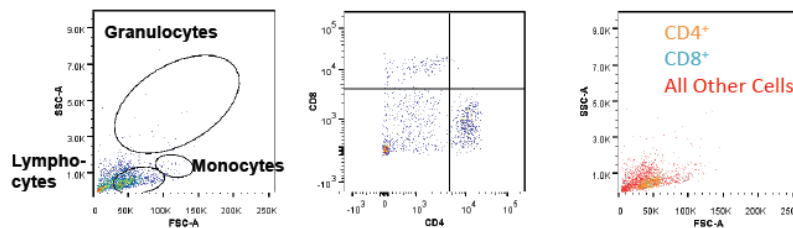
3.2. Gating Results for Various Lymphocyte Populations

Lymphocyte cell populations were gated according to their SSC-A^{low} /FSC-A^{low} profiles (**Fig. 2 and 3**). Five

major lymphocyte populations were analyzed: Cells with positive fluorescence for mAb recognizing CD4 (CD4⁺ T cells), CD8 (CD8⁺ T cells), CD4/CD8 double positive T cells, CD4/CD8 double negative T cells and WC1 positive T cells ($\gamma\delta$ T cells). CD335 was used as a marker for NKT cells, but only trace amounts were present and thus were not included in this analysis. Data for cells with positive surface expression for CD4 and/or CD8 are shown in **Fig. 2**, while those for WC1 and/or CD4 are shown in **Fig. 3**.

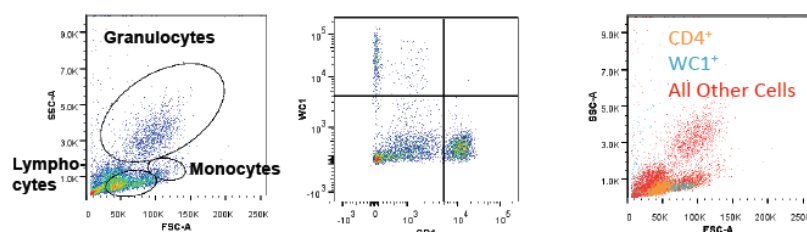
Table 2. Statistical analyses of potential breed differences of average cell amounts of peripheral white blood cell populations for the two different time points and two different panels at week 3 (F: F test statistic for oneway ANOVA, BH p-value represents Benjamini-Hochberg adjusted p-value)

Cell type	Time point	Alpine	Anglo-Nubian	Saanen	F	P-value	BH p-value
Granulocytes	Week 3	13.6600	15.1700	22.0000	1.681	0.210	0.437
Granulocytes	Week 5	8.8860	11.4600	6.7900	0.838	0.446	0.613
Lymphocytes	Week 3	46.9210	43.6570	41.6670	0.276	0.762	0.762
Lymphocytes	Week 5	51.5500	39.6100	38.7970	2.414	0.114	0.344
Monocytes	Week 3	4.2280	2.7300	2.6500	2.634	0.095	0.344
Monocytes	Week 5	3.7510	2.2030	2.7530	2.404	0.115	0.344



Tag #	Breed	CD4+	CD8+	CD4+/CD8+	CD4-/CD8-
6578	Alpine	39.9	11.6	0.49	48
6583	Alpine	36.9	13	0.39	49.8
6593	Alpine	50.1	17.9	0.4	31.7
6617	Alpine	41.3	15.6	0.65	42.5
6618	Alpine	40.4	13.5	0	46.2
6621	Alpine	37.8	6.08	0.1	56
6629	Alpine	59.9	14.3	0.47	25.4
6630	Alpine	43.5	16.3	0.25	39.9
6631	Alpine	50.6	12.6	0.73	36
6632	Alpine	42.8	17.8	0.64	38.7
6670	Alpine	22.2	5.61	0.35	71.9
6673	Alpine	44.9	14.5	0.47	40.1
6677	Alpine	44.8	16.9	0.89	37.5
6678	Alpine	52	19.2	0.51	28.3
Average	Alpine	43.364	13.921	0.453	42.286
6616	Anglo-Nubian	35.5	22.9	0.39	41.2
6622	Anglo-Nubian	32.1	13.6	0.097	54.2
6623	Anglo-Nubian	39.2	10.6	0.93	49.3
6625	Anglo-Nubian	53.6	14.6	0.66	31.1
6679	Anglo-Nubian	45.2	8.31	0.47	46
6682	Anglo-Nubian	40.1	10.2	0.92	48.8
6686	Anglo-Nubian	18.5	8.81	0	72.7
Average	Anglo-Nubian	37.743	12.717	0.495	49.043
6620	Saanen	53.4	14.7	1.03	30.9
6624	Saanen	34.1	17	0.46	48.4
6671	Saanen	42.5	7.58	0.18	49.7
Average	Saanen	43.333	13.093	0.556	43.00
6619	LaMancha	44.9	13.6	0	41.5
6627	Toggenburg	48.7	12.9	0.41	38

Fig. 2. Flow cytometry plots of goat #6593 (as one example for the gating strategy) at age 3.5 weeks (second set). Left plot represents cell sorting according to cell size (FSC-A) and granulation (SSC-A). Lymphocytes are encircled as cells small in size and low in granulation. This cell population was used for analysis for markers for CD4 (x-axis) and CD8 (y-axis) (middle plot). Cells were then back-gated to the original FSC-A/SSC-A plot to identify the correct population of lymphocytes (plot at the right). The table below the flow cytometry plots contains data for the various cell subpopulations associated with the two markers for each goat as well as the average of each breed



Tag #	Breed	CD4+	WC1+	CD4+/WC1+	CD4-/WC1-
6578	Alpine	33.7	9.62	0.043	56.7
6583	Alpine	28	25.6	0.19	46.2
6593	Alpine	40.4	6.91	0.14	52.5
6617	Alpine	29.9	27.1	0.13	42.8
6618	Alpine	31.8	10.7	0	57.6
6621	Alpine	31.8	16.4	0.15	51.7
6629	Alpine	51.7	13.1	0.089	35.1
6630	Alpine	34.1	26.8	0.17	38.9
6631	Alpine	43.8	8.07	0.08	48
6632	Alpine	35.9	21.7	0.11	42.3
6670	Alpine	19.3	33	0	47.7
6673	Alpine	36.1	24.2	0.082	39.6
6677	Alpine	42	18	0.093	39.9
6678	Alpine	31	21.8	0.21	47
Average	Alpine	34.964	18.786	0.106	46.143
6616	Anglo-Nubian	31.2	14.4	0.11	54.3
6622	Anglo-Nubian	29.4	17.2	0.36	53
6623	Anglo-Nubian	33.2	13.1	0.11	53.5
6625	Anglo-Nubian	47.1	9.98	0.22	42.7
6679	Anglo-Nubian	13.3	15.5	0	71.1
6682	Anglo-Nubian	30.3	29.9	0.17	39.7
6686	Anglo-Nubian	33	32.6	0.12	34.2
Average	Anglo-Nubian	31.071	18.954	0.156	49.786
6620	Saanen	45.5	15	0.15	39.3
6624	Saanen	31.1	12.1	0.09	56.7
6671	Saanen	35	17.3	0.19	47.5
Average	Saanen	37.2	14.8	0.143	47.833
6619	LaMancha	37.9	14.9	0.27	46.9
6627	Toggenburg	40.5	17.9	0.11	41.4

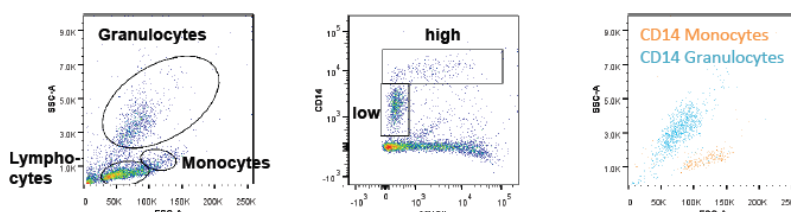
Fig. 3. Flow cytometry plots of goat #6593 (as one example for the gating strategy) at age 3.5 weeks. Left plot represents cell sorting according to cell size (FSC-A) and granulation (SSC-A). Lymphocytes are encircled as cells small in size and low in granulation. This cell population was used for markers for CD4 (x-axis) and WC1 (y-axis) (middle plot). Cells were then back-gated to the original FSC-A/SSC-A plot to identify the correct population of lymphocytes (plot at the right). The table below the flow cytometry plots contains data for the various cell subpopulations associated with the two markers for each goat as well as the average of each breed

No statistical significant differences between the three breeds were found for any T cell population (**Table 3**).

3.3. Gating Results for CD14 Bearing Cells and Antigen-Presenting Cells

Whole lysed blood cell samples obtained from each goat at 5.5 weeks of age were used to identify cells with the CD14 marker (monocytes, granulocytes) in combination with molecules necessary for antigen

presentation (CD1, MHC class II). While most of these cells should be monocytes, a significant portion was identified to be within the granulocyte population (**Fig. 4**). Interestingly, while all granulocytes bear CD14, only a portion of the monocytes bears this marker (32 to 92%). No statistical significances for breed differences were detected (**Table 4**). MHC class II and CD1 molecules are antigen presenting cell surface molecules present on antigen-presenting cells (macrophages, dendritic cells).



Tag #	Breed	Lymphocytes	Mono-cytes	Granulo-cytes	CD14 within monocytes	CD14 within granulocytes
6578	Alpine	44.9	2.88	11	2	11.2
6583	Alpine	60	7.59	10.2	2.42	10.4
6593	Alpine	53.6	5.97	16.7	4.41	16.4
6617	Alpine	41.4	2.83	6.1	1.18	6.29
6618	Alpine	60.6	5.07	7.45	2.07	8.87
6621	Alpine	55.1	4.26	15.6	2.84	16.3
6629	Alpine	40.7	2.17	9.38	1.42	10.4
6630	Alpine	30.3	2.09	3.65	0.97	4.24
6631	Alpine	51.5	5.12	16.3	3.43	15.9
6632	Alpine	64.7	2.77	5.23	1.36	5.68
6670	Alpine	52.8	2.95	4.97	2.06	6.66
6673	Alpine	49.5	2.29	5.91	1.18	7.6
6677	Alpine	53.2	3.05	2.86	2.18	4.16
6678	Alpine	63.4	3.47	9.06	1.9	9.64
Average	Alpine	51.55	3.75	8.89	2.101	9.553
6616	Anglo-Nubian	17	0.78	3.61	0.72	4.15
6622	Anglo-Nubian	41.2	4.12	15	3.04	17.7
6623	Anglo-Nubian	31.8	2.56	23.9	2.04	25.2
6625	Anglo-Nubian	36.3	3.22	16	2.57	17
6679	Anglo-Nubian	52.6	0.67	3.62	0.64	4.1
6682	Anglo-Nubian	62	1.84	6.51	1.65	6.85
6686	Anglo-Nubian	36.4	2.23	11.6	1.89	13.2
Average	Anglo-Nubian	39.61	2.2	11.46	1.793	12.6
6620	Saanen	50.5	3.76	9.25	1.85	8.89
6624	Saanen	9.59	0.53	0.53	0.34	0.95
6671	Saanen	56.3	3.97	10.6	2.34	11.8
Average	Saanen	38.8	2.75	6.79	1.51	7.213
6619	La Mancha	32.9	4.64	19.1	5.56	21
6627	Toggenburg	35.6	5.53	24.6	5.91	25.9

Fig. 4. Flow cytometry plots of goat #6593 (as one example for the gating strategy) at age 5.5 weeks. Left plot represents cell sorting according to cell size (FSC-A) and granulation (SSC-A). Monocytes are encircled as cells larger in size and low in granulation, while granulocytes were detected as cells with strong granulation and variable sizes. These cell populations were used for analysis for markers for MHC II (x-axis) and CD14 (y-axis) (middle plot). CD14+ cells were divided into cells with high amount of CD14 and those with lesser amounts (boxed accordingly). CD14 positive cells were then back-gated shown in the plot to the right (top box: CD14 high; bottom left box: CD14 low) and associated to their respective original cell population as monocytes and granulocytes, respectively. The table below the flow cytometry plots contains data for the various cell subpopulations associated with the two markers for each goat as well as the average of each breed

Table 3. Statistical analyses of potential breed differences of average cell amounts of peripheral T cell populations (F: F test statistic for one-way ANOVA, BH p-value represents Benjamini-Hochberg adjusted pvalue)

Cell type	Alpine	Anglo-Nubian	Saanen	F	P-value	BH p-value
Cd4+ Panel 1	34.9640	31.0710	37.2000	0.728	0.495	0.809
CD8+	13.9210	12.7170	13.0930	0.182	0.835	0.902
CD4+/CD8+	0.4530	0.4950	0.5560	0.161	0.852	0.902
CD4-/CD8-1	42.2860	49.0430	43.0000	0.755	0.482	0.809
CD4+ Panel 2	43.3640	37.7430	43.3330	0.862	0.437	0.809
WCI+	18.7860	18.9540	14.8000	0.336	0.718	0.902

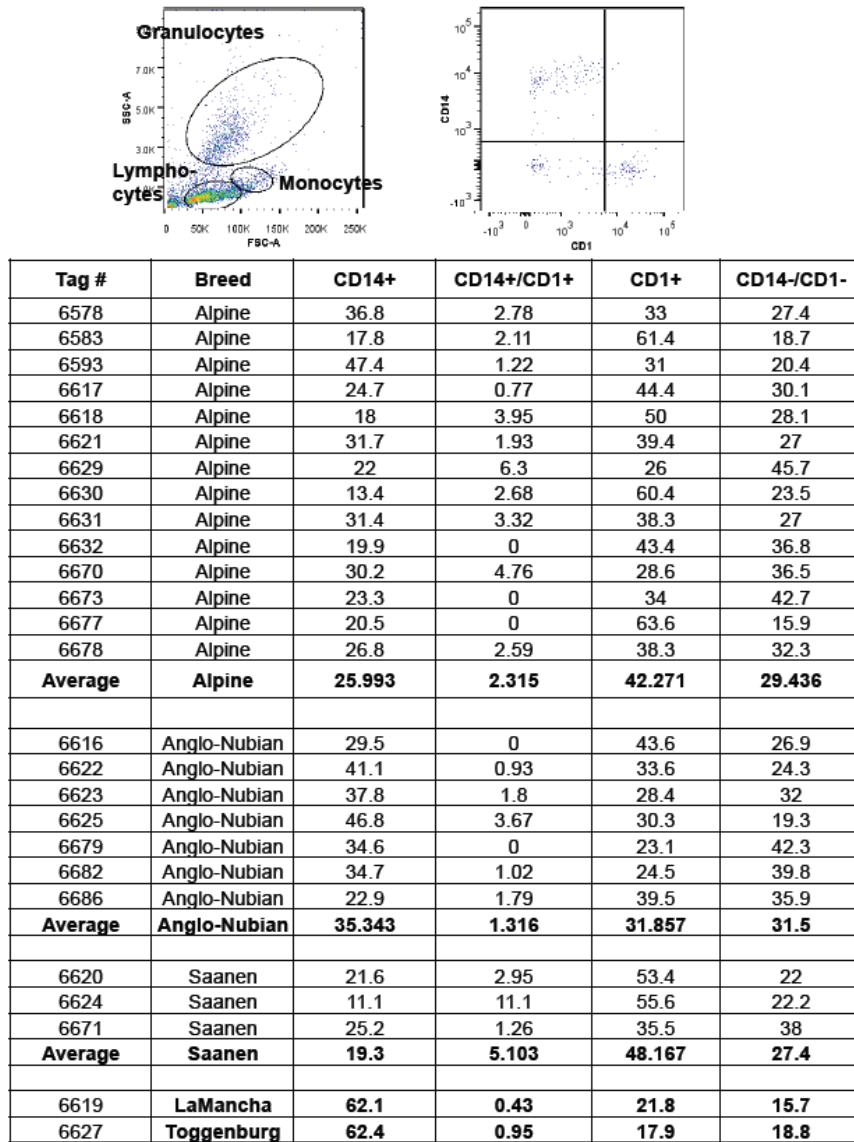


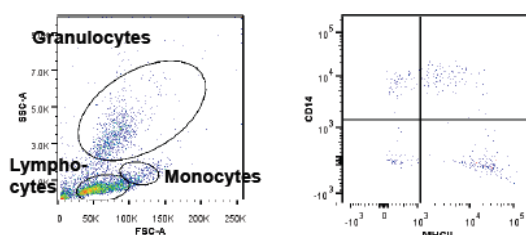
Fig. 5. Flow cytometry plots of goat #6593 (as one example for the gating strategy) at age 5.5 weeks. Left plot represents cell sorting according to cell size (FSC-A) and granulation (SSC-A). Monocytes are encircled as cells larger in size and low in granulation. This cell population was used for analysis for markers for CD14 (y-axis) and CD1 (x-axis) (right plot). The table below the flow cytometry plots contains data for the various cell subpopulations associated with the two markers for each goat as well as the average of each breed

Table 4. Statistical analyses of potential breed differences of average amounts of peripheral CD14 bearing cell populations (F: F test statistic for one-way ANOVA, BH p-value represents Benjamini-Hochberg adjusted p-value)

Cell type	Alpine	Anglo-Nubian	Saanen	F	P-value	BH p-value
CD14+ Monocytes	2.101	1.793	1.510	0.601	0.557	0.836
CD14+ Granulocytes	9.553	12.60	7.213	1.130	0.342	0.809

Table 5. Statistical analyses of potential breed differences of average amounts of peripheral antigenpresenting cell populations (F: F test statistic for one-way ANOVA, BH p-value represents Benjamini-Hochberg adjusted p-value)

Cell type	Alpine	Anglo-Nubian	Saanen	F	P-value	BH p-value
CD14+ Panel 1	25.9930	35.3430	19.3000	4.610	0.022	0.197
CD1+	42.2710	31.8570	48.1670	3.002	0.071	0.358
CD14+ /CD1+	2.3150	1.3160	5.1030	2.860	0.080	0.358
CD14-/CD1-	29.4360	31.5000	27.4000	0.261	0.773	0.902
CD14+ Panel 2	8.7210	13.4770	5.9870	6.671	0.006	0.102
MHC II+	54.2570	44.8860	57.4000	2.420	0.113	0.408
CD14+/MHC II+	18.5360	22.5280	17.7670	0.840	0.446	0.800
CD14-/MHC II-	18.4830	19.1000	18.8670	0.017	0.983	0.983



Tag #	Breed	CD14+	CD14+/MHC II +	MHC II+	CD14-/MHC II-
6578	Alpine	6.94	31.6	42.7	18.8
6583	Alpine	10.8	7.77	70.4	11.1
6593	Alpine	16.4	30.4	40.7	12.5
6617	Alpine	5.79	19.3	57.5	17.4
6618	Alpine	5.26	15.4	65.8	13.6
6621	Alpine	11.6	22	52.1	14.3
6629	Alpine	7.87	17.3	33.9	40.9
6630	Alpine	8.72	6.04	67.1	18.1
6631	Alpine	8.59	25.6	50	15.8
6632	Alpine	5.15	14.7	58.8	21.3
6670	Alpine	11.1	23.8	44.4	20.6
6673	Alpine	5.83	15.5	57.3	21.4
6677	Alpine	7.95	12.5	69.9	9.66
6678	Alpine	10.1	17.6	49	23.3
Average	Alpine	8.721	18.536	54.257	18.483
6616	Anglo-Nubian	7.69	21.8	55.1	15.4
6622	Anglo-Nubian	9.35	31.8	47.7	11.2
6623	Anglo-Nubian	17.6	21.6	41.4	19.4
6625	Anglo-Nubian	15.6	33	38.5	12.8
6679	Anglo-Nubian	15.4	19.2	46.2	19.2
6682	Anglo-Nubian	18.4	17.3	33.7	30.6
6686	Anglo-Nubian	10.3	13	51.6	25.1
Average	Anglo-Nubian	13.477	22.528	44.886	19.1
6620	Saanen	7.21	16.4	62.3	14.1
6624	Saanen	3.7	18.5	63	14.8
6671	Saanen	7.05	18.4	46.9	27.7
Average	Saanen	5.987	17.767	57.4	18.867
6619	LaMancha	20.5	41.2	31	7.33
6627	Toggenburg	40.2	21.7	24.5	13.6

Fig. 6. Flow cytometry plots of goat #6593 (as one example for the gating strategy) at age 5.5 weeks. Left plot represents cell sorting according to cell size (FSC-A) and granulation (SSC-A). Monocytes are encircled as cells larger in size and low in granulation. This cell population was used for analysis for markers for CD14 (y-axis) and MHC II (x-axis) (right plot). The table below the flow cytometry plots contains data for the various cell subpopulations associates with the two markers for each goat as well as the average of each breed

Figure 5 shows the cells bearing CD1 and/or CD14, while **Fig. 6** presents cells bearing MHC class II and/or CD14 molecules. These cells were characterized by their cell surface expression of CD14, CD1 and MHC class II molecules. Although no statistical significances were found for any cell type after correcting for multiple testing, CD14+ Panel 1 and Panel 2 both have unadjusted p-values < 0.05 (**Table 5**). For CD14+ from both panels Anglo Nubian goats had higher means compared to Alpine and Saanen goats.

4. DISCUSSION

Goats are excellent animal models for chronic infectious diseases, such as mycobacterial diseases (Johne's disease, bovine and human tuberculosis), melioidosis, or brucellosis (Soffler *et al.*, 2012; Kahl-McDonagh *et al.*, 2006; Stewart *et al.*, 2006; Bezos *et al.*, 2010; Perez *et al.*, 2011). Unfortunately, the goat model is rarely used due to lack of goat-specific antibodies, information on cross-reactivities of bovine, ovine, murine and human antibodies and baseline data on (1) peripheral leukocyte population determined by flow cytometry, (2) potential breed differences and (3) available conjugated antibodies for cell surface markers of various peripheral blood cell populations. For any study using the goat model, it is important to know potential differences between breeds if single breeds are not available. In our case, we received 26 goat kids of various breeds. Most of them were Alpine, Saanen and Anglo-Nubian, however, we also received one LaMancha and one Toggenburg. The main question was if differences in cell surface markers are present between the three major breeds investigated.

The analyses were performed for peripheral blood leukocytes at the age of 3.5 weeks (emphasis on lymphoid cells) and 5.5 weeks (emphasis on myeloid cells). There was a clear pattern that distinguished the various cell populations from each other. While granulocytes were classified as high in granulation, lymphocytes and monocytes were detected-as expected-as low in granulation. Lymphocytes and monocytes could be clearly separated by cell size.

While we analyzed cell populations as early as 3.5 weeks, Stabel and Robbe-Austerman (2011) described their analyses of calf PBMCs at an earlier age (1 week) without any difficulties. Somvanshi *et al.* (1987) described the development and changes of the whole hematology of Cashmere goats at different ages from less than one month to 10 years. While those are primarily fiber goats, they found slightly different data

for lymphocytes (52.63% for male and 57% for female) and monocytes (4.81% for male and 4.02% for female) compared to our dairy goat breed data with lymphocytes ranging from 41.58 to 57.02% and monocytes ranging from 1.42 to 4.23%. Clearly, our numbers for granulocytes differ from those determined by Somvanshi *et al.* (1987). While our numbers are primarily in the single digits or teens, their numbers range between 38.69 and 42.56%. Different breeds, higher elevation, or different methods could have attributed to varying granulocyte amounts. While they discussed their different data for erythrocytes as compared to European goats, type of breed and different nutrition were mentioned as plausible causes for those differences. Unfortunately, these seem to be the only intensive descriptions of early white cell populations and immune markers in goat kids.

Most of the cell population analyzed for the two different time points did not exhibit any significant differences due to time points or breeds. This is to our knowledge the first report on such differences. Goat kids start their lives with high numbers of lymphocytes and lower numbers of granulocytes and the number of granulocytes increases throughout the first months of life (Mbassa and Poulsen, 1991). Indeed, we see those changes in the granulocyte populations between the weeks of 3.5 and 5.5 of age. However, lymphocytes are still the major cell population within the PBMCs of goats (Eiselt *et al.*, 2011). While the main purpose of this study was to determine potential breed difference, we think that this was noteworthy.

While one could assume that there might be breed-specific differences in lymphocytes, granulocytes and/or monocytes, none have been reported yet. Breed-specific leukocyte differences were detected for three cattle breeds (Friesian, Red Danish, Jersey cattle) with statistical significance (Flensburg and Willeberg, 1976). Also, MHC class I reactivity to various antigens differs significantly between breeds of cattle in Australia (Stear *et al.*, 1987). None were reported so far for the three major dairy goat breeds used in this study.

In our study, we used several antibodies that cross-reacted with the caprine system. The reactivities of these antibodies were against bovine (three), ovine (three) and human (one); however, all of the tested antibodies demonstrated strong cross-reactivity with the caprine system. Interestingly, Dagleish *et al.* (2012) described crossreactivities of cell surface marker antibodies for the ovine system with the cervine system (European Red Deer) (CD3, CD4, CD8, MHC II, $\gamma\delta$ TCR, CD21, CD79 α). This seems logical since ruminants are

closely related species; however, we did not see any useful cross-reactivity for the highly species-specific lymphocyte marker CD3. Neither the bovine nor the ovine antibodies cross-reacted. Thus, it seems essential to generate such antibody for further studies. In an earlier study, Naessens *et al.* (1993) analyzed 189 antibodies for cell surface markers towards their potential of cross reactivity with domestic and wild ruminant species including cows, sheep, goats, buffalo and waterbuck. Monoclonal antibodies for CD3 demonstrated cross-reactivity for the bovine system as well as for the buffalo and waterbuck systems but not for the ovine or caprine system. Of the markers of key interest for studies on the immune system of goats CD1w3, CD2, CD4, CD5, CD6, CD8, CD11a, CD11b, CD11c, CD18, CD25, WC1 and CD44 demonstrated strong cross-reactivity with the caprine system. Although the clones for those specific antibodies are not commercially available we can confirm that the commercially available antibodies used in our study have similar cross-reactivities as described by Naessens *et al.* (1993). Furthermore, while several follow-up studies analyzed newly designed antibodies, they were only tested for the bovine system and not the ovine or caprine system (Sopp and Howard, 1997; Sopp *et al.*, 2001; 2007). The most comprehensive analyses of antibody cross-reactivities were performed by the laboratory of Dr. Davis, in which most bovine antibodies currently in use were generated (Mosaad *et al.*, 2006). Their intensive analysis confirmed many cross-reactivities but also ensured the lack of a specific antibody for CD3 marker in goat that could be used in caprine studies.

While the main purpose in this study was to obtain baseline profiles for peripheral blood leukocytes and to define potential differences in granulocytes, lymphocytes and monocytes due to breeds, we also analyzed the lymphocyte subpopulations of goat kids. There are only few published studies targeting subpopulation analyses at an early age in goats. Navarro *et al.* (1996) studied four seven-month old Murciano-Granadina goats (dairy goats breed) for the presence of CD2, CD4, CD8, MHC I, MHC II, WC1 and CD25 in peripheral blood, lymph nodes, spleen and ileal Peyer's patches. No statistical analyses were performed due to the small number of animals involved.

Since the presence of CD4+ and CD8+ T cells is well documented in various analyses, CD4/CD8 double positive cells are mostly seen as undifferentiated T cells, which later will become either CD4+ or CD8+. Although in the past the importance of CD4/CD8 double negative T cells was dubious, over the last decade, it was shown

that they might play a key role in local defense systems in the intestinal system with their own stimulation through the MR1 bearing antigen-presenting cells. This newly identified immune defense cascade seems to eliminate bacterial and viral pathogens during early infection. Interestingly, in HIV infected people, these cells become exhausted over time, marking disease progression. This observation might be important for various chronic infectious diseases that have their initial start in the intestinal system. Thus, it seems even more logical to analyze potential differences of CD4/CD8 double negative cells in various breeds. No statistically significant differences in CD4/CD8 double negative cell populations were observed between the three breeds.

Although data cannot be compared due to the age differences of the animals it is clear that our goat kids had higher CD4+ and CD4+/CD8+, as well as lower CD8+ number compared to those determined by Navarro *et al.* (1996). Although different antibodies were used in our study and in the study by Navarro *et al.* (1996), it seems that the amount of $\gamma\delta$ T cells are similar throughout the first seven months. The same group published a follow-up study on the postnatal evolution of lymphocyte subpopulations in peripheral blood of goats (Caro *et al.*, 1998). They analyzed the same goat breed at various time points throughout the first seven months for the same markers. Interestingly, their numbers strongly vary from our data even for 1 month of age, suggesting differences between the goat breeds involved. The final study on subpopulations of lymphocytes was performed by Winnicka *et al.* (1999) on 18 Saanen breed goats from 1 day of age to 1.5 years of age. No specific numbers for CD4+, CD8+ and CD4+/CD8+ cells were provided during their discussion. Although they did not analyze lymphocyte subpopulations at 1 month, it seems when analyzing **Fig. 3** in their study, however, that the CD8+ cell amount is similar to the number in our study. This last study on Saanen goats used a statistically significant amount of goats while the other two studies used only four animals. In comparison, our study included 14 goats of the Alpine breed, six Saanen goats and only four Anglo-Nubian goats.

5. CONCLUSION

While the study has its limitations by the number of goat kids per breed involved in the analyses and by the inclusion of only male goat kids, it still provides excellent baseline data on peripheral blood leukocytes and profiles of various T cell population and antigen-

presenting cell surface marker population profiles. From our statistical analyses, two additional conclusions could be made: (1) any goat study could include goats from the three dairy goat breeds used in our analyses (Alpine, Anglo-Nubian, Saanen) and (2) many antibodies for various T cells and cell surface markers generated for cows, sheep, or humans could be successfully used in the goat model.

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