

## Use of flow cytometry to develop and characterize a set of monoclonal antibodies specific for rabbit leukocyte differentiation molecules

William C. Davis\*, Mary Jo Hamilton

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

Flow cytometry was used to identify and characterize monoclonal antibodies (mAbs) that react with rabbit leukocyte differentiation molecules (LDM). Screening sets of mAbs, developed against LDM in other species, for reactivity with rabbit LDM yielded 11 mAbs that recognize conserved epitopes on rabbit LDM orthologues and multiple mAbs that recognize epitopes expressed on the major histocompatibility class I or class II molecules. Screening of mAbs submitted to the Animal Homologues Section of the Eighth Human Leukocyte Differentiation Workshop yielded 7 additional mAbs. Screening of mAbs generated from mice immunized with leukocytes from rabbit thymus or spleen or concanavalin A activated peripheral blood and/or spleen lymphocytes has yielded 42 mAbs that recognize species restricted epitopes expressed on one or more lineages of leukocytes. Screening of the anti-rabbit mAbs against leukocytes from other species yielded one additional mAb. The studies show that screening of existing sets of mAbs for reactivity with rabbit LDM will not be productive and that a direct approach will be needed to develop mAbs for research in rabbits. The flow cytometric approach we developed to screen for mAbs of interest offers a way for individual laboratories to identify and characterize mAbs to LDM in rabbits and other species. A web-based program we developed provides a source of information that will facilitate analysis. It contains a searchable data base on known CD molecules and a data base on mAbs, known to react with LDM in one or more species of artiodactyla, equidae, carnivora, and or lagomorpha.

**Keywords:** leukocyte differentiation molecules, monoclonal antibodies, rabbit

### Introduction

Over the past years, development and characterization of mAbs developed against leukocyte differentiation molecules (LDM) in humans has been facilitated by the convening of international workshops to compare the reactivity of mAbs developed in different laboratories [66]. Similar workshops have been convened for characterization of mAbs to LDM in ruminants [29,30,46], pigs [23,38,52,55], horses [33,36], and dogs [8]. However, progress has been much slower owing to limited number of laboratories participating in the workshops and the smaller number of mAbs submitted for analysis. In effort to accelerate identification of important mAbs, investigators have explored the possibility that many of the well characterized mAbs to human LDM might recognize epitopes conserved on orthologous LDM in other species. Although some useful cross reactive mAbs have been identified [56–58], recent results from analysis of a large set of anti-human LDM mAbs submitted to the Animal Homologues Section of the eighth human LDM workshop [54] and results reported in the ruminant and pig workshops [29,30,46,56–58] have shown the probability of finding a mAb that recognizes an epitope conserved on orthologous LDM is greater between closely related species than between distantly related species [4] for example, between cattle, bison, water buffalo, Cape buffalo, goats, sheep, and camelids [28,44,45,47,61]. The most successful approach for identifying mAbs to LDM in the species of interest has remained a focused effort on developing mAbs to LDM in that species, taking advantage of cross reactive mAbs whenever they are found to facilitate characterization of new mAbs [14].

The rabbit is an example of a species where there is a critical need for mAb reagents (NCBI Rabbit Genome Resources, USA). To date, however, only a few mAbs have been developed to meet this need. Efforts to expand the available sets of mAbs with cross reactive mAbs generated against LDM in other species has only yielded a few mAbs. The mAbs found in our sets of mAbs (this report) and

\*Corresponding author

Tel: +1-509-335-6051; Fax: +1-509-335-8328

E-mail: davisw@vetmed.wsu.edu

mAbs submitted to the Animal Homologues Section of the HLDA8 have been specific for major histocompatibility (MHC) I and II molecules, CD7, CD9, CD14, CD21, CD11a, CD18, CD44, CD45RB, CD49d, CD209 [54]. In light of these findings, it is apparent that a more direct approach will be required to identify mAbs for research in rabbits. As part of our continued effort to develop mAbs critical to our research efforts in ruminants, we have developed a flow cytometric approach for initial identification and characterization of mAbs to LDM [11]. Previous studies have shown that two parameter single fluorescence flow cytometry can be used to cluster mAb that recognize the same or different epitopes on the same LDM, based on the pattern of expression of the molecule on one or more lineages of leukocytes [11,16,35]. Comparative studies have shown this method can also be used to identify and tentatively cluster mAbs that recognize epitopes on orthologous LDM based on the similarity of the pattern of expression of the LDM on leukocytes in different species. Our studies have revealed the pattern of expression of many orthologous LDM has been conserved cross species. This observation has proven useful, especially in the characterization of mAbs specific for LDM in less well studied species [13-15,59,60]. It has also proven useful in determining whether mAbs that cross react with LDM in one or more species recognize an epitope conserved on bona fide LDM orthologues. Specificity has also been documented by cloning and expression of LDM initially identified with cross reactive mAbs [59]. To aid others as well as ourselves, we have also developed a web based program, the Taxonomic Key Program (TKP; College of Veterinary Medicine, Washington State University, USA), to facilitate characterization of mAbs generated against LDM in less well studied species. The program contains a searchable database on known CD molecules and a database containing a catalog of mAbs known to react with LDM in one or more of the less well studied species. In the present report we summarize the results we have obtained thus far, in our efforts to develop mAbs for use in immunological investigations in the rabbit. Information on the mAbs recognizing rabbit LDMs are listed under reactivity of antibodies in the TKP program (NCBI Rabbit Genome Resources, USA).

## Materials and Methods

### Animals

Rabbits being used in other studies were used as a source of blood and tissues. They were housed and maintained according to the Institutional Animal Care and Use committee guidelines and Association for Assessment and Accreditation of Laboratory Animal Care (USA). Both male and female rabbits were used since initial studies did not reveal any apparent differences in the frequency of

leukocyte subsets. The age of the rabbits varied from six months to about two years.

### Preparation of leukocytes for flow cytometry

Because of the tendency for T lymphocytes to bind to erythrocytes, separation medium could not be used to isolate leukocytes. Whole blood, collected in anti-coagulant citrate-dextrose (ACD), was used with a fix-lyse solution to obtain leukocytes for analysis. For single color flow cytometry (FC), 50  $\mu$ l of blood was distributed in conical bottom 96-well microtiter plates (Corning, USA) containing 50  $\mu$ l of optimally diluted mouse mAbs and then incubated for 15 min on ice. Following centrifugation, the supernatants were removed by aspiration. The lymphocytes were subjected to 3 cycles of centrifugation and washing in FC first wash buffer (FWB, PBS containing 20% ACD and 0.5% horse serum) and then incubated with a second step fluorescein conjugated polyclonal goat anti-mouse IgG/IgM second step reagent (Caltag Laboratories, USA) for an additional 15 min. Following 2 cycles of centrifugation and washing in FC second wash buffer (PBS-20% ACD) the lymphocytes were resuspended in FACS lysing solution (Becton Dickinson, USA) to lyse erythrocytes. The lymphocytes were then centrifuged and resuspended in 2% PBS-buffered formaldehyde and kept in the refrigerator until examined. For multi-color FC, blood was distributed in microtiter plates containing 2 or 3 mAbs and incubated as described. Following centrifugation and 3 cycles of washing, the lymphocytes were incubated with second step reagents. For most of the studies, combinations of mAbs of different isotype were used with isotype specific goat anti-mouse immunoglobulins conjugated with fluorescein (FL), phycoerythrin (PE), PE-Cy5, or Cy5 (Caltag Laboratories, USA). Where the mAbs of interest were the same isotype, Zenon Fab fragments of goat isotype specific anti-mouse antibody, conjugated with different fluorochromes, were used according to the manufacturers' instructions (Invitrogen, USA). One  $\mu$ g of each mAb in 20  $\mu$ l of FWB were incubated separately with 5  $\mu$ l of Zenon-Fab reagent conjugated with different fluorochromes (FL, PE, PE-Cy5, or Cy5) for 5 min at room temperature as recommended by the manufacturers. The mixtures were then incubated with 5  $\mu$ l of blocking reagent (mouse immunoglobulin) for an additional 5 min. The labeled antibodies were then added to the lymphocyte preparations under study. Following 15 min of incubation on ice, the lymphocytes were processed as described and fixed in 2% buffered formaldehyde.

Peripheral blood mononuclear lymphocytes (PBMC) and spleen lymphocytes stimulated with concanavalin A (ConA) were used for immunization and identification of mAbs that recognize molecules upregulated on activated lymphocytes (rabbit activation molecules, RACT). To simplify initial screening of supernatants from primary cultures of hybridomas for the presence of a mAb that

recognizes a RACT, spleen lymphocytes stimulated with ConA (5 µg/ml) for 24 to 48 h were incubated with hydroethidine (250 µg/ml in tissue culture medium), a vital dye that is selectively taken up by live cells. Hydroethidine (Polysciences, USA) intercalates into DNA similar to propidium iodide. It is excited at 488 nm and emits at high wave lengths (580 nm and higher). Following 8 min incubation at 37°C, the cells were subjected to 2 cycles of washing by centrifugation and re-suspension in medium and then added to an equivalent concentration of unstimulated cells. The mixed populations of cells were then incubated with tissue culture supernatants on ice as described and prepared for FC. Screening was performed with live cells immediately after labeling.

For further analysis of the pattern of expression of mAb-defined LDM, cells were obtained from thymus, spleen, and appendix at the time of necropsy. Cells from the respective tissues were isolated by mincing the tissues with a scissors and then passing the tissue preparation through a 100 mesh stainless steel sieve and suspended in PBS. Cells were used immediately or cryopreserved for later use. For cryopreservation,  $10^7$  to  $10^8$  cells were resuspended in bovine calf serum containing 10% DMSO and kept in a liquid nitrogen freezer.

### Development of mAbs to rabbit LDM

Five fusions were made with groups of 5 mice hyper-immunized with thymus (RT and RTH), ConA stimulated spleen cells (ISC), resting and ConA stimulated spleen and PBMC (MRB), or ConA stimulated PBMC (RACT) as previously described [22]. The general protocol was to immunize mice 5 times subcutaneously with  $\sim 5 \times 10^6$  cells per mouse. Seventy two hours before fusion, mice were injected i.v. through the tail vein with approximately  $3 \times 10^6$  cells. After 72 h, spleen cells were harvested and pooled.  $10^8$  lymphocytes were fused with  $4 \times 10^7$  X63 myeloma cells as previously described [22] and then distributed into ten 96 well culture plates. The rest of the lymphocytes were cryopreserved for use in additional fusions. At 8 days, supernatants were collected and screened by FC for the presence of antibody, using blood or unstimulated and ConA stimulated spleen cells as described above.

Supernatants from primary cultures of hybridomas were screened for the presence of mAb specific for LDM using FC with whole blood. Positive cultures were expanded in 12 well culture plates. Supernatants were collected for further analysis and the cells cryopreserved. Since there was limited information on the pattern of reactivity of known LDM expressed on rabbit leukocytes, all hybridomas producing mAb were cryopreserved. This included hybridomas identified in screening experiments where hydroethidine was used to identify hybridomas producing mAbs to activation molecules.

### Antibodies

Cross reactive and new mAbs developed in our laboratory are shown in Table 1. mAbs specific for CD4 (Ken4) [31], CD11b (mAb 198) [65], CD11c (mAb 3/22, no longer listed by AbD Serotec [NC]), CD45 (mAb L12/201) [65], CD58 (VC21) [64] were purchased from AbD Serotec (USA). A mAb thought to react with rabbit CD5 (Ken5; BioSource, USA) [31]. CD8 (12.C7) [18] was purchased from Abcam (USA). mAbs specific for CD11a (Ken11) [31] and CD25 (Kei- $\alpha$ 1) [32] were purchased from BD Pharmingen (USA). Fluorescein conjugated anti-rabbit Ig was purchased from Zymed (USA).

### Clustering and Characterization of mAbs

All hybridomas producing mAbs to LDM expressed on lymphocytes or granulocytes were cloned. Hybridomas producing mAbs to LDM expressed on multiple lineages of leukocytes were first clustered based on the unique patterns of expression of the molecule on leukocytes, as detected by 2 parameter FC (SSC vs fluorescence). Two or three hybridomas were selected from each distinct cluster for cloning and further analysis. Hybridomas producing mAbs that yielded profiles similar to MHC class I and II molecules were set aside for later analysis. For further characterization, FC dot plot profiles of whole blood preparations of leukocytes labeled with new mAbs were compared to each other and with profiles obtained with the cross reactive mAbs or commercially available mAbs specific for rabbit LDM. Two color FC analysis was performed to determine whether mAbs in a cluster recognized the same or different molecules. Two mAbs were considered to recognize the same molecule if one of the mAb blocked labeling by the other or if the mAbs being compared yielded a diagonal pattern of labeling [11,34,35]. Pairs of mAbs yielding a diffuse pattern of labeling were considered to recognize different molecules on the same population of lymphocytes.

### Flow cytometry

A Becton Dickinson FACSsort equipped with a MAC computer and Cell Quest software (BD Immunocytometry Systems, USA) were used to collect data.

### Data analysis

Cell Quest and FCS Express software (DeNovo Software, USA) were used to analyze the data.

## Results

### Identification of cross-reactive mAbs that recognize conserved epitopes expressed on orthologous LDM in rabbits

At the initiation of the study, we screened sets of mAbs we developed against LDM in cattle, goats, sheep, horses,

pigs, cats, and dogs for mAb that cross reacted with rabbit LDM. We also screened additional sets of mAbs we developed during the course of the study for cross reactivity. Several strategies were used to increase the potential of generating mAbs that react with conserved determinants. These included hyperimmunization with leukocytes from multiple species and then selecting a single species to screen supernatants from primary cultures of freshly prepared hybridomas, hyperimmunization with leukocytes from a single species and screening for mAbs reactive with leukocytes from another species of interest, hyperimmunizing with leukocytes from a single species and screening for all mAbs that reacted with LDM from the same species and then screening for cross reactivity with LDM in other species. Although not used extensively for identification of cross reactive mAbs, simultaneous examination of primary cultures for mAb that recognized epitopes conserved on LDM in two species, using hydroethidine to mark one set of cells, showed that cross reactive mAbs could be identified directly. Cross reactive mAbs to bovine, caprine, and ovine CD4, CD8, CD45R, and CD45R0 were identified by this method [11,28]. Regardless of the strategy used for immunization, the most frequently encountered cross reactive mAbs were specific for MHC class I and II molecules. Other mAbs of interest that were identified by single fluorescence analysis recognized epitopes only conserved on orthologous molecules in closely related species e.g.: epitopes conserved on orthologous LDM in bison, water buffalo, Cape buffalo, goats, and sheep, with highest conservation noted between orthologues in cattle and bison [43]. Some of the epitopes recognized by mAbs were highly conserved and expressed on LDM in closely and distantly related species [12,54] (Table 1).

The screening of several hundred mAbs developed in our laboratory yielded 13 mAbs that recognize conserved epitopes expressed on rabbit LDM. The specificity of 10 of the mAbs (RH1A and LT86A [CD9]; HUH73A [CD11a]; CAM36A [CD14]; H20A, BAQ30A, and HUH82A [CD18]; and BAG40A and LT41A [CD44] ) was validated in the Animal Homologues section of the HLDA8 (Table 1, Fig. 1) [12,54]. Two additional mAbs, RACT48A and GBSP71A submitted to the workshop reacted with molecules expressed on multiple lineages of leukocytes in humans and other species. No clear match was obtained with standard panels of human leukocytes or cell lines transfected with known CD molecules. BAQ44A and CADO34A were not submitted to the HLDA8 workshop since they did not react with leukocytes from humans. However, the mAb-defined epitope recognized by BAQ44A is expressed on B lymphocytes in multiple species of ruminants. The epitope recognized by CADO34A is expressed on granulocytes, B lymphocytes and subsets of T lymphocytes in dogs and cats. Multiple mAbs were identified that reacted with rabbit MHC I and II molecules. The best characterized

mAbs are listed in Table 1. Analysis of the specificities of TH14B and TH81A5 have shown they recognize epitopes conserved on the orthologues of HLA-DR and HLA-DQ, respectively [1].

### **Identification of mAbs that recognize LDM expressed on T lymphocytes**

Screening of the mAb sets obtained from the different fusions yielded multiple mAbs that recognize LDM expressed on all lymphocytes or subsets of lymphocytes. These were further analyzed to determine which mAbs detected LDM expressed on T lymphocytes, B lymphocytes, or T and B lymphocytes using 2 color FC. Fluorescein conjugated anti-rabbit Ig was used to identify mAbs recognizing LDM on B lymphocytes. Ken4 (CD4) and Ken5 (pan T) were used to identify mAbs recognizing LDM on T lymphocytes. 12.C7 (CD8) was used to verify specificity of mAbs reacting with CD8 [18]. As summarized in Tables 1 and 2 and fig. 1A, 1B, 8 mAbs were identified that recognize LDM expressed on all T lymphocytes (MRB61A, RT22A, RTH2A, RTH21A, RTH26A, RTH65A, RTH230A, and RACT53A). Cross comparison of the patterns of reactivity of the mAbs using 2 color FC showed RTH2A and RTH230A; RT21A and RTH21A; and RTH26A, RTH65A, and Ken5; recognize Pan T1, Pan T2, and Pan T4 LDM respectively. Zenon second step antibodies were used to demonstrate RTH2A (IgG1) and RTH230A (IgG1) recognize the same LDM (Fig. 2). RACT53A (PanT5) recognizes an additional molecule expressed on all T cells (Fig. 3). Analysis of the reactivity of MRB61A (Pan T3) revealed it detects a LDM expressed on all T lymphocytes and basophils (Fig 1 #7, two color labeling not shown).

Seven mAbs were identified that recognize LDM expressed on T lymphocyte subsets. Comparison of labeling with Ken 4 and 12.C7 demonstrated that RTH1A recognizes CD4 [41] and that ISC16A, ISC27A, ISC29A, ISC38A, and RT1A recognize CD8 [18] (Table 1, Fig. 1 #9 & #10, FC two color comparisons not shown). No information was obtained on whether the CD8 mAbs recognize epitopes on CD8 $\alpha$  or CD8 $\beta$ .

Comparison of labeling with RACT19A (Fig. 1 #12) with PanT1, RTH1A and ISC38A revealed the molecule detected is expressed on a large subset of CD4 and the majority of CD8 lymphocytes (Fig. 4).

### **Identification of mAbs that recognize LDM expressed on B lymphocytes**

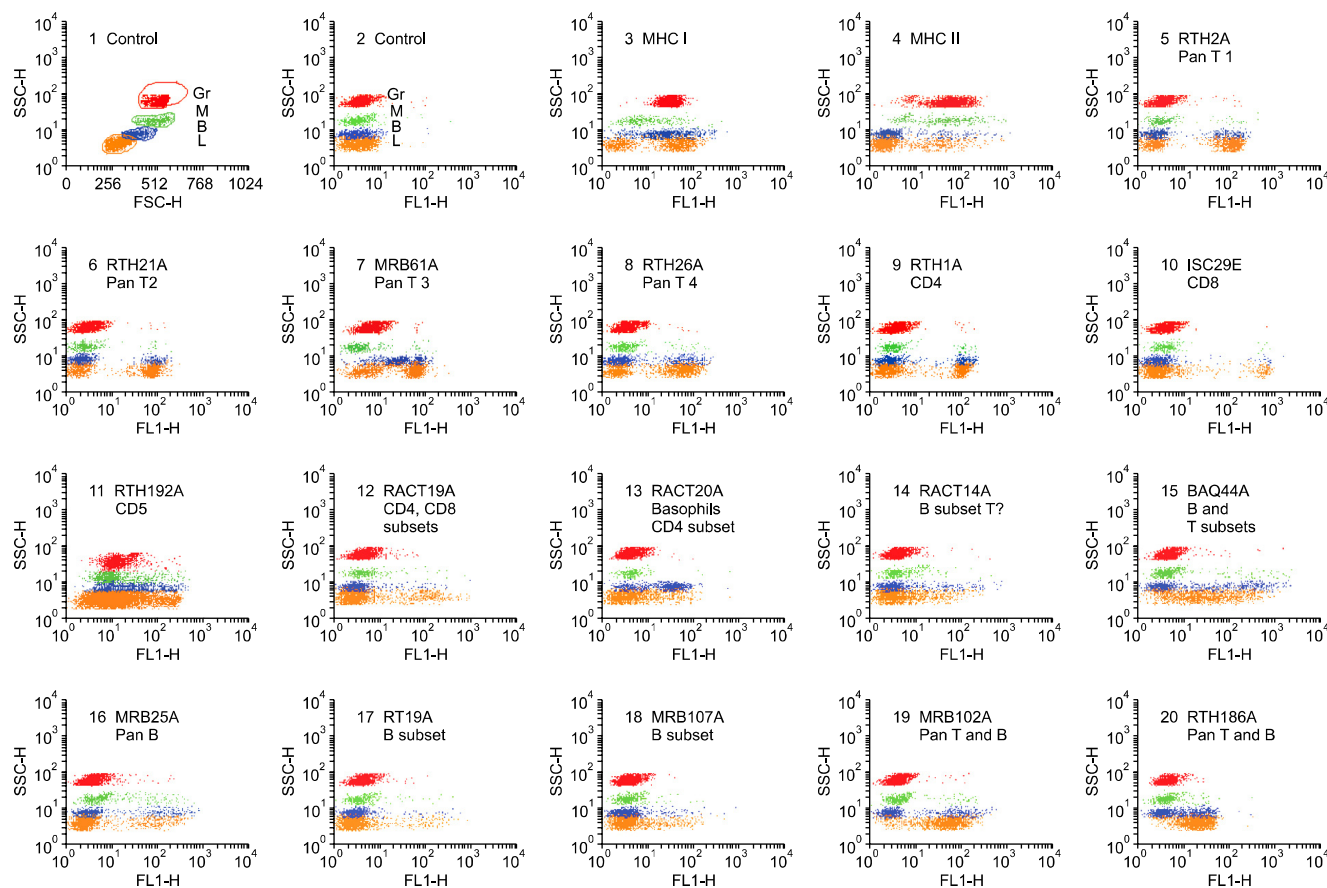
Eleven mAbs were identified that recognize LDM expressed on B lymphocytes (Tables 1 and 2, Fig. 1 #16, #17 & #18). Comparison of labeling with fluorescein conjugated polyclonal anti-rabbit immunoglobulin (Ig), RACT30A (Fig. 5) and PanT5 (Fig. 3) were used to demonstrate that MRB25, MRB29A, and MRB143A recognize one or more molecules expressed on all B

**Table 1.** Monoclonal antibodies reactive with rabbit mhc and leukocyte differentiation molecules

MoAb	Ig isotype	Specificity
H1A	IgG2a	MHC CL I
H58A	IgG2a	MHC CL I
TH14B	IgG2a	MHC CL II HLA-DR equivalent
TH81A5	IgG2a	MHC CL II HLA-DQ equivalent (polymorphic determinant)
RTH2A	IgG1	Pan T1
RTH230A	IgG1	= Pan T1
RTH21A	IgG1	Pan T2
RT22A	IgM	= Pan T2 (blocked by RTH21A)
MRB61A	IgG1	Pan T3 (also expressed on basophils)
RTH26A	IgG2a	Pan T4 = Serotec Ken 5 (diagonal co-labeling)
RTH65A	IgM	Pan T4 = RTH26A (diagonal co-labeling)
RACT53A	IgG1	Pan T5
RTH1A	IgG1	CD4 = Serotec Ken 4 (diagonal co-labeling)
RTH192A	IgG1	CD5 (inferred from pattern of FC labeling)
ISC16A	IgM	CD8 (diagonal co-labeling with ISC27A, ISC29A, ISC38A)
ISC27A	IgG2a	CD8 (diagonal co-labeling with 12.C7, ISC29A, ISC38A, RT1A)
ISC29E	IgG1	CD8
ISC38A	IgG1	CD8
RT1A	IgM	CD8
RACT19A	IgM	CD4 and CD8 subpopulations
RACT20A	IgG1	Basophils and subpopulation of CD4 <sup>+</sup> T lymphocytes
MRB120A	IgG1	Granulocytes, basophils, and monocytes
RACT14A	IgM	Subpopulations of B and T lymphocytes
RACT21A	IgM	Subpopulations of B and T lymphocytes
RACT30A	IgM	Pan B (expressed on some T lymphocytes?)
MRB25A	IgM	Pan B
MRB29A	IgM	Pan B
MRB143A	IgM	Pan B
BAQ44A	IgM	Pan B and subpopulation of CD4 and CD8 T lymphocytes
CADO34A	IgM	Pan B and subpopulation of CD4 and CD8 T lymphocytes
RT19A	IgM	B subpopulation
MRB107A	IgG1	B subpopulation
MRB102A	IgM	Pan lymphocyte
RTH186A	IgG1	Pan lymphocyte
RH1A	IgG3	CD9
LT86A	IgG2a	CD9
RACT48A	IgG1	CD11a = Serotec CD11a
HUH73A	IgG1	CD11a = RACT48A
RTH161A	IgG1	CD11a = HUH73A = RACT48A
RT18A	IgM	CD11b = Serotec CD11b
RT3A	IgM	CD11c = Serotec CD11c
CAM36A	IgG1	CD14
H20A	IgG1	CD18
HUH82A	IgG2a	CD18
BAQ30A	IgG1	CD18
25-32	IgG1	CD44
BAG40A	IgG3	CD44
LT41A	IgG2a	CD44 = BAG40A
ISC18A	IgG2a	CD45 = Serotec

**Table 1.** Continued

MoAb	Ig isotype	Specificity
ISC39A	IgG1	CD45 = ISC18A, ISC76A
ISC76A	IgM	CD45 = Serotec CD45 (blocks labeling with Serotec CD45)
ISC4A	IgG3	Pan T + granulocytes
ISC24A	IgM	Pan T + granulocytes (diagonal with ISC4A)
RTH32A	IgM	CD58 = Serotec CD58
RTH33A	IgG1	CD58 = RTH32A = Serotec CD58
RACT43A	IgM	Granulocytes
RACT44A	IgM	Granulocytes = RACT43A?
RACT38A	IgG1	Pan leukocyte
RT23A	IgM	Pan leukocyte
RACT1A	IgG1	ACT1
RACT4A	IgG1	ACT2
RACT12A	IgG1	ACT3



**Fig. 1.** Representative dot plot profiles of peripheral blood leukocytes labeled with the mAbs indicated. A single representative profile is shown for mAbs that recognize the same or different epitopes on the same subset of cells. A side light scatter (SSC) vs forward light scatter dot plot was used to gate and color code the major populations of leukocytes: red for granulocytes, green for monocytes, blue for basophils, and orange for lymphocytes. Note that in contrast to other species, rabbits have a relatively large population of basophils in blood. It was necessary to label leukocytes in blood and use a fix lyse solution to isolate and analyze the composition leukocytes in peripheral blood. T lymphocytes bind to erythrocytes and are lost when leukocytes are separated using density gradient separation media.

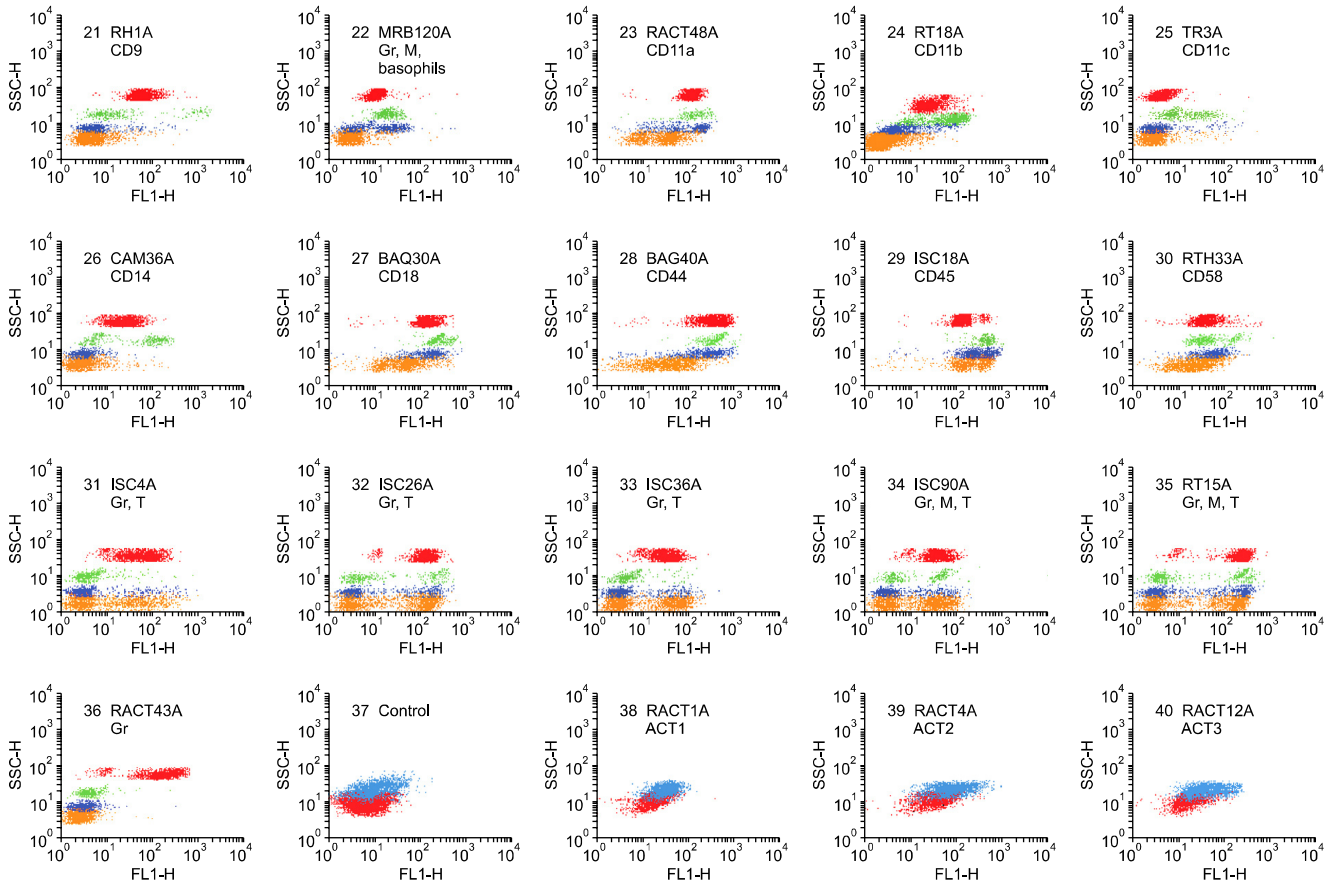


Fig. 1. Continued.

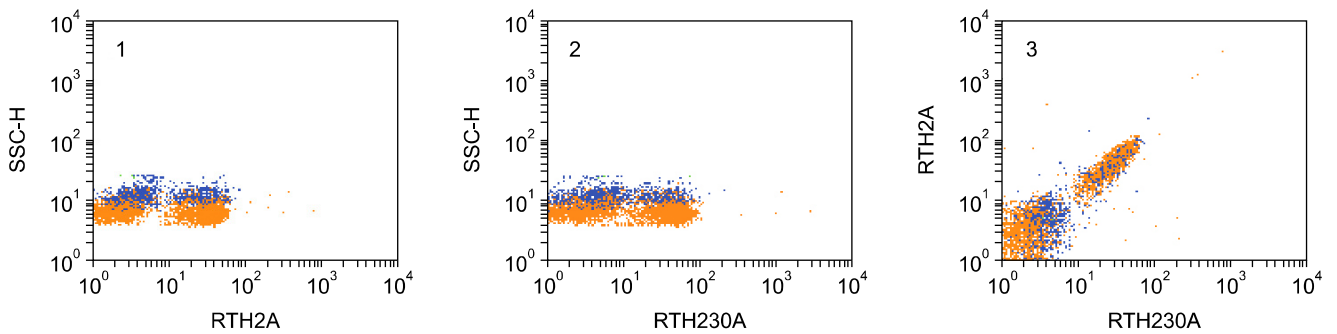
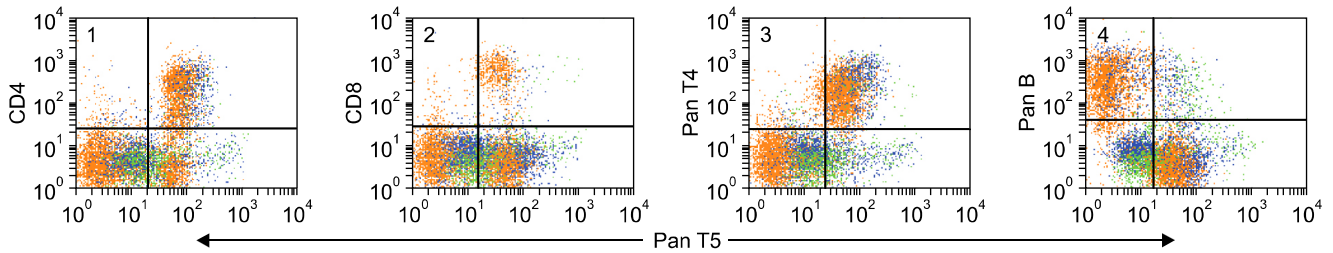


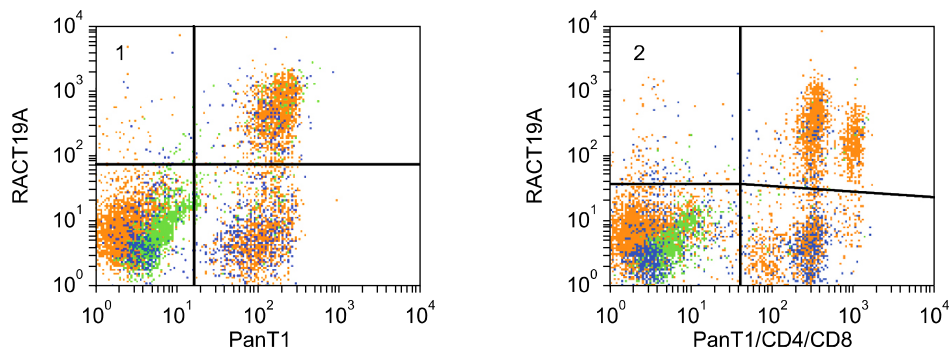
Fig. 2. Two color FC analysis of labeling with mAbs that recognize different epitopes expressed on the same molecule. mAbs that recognize epitopes on the same molecule yield a diagonal pattern of labeling if the epitopes are sterically distant from each other. If the mAbs recognize the same epitope or epitopes that are sterically close, labeling with one mAb will block labeling with the second mAb. RTH2A and RTH230A recognize different epitopes expressed on a molecule expressed on all T lymphocytes.

lymphocytes (dot plots not shown). Comparison of labeling of MRB107A with MRB25A and BAQ44A demonstrated that MRB107A recognizes a LDM expressed on a subset of B lymphocytes. The molecule detected is only expressed on a subset of MRB25<sup>+</sup> B lymphocytes. The whole population is included in the BAQ44A positive

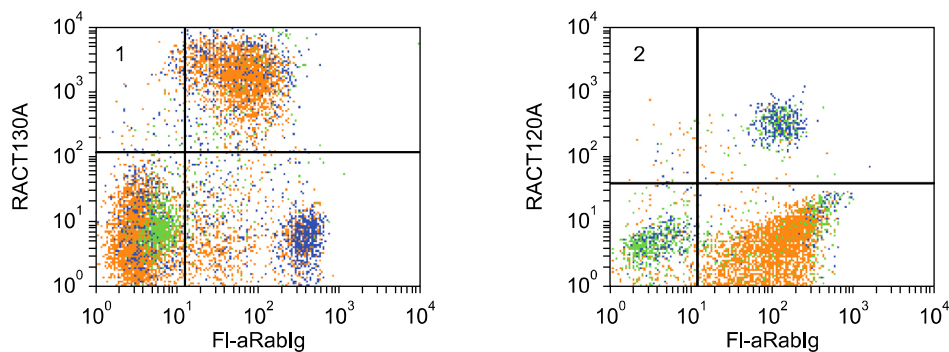
population of B lymphocytes (Fig. 6). As shown in Table 2, the level of expression of the pan B mAb-defined LDM(s) were similar in peripheral blood, thymus and spleen. However, other mAbs that recognize LDM expressed on subsets of B lymphocytes exhibited different patterns of expression (Table 2, FC for thymus and spleen not shown).



**Fig. 3.** Two color FC analysis showing the pattern of labeling obtained with mAbs that recognize different molecules only expressed on T lymphocytes. The subsets labeled with anti-CD4 and CD8 mAbs are included in the population labeled with a pan T mAb, panels 1 and 2. Labeling with the two anti-pan T mAbs yields a diffuse pattern of labeling, panel 3. Mutually exclusive populations of lymphocytes are labeled with mAbs specific for T and B lymphocytes, panel 4. The example presented here suggests a small subset of B lymphocytes may express the pan T4-defined T lymphocyte molecule.



**Fig. 4.** Two color FC analysis showing RACT19A recognizes a molecule expressed on a major subset of T lymphocytes, panel 1. mAbs specific for PanT1, CD4, and CD8 were combined to show the molecule is expressed on a large subset of CD4 T lymphocytes and most CD8 lymphocytes. The level of expression of the RACT19A-defined molecule on CD4 lymphocytes is less than the level of expression on CD8 lymphocytes.



**Fig. 5.** Two color FC analysis demonstrating that RACT30A recognizes a molecule expressed on all B cells, panel 1. As shown in panel 2, immunoglobulin detected with polyclonal anti-rabbit Ig is also present on basophils. RACT20A recognizes a molecule expressed on basophils and a subset of CD4 T lymphocytes.

The subset of B lymphocytes detected with RACT14A and RACT21A was in low frequency in peripheral blood and in high frequency in spleen and appendix. The subset detected with RTH72A was low in peripheral blood, thymus, and appendix and high in spleen. The subset detected with mAbs RT19A and RTH172A was low in peripheral blood but high in thymus, spleen, and appendix.

### Identification of mAbs that recognize LDM expressed on T and B lymphocytes

Four mAbs were identified that recognize LDM expressed on T and B lymphocytes, MRB102A, RTH186A, RTH192A, and BAQ44A (a cross reactive mAb) (Fig. 1 #19, #20, #11 & #15, respectively). The level of expression of the LDM detected with MRB102A on lymphocytes was higher than

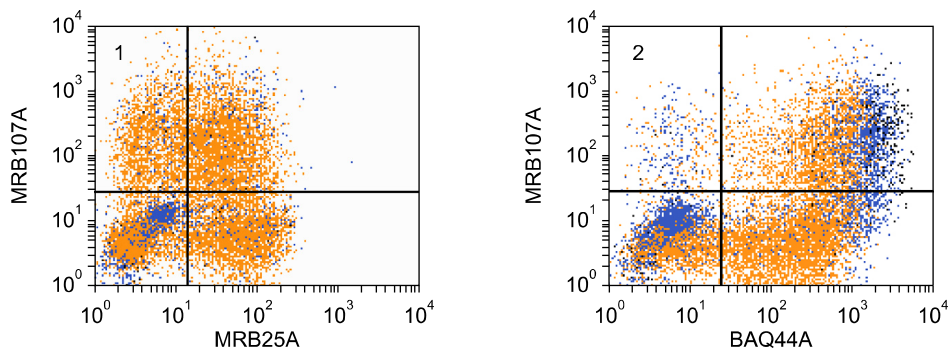


**Table 2.** Reactivity of monoclonal antibodies with leukocyte from blood and primary and secondary lymphoid organs

mAb	% + Peripheral blood	%+ Thymus	%+ Spleen	%+ Appendix
H1A	86	57	79	8
H58A	82	46	75	7
TH14B	57	63	50	99
TH81A5	58	52	47	99
RTH2A	27	37	37	4
RTH230A	28	37	40	4
RTH21A	26	92	45	4
RT22A	27	97	44	4
MRB61A	40	99	45	8
RTH26A	28	99	48	5
RTH65A	26	99	36	5
RACT53A	30	28	59	11
RTH1A	24	87	20	3
RTH192A	50	16	57	97
ISC16A	4	83	9	1
ISC27A	4	84	12	1
ISC29E	4	75	9	1
ISC38A	4	86	9	1
RT1A	4	84	10	1
RACT19A	8	6	15	13
RACT20A	18	2	4	3
MRB120A	22	1	6	20
RACT30A	26	11	45	94
MRB25A	14	10	44	82
MRB29A	15	6	45	77
MRB143A	15	5	38	77
RACT14A	9	14	45	69
RACT21A	11	13	48	69
RT19A	5	50	45	56
RTH72A	3	8	31	5
RTH172A	7	59	44	61

**Table 2.** Continued

mAb	% + Peripheral blood	%+ Thymus	%+ Spleen	%+ Appendix
MRB107A	7	4	18	3
MRB102A	38	75	69	79
RTH186A	40	14	76	8
BAQ44A	34	40	59	82
CADO34A	12	3	64	90
RACT48A	99	97	99	99
HUH73A	99	NT	NT	NT
RTH161A	99	99	99	99
RT18A	28	5	53	4
RT3A	7	1	20	4
MRB128A	8	2	36	7
CAM36A	8	1	5	2
H20A	99	97	73	99
HUH82A	99	NT	NT	NT
BAQ30A	99	99	76	99
BAG40A	93	15	85	99
LT41A	99	NT	NT	NT
ISC18A	99	90	92	97
ISC39A	99	NT	NT	NT
ISC76A	99	NT	NT	NT
ISC4A	20	49	54	5
ISC24A	15	91	48	5
ISC26A	34	97	81	52
ISC36A	24	98	70	9
ISC90A	28	97	82	93
RT15A	33	97	85	87
RTH33A	99	96	99	31
RACT43A	32	9	26	5
RACT44A	27	9	28	5
RACT38A	99	99	88	99
RT23A	99	95	95	96



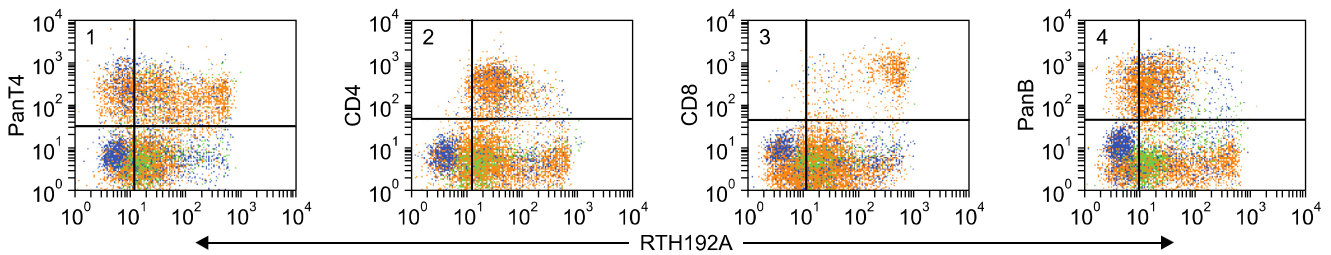
**Fig. 6.** Two color FC analysis of the expression of a molecule detected with MRB107A that is expressed on a subset of B lymphocytes. The molecule is expressed on a subset of MRB25A+ B lymphocytes, panel 1. All the MRB107A<sup>+</sup> lymphocytes co-express the molecule detected with BAQ44A, panel 2.

the LDM detected with RTH186A. However, two color analysis showed the level of expression of both LDMs on CD4 and CD8 T and B lymphocyte subsets was similar (FC not shown). The level of expression of the MRB102A-defined LDM was also high on lymphocytes in the thymus, spleen, and appendix. In contrast, the RTH186A defined LDM was only expressed on a few lymphocytes in the thymus and appendix. It was expressed on a large population of lymphocytes in the spleen (Table 2, FC not shown).

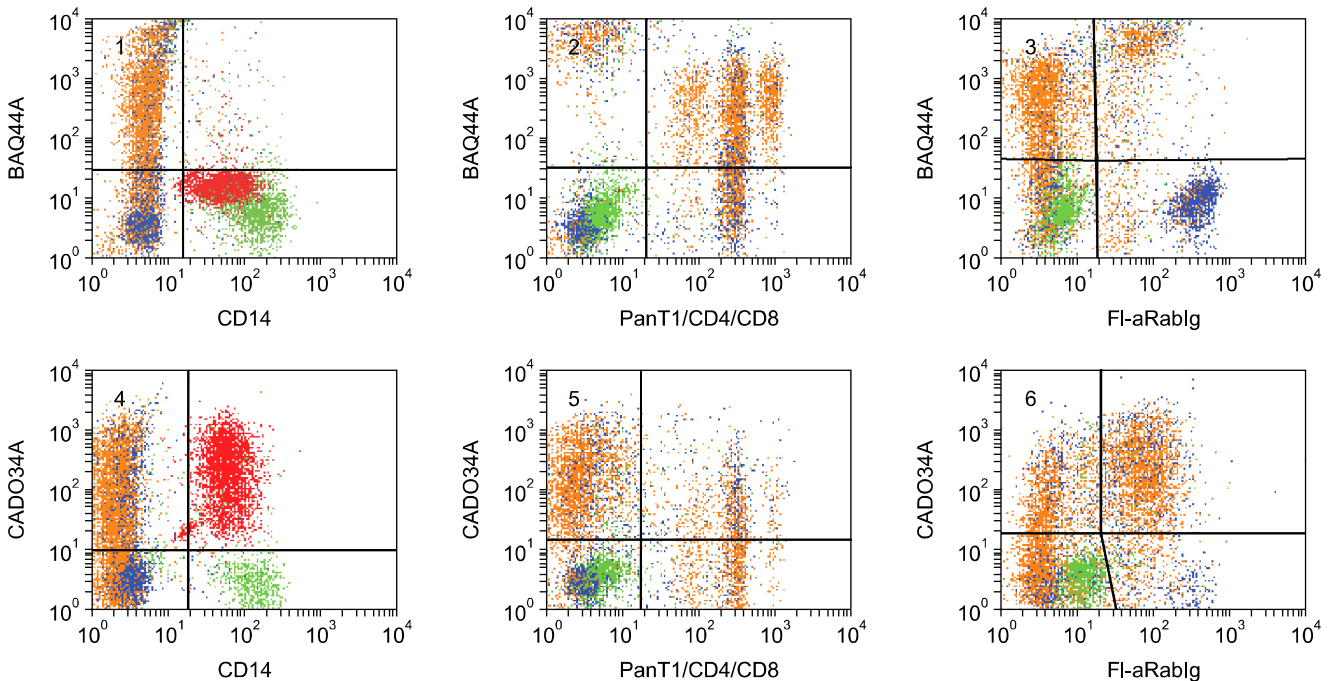
The level of expression of the LDM detected with RTH192A and BAQ44A differed on CD4 and CD8 T and B lymphocytes. The level of expression of the RTH192A-

defined LDM was variable on PanT<sup>1+</sup> lymphocytes (Fig. 7). It was low on CD4 T and B lymphocytes (Fig. 7). It was high on CD8 T lymphocytes (Fig. 7). It was only expressed on a few thymocytes. It was expressed at a high level on about 50% of lymphocytes in the spleen and essentially all lymphocytes in the appendix (Table 2, FC not shown). The pattern of expression on T and B lymphocytes suggests the LDM detected is CD5 [41,49-51].

The level of expression of the LDM detected with BAQ44A also differed on CD4 and CD8 T and B lymphocytes (Fig. 8). Simultaneous labeling with Pan T1, CD4, and CD8 mAbs and anti-rabbit Ig demonstrated that the LDM is



**Fig. 7.** Two color FC analysis of the expression of RTH192A on T and B lymphocytes. The level of expression of PanT4 on RTH192A<sup>+</sup> lymphocytes was variable from high to low, panel 1. Expression of CD4 and the MRB25A-defined B molecule were also low, panels 2 and 4. Expression of the TH192A-defined molecule was invariably higher on CD8 lymphocytes than expression on the other mAb-defined populations, panel 3.



**Fig. 8.** Two color FC analysis of the expression of BAQ44A- and CADO34A-defined molecules. The BAQ44A-defined molecule was not expressed on granulocytes or monocytes. Comparison of labeling with BAQ44A in combination with mAbs to PanT1, CD4, and CD8 showed subsets of CD4 and CD8 co-expressed the BAQ44A-defined molecule, panel 2. The molecule was not expressed on basophils, panel 4. The pattern of labeling indicate a subset of Pan T<sup>+</sup> CD4<sup>+</sup> CD8<sup>+</sup> also express the BAQ44A-difined molecule. B cells also co-expressed the molecule. The molecule was not expressed on basophils, panel 3. A similar pattern of labeling was observed with the CADO34A-defined molecule, Panels 2 and 3. The molecule was also expressed on granulocytes, panel 1.

highly expressed on B lymphocytes, a subset of CD4 and CD8 negative T lymphocytes, a large subset of CD4 T lymphocytes and the majority of CD8 T lymphocytes. The BAQ44A-defined LDM was expressed on large populations of lymphocytes in the thymus, spleen and appendix (Table 2, FC not shown).

### Identification of a mAb that recognizes a LDM expressed on granulocytes, T and B lymphocytes

One cross reactive mAb, CADO34A, was identified that recognizes a LDM expressed on granulocytes as well as T and B lymphocytes (Fig. 8). Comparison of labeling with CADO34A to labeling with BAQ44A revealed the pattern of labeling is similar to the labeling pattern obtained with BAQ44A for T and B lymphocytes. Simultaneous labeling with Pan T1, CD4, and CD8 mAbs demonstrated the LDM is expressed on a subset of CD4 and CD8 negative lymphocytes, a large subset of CD4 and the majority of CD8 T lymphocytes. The LDM was only expressed on a few thymocytes. It was expressed on a large population of lymphocytes in the spleen and most of the lymphocytes in the appendix (Table 2, FC not shown).

### Identification of a mAbs that recognize a LDM expressed on granulocytes and monocytes, granulocytes, monocytes and basophils, or basophils

Three mAb were identified that detect LDM expressed on granulocytes and monocytes, granulocytes, monocytes and basophils or basophils (CAM36A, MRB120A, and RACT20A, Fig. 1 #26, #22 & #13, respectively and Fig. 9). CAM36A recognizes a conserved epitope expressed on CD14. In contrast to some species, expression of CD14 is high on rabbit granulocytes. Two color analyses showed CD14 is not expressed on basophils (Fig. 9). Two color analyses showed the MRB120A recognizes a LDM expressed on granulocytes, monocytes and basophils while the RACT20A recognizes a LDM only expressed on

basophils and a subset of CD4<sup>+</sup> lymphocytes (Fig. 9). Expression on CD8 lymphocytes is low or absent. Two color analyses were not performed to determine whether expression of the RACT20A-defined LDM on small populations of cells detected in the thymus, spleen and appendix were basophils or T lymphocytes (Table 2).

### Identification of mAbs recognizing LDM expressed on granulocytes

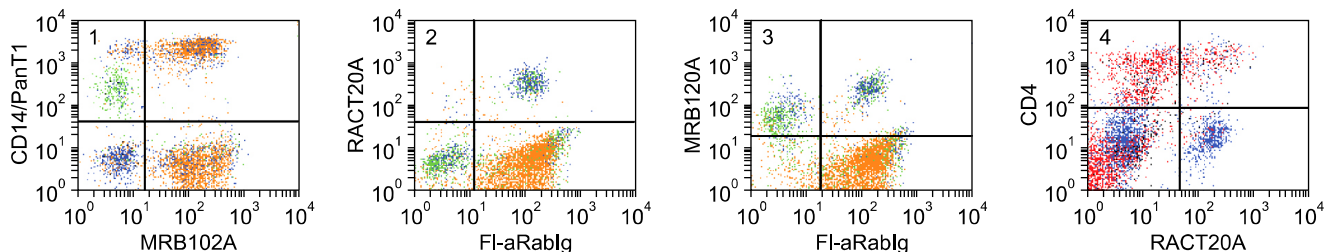
Two mAbs (RACT43A and RACT44A Table 1, Fig. 1 #36) were identified that recognize a LDM expressed on granulocytes. The similarity of the pattern of labeling with the mAbs suggests they may recognize the same LDM.

### Identification of mAbs recognizing CD9

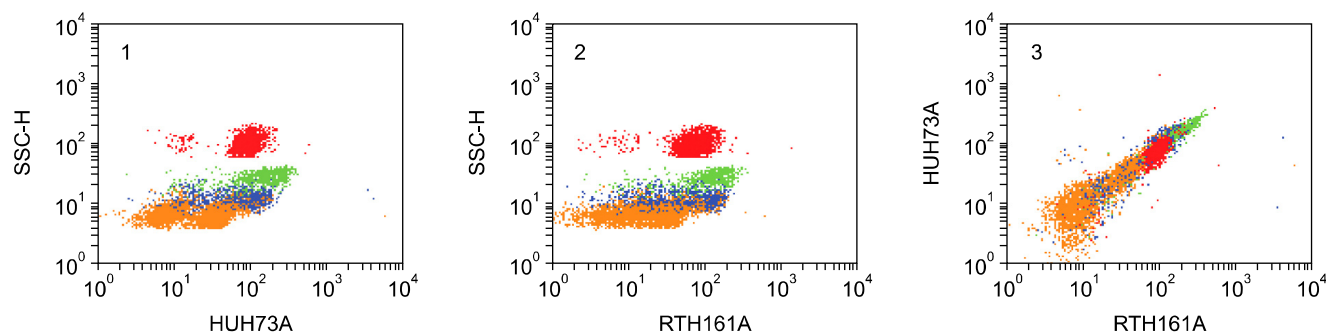
Comparison of the labeling patterns with cross reactive RH1A and LT86A (Table 1, Fig. 1 #21) showed they recognize CD9 in rabbits.

### Identification of mAbs recognizing CD11a, CD11b, CD11c, and CD18

mAbs that recognize CD11a, CD11b, CD11c, and CD18 (Table 1, Fig. 1 #23, #24, #25 & #27 respectively) were identified by two color FC with mAbs that recognize epitopes conserved on orthologues in one or more species or with commercially available mAbs generated against the rabbit orthologues. Comparison of the patterns of labeling obtained with RACT48A and RTH161A with commercially available anti-CD11a (Ken11) [31] suggested these mAbs recognize CD11a. Subsequent comparative two color FC analysis with HUH73A, a mAb demonstrated to recognize a conserved epitope on the CD11a orthologue in the Animal Homologues section of the HLA8 workshop [54], verified these mAbs recognize CD11a (Fig. 10). The studies also demonstrated RTH161A recognizes a species restricted epitope. Comparison of the patterns of labeling obtained with RT18A with anti-CD11b (198) and subsequent



**Fig. 9.** Two color analysis of the expression of LDM detected with RACT20A and MRB120A on basophils, monocytes, and CD4 T lymphocytes. mAbs to CD14 and Pan T1 were combined and used to distinguish monocytes and T lymphocytes simultaneously. MRB102A was used to identify all T and B lymphocytes. Polyclonal anti-Ig was used to distinguish B lymphocytes. Panel 1 shows the populations present in PBMC: basophils, lower left quadrant; monocytes, upper left quadrant; T lymphocytes, upper right quadrant; B lymphocytes, lower right quadrant. Panel 2 shows the LDM recognized by RACT20A is only expressed on basophils, upper right quadrant. As noted, Ig is present on basophils. Panel 3 shows the LDM recognized by MRB120A is expressed on monocytes, upper left quadrant and basophils, upper right quadrant. Panel 4 shows the LDM recognized by RACT20A is expressed on basophils, lower right quadrant and also a subset of CD4 T lymphocytes, upper right quadrant.



**Fig. 10.** Two color analysis of HUH73A and MRB161A. The similarity in the pattern of labeling obtained with both mAbs indicates they recognize the same molecule, panels 1 and 2. The diagonal pattern of labeling indicates the mAbs recognize epitopes on the same molecule. Based on findings with HUH73A in the animals homologues section of the 8th human leukocyte antigen workshop, the molecule identified is the rabbit orthologue of CD11a.

2 color FC showed RT18A recognizes CD11b (Fig. 1 #24). Similar studies comparing RT3A with 3/22 showed RT3A recognizes CD11c (Fig. 1 #25). Three mAbs demonstrated to recognize conserved epitopes on orthologues of CD18 (Fig. 1 #27), in 2 or more species were shown to recognize rabbit CD18. The pattern of expression of the rabbit orthologues for each molecule was similar to that noted in other species.

#### Identification of mAbs that recognize CD44, CD45, and CD58

Screening of a large series of mAbs that recognize conserved epitopes on CD44 in 2 or more species, including humans, showed 25-32 [40], BAG40A, and LT41A (Table 1, Fig. 1 #28) recognize epitopes expressed on rabbit CD44 [14,24,54]. The pattern of expression of rabbit CD44 was similar to that noted in other species. Comparison of the pattern of labeling obtained with L12/201 (CD45) with the panels of mAbs developed against rabbit LDM revealed several mAbs yielded similar patterns of labeling (Table 1, Fig. 1 #29). Two color flow cytometry yielded diagonal patterns of labeling or blocking, indicating the mAbs recognize rabbit CD45 (FC not shown). Comparison of the labeling pattern obtained with VC21 (CD58) revealed two mAbs (RTH32A, RTH33A, Table 1, Fig. 1 #30) yielded similar patterns of labeling. Two color comparisons yielded diagonal patterns of labeling indicating the mAbs recognize CD58 (FC not shown).

#### Identification of mAbs that recognize LDM expressed on granulocytes and lymphocytes

Six mAbs were identified that recognize LDM expressed on granulocytes and lymphocytes ISC4A, ISC24A, ISC26A, ISC36A, ISC90A, and RT15A (Table 1, Fig. 1 #31, #26, #36, #90 and #35). Two color analysis showed ISC4A and ISC24A recognize the same molecule (FC not shown). The others identified different molecules. The molecules identified with ISC90A and RT15A are also expressed on

some monocytes. The molecule identified with RT15A is highly expressed on thymocytes, spleen lymphocytes, and lymphocytes in the appendix (Table 2). Two color analysis with anti-CD4 and -CD8 mAbs indicate none of the mAbs recognize CD45R0. The studies completed thus far indicate there are no clearly defined subsets of CD4 and CD8 negative for these LDM (FC not shown).

#### Identification of mAbs that recognize LDM expressed on all leukocytes

Three mAbs under investigation identify LDM expressed on all leukocytes RACT38A, RT23A, and GBSP71A (Table 1, FC not shown). Comparison of the flow cytometric profiles with those of known CD molecules has thus far not suggested which molecules are recognized by these mAbs.

#### Identification of mAbs that recognize LDM expressed on activated lymphocytes

Three mAbs were identified that recognize mAbs upregulated on ConA activated lymphocytes RACT1A, RACT4A, RACT12A (Table 1, Fig. 1 #38, #39 & #40). Two color analysis with mAbs specific for ConA and CD25 [32] demonstrated the mAbs recognize different LDM (FC not shown).

### Discussion

Cumulative data obtained from international workshops convened to complete characterization of mAbs to LDM in humans have shown flow cytometry can be used to compare and cluster mAbs that appear to recognize the same LDM for further analysis [34]. Our studies and studies conducted as part of workshops convened to characterize mAb-defined LDM in ruminants, swine, horses, and dogs have shown that the pattern of expression of many orthologous of CD molecules is conserved cross species [11]. These findings have afforded an opportunity to devise a strategy for characterizing mAb to LDM in

additional less well studied species. mAb that identify LDM with patterns of expression similar to the patterns of expression of known Human Cell Differentiation Molecules (HCDM) can be clustered for further analysis. The TKP can be used to facilitate determining whether a mAb recognizes a new or known HCDM where flow cytometric data are not conclusive. Where cross reactive mAbs are identified, they can be used to validate the specificity of the mAbs recognizing species restricted epitopes using two color FC. A diagonal pattern of labeling implies the epitopes detected are present on the same molecule. Complete or partial blocking of labeling with one of the mAbs indicates the epitopes detected are sterically close on the same molecule, with the binding of one mAb interfering with the binding of the second mAb. These methods of analysis have been used effectively to develop a set of mAbs for use in alpacas and llamas [14] and, as demonstrated in the present report, rabbits. Cross reactive mAbs allowed us to identify mAbs specific for MHC class I and II and several CD molecules early in the course of the studies. Two color analyses with commercially available mAbs to some rabbit LDM facilitated validation of the specificity of additional mAbs generated in our laboratory. To date, we have identified mAbs to MHC class I and II molecules and 16 known LDM. Additional mAbs to T and B lymphocytes have been identified that require further characterization to determine their relation to known mAb-defined HCDM.

Until now the lack of mAbs to rabbit LDM and MHC has made it difficult to compare the immune system of rabbits to those characterized in other species. Comparative studies have shown the immune systems of different orders and species of vertebrates are similar but not identical. Differences have been noted in the evolution and expression of immunoglobulin genes with expansion of the IgA genes a unique feature of rabbits [41] and the development of IgG heavy chain genes a unique feature of camelids [21,41]. The expression of  $\alpha\beta$  CD4 and CD8 T cell subsets have appeared similar with an exception in swine. There is a large population of CD4/CD8 double positive cells in peripheral blood. Analysis has shown the proportion of double positive cells increases with age and is correlated with the appearance of the majority of memory T cells in this population [67,68]. The most striking difference noted is in the abundance of  $\gamma\delta$  T cells in some species [20].  $\gamma\delta$  T cells comprise a high proportion of lymphocytes in peripheral blood of chickens [5,9], swine [2,3,17] and ruminants [19,25,39]. The relation between the large population of  $\gamma\delta$  T cells that have evolved in chickens and the one in pigs and ruminants is not clear. However, recent studies have provided an explanation for the abundance in the latter species. Abundance is attributable to the presence of a unique subset of  $\gamma\delta$  T cells that has only been found in artiodactyla. The subset is characterized by the expression

of a molecule referred to as workshop cluster 1 (WC1) in ruminants [42]. The WC1<sup>+</sup> population may comprise 50% or more of T lymphocytes in the blood of young ruminants [62,63]. A WC1- population is also present but comprises ~5% of  $\gamma\delta$  T cells in blood [10]. The large subset identified in swine expresses the orthologue of WC1 [7,17]. The subset expressing the orthologue has been identified in camelids also [14].

An additional difference noted in the comparative studies is in the expression of MHC II. In humans, mice, and ruminants MHC II is expressed primarily on monocytes and B lymphocytes in blood. MHC II is upregulated on T cells following activation. MHC II is expressed on monocytes, B cells and resting T cells in horses [37], swine [48], dogs [8], and cats (personal observation).

As mentioned, the flow cytometric approach to identifying and characterizing mAbs to rabbit LDMs has shown the pattern of expression of rabbit orthologues of known hCDLDMs have proven, thus far, to be similar. The composition of lymphocyte subsets also appear more similar to that of humans than ruminants and swine. Analysis of T lymphocytes in the rabbit shows  $\alpha\beta$  T cells are the major population present in blood. Comparison of the percentages of cells expressing LDM present on all T cells with subsets of cells expressing CD4 or CD8 in two color flow cytometry has not revealed any CD4 CD8 double negative subset. Likewise comparison of the percentages of lymphocytes expressing LDM on T cells with those expressing LDM on B cells has not revealed the presence of a clearly defined subset of lymphocytes negative for T or B cell LDM. The findings suggest  $\gamma\delta$  T cells comprise a small percentage of lymphocytes in blood. Further studies are needed to identify rabbit  $\gamma\delta$  T cells.

The unique differences that have been noted are with the expression of MHC II on granulocytes, expression of certain mAb-defined LDM, and presence of immunoglobulin on basophils. Multiple mAbs of different isotype to MHC II were used to confirm expression of MHC II on granulocytes. Both IgG1 and IgG2a isotype mAbs yielded identical patterns of labeling. As in humans and mice, expression of MHC II on monocytes and B cells is similar. It is upregulated on activated T cells. Basophils comprise 5% to 30% of blood leukocytes in rabbits. Two mAbs were identified that identify molecules expressed on all T cells and basophils (MRB61A) and basophils and a subset of CD4 T cells (RACT20A). No information has been obtained on the functional activity of these LDM. Two color analyses have demonstrated that immunoglobulin is present on basophils. This finding is in agreement with an earlier study demonstrating the presence of multiple immunoglobulin isotypes on basophils, presumably binding to basophils through Fc receptors [6].

In summary, the use of flow cytometry has provided an approach to the identification and characterization of

mAbs to MHC I and MHC II molecules and LDM in less well studied species. mAbs that recognize the same LDM can be clustered based on the similarity of the pattern of expression and further characterized by comparing the pattern of expression with that of known LDM. When available, identity can be verified by comparison with a mAb that recognizes a conserved epitope on orthologous molecules. Where needed, mAb cluster-defined LDM can be subjected to immunoprecipitation and micro sequencing. We have used this approach successfully when participating in the international workshops of LDM in ruminants [26,27,46], swine [23,38,53], horses [33,36], dogs [8], the homologues section of the HLDA8 [54,66] and independently in the characterization of mAbs to LDM in lammas [14] and rabbits. The mAbs characterized here should facilitate characterization of the immune system in rabbits and the use of rabbits in immunological investigations.

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