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Flow cytometric analysis of an immunodeficiency disorder affecting juvenile llamas

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Abstract

The present study was undertaken to characterize the immune system of llamas and alpacas and establish the basis for an immunodeficiency disorder affecting juvenile llamas. Flow cytometric (FC) analysis of the immune system with a panel of monoclonal antibodies (mAbs) revealed the immune system of llamas and alpacas is similar in leukocyte subset composition to that in ruminants. Peripheral blood mononuclear cells in adults are comprised of surface immunoglobulin (sIg^+) B-cells (31%±8 S.D.), αβ T-cells (27%±12 S.D.), $WC1^+$ γδ T-cells (16%±11 S.D.), and 5–16% monocytes. In contrast to cattle, goats, and sheep, however, the frequency of $WC1^+$ γδ T-cells is not high in juveniles but similar to the frequency in adults. Also, sIg^+ B-cells are present in high concentration in juveniles (43%±11 S.D.). Expression of major histocompatibility class II molecules on resting T-cells was low or absent. Comparative analysis of peripheral blood lymphocyte composition in normal juvenile llamas and llamas presenting with the signs of the juvenile llama immunodeficiency syndrome (JLIDS) revealed the concentration of B-cells is extremely low (1–5%) in affected animals. The findings suggest JLIDS is attributable to an autosomal recessive genetic defect in the development of B-cells.

Keywords: Llama; Alpaca; Immunodeficiency; Monoclonal antibodies; Leukocyte differentiation molecules; B-lymphocytes

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[1] World Wide Web: http://www.vetmed.wsu.edu/depts-MoAb/index.htm

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1. Introduction

Deficiencies in the function of the immune system and immunodeficiency syndromes in juvenile and adult llamas have been reported over the past few years (Hutchinson et al., 1992; Hutchinson and Garry, 1994; Barrington et al., 1997). Affected animals present with weight loss, poor growth, anemia and persistent and/or recurrent infections. Infections are frequently caused by organisms that are not commonly pathogenic to normal animals. Infections often fail to respond to conventional treatment, inevitably leading to premature death of the animal. The juvenile form of the disease has a median age of onset of 11.6 months (range 2.3–30.1 months) (Hutchinson et al., 1995). A clinically similar syndrome was recently described in a 3.5-year-old adult female llama (Barrington et al., 1997). The etiological basis of these syndromes is unknown. However, genetic, infectious and environmental factors have been considered. The difficulty in distinguishing between these possibilities has been due to the lack of knowledge of the composition and function of the immune system as well as the lack of diagnostic assays to distinguish between immunodeficiency disorders caused by infectious agents and those caused by abnormal development and function of different subpopulations of lymphocytes. To address these problems, we have developed and used a panel of mAbs to major histocompatibility complex (MHC) and leukocyte differentiation molecules to characterize the immune system in llamas and alpacas. In the present report, we show that the immune system of camelids is similar in composition to that of ruminants, and also that the basis for one immunodeficiency disorder, JLIDS, is most likely attributable to a heritable defect in the development of B-lymphocytes.

2. Material and methods

2.1. Animals

Llamas and alpacas used in the present study were from multiple sources: the College of Veterinary Medicine teaching herd; private herds; and animals being examined by clinicians at veterinary clinics in Washington, Oregon, Nevada, Ohio, Colorado, and Canada. Tissue was obtained from animals at the time of necropsy. During the course of the study, blood was obtained from seven llamas with clinical symptoms of JLIDS. Holstein steers used as a source of blood were being maintained for other experiments. BALB/c mice used in the study were obtained from a mouse colony maintained by the WSU Monoclonal Antibody Center. All animals at the college were cared for according to the guidelines of AAALAC and the Institutional Animal Care and Use Committee. The term lama will be used when referring to llamas and alpacas generically. The common species names, llama (*Lama glama*) and alpaca (*Lama pacos*), will be used when referring to specific species.

2.2. Monoclonal antibodies

The mAbs used to characterize the immune system of lamas are listed in Table 1. The panel is comprised of mAbs that recognize conserved determinants on orthologous

Table 1
Monoclonal antibodies reactive with llama major histocompatibility and leukocyte differentiation antigens[a]

Cluster	mAb	Ig Isotype	Species[b] reactivity				Predominant pattern of cell expression
			L	B	O	C	
MHC I	H58A	IgG2a	+	+	+	+	Pan leukocyte, R.B.C., platelets
	H11A	IgG2a	+	+	+	+	
	PT85A	IgG2a	+	+	+	+	
MHC II	TH14B	IgG2a	+	+	+	+	B-lymphocytes, monocytes/macrophages
	TH85A	IgG2a	+	+	+	+	Activated T-lymphocytes
	H34A	IgG2b	+	+	+	+	
	H42A	IgG2a	+	+	+	+	
	TH21A	IgG2b	+	+	+	+	
	TH22A	IgG2a	+	+	+	+	
	CF75A	IgM	+	+	+	+	
	LT1A	IgG2a	+	+	+	+	
	LT6A	IgM	+	+	+	+	
LaCD4	GC50A	IgM	+	+	+	+	T-lymphocyte (helper/inducer)
LaCD5	LT3A	IgG1	+	−	N	N	αβ and γδ T-lymphocytes, low level expression on B-lymphocytes
	LT4A	IgG2a	+	−	N	N	
	LT7A	IgG3	+	−	N	N	
LaCD8	LT5A	IgG2a	+	−	N	N	T-lymphocyte (cytotoxic/suppressor)
LaCD11a	LT35	IgG1	+	−	N	N	Pan leukocyte (similar to CD18)
	LT48A	IgG2a	+	−	N	N	
LaCD11c	LT93A	IgG2a	+	−	N	N	Granulocyte, monocyte (variable level of expression on granulocytes in different species)
	LT24	IgG2a	+	−	N	N	
LaCD18	BAQ30A	IgG1	+	+	+	+	Pan leukocyte
	LT43	IgG2a	+	−	N	N	
LaCD14	CAM36A	IgG1	+	+	+	+	Monocytes/macrophages (variable level of expression on granulocytes in different species)
LaCD44	LT36A[c]	IgG2b	+	+	+	N	Pan leukocytes, R.B.C., platelets
	LT41A[c]	IgG2a	+	+	+	N	
	LT57	IgG1	+	−	N	N	
	LT63	IgG1	+	−	N	N	
	LT89[b]	IgG2b	+	+	+	N	
LaCD45	LT12A	IgG2a	+	−	N	N	Pan leukocyte
	LT13A	IgG3	+	−	N	N	
	LT14	IgG2a	+	−	N	N	
	LT15	IgG1	+	−	N	N	
BoWC1[c]	GB45A	IgG1	+	+	+	+	Subset of γδ T-lymphocytes
	CACTB28A	IgG1	+	+	+	+	
	BAQ128A	IgG1	+	+	+	+	

Table 1 (*Continued*)

Cluster	mAb	Ig Isotype	Species[b] reactivity				Predominant pattern of cell expression
			L	B	O	C	
BoWC9	RH1A	IgG3	+	+	N	N	Granulocytes, lymphocytes (low expression), monocytes, platelets
	LT86A	IgG2a	+	+	N	N	
SWC3[d]	DH59B	IgG1	+	+	+	+	Granulocytes, monocytes/macrophages
	LT18	IgG1	+	−	N	N	
LC1	LT10A	IgG2a	+	−	N	N	αβ T-lymphocytes (CD2, CD6?), low level expression on B-lymphocytes
	LH43A	IgG1	+	−	N	N	
LC2	LT97A	IgG2b	+	−	N	N	αβ and γδ T-cells (CD3?)
	LT98A	IgG2b	+	−	N	N	
LC3	LT20	IgG1	+	−	N	N	Granulocytes, lymphocyte subpopulation, R.B.C.
	LT25	IgG1	+	−	N	N	
	LT28	IgG1	+	−	N	N	
LC4	TH17A	IgM	+	+	+	+	Pan leukocyte, R.B.C., platelets
	GC62A	IgM	+	+	+	N	
	LT56	IgM	+	+	−	N	
	LT91	IgG2a	+	+	−	N	
LC5	LT34	IgG2a	+	−	N	N	Pan leukocyte, R.B.C., platelets
	LT38	IgG1	+	−	N	N	
	LT53	IgG1	+	−	N	N	
	LT61	IgG2a	+	N	N	N	
	LT67	IgG1	+	−	N	N	
	LT69A[c]	IgG1	+	+	+	N	
	LT70	IgG1	+	−	N	N	
	LT72	IgG1	+	−	N	N	
	LT80A[c]	IgG1	+	+	+	N	
	LT81	IgG1	+	−	N	N	
	LT82	IgG1	+	−	N	N	
	LT83	IgG1	+	−	N	N	
No cluster	LT78A	IgM	+	−	N	N	B-lymphocyte subpopulation
No cluster	LT79A	IgG3	+	−	N	N	B-lymphocyte subpopulation
No cluster	LH7A	IgM	+	−	N	N	B-lymphocyte subpopulation
No cluster	LH41A	IgM	+	−	N	N	B-lymphocytes
No cluster	GB26A	IgM	+	+	+	+	B-lymphocytes, subpopulation of T-lymphocytes
No cluster	LH4A	IgG2b	+	N	N	N	Activation (pan lymphocyte)
No cluster	LH9A	IgM	+	N	N	N	Activation (subpopulation)
No cluster	LH10A	IgG1	+	N	N	N	Activation (pan lymphocyte)

[a] Antibodies that cross-react with orthologous molecules in cattle.
[b] L, llama; B, bovine; O, ovine; C, caprine.
[c] Bovine workshop cluster.
[d] Swine workshop cluster.

molecules in two or more species and mAbs developed from mice immunized with llama peripheral blood leukocytes, thymocytes, or cultured lymphocytes stimulated with concanavalin A by previously described procedures (Hamilton and Davis, 1995). Flow cytometry was used to identify and cluster mAbs that recognize the same molecule (Davis et al., 1995).

To minimize confusion of terminology, the nomenclature adopted for mAb-cluster defined molecules (CD) in humans has been used to name orthologous molecules in lamas. Where clusters of mAbs clearly recognized molecules equivalent to those defined in the human clusters, the cluster has been designated LaCD. Where identity of the mAb-defined molecule was uncertain, the clusters have been given a temporary designation of LC. Some clusters include mAbs in bovine and porcine workshop clusters where the human orthologue has not been identified. The bovine and porcine workshop cluster designations have been used as temporary designations for these llama clusters. A LC number was given if two or more mAbs were identified that yielded the same pattern of labeling. Single mAbs of interest were not given cluster numbers. In the text where the lama orthologue is being discussed, the CD and WC designations are used as generic terms.

2.3. Flow cytometry

Single and two-color flow cytometry (FC) were performed to characterize the immune system of lamas. Leukocytes were obtained from blood collected in acid citrate dextrose, using high density separation medium (1.119 Histopaque, Sigma, St. Louis MO) to obtain preparations of granulocytes, monocytes, and lymphocytes and low density medium (1.086, Accu-Paque, Accurate Chem. and Scientific, Westbury NY) to obtain preparations of lymphocytes and monocytes (Davis et al., 1995). In some cases, erythrocytes and platelets were added back to cell preparations containing all leukocytes. These preparations were used to determine the complete pattern of expression of leukocyte differentiation molecules. Activated lymphocytes were obtained from cultures of lymphocytes stimulated with ConA for 24 h as previously described (Davis et al., 1996a). In most experiments, cells were labelled in 96 well conical base microtiter plates (Davis et al., 1995). One million cells were incubated for 15 min (4°C) with one or two mAbs (50 μl each, 15 μg/ml) in 100 or 150 μl of phosphate buffered saline containing 0.5% immunoglobulin free horse serum (GIBCO/BRL Life Technologies, Gaithersburg, MD) and 0.02% azide. Following washing, cells were incubated for 15 min (4°C) with 100 μl of appropriately diluted fluorescein conjugated polyclonal goat anti-mouse IgG/IgM antibody or goat isotype specific antibodies conjugated with fluorescein or phycoerytherin (Caltag, Burlingame, CA). Fluoresceinated polyclonal goat anti-lama Ig (Triple J Farms, Redmond, WA) was used in single and two color FC. In two color FC, the fluorescein conjugated anti-lama Ig was added at the same time as second step reagents. After incubation, the cells were washed and fixed in 2% formaldehyde in PBS and stored in the refrigerator until examined. Becton Dickinson FACScan and FACSort flow cytometers equipped with CELL QUEST software were used to collect data. Data were analyzed with CELL QUEST and QUATTRO PRO software.

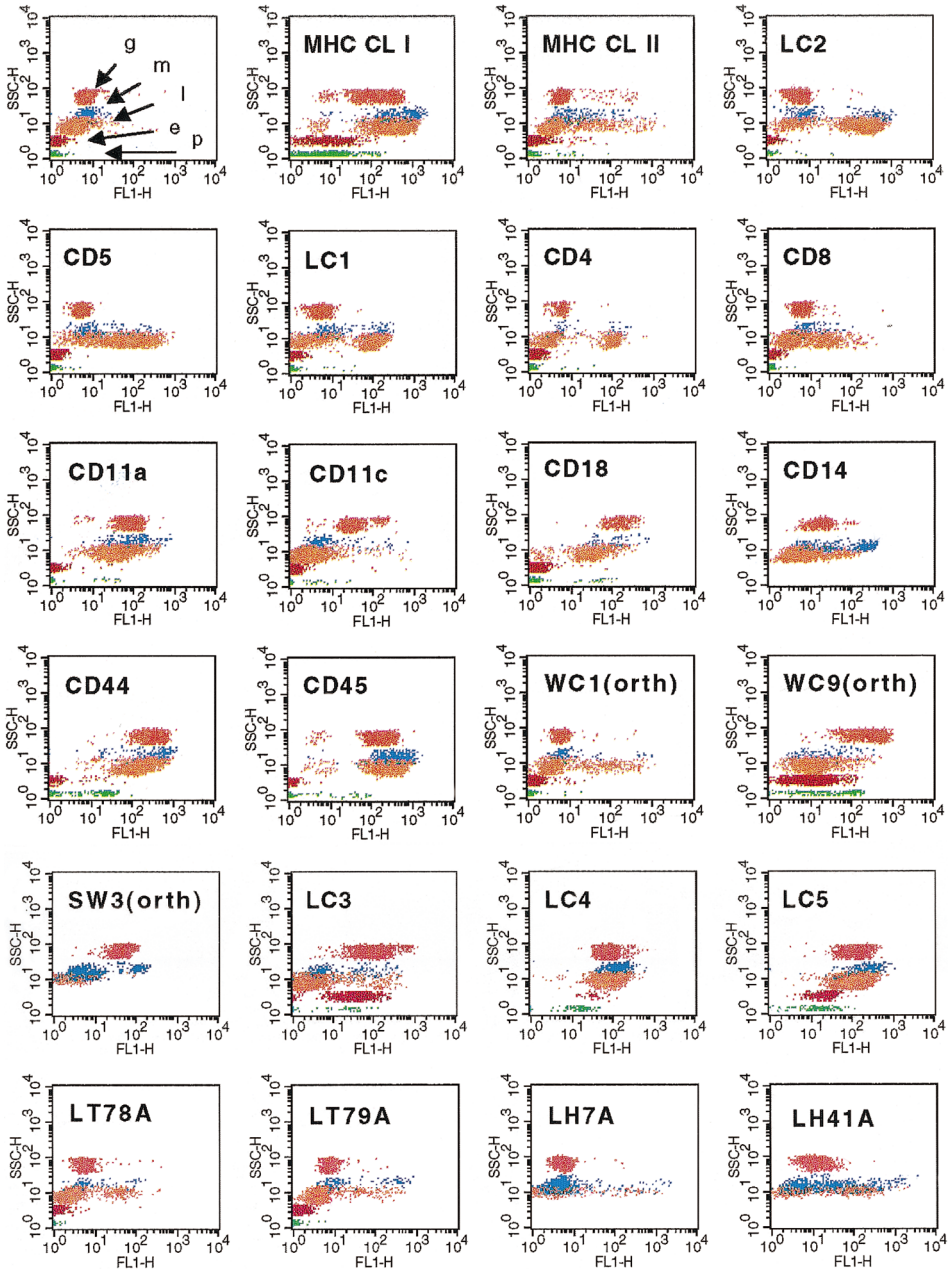

Fig. 1. Representative dual parameter, single fluorescent, dot plot profiles of a leukocyte preparation from a llama that contained all leukocytes, with added platelets and erythrocytes. The cells in the first panel in the upper left corner were reacted with second step reagent alone. The uppermost set of dots represents granulocytes (red, g), the second set down represents mononuclear cells including lymphocytes (orange dots, l) and monocytes (blue dots, m), the third set represents erythrocytes (red dots, e), and the lowest set represents platelets (green

2.4. Tissue culture

To assess functional activity of the different populations of lymphocytes in animals with the immunodeficiency syndrome, cultures of buffy coat cells and PBMC, separated with Accu-Paque (1.086), were cultured in Petri dishes (2×10^6 cells/ml) and stimulated with concanavalin A (ConA) for 8–24 h. The cells were then collected and labeled for flow cytometry. Three mAbs that identify molecules only expressed on activated lymphocytes were used in conjunction with mAbs that recognize T-cell subsets and B-cells to assess the proliferative response to ConA. The mAbs were also used to assess whether there was any difference in the proliferative response of lymphocytes from animals affected by JLIDS.

3. Results

3.1. Monoclonal antibodies reactive with MHC classes I and II molecules and leukocyte differentiation molecules of llamas

To initiate the studies, panels of mAbs developed for studies in cattle, goats, and sheep were screened to identify mAbs that react with antigenic determinants conserved on orthologous molecules in llamas. Additional mAbs were developed by hyperimmunizing mice with peripheral blood leukocytes and/or thymocytes by previously described methods (Hamilton and Davis, 1995). One group of mice was immunized with lymphocytes stimulated with ConA. Flow cytometry was used to identify and cluster mAbs reactive with MHC and leukocyte differentiation molecules according to the methods used in human, ruminant, and porcine leukocyte differentiation molecule workshops (Howard et al., 1991; Howard and Naessens, 1993; Carr et al., 1994; Naessens and Hopkins, 1996; Saalmüller et al., 1998). The specificity of the mAbs reactive with species restricted determinants was established by (1) comparison of the patterns of labeling of llama leukocytes with the pattern of labeling obtained with mAbs of known specificity in other species, using single color FC analysis with preparations of leukocytes containing all leukocytes, platelets and erythrocytes, and (2) direct comparison of labeling with mAbs that recognize determinants conserved on orthologous molecules using single and two color FC analysis (Davis et al., 1995; Davis et al., 1987). The data are summarized in Table 1 and Fig. 1. As noted in previous leukocyte workshop studies, it was possible to identify and cluster antibodies based on the similarity of the pattern of expression obtained with the different mAbs. The patterns of expression of MHC classes I and II molecules were identical to those observed in ruminants. MHC Class II molecules were not expressed on resting T-lymphocytes as in humans and mice.

←

dots, p). The remainder of the panels are representative profiles of cell preparations labeled with the indicated mAbs. The patterns of labeling were used to cluster mAbs that yielded the same pattern of labeling. The profiles obtained with mAbs CD14, SWC3 (orth), LH7A, and LH41A were obtained from a data set of cell profiles that did not include erythrocytes or platelets.

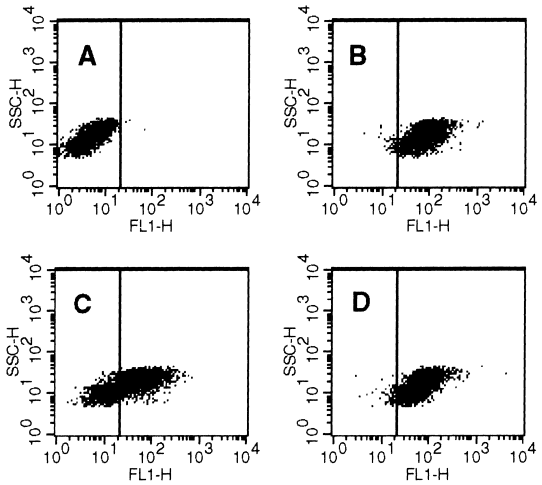

Fig. 2. Representative dual parameter, single fluorescent, dot plot profiles of activated lymphocytes reacted with mAbs specific for molecules only expressed on activated lymphocytes. (A) Represents cells only reacted with second step reagent. (B) Represents cells labeled with LH4A. (C) Represents cells labeled with LH9A. (D) Represents cells labeled with LH10A. LH9A recognizes a molecule expressed on a subset of activated lymphocytes. LH4A and LH10A appear to recognize the same molecule. The mAbs did not label resting lymphocytes.

Expression of leukocyte differentiation molecules was similar or identical to the patterns of expression observed in ruminants and other species. In some instances, specificity could be validated by the pattern of expression and two-color analysis with cross-reactive antibodies. Sequential labeling with mAbs that recognize determinants on the same molecule yielded a diagonal pattern of labeling or unidirectional or reciprocal bi-directional blocking of labeling with one of the mAbs. These criteria for determining specificity were used to cluster mAbs that recognize the same molecule.

The data are summarized in Table 1 and Figs. 1–3. Screening of mAbs developed against ruminant or swine MHC and leukocyte differentiation molecules yielded two mAbs specific for MHC Class I and 9 for MHC Class II molecules. Single mAbs were identified that were specific for CD4, CD14, and CD18. mAbs were also identified that were specific for molecules given temporary workshop cluster designations in the bovine or porcine workshops: BoWC1; BoWC9; and SWC3 (Howard et al., 1991; Morrison and Davis, 1991; Wijngaard et al., 1992; Howard and Naessens, 1993; Lunney et al., 1994). Screening of 158 new mAbs developed against llama leukocytes yielded two additional mAbs specific for MHC Class II molecules, three putatively specific for CD5 (referred to as CD5-like), one for CD8, three for CD11a, three for CD11c, 12 for CD44, four for CD45, and five clusters that could not be assigned based on the pattern of expression of the molecule. The CD5-like mAbs recognize a molecule expressed on $\alpha\beta$ and $\gamma\delta$ T-cells and some B-cells. The mAb assigned to CD8, LT5A, recognizes a molecule expressed on a subset of $\alpha\beta$ T-cells and 1–5% of $CD4^+$ T-cells. It is not expressed on $WC1^+$ cells. The mAbs in the CD11a, CD11c, and CD18 clusters yield patterns of labeling consistent with

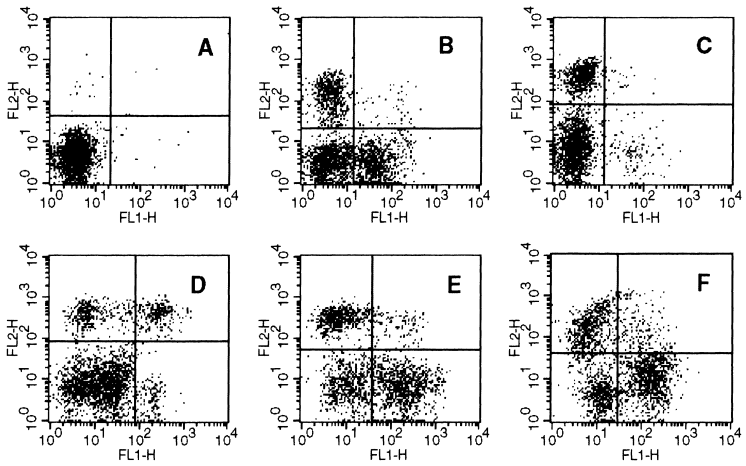

Fig. 3. Representative dual parameter, two color fluorescent, dot plot profiles of PBMC from a normal juvenile llama labeled with mAbs specific for molecules expressed on lymphocyte subsets. (A) Represents cells reacted only with second step reagent. (B) Represents cells labeled with anti-CD8 (LT5A) *Y*-axis (22%) and anti-CD4 (GC50A) *X*-axis (38%). Approximately 2% of cells coexpress CD4 and CD8. (C) Represents cells labeled with mAbs specific for αβ T-cells (LT10A) *Y*-axis (26%) and WC1 (GB45A), *X*-axis (8%). (D) Represents cells labeled with LT10A *Y*-axis (10%) and a mAb that reacts with a CD5-like molecule (LT3A) *X*-axis upper right quadrant. Approximately 13% coexpress both molecules. The $WC1^+$ cells, lower right quadrant (8%) coexpress the CD5-like molecule, but not the molecule defined with LT10A. (E) Represents cells labeled with LT10A *Y*-axis (26%) and a mAb (LH41A) specific for a molecule expressed on all B-cells *X*-axis (42%). (F) Represents cells labeled with LT10A *Y*-axis upper left quadrant (27%) and polyclonal anti-lama Ig *X*-axis lower right quadrant (49%).

the patterns of labeling observed in cattle, goats, and sheep. The mAbs in LC1 recognize a molecule expressed on αβ T-cells. It is not expressed on $WC1^+$ γδ T-cells but may be expressed at low density on some B-cells. These mAbs may recognize CD2 or CD6. The mAbs in LC2 recognize a molecule expressed on αβ and γδ T-cells. The pattern of labeling is reminiscent of the pattern of expression of CD3 (Fig. 1). The remaining three clusters of mAbs yielded distinct patterns of labeling that could not be matched with patterns of labeling observed with mAbs specific for human, ruminant, or porcine leukocyte differentiation molecules. LC4 contains two cross-reactive mAbs generated against ruminant leukocyte molecules (TH17A and GC62A). LC5 contains two mAbs that cross-react with the orthologue in cattle (LT69A and LT80A). mAbs in each of these clusters yielded identical patterns of labeling in single color FC and a diagonal pattern of labeling in two color FC in lamas and cattle.

Single mAbs were identified that react with all or subsets of B-cells. Three mAbs were also identified that react with molecules expressed on ConA-activated lymphocytes (Table 1 and Fig. 2). Two of these mAbs recognize a molecule or molecules expressed on all activated lymphocytes. One recognizes a molecule expressed on a subset of activated lymphocytes. Further characterization of the new mAbs will require determination of molecular weights and analysis of functional activity.

Table 2
Composition and phenotype of peripheral blood mononuclear cells in normal adult llamas (1–8 years of age N=16)

mAb	Specificity	Mean	STD	Range
TH14B	MHC Class II	39	13	21–65
LT3A	CD5-like	45	11	22–61
LT10A	αβ T-cells	27	12	7–52
GC50A	CD4	13	10	4–34
LT5A	CD8	13	5	5–20
GB45A	WC1	16	11	4–40
GB26A	B- and T-subpopulation	29	7	16–52
LH41A	B	31	8	20–51
LT78A	B-subpopulation	19	9	14–44
LH7A	B-subpopulation	11	5	5–26
DH59B	Gr[a]/M[b]	10	5	5–16
Anti-Ig	B	28	7	15–42

[a] Granulocytes.
[b] Monocytes.

3.2. Characterization of the llama immune system

A set of mAbs was selected for analysis of the composition of peripheral blood leukocytes (PBL) in adult (1–8 years of age) and young (1–4 months of age) llamas (Table 2). The mAbs that clustered with LT3A and LT10A (LT4A and LH43A, respectively) were also used. There was good agreement in the percent of cells labeled with each set of mAbs. Initial studies showed granulocytes comprised 60–80% of PBL in all age groups. In this study, it was not possible to distinguish neutrophils from eosinophils based on expression of lineage-restricted molecules. In further studies, data were only collected on peripheral blood mononuclear cells (PBMC). Considerable variation was noted in the overall composition of PBMC. The relative proportion of monocytes in adult animals ranged from 5 to 50% in apparently healthy animals. The range for the majority of animals, however, was 5–16% (Table 2). The range in young animals was similar (3–20%). Only animals with this proportion of monocytes were used to determine the composition of PBMC in animals with no apparent clinical signs of disease. Similar variations were noted in the composition of T- and B-cell populations. In adults the proportion of T-cells varied from 21 to 61% and B-cells from 15 to 41% based on expression of surface Ig and from 20 to 51% based on expression of a lineage restricted molecule detected with LH41A. Molecules expressed on subsets of B-cells detected with LH7A and LT78A showed parallel variations in the percent of labeled cells. Populations labeled with these mAbs were always lower than the total population identified with LH41A. The ratio of CD4 to CD8 T-cells varied but was close to 1. In some animals, the ratio was inverted with CD8-positive cells predominant. The proportion of cells positive for both CD4 and CD8 was usually <1%. However, the proportion of double positive cells approached 5% in some animals. The relative proportion of γδ T-cells positive for the orthologue of WC1 and negative for CD4, CD8, and LT10A varied from 4 to 40%. As noted in cattle and pigs, the level of expression of

Table 3
Composition and phenotype of peripheral blood mononuclear cells in normal young llamas (1–4 months of age N=13)

mAb	Specificity	Mean	STD	Range
TH14B	MHC Class II	52	12	32–75
LT3A	CD5-like	31	9	21–49
LT10A	αβ T-cells	18	6	9–30
GC50A	CD4	5	2	3–10
LT5A	CD8	10	4	4–19
GB45A	WC1	16	9	7–35
GB26A	B- and T-subpopulation	28	7	14–39
LH41A	B	43	11	24–67
LT78A	B-subpopulation	31	8	16–40
LH7A	B-subpopulation	22	5	16–33
CAM36A	CD14	9	5	3–20
Anti-Ig	B	41	9	27–53

CD5 on γδ T-cells was lower than the level of expression on αβ T-cells (data not shown). GB45A (anti-BoWC1) appeared to react with the majority of WC1 positive γδ T-cells. The proportion of WC1 negative γδ T-cells could not be determined.

The composition and variation of T- and B-lymphocyte subpopulations in young llamas was similar to that of adults (Table 3 and Fig. 3). In contrast to cattle, goats, and sheep, however, the WC1 positive population was not elevated. Also, the relative proportion of B-cells was much higher than seen in young calves of similar age.

3.3. Characterization of the alpaca immune system

The mAbs reactive with llama leukocytes were examined to see which ones reacted with orthologous molecules in alpacas. All the mAbs yielded patterns of labeling identical to those obtained with llama leukocytes. Analysis of PBL and PBMC with the panel of mAbs in Table 2 revealed the composition of the leukocyte populations in adult and young alpacas is identical to that of llamas, consistent with the close genetic relationship (data not shown).

3.4. Analysis of animals with the juvenile llama immunodeficiency syndrome

During the course of the study, a number of animals, 10 months to 2 years of age, were referred through the WSU clinic with apparent abnormalities in their immune system that included low immunoglobulin levels, poor thrift, opportunistic infections, and atrophic lymph nodes. Blood from these and normal animals were compared by two color FC to determine if there were any differences in the composition of PBMC. PBMC from some animals were stimulated with a polyclonal activator, ConA, and examined to see if there were any alterations in the proliferative response. Seven animals were identified by FC that had JLIDS. As shown in Table 4 and Fig. 4, all affected animals had a marked deficiency in B-cells (<5% of PBMC) and a high frequency of $CD8^+$ T-cells (up to 50%). Three cases were offspring from the same sire and three different dams. Two of these

Table 4
Composition and phenotype of peripheral blood mononuclear cells in normal llamas with JLIDS (1–2 years of age N=4)

mAb	Specificity	Mean	STD	Range
TH14B	MHC Class II	3.5	1	3–8
LT3A	CD5-like	58	21	31–80
LT10A	αβ T-cells	66	16	45–82
GC50A	CD4	11	6	3–19
LT5A	CD8	50	22	24–76
GB45A	WC1	6	2	4–9
GB26A	B- and T-subpopulation	29	7	21–40
LH41A	B	5	2	2–7
LT78A	B-subpopulation	5	1	2–11
LH7A	B-subpopulation	2	3	1–2
CAM36A	CD14	28	13	11–42
Anti-Ig	B	2	1	1–2

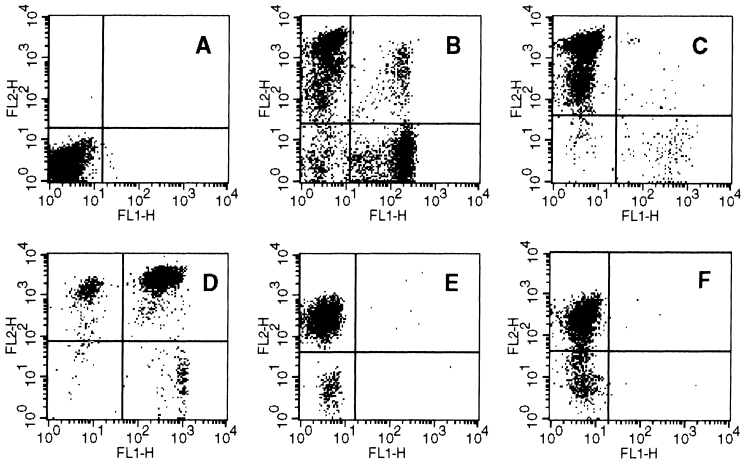

Fig. 4. Representative dual parameter, two color fluorescent, dot plot profiles of PBMC from a llama with JLIDS labeled with mAbs specific for molecules expressed on lymphocyte subsets. (A) Represents cells reacted only with second step reagent. (B) Represents cells labeled with anti-CD8 (LT5A) *Y*-axis upper left quadrant (47%) and anti-CD4 (GC50A) *X*-axis lower right quadrant (40%). Approximately 5% of cells coexpress CD4 and CD8 (upper right quadrant). (C) Represents cells labeled with mAbs specific for αβ T-cells (LT10A) *Y*-axis (95%) and WC1 (GB45A), *X*-axis lower right quadrant (3%). (D) Represents cells labeled with LT10A *Y*-axis upper left (7%) and right quadrants (89%) and a mAb that reacts with a CD5-like molecule (LT3A) *X*-axis lower and upper right quadrants. The upper right quadrant contains cells that coexpress the molecules detected with LT10A and LT3A. The lower right quadrant contains the $WC1^{+}$ population that is negative for the LT10A-defined molecule. (E) Represents cells labeled with an anti-pan T mAb (LT97A) *Y*-axis (94%) and a mAb (LH41A) specific for a molecule expressed on all B-cells *X*-axis lower right quadrant (<1%). (F) Represents cells labeled with LT97A (90%) and polyclonal anti-lama Ig. The absence of B-cells is evident in D (lower left quadrant), E, and F (lower right quadrants). There were <1% B-cells in PBMC from this affected animal.

three animals were diagnosed as being affected by JLIDS based on clinical symptoms and FC. The third animal had no history of illness at the time of initial examination. It was identified by FC as a possible case of JLIDS out of 10 additional offspring from different females mated to the same male mentioned above. B-cells in this animal were comparable in concentration to that in animals with clinical signs of illness. Also, $CD8^+$ T-cells were elevated as noted in other affected animals. At ca. 2 years of age the animal developed clinical signs of JLIDS and died of upper respiratory infection. Death was precipitous so no additional clinical tests could be performed.

4. Discussion

4.1. Strategies for comparative analysis of MHC and leukocyte differentiation molecules

Comparative studies have shown the structure, function, and pattern of expression of MHC and leukocyte differentiation molecules are highly conserved cross species with only a few exceptions. The studies have also shown that the immune systems of different species are similar but not identical in composition. Studies by flow cytometry have shown that mAbs specific for MHC and leukocyte differentiation molecules can be tentatively clustered and assigned to known mAb-defined clusters by comparing the patterns of expression of the mAb-defined molecules under study with those of the human orthologues (Lanier et al., 1983; Lanier et al., 1986; Davis et al., 1987). This includes MHC Class I, CD2, CD3, CD4, CD5, CD6, CD8, CD11a, CD11b, CD11c, CD18, CD14, CD44, CD45, CD45R, CD45R0, and CD58. Exceptions to this have been expression of MHC Class II, CD4, and CD90 (Thy-1) (Davis and Hamilton, 1998). MHC Class II is expressed on both resting and activated T-cells in some species as well as on B-cells and monocytes. CD4 is uniformly expressed on T-helper/inducer cells in all species. It has been identified on monocyte/macrophages and eosinophils in some species and on neutrophils in dogs (Moore et al., 1992). Expression of CD90 varies considerably between species (Davis et al., 1987). The prominent differences noted in the immune system have been in $\alpha\beta$ T-cells in pigs and $\gamma\delta$ T-cells in both pigs and ruminants. A subpopulation of T-cells is present in pigs that coexpresses CD4 and CD8. It appears to be comprised primarily of the memory T-cells (Zuckermann and Gaskins, 1996; Zuckermann and Husmann, 1996). The proportion of double positive cells increases with age.

Analysis of the composition of the $\gamma\delta$ T-cell population in ruminants and pigs, members of the order Artiodactyla (suborders Ruminantia and Suiformes, respectively), have shown it is comprised of two complex subpopulations that differ in tissue distribution and possibly function. One is similar in phenotype to $\gamma\delta$ T-cells described in humans and rodents. It is characterized by the expression of CD2, CD3, CD5, and CD6. A subset of this population coexpresses CD8. The second population is characterized by the absence of CD2 and CD6 and the expression of two unique molecules, WC1 and GD3.5 in cattle and the orthologue of WC1 and SWC6 in pigs (Davis et al., 1998a). Limited information is available on GD3.5 and SWC6 (Binns, 1994; Jones et al., 1996; Davis et al., 1998b). WC1 is a member of the B subfamily of the scavenger receptor cysteine rich superfamily (SRCRSF) of molecules that contain one or more copies of a

highly conserved ~110 aa motif. CD5 and CD6 are also members of the B subfamily of SRCRSF molecules (Wijngaard et al., 1994; Aruffo et al., 1997). Analysis in cattle has shown that multiple isoforms of the molecule, encoded by different members of the WC1 family of genes, exist and may be expressed on mutually exclusive or overlapping subsets of $WC1^+$ cells (MacHugh et al., 1993). Analysis of V_γ gene segment usage indicates the $WC1^+$ population is a distinct lineage (Davis et al., 1996b; Hein and Dudler, 1997; MacHugh et al., 1997). Comparative studies have shown the populations of $WC1^+$ cells in ruminants and the $WC1^+$ orthologue in pigs are high in peripheral blood (30–60%) of young animals and low in secondary lymphoid tissue (5–10%). The $WC1^-$ population is low in peripheral blood (3–5%) and high in spleen. The distribution is similar to the $WC1^+$ cells in other lymphoid organs.

4.2. Camelid immune system

Camelids are the only surviving lineage of Artiodactyla suborder Tylopoda (Hamers and Muyldermans, 1998). It was not known whether the immune system of camelids would resemble the immune system of ruminants (suborder Ruminantia), pigs (suborder Suiformes), or humans and rodents. Although there are unique differences, as shown here with lamas, the camelid immune system is most similar in composition to the ruminant immune system. mAbs that identify the apparent orthologues of known CD molecules exhibit the same patterns of expression as those noted in cattle. Where cross-reactive mAbs were available, specificity could be confirmed by two color labeling. CD4 and CD8 are expressed mainly on mutually exclusive populations of T-cells. Only a small percentage of cells are double positive. $CD4^+$ and $CD8^+$ T-cells coexpress LC1, LC2, and the CD5-like molecule. The patterns of expression of LC1 and LC2 suggest they might be the orthologues of CD2 or CD6 and CD3, respectively. The pattern of expression of the CD5-like molecule is similar to the pattern of expression of CD5 noted in cattle and pigs, bright expression on $LC1^+$ $CD4^+$ and $LC1^+$ $CD8^+$ ($\alpha\beta$ T-cells) and dim expression on cells that express the orthologue of $WC1^+$ ($\gamma\delta$ T-cells). One unexplained difference is the presence of a subset of $LC1^+$ T-cells that do not express the CD5-like molecule. The $CD5^-$ cells comprise 3–7% of PBMC. The pattern of expression of the WC1 orthologue is consistent with expression in cattle and pigs with the exception that it appears not to be elevated in concentration in young lamas. Further studies need to be conducted to confirm the existence of a $WC1^-$ population. The three mAbs that recognize the orthologue of WC1 recognize conserved determinants that are expressed on the majority of $WC1^+$ $\gamma\delta$ T-cells in cattle. The mAbs appear to detect all cells that express the WC1 orthologue.

As in ruminants and pigs, the majority of B-cells express surface immunoglobulin. Since a polyclonal anti-Ig was used, it was not possible to determine whether the majority of B-cells express sIgM. It is possible some B-cells might express other classes of Ig. In addition to the classic four chain Igs IgM, IgG_1, IgA, and IgD, camelids have two unique classes of two heavy chain Igs devoid of light chains IgG_2 and IgG_3 (Hamers and Muyldermans, 1998). These Igs are missing the C1 domain that binds light chains, (V_H, C_H2, C_H3). Approximately 75% of antibodies in serum are comprised of these Igs. Based on the observation that different sets of V_H gene segments are used by four and two chain

Igs, it has been postulated that two lineages of B-cells exist that secrete the different forms of antibodies. In contrast to cattle, the percentage of B-cells is similar in young and adult animals.

4.3. JLIDS

The findings obtained in the present study indicate JLIDS is attributable to a defect in the development of B-cells. The concentration of B-cells was between 1 and 5% in affected animals compared to 27–53% in normal young animals. This accounts for the low levels of Ig present in serum and the poor antibody response to test antigens (Hutchinson et al., 1995). If, as postulated, there are two lineages of B-cells that produce the four and two chain forms of immunoglobulin, the defect in B-cell maturation in JLIDS would be before lineage commitment. The only other consistent difference noted in the cell profile was elevation in the concentration of $CD8^+$ T-cells. No difference was noted in the proliferative response of PBMC to ConA. Although there may be other immunodeficiency syndromes in lamas, this form of JLIDS appears to be associated with an autosomal genetic defect that affects B-cell development. JILDS occurs in both sexes and, as reported here, three affected offspring were progeny from three different females sired by a single male. Of interest, examination of the PBMC profile of the parents of affected animals did not reveal any discernable abnormalities in the concentration of B-cells and $CD8^+$ T-cells. Thus, carriers of the defective gene cannot be identified by FC at this time.

At this juncture, it is not known whether JLIDS occurs in alpacas. There is a report, however, of an alpaca with clinical signs of JLIDS. The animal died before clinical studies could be completed (Constable et al., 1999).

The occurrence of JLIDS in animals after the neonatal period has suggested that there may be more than one form of immunodeficiency presenting with similar clinical signs. Although this remains a possibility, review of other B-cell immunodeficiency syndromes in humans and horses suggests the differences in age of onset are attributable to husbandry and compensatory changes in the immune system that permit some animals to survive longer than others. These diseases are similar in clinical presentation to JLIDS (Perryman and McGuire, 1980; Fischer and Arnaiz-Villena, 1995; Conley and Cooper, 1998). They also present as ‘failure to thrive’ syndrome with failure to grow, weight loss, persistent, recurrent infections and anemia. Some of the affected animals/humans are presented for evaluation long after the neonatal period. There is an X-linked agammaglobulinemia in humans which is caused by a defect in Bruton’s tyrosine kinase (btk) gene. These individuals have a complete lack of mature B-cells. Exactly how the defect in the tyrosine kinase causes a defect in B-cell development is unknown at this time (Fischer and Arnaiz-Villena, 1995). There is also an autosomal recessive agammaglobulinemia in humans (Conley and Sweinberg, 1992). This defect may be caused by a defect in the μ-heavy chain gene in some patients. This prevents the production of all immunoglobulins due to a defect in the production of the initial immunoglobulin heavy chain (Yel et al., 1996). In other patients, it may be caused by a defect in the Pax5 gene, which interferes with maturation of early B-cell progenitor cells (Meffre et al., 1996).

There is another type of immunodeficiency involving B-cells in humans called common variable immundeficiency (CVID) (Spickett et al., 1997). It has been tentatively postulated that the syndrome is actually made of four different disorders but due to the similarity of clinical signs, they are grouped as one disease entity (Spickett et al., 1997). These disorders vary from a complete lack of B-cells and agammaglobulinemia to normal numbers of B-cells with a variable immunoglobulin deficiency (Spickett et al., 1997).

In horses, a rare agammaglobulinemia has been reported in Thoroughbreds and Standardbreds. All the reported cases of agammaglobulinemia have been males, indicating this may be an X-linked trait, such as that found in humans (Perryman and McGuire, 1980). Of interest here is the fact that animals developed clinical signs of disease at various ages extending beyond the neonatal period.

Further studies are needed to complete characterization of the immune system in lamas and determine whether the genetic defect that causes JLIDS is attributable to an undescribed mutation or is identical to defects previously identified in humans and horses. At this juncture, however, sufficient data have been obtained to establish the genetic basis for JLIDS and in addition, show affected animals can be identified by phenotyping PBMC shortly after the time of birth, before the appearance of clinical signs. The concentration of B-cells is very low at the time of birth and readily detected by FC with polyclonal anti-Ig antibodies and mAbs specific for T- and B-cells.

The availability of a set of mAbs to MHC and leukocyte differentiation molecules in lamas now affords an opportunity to conduct functional studies as well as develop diagnostic tests that can be used to identify animals with JLIDS and other immunodeficiencies associated with functional or maturational defects in leukocytes.

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